Molecular, biochemical and physiological characteristics associated with quality and tolerance to drought and low temperature in tea (*Camellia sinensis* (L) O. Kuntze)

by

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DECLARATION

I declare that the thesis, which I hereby submit for the degree of Doctor of Philosophy (Horticultural Science) at the University of Pretoria, is my own work and has not previously been submitted by me for a degree at another university. Where secondary material is used, this has been carefully acknowledged and referenced in accordance with university requirements. I am aware of university policy and implications regarding plagiarism.

Signed:_____

Nicholas Ishumael Kosamu Mphangwe

DEDICATION

To my daughters **Grace** and **Lisa Mphangwe**

It is by the abundant **grace** of God Almighty that I have reached this far

Praise God from whom all blessings flow

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LIST OF ABBREVIATIONS

ABA	-	Abscisic acid
AFLP	-	Amplified fragment length polymorphism
BR	-	Brightness
bp	-	Base pairs
CAP	-	Cleaved amplified polymorphism
CET	-	Cultivar evaluation trial
CFC	-	Crude fibre content
cm	-	Centimetre
DArT	-	Diversity array technology
DNA	-	Deoxy-ribonucleic acid
DSI		Drought susceptibility index
EC	-	(-)-epicatechin
ECM	-	Environmentally controlled miniature tea processing unit
ECG	-	(-)- epicatechin gallate
EGC	-	(-)-epigallo-catechin
EGCG	-	epigallo-catechin gallate
F_1	-	First filial generation
Fm	-	Fresh mass

F_m	-	Maximum fluorescence
Fo	-	Initial fluorescence
Fv	-	Variable fluorescence
H_0	-	Null hypothesis
ha	-	Hectare
ha^{-1}	-	Per hectare
IAA	-	Indole-acetic acid
ISSR	-	Inter-simple sequence repeats
L	-	Litre
m	-	Metre
MAS	-	Marker assisted selection
min	-	Minute
mL	-	Millilitre
mm	-	Millimetre
mM	-	Millimole
MTRS	-	Mimosa Tea Research Station
OA	-	Osmotic adjustment
PAL	-	Phenylalanine ammonia lyase

PC	-	Progeny cultivar
PCR	-	Polymerase chain reaction
pH	-	Scale of acidity (0-14)
PPO	-	Polyphenol oxidase
POP	-	Preliminary observation plot
QTL	-	Quantitative trait loci
RAPD	-	Randomly amplified polymorphic DNAs
RFLP	-	Restriction fragment length polymorphism
ROS	-	Reactive oxygen species
RWC	-	Relative water content
SCAR	-	Sequence characterized amplified region
s	-	Second
SFS	-	Swazi field selection
SNP	-	Single nucleotide polymorphism
SSR	-	Simple sequence repeats
SVPD	-	Saturated vapour pressure deficit
t	-	Tonne
T_b	-	Base temperature

TC	-	Total colour
TF	-	Theaflavin
Tm	-	Turgid mass
TR	-	Thearubigin
TRFCA	-	Tea Research Foundation of Central Africa
TSS	-	Total soluble solids
VP	-	Vegetatively propagated
°C	-	Degrees centigrade
°E	-	Degrees East
°N	-	Degrees North
°S	-	Degrees South
°W	-	Degrees West
(-)	-	Absent
≤	-	Equal to or less than
µL	-	Micro litre
µM	-	Micro mole
v/v	-	Volume per volume
w/v	-	Weight per volume

%	-	Per cent
(+)	-	Present

ABSTRACT

Tea cultivars that make high black tea quality and are drought or low temperature tolerant are needed for sustainable tea cultivation. However, there is still a lack of precise, time- and cost-effective selection criteria for these desired traits in tea breeding programmes. The aim of this study was to identify molecular, physiological and biochemical characteristics associated with black tea quality and tolerance to drought or low temperature in order to establish objective selection criteria for these traits. Cultivars that were pre-classified for absence or presence of each trait were used in the study. Using the random amplified polymorphic DNA (RAPD) technique, six specific RAPD bands were identified that closely associated with black tea quality (three bands), tolerance to drought (two bands) and low temperature (one band). These RAPD bands can be used as markers that will facilitate identification of elite breeding stocks or genotypes at an early stage. In the study on drought tolerance, individual parameters could not clearly separate the tolerant and susceptible cultivars, although a trend suggesting differences between the two groups was observed. The univariate analysis probably failed to show significance between the two groups due to the relatively small sample sizes used. This was to some extent confirmed by multivariate analysis in which it was established that high relative water content (RWC) and antioxidant activity can jointly be useful indicators of drought tolerance in tea. Drought tolerant cultivars maintained high relative water content and antioxidant activity in order to optimize water use and reduce oxidative stress. The study on low temperature tolerance showed that shoot extension was faster and total polyphenol content (TPC) and antioxidant activity were higher in tolerant than in susceptible cultivars. The small sample size may also have affected the ability to identify individual characteristics that associate with the trait. In conclusion, RAPD bands that closely associate with high black tea quality and drought or low temperature tolerance, relative water content and antioxidant activity during drought and total polyphenol content and antioxidant activity under low temperature stress could be used in the selection of elite tea cultivars. The RAPD technique and measurements of relative water content, total polyphenols and antioxidant activity are easy and inexpensive and can easily be incorporated in routine selection to save costs and time, and to improve selection precision and success of breeding programmes. The limitations associated with RAPD should be taken into account when using the technique to ensure consistence and reproducibility of the identified markers.

CHAPTER 1

GENERAL INTRODUCTION

Tea (*Camellia sinensis* (L) O. Kuntze) is a widely consumed non-alcoholic beverage in the world, coming second after water in terms of volume consumed (Anesini *et al.,* 2008). The species *sinensis* is the most important crop species in the genus *Camellia* (Chen *et al.,* 2005; Prabu & Mondal, 2010). The tea plant has been cultivated and used in many forms for almost 5000 years (Wright *et al*, 2002). Currently, the commonly used forms of tea include black, green and oolong tea. Tea cultivation is thought to have started to spread worldwide from about 1823, when an indigenous Assam variety of tea was discovered (Bezbaruah, 1975). Recent reports indicate that South-East Asia and Eastern Africa are the major tea producing regions of the world (De Costa *et al.*, 2007). It is also reported that tea growing areas stretch between latitudes 49°N (outer-Carpathians, U.S.S.R.) and 34°S (KwaZulu-Natal, South Africa) (Shoubo, 1989; Mondal *et.al.,* 2004; Vyas & Kumar, 2005) and longitudes 150°E in New Guinea to 60°W in Argentina (Bezbaruah, 1975). This shows that tea is cultivated under a wide range of climatic conditions which vary from Mediterranean climates to the warm, humid tropics (De Costa *et al.,* 2007). The climatic conditions in most of these regions are similar to the cool and tropical conditions experienced in China and the Indian sub-continent where tea is believed to have originated (Kingdon-Ward, 1950; Chen *et al.,* 2005; Carr, 2010).

The tea plant thrives in different tea growing areas by adapting to varied environments. In order to ensure good adaptation, most tea producing countries have breeding programmes that develop improved cultivars that are adapted to local growing conditions. For instance, the tea breeding programme in Malawi that started in the 1960s has developed cultivars,

some of which are adapted to low temperatures (May to August) and hot, dry conditions (September to November) that prevail in the region (Ellis & Nyirenda, 1995; TRFCA, 2000).

In a conventional tea breeding and selection programme, it takes 15–20 years to develop a new tea cultivar (Ellis & Nyirenda, 1995). The long breeding and selection cycle in tea is the result of several factors. One such factor is that most of the economically important traits cannot be selected on single and young tea bushes using conventional selection methods (Riley, 1989). This means that selection for such traits can only start in the later stages of the selection process. For example, in the breeding programme at the Tea Research Foundation of Central Africa (TRFCA), assessment of black tea quality starts six years from the year of making the cross pollinations (Nyirenda, 1993). This is because each selection must first be bulked-up and planted in 16 bush field plots that can produce about 300 g of green leaf required for mini-manufacturing of black tea samples (Ellis & Nyirenda, 1995). For some complex traits such as drought tolerance, there can be an imperfect correlation between the juvenile and mature stages of the plant. This implies that early selection can result in inordinate risks of reducing any genetic gains that can be made (Nyirenda & Mphangwe, 2008). In addition, most traits take several years to develop and long-term field testing is required to establish a consistent trend. This makes selection a long-term and expensive venture (Henry, 1997).

The occurrence of superior genotypes exhibiting a desirable trait, like yield, in a seedling population can be as low as 0.0025% (Wight, 1959) or even lower for genotypes that possess a combination of desirable traits such as yield and quality (Vinod & Suryakumar, 2004). This implies that selection for desired traits using conventional methods can only be effective if done on a large and variable population of plants. This can be created by pollinating many flowers for each combination of selected parents. The resultant seedlings still have to be

grown to a certain stage before selection can start. In addition, there is need for large areas of land on which to grow and evaluate the large number of selections for field performance (Riley, 1989; Vinod & Suryakumar, 2004).

In order to make breeding and selection more successful, there is need to develop objective and reliable selection criteria for each of the desirable traits. Such criteria should be amenable to large-scale use and applicable in the early stages of the selection programme in order to increase precision and efficiency of the selection methods.

Use of molecular markers associated with important traits can make plant breeding more precise, rapid and probably cost-effective in comparison to phenotypic selection (Henry, 1997; Ni *et al.*, 2008). Some of the DNA based markers that have been extensively studied include Restriction Fragment Length Polymorphism (RFLP), Amplified Fragment Length Polymorphism (AFLP), Randomly Amplified Polymorphic DNAs (RAPD) and Simple Sequence Repeats (SSR) (Zhao *et al.*, 2008). Molecular markers also hold great potential for increasing the speed of cultivar improvement programmes in perennial crops such as tea.

Objective and reliable selection criteria can also be developed by understanding the biochemical and/or physiological mechanisms underlying some traits of interest. Currently, the mechanisms that underlie complex traits such as tolerance to drought and low temperature are still not very well understood. This makes indirect and/or targeted selection of breeding stocks in the early stages of breeding difficult, if not impossible. A clear understanding of these mechanisms is therefore a critical step in devising selection methods that are more effective and amenable to early selection of elite plant material.

1.1 Problem statement

Tea growth and productivity are affected by several factors which vary according to the region in which the tea crop is grown. In central Africa, recurrent droughts, extremes in

temperature in some months of the year, and incidences of pests and diseases are some of the most important factors affecting tea growth and productivity. These factors have a negative impact on both tea yield and quality and therefore threaten long-term viability and sustainability of tea cultivation in this region. Tea breeding and selection can help in producing cultivars that are adapted to some of these unfavourable environmental factors that prevail in the region (Nyirenda *et al.*, 2009). However, successful development of elite tea cultivars largely depends on availability and use of objective, reliable and cost-effective selection criteria for the desired traits. This is one of the main challenges to genetic improvement of the tea crop because most tea breeding programmes rely solely on conventional selection methods for most of the desirable traits. Most conventional selection methods are subjective, time consuming, expensive and not amenable to use on large-scale and/or in the early stages of selection (Banerjee, 1992; Mondal *et al.*,2004; Cancado *et al.*, 2013). Budgetary constraints at some research institutions may also hinder use of expensive assays, more especially in the early stages of selection where there are large numbers of selections (TRFCA, 2009). Selection for complex traits like drought tolerance, also necessitate establishment of long-term, replicated field trials at different locations that adds further to the cost of selection (Henry, 1997). Furthermore, screening tea germplasm for some of these traits under field conditions where there is no control over the severity, duration and intensity of the stress factors greatly compromises the reliability and objectivity of the assessments (Larkindale *et al.*, 2005).

The afore-mentioned shortcomings associated with conventional selection criteria for most of the desirable traits in tea are a major impediment to genetic improvement of the crop. These need to be addressed in order to have a more precise, time- and cost- effective breeding and selection programme. A clear understanding of the molecular, biochemical and physiological characteristics associated with the traits of interest holds more potential in addressing some of

the current hurdles in tea breeding and selection. This was the main motivation for the present study.

1.2 Aim of the study

High black tea quality and tolerance to drought and low temperature conditions are some of the most important desired traits for tea grown in central Africa. The primary aim of this study was therefore to identify molecular, physiological and biochemical characteristics associated with black tea quality, and tolerance to drought and low temperatures. This was expected to facilitate establishment of marker assisted and objective selection criteria for desired tea traits. The study had two specific objectives:

1. To identify random amplified polymorphic DNA (RAPD) molecular markers that associate with black tea quality and, drought and low temperature tolerance.

2. To establish biochemical and physiological characteristics that associate with tolerance to drought or low temperature in tea.

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CHAPTER 2

LITERATURE REVIEW

2.1 Origin of tea and global spread of tea cultivation

The tea plant (*Camellia sinensis* (L) O. Kuntze) has been cultivated and used in one form or another in many parts of the world for about 2000 – 5000 years (Kaundun & Matsumoto, 2002; Wright, *et al.*, 2002). However, its origin and earliest home as a wild plant has been a matter of some speculation and debate. The Va-Ye or Bohea mountain of Fu-Kein province of China is considered by some authors to be the place of origin where tea was first discovered (Saha & Gazi, 1994). However, other reports indicate that the cultivated tea originated between China and the Indian sub-continent, on the India/Myanmar border region (Chen *et al.*, 2005; Carr, 2010).

Tea cultivation gradually started to spread to other parts of world following the discovery of an Assam type of tea (Bezbaruah, 1975). At present, tea is grown in 52 countries across the world, mainly in South-East Asia and Eastern Africa (De Costa *et al.*, 2007). Most tea producing areas experience Mediterranean or hot, humid climates in the tropics (Carr, 2010). The tea plant therefore thrives in these areas because they receive adequate amounts of precipitation (≥1000 mm per year) and favourable temperatures (from ≥12.5 to 30°C) for optimal growth and productivity. These conditions are similar to the warm, wet summer and cool, dry winter conditions experienced in areas where tea had originated (De Costa *et al.*, 2007), notwithstanding the micro-climatic variations within and among regions (Kingdon-Ward, 1950; Netto, *et al.*, 2010). In Africa, tea was first introduced in Morocco in 1854 by the British. However, the first commercial cultivation of tea in Africa started in Malawi in 1878 (Anonymous, 2008), after which cultivation later spread to Kenya, Uganda and northern

Tanzania in the 1930s (Palmer, 1985). The plantations in Malawi were established from seed imported from India and China (Carr, 2010).

2.2 Tea classification and taxonomy

The cultivated tea belongs to the family *Theaceae,* and genus *Camellia* (Chen *et al.,* 2005; Sabhapondit *et al.,* 2012). The genus *Camellia* comprises over 80 taxa but only *C. sinensis* (L) O. Kuntze is commercially used to produce beverage tea (Latip *et al.,* 2010). Several eco-types have evolved over time. Tea classification has been revised several times by various researchers. Wight (1962) revised the genus *Camellia,* based on differences in reproductive structures and assigned specific status to *var. sinensis*, *var. Assamica,* and the Southern or Cambod form of *Camellia assamica* (Masters) Wight, which was classified as *Camellia assamica ssp. lasiocalyx* (Planchon ex. Watt) (Kaundun *et al.*, 2000; Premkumar *et al*., 2008). All commercially-grown teas are thought to be hybrids of the *Assam*-type (also known as '*Assam-jat'*) and China-type (*China-jat*) (Wight, 1959; Anesini *et al.,* 2008). Despite several taxonomic revisions of the genus *Camellia,* all teas are generally grouped under *Camellia sinensis,* regardless of their taxonomic differences. However, some tea varieties are still described as of Chinary (*Camellia sinensis*), Assam (*Camellia assamica*) or Cambod *(Camellia assamica ssp. Lasiocalyx)* species, based on various morphological and biochemical parameters (Kaundun *et al.,* 2000; Kaundun & Park, 2002).

The Chinary-type (*var. sinensis*) is characterized by small (3-6 cm long), relatively erect, dark-green leaves and it only grows into a shrub of up to two metres high and one metre in diameter (Hadfield, 1975; De Costa *et al.,* 2007). On the other hand, the Assam-type (*var. assamica*) has larger (15-20 cm long) and light-green leaves with a glossy surface and may naturally grow into a tree of up to 10 m high and 6 m in diameter (Hadfield, 1975; Takeda,

1994; De Costa *et al.*, 2007). The Assam-type is believed to have originated under the shade of humid, tropical forests, whereas the Chinary-type is thought to have originated under open conditions in the cool, humid tropics (Carr & Stephens, 1992). The performance of these eco-types in some environments has been related to the conditions that prevailed in areas of their origin.

2.3 Economic importance of tea

Tea plays a very important role in the economies of many producing countries in Africa and Asia (Kaundun & Park, 2002; Zhao *et al.*, 2008). This is because tea exports are a major source of foreign exchange for most tea producing countries. In addition, tea cultivation creates employment opportunities for the population living around the tea growing areas (Damayanthi *et al.*, 2010). Tea has gained further popularity because of the potential health benefits associated with drinking tea (Mondal *et al.*, 2004; Bharadwaz & Bhattacharjee, 2012). The relative economic importance of tea varies between producing countries, depending on the size of the tea industry, as well as other drivers of the economies of individual countries.

In Malawi, tea is the third most important export cash crop that contributes about 9%to the country's foreign exchange earnings (Anonymous, 2008). The Malawi tea industry covers about 20,000 ha planted to both seedling and improved vegetatively propagated (VP) tea cultivars. The industry is dominated by the commercial estate sector, with smallholder farms constituting only about 14% of the total tea area (Van der Wal, 2008). On a global scale, about 2.7 million ha of cultivatable land is planted to tea (Mondal *et* al., 2004).

Tea is mainly produced and consumed as black, green or oolong tea, with each type constituting 78, 20 and 2 % respectively, of all the teas produced in the world (Chan *et al.*,

2007). Malawi is Africa's second largest producer and exporter of black tea, after Kenya. However, Malawi black tea exports account for only about 4% of the world black tea production (Van der Wal, 2008). The Malawi tea industry produces about 45 million kg of black tea per annum when growing conditions are good. The national average yield of black tea is 2.5 t ha^{-1} but some estates have reported average yields of over 5 t ha^{-1} where elite cultivars and improved field practices have been implemented (Ellis & Nyirenda, 1995).

2.4 Factors affecting tea production in central Africa (Malawi and Zimbabwe)

Tea production is influenced by several climatic and environmental factors, but the most important factors are rainfall and temperature, as changes in both these factors impact tea growth and productivity. Tea requires a minimum of between 1000-1400 mm per annum of evenly distributed rainfall for optimum growth and production (Shoubo, 1989). The tea growing areas in central Africa receive low and uni-modal type of rainfall (TRFCA, 2009). For instance, the Mimosa Tea Research Station in Mulanje, Malawi, gets an average total rainfall of about 1200 mm per annum, which is mainly spread out between mid-November and early April (Squire, 1976; Kumwenda *et al.*, 2011). As a result, tea production in Malawi is largely seasonal and about 80% of the crop is harvested during these warm and wet months (Squire, 1976; Palmer, 1985; Carr & Stephens, 1992). During the period May to October there is very little or no rain at all and the total monthly precipitation may be ≤20 mm in these months (TRFCA, 1994/95). This is far below the 100–150 mm of rain per month needed to sustain optimum growth and development of tea plants in areas where the maximum temperatures do not exceed 35°C (Shoubo, 1989). This means that tea plants in Malawi are subjected to sub-optimum soil water conditions for almost half of the year. Recent reports indicate that the situation is deteriorating and that in some years the main rainfall season in Malawi now only lasts for four months (Kumwenda *et al.*, 2011).

Temperatures also fluctuate significantly within the season and between different tea growing areas. Extremes in both minimum and maximum ambient temperatures negatively impact on tea growth and production. For instance, some tea estates in Zimbabwe experience lower temperatures during the cool season than estates in Malawi. However, both countries experience mean ambient temperatures that are lower than the base temperature for tea growth (12.5°C) during the cool dry months (April – August). In the hot and dry season (September – November), mean daily maximum temperatures at some locations in Malawi exceed 35°C. These temperatures are out of the optimum temperature window of 12.5°C (for shoot growth and extension) and 30°C (for dry matter accumulation) of tea (Eden, 1976; Herd, 1976; Carr, 2010). Extremely high temperatures are also associated with high saturated vapour pressure deficits (SVPD) of the air, which adversely affect stomatal conductance, transpiration and photosynthesis of plants and consequently their response to drought (Carr & Stephens, 1992; Karunaratne *et al.*, 1999). Prevalence of unfavourable temperatures and drought hinder active growth of tea shoots, resulting in month to month variations in the amount of tea leaf harvested. At farm level, uneven distribution of the crop between months presents practical operational problems. For example, during the lean harvesting months, the tea factories and field labour are underutilized. On the other hand, during the peak harvesting months, leaf handling both in the field and factory becomes a problem, which can lead to reduction in quality of the processed tea (Mashingaidze & Tomlins, 1997). The weather pattern also affects the quality of processed tea, because during the hot and wet months (main growing season) there is rapid growth of tea shoots that results in lowering of the simple catechins to gallo-catechins ratio, which is associated with low black tea quality (Ellis & Nyirenda, 1995).

2.5 Tea breeding and selection at the TRFCA

The tea plant is a highly out-crossing and strongly self-incompatible tree species (Chen & Zhou, 2005). As a result, tea is highly heterogeneous and heterozygous (Banerjee, 1992; Wachira & Kamunya, 2005). Tea has a juvenile period of between three to five years from planting to flowering, when cross pollinations can start (Mondal *et al.*, 2004; Kamunya, 2010). These factors contribute to a long breeding programme.

The TRFCA breeding and selection programme started in 1956 (Ellis & Nyirenda, 1995). The early stages concentrated on producing improved seed in order to meet the high demand from a rapidly expanding industry. Improved seed was produced in seed gardens that comprised at least five different clones that were planted to facilitate open cross pollination and create genetic variation. The resultant seed varieties showed improvement in yield potential and some uniformity in leaf morphology (TRFCA, 1990). Uniformity of seedling tea varieties was enhanced further by establishing biclonal seed gardens.

Clonal development started with the selection of single bushes from seedling tea fields, for high yield potential, tolerance to water stress and black tea quality (Ellis & Nyirenda, 1995). Bushes that exhibited higher yield and black tea quality potential than the unselected seedling varieties were recommended as clonal cultivars for commercial use, e.g. cultivar SFS 150. The emphasis during selection later shifted to potential for high black tea quality and total value of the crop (a product of yield and crop value). This led to the release of cultivars with above average potential for yield and black tea quality, for example PC 81. Low or average yielding cultivars with very high black tea quality potential, for example SFS 204 and PC 105, were used as scion cultivars for grafting onto invigorating clonal rootstocks (Whittle & Nyirenda, 1995; TRFCA 2009).

A formal hybridization (cross pollination) programme for VP cultivars started in 1962 with emphasis on improving black tea quality (Ellis & Nyirenda, 1995). Selected parents were hand-pollinated annually to create genetic variation in seedling population from which VP cultivars with the desirable traits were selected. For many years, the TRFCA breeding programme served tea growing areas in central and southern Africa, a region that encompasses a wide range of agro-ecological zones. As such the programme had to develop cultivars that could adapt to many and diverse environmental factors (Ellis & Nyirenda, 1995).

The TRFCA breeding and clonal selection strategy involves four main stages: controlled cross pollination, preliminary selection of promising genotypes, evaluation for field performance and release of improved cultivars (Nyirenda, 1993; Mphangwe & Nyirenda, 2001; Wium, 2009). Activities done at the various stages have been previously described (Nyirenda, 1993; Wium, 2009) (Appendix 2.1). In the first step, parental stocks with contrasting but complementary traits, primarily high black tea quality and yield, are chosen (Mphangwe & Nyirenda, 2001). This is followed by cross pollination involving different parental combinations in order to create sufficient genetic variation in the progeny. The F_1 (first filial generation) seeds from each parental combination are harvested, bulked-up and germinated. The resultant seedlings are raised in a nursery for 15 – 18 months during which they are assessed for growth vigour before planting out the vigorous plants in the field as F_1 family-blocks. Selection is done within and among the F_1 families, based on bush size, growth vigour, tolerance to major insect pests (e.g. *Helopeltis schoutedeni* – mosquito bug), shoot size, ability to recover from a prune (Nyirenda, 1993) and black tea quality potential. At this stage, quality is assessed using the chloroform test for fermentation ability (Sanderson, 1963), because the single plants cannot produce enough green leaf for mini-processing. Bushes with the desirable traits are shortlisted and assessed for ease of rooting

using stem cuttings, growth vigour and tolerance to major pests and diseases in the nursery (Nyirenda & Mphangwe, 1998). The most promising seedling bushes are vegetatively propagated (cloned). The VP lines are tested for field performance, first in un-replicated field plots of 16 bushes, followed by further evaluation in multi-location cultivar evaluation trials (CET) that follow standard statistical designs.

2.6 Traits of economic importance for teas produced in central Africa

Economic importance of traits may differ between tea growing regions. For the teas produced in central Africa, some of the desirable traits are high quality and yield, and tolerance to biotic (insect pests and diseases) and abiotic stresses (e.g. drought, low and high temperatures). In the present study a total of seven traits were initially considered and from these, three traits, black tea quality and tolerance to drought or low temperature, were chosen for further investigation.

2.6.1. Black tea quality

Quality is usually one of the most important characteristics of any product and tea is no exception (Ramasinghe *et al.,* 2005). This is why improvement of black tea quality is the main objective of most tea breeding programmes (Seurei, 1997; Wachira, 1990; Ngure *et al.,* 2009). Tea quality is affected by environmental factors, type of plant material (genotype), agronomic and manufacturing practices and market requirements (Odhiambo *et al.,* 1988; Tudu *et al., 2*009). The relative effects of each of these factors on quality can also vary within and between tea producing countries.

Variations in rainfall, temperature and humidity experienced in different tea growing regions are reflected in some quality attributes of tea (Bhuyan *et al.,* 2009). As a result, tea buyers

often associate some tea quality attributes with certain production regions. For instance, black teas produced in most African countries are considered as 'plain' teas that lack aroma (Ellis & Nyirenda, 1995; Owuor & Obanda, 1998). This is partly because most African tea producing countries experience wide variations in temperature and/or rainfall, both of which affect the rate of shoot growth and consequently the amounts of biochemical compounds that accumulate in the shoots, which ultimately influence quality. For this reason, tea products usually have labels that show their region of production because it influences the consumers' choice (Kovacs *et al.,* 2010). High rainfall is associated with a decline in aroma and thus low quality, flat teas (Odiambo *et al*., 1988). This is probably because under high rainfall the tea shoots remain succulent and if temperatures are sufficiently warm they grow very fast, resulting in lower accumulation of the compounds that influence black tea quality. Drought can also lead to a decline in the content of some of these compounds in the leaf, influencing tea quality, mainly because cell desiccation due to drought reduces the activities of phenylalanine ammonia lyase (PAL), polyphenol oxidase (PPO) and peroxidase (Chakraborty *et al*., 2002). Low activity of PAL leads to reduced synthesis of some quality influencing parameters such as epigallocatechin gallate (EGCG) and epicatechin gallate (ECG) (Waheed *et al.,* 2012). These compounds are precursors of theaflavins (TF) which correlate with black tea quality in central Africa (Ellis & Nyirenda, 1995; Owuour *et al.*, 2006). Changes in the biochemical composition of the shoots due to drought lead to seasonal variations in tea quality, particularly in areas that experience extended dry periods.

Temperature affects quality of black tea by influencing growth rate of the shoots, which has an effect on the biochemical composition of the shoots (Robertson, 1992; Erturk *et al.*, 2010). Warm conditions cause rapid shoot growth that is associated with poor black tea quality (Odiambo *et al,* 1988). Cool ambient temperatures and long days, with a greater number of sunshine hours can enhance the synthesis of catechins in tea shoots and thus

improve black tea quality (Ercisli *et al.*, 2008; Kottur *et al.*, 2010). However, more sunshine can also lead to a decline in caffeine and theanine synthesis, which seem to increase under shaded conditions (Song *et al.*, 2012). Long days might therefore have a negative impact on quality if high caffeine content is desired.

The type of plant material (genotype) affects the quality of processed tea. The two commonly cultivated varieties of tea, *C. assamica* and *C. sinensis*, show significant differences in polyphenol content, flavour and total catechin content (Ellis & Nyirenda, 1995; Apostolides, 1999; Gulati *et al.*, 2009). Assam-type cultivars generally have higher polyphenol content than Chinary-type cultivars, which in turn contain quercetin and kaemoferol-3-glucosides that are absent in the Assam cultivars (Sabhapondit *et al*., 2012). It has also been previously established that VP cultivars (clonal varieties) show significant differences in black tea quality (Owuor, 1989; Hampton, 1992; Ellis & Nyirenda, 1995). This is as a result of their inherent differences in accumulation of major compounds that influence plain black tea quality. The wide variation in quality between clonal varieties suggests that tea quality can be improved through breeding and selection of clonal varieties with high quality potential.

Biochemical compounds that influence tea quality are the catechins (commonly known as polyphenols), caffeine and L-theanine (Anesini *et al.*, 2008; Song *et al.*, 2012). Catechins in the green leaf are primarily composed of (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epigallocatechin gallate (EGCG) and (-)-epicatechin gallate (ECG) (Saravanan *et al.*, 2005). The chemical structures of these major catechins are shown in Figure 2.1. Total polyphenol content positively correlates with black tea quality (Anesini *et al.*, 2008; Ercisli *et al.*, 2008). The desired colour of the liquor and briskness depend on the oxidation of polyphenols to form theaflavins (TF) and thearubigins (TR) (Lopez *et al.*, 2005;

Sabhapondit *et al.*, 2012). In central Africa, high TF content is associated with high black tea quality (Ellis & Nyirenda, 1995). Since TFs are oxidative products of catechins, the presence of the PPO enzyme is crucial. The ratio between PPO and the catechins has been suggested as an important quality parameter (Lopez *et al.*, 2005). The level of the individual catechins in tea shoots and their influence on tea quality tends to vary with seasons and region of production. For instance, teas produced in North East India have about half of their total catechins as EGCG, which is therefore an important biochemical marker for quality of teas produced in this region. EGCGs are also widely known for flavour characteristics of tea (Ellis & Nyirenda, 1995; Ercisli *et al.*, 2008). In Malawi, teas produced during the cold season have low levels of EGCG and high levels of non-gallated catechins (Robertson, 1992). A high ratio of the non-gallated to the gallated catechins has been associated with high quality of teas produced during the cold season (Ellis & Nyirenda, 1995; Wright, 2002). This ratio declines during the warm and wet months, resulting in production of tea that are of low quality (Ellis & Nyirenda, 1995). In China, a catechin index ([(EC+ECG)/ (EGC+EGCG)] has been suggested as possible indicator of high black tea quality (Chen &Zhou, 2005).

Other factors that affect black tea quality are agronomic and manufacturing practices. Agronomic practices that affect black tea quality include plucking methods and standards (Mahanta, 1988; Nyasulu, 2006; Owuor *et al.*, 2009) and fertilizer rates and types (Owuor & Othieno, 1996; Jayaganesh & Venkatesan, 2010). Plucking standard affects black tea quality because the biochemical content of tea leaves varies with age. The content of compounds that influence tea quality is highest in the bud (youngest leaf) and decreases progressively from the first leaf downwards (Robertson, 1992). A plucking method that increases the proportion of older leaves in the plucked tea will therefore lead to poor black tea quality. This is why quality can deteriorate when non-selective machines are used for plucking

(Ravichandran & Parthiban, 1988; Robertson, 1992). In central and southern Africa, a plucking standard of a shoot with two or three leaves plus a terminal bud is considered good for optimizing both tea quality and yield (TRFCA, 1990; Ellis & Nyirenda, 1995).

The influence of manufacturing processes, including withering, maceration, fermentation, drying and sorting, on black tea quality has been extensively reported (Subramanian *et al.*, 1999; Baruah *et al.*, 2008; Dhar, 2009; Joshi & Ganguli, 2008; Ngure *et al.*, 2009; Ölmez & Yilmaz, 2010). All these processes influence the production of quality compounds and thus affect quality of the dried black tea.

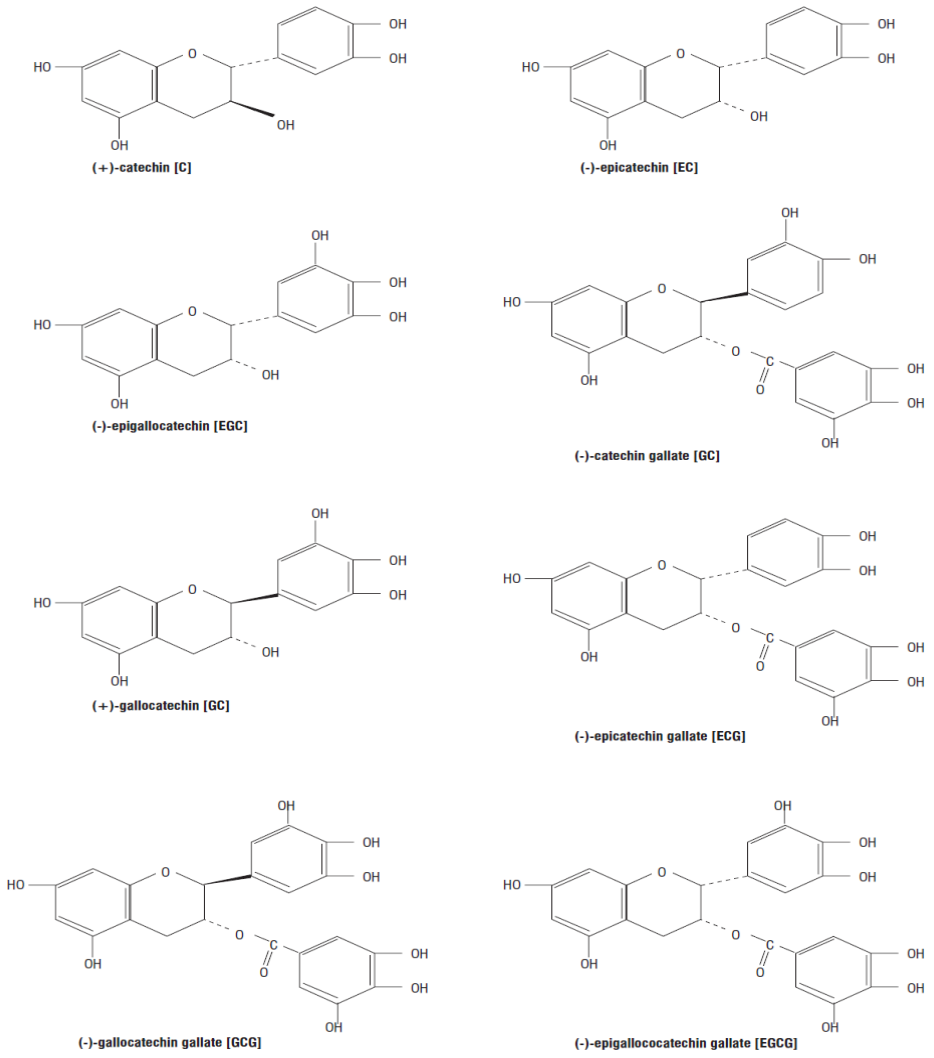

Figure 2.1: Chemical structures for major catechins in green tea (Gramza *et al.*, 2005)

2.6.2.Drought tolerance

Drought tolerance is a very complex trait whose effects are usually aggravated by other stresses such as high temperature, low relative humidity and irradiance (Boussadia *et al.*, 2008; Berger *et al.*, 2010; Carr & Lockwood, 2011). Plant water stress can result from insufficient precipitation, high saturation vapour deficit of the air or inability of plants to efficiently use available soil water (Waheed *et al.*, 2012). Tea needs adequate moisture (100-150 mm of rain every month) to sustain normal growth and development throughout the season (Shoubo, 1989). However, in some tea growing areas, e.g. in Malawi, tea plants experience water stress during about half of the year, which confines the main-harvesting season to the wet months of the year (Squire, 1976; Palmer, 1985; Carr & Stephens, 1992). The dry conditions in the field provide an opportunity for selecting for tolerance to water stress. However, seasonal and intra-seasonal variations in rainfall can affect selection of drought tolerant cultivars under field conditions, where severity of stress is difficult to quantify and control.

Tea response to drought depends on several factors, including type of planting material (genotype), age of the bushes, agronomic practices and severity of drought. Different genotypes have different inherent characteristics that impart drought tolerance. For example, Chinary tea varieties are more tolerant to drought than the Assam-type varieties (De Costa *et al.*, 2007). VP cultivars also exhibit different responses to drought, although the reasons for such differences have not been fully established (Carr & Stephens, 1992). Differences between cultivars suggest that the response to drought is under genetic control, which can be manipulated through breeding and selection to develop drought tolerant cultivars. Tea bushes respond differently to drought at different ages. Mature tea bushes are more tolerant to water stress than young tea bushes, probably because mature bushes develop an extensive and deep root systems as they mature, which enable the plants to exploit more plant available soil

water (Karunaratne *et al*., 1999; Mc Dowell *et al.*, 2008; TRFCA, 2009; Waheed *et al.*, 2012). In coffee, differences in rooting depth were also related to relative drought tolerance of some clones, with drought tolerant clones having greater rooting depth than the susceptible ones (Pinheiro *et al.*, 2005). Several agronomic practices, e.g. mulching of young tea, time of pruning and irrigation, can affect tea response to drought (TRFCA, 1990). Most plants can tolerate short-term drought but the less tolerant bushes succumb to prolonged and more severe droughts. Plant response to drought may also depend on the duration, severity and rate at which the drought stress is imposed (Cavatte *et al.*, 2012). This makes it difficult to predict when plant stress will exceed a threshold and cause widespread mortality under field conditions (Mc Dowell *et al.*, 2008). The response to drought is complex and can involve changes in morphological, physiological, biochemical as well as molecular parameters (Cellier *et al.*, 1998; Cavatte *et al.*, 2012; Gupta *et al.*, 2012; Upadhyaya *et al.*, 2012). This is probably why most of the mechanisms affecting plant survival and mortality under drought are still poorly understood. The various factors also interact in different ways, which makes it difficult in some cases to identify the main factors that confer tolerance to drought (Chapman, 2008). Under field conditions, this may be complicated further by simultaneous occurrence of other stresses in addition to drought.

Several morphological characteristics have been associated with tolerance/susceptibility to drought. As mentioned above, some tea cultivars adapt to soil water stress by developing a deeper and extensive root system (Nagarajah & Ratnasuriya, 1981; Bruce *et al.*, 2002; Shao, *et al.*, 2009). In rice, rooting depth has also been associated with drought tolerance (Kato *et al.*, 2008). In other plants, improved size, architecture or hydraulic conductance of the roots can help plants to maintain transpiration rate under drought (Collins *et al.*, 2008). Some tea cultivars show dull and droopy leaves, followed by wilting when exposed to drought (Gupta *et al.*, 2012). How early such symptoms appear may be related to the degree of tolerance to

drought and the ability to maintain plant water status. The differences in wilting between cultivars can be used in screening for tolerance to water stress.

A number of physiological changes occur in plants when subjected to drought. The ability of some genotypes to maintain high relative water content (RWC) has been correlated to drought tolerance as it indicates the status of plant water balance (Sandanam *et al.*, 1981; Chakraborty *et al.*, 2002). In olive trees, for example, low RWC resulted in reduced photosynthesis and stomatal conductance (Jones, 2007; Gupta *et al.*, 2012). Drought tolerant tea cultivars can conserve water through efficient stomatal control, reduction in transpiration rates and photosynthesis (Squire, 1978; Hajra & Kumar, 1999; Netto *et al.*, 2010). Transpiration rate and stomatal conductance of leaves can therefore be used as indicators to screen tea cultivars for drought tolerance (Karunaratne *et al.*, 1999; Netto *et al.*, 2010). It must be noted, however, that stomatal closure, reduced photosynthesis and low transpiration rates under drought is at the expense of productivity (Ma, 2004), but these mechanisms can help the plants to survive and go through short-term drought. Accumulation of osmotically active solutes in plant tissues can enable the plant to make osmotic adjustments through maintenance of turgor that enhances absorption of water from drier soils during drought (Blum, 2005). Plants under water stress can also accumulate osmotically active solutes such as sugars, polyols, betaines and amino acids in their tissues and make osmotic adjustments that enable the plant to absorb water from drying soil during drought (Blum, 2005; Genga *et al.*, 2011).

Water stress can also alter different biochemical processes in plants. In tea, phenolic content and activities of PAL, PPO and peroxidase may initially increase, but decrease during extended drought (Chakraborty *et al.*, 2002; Upadhyaya *et al.*, 2012). Reduced enzyme activity may also lead to reduced synthesis of flavonoids such as EGCG and ECG (Waheed *et*

al., 2012) and thereby reduce the quality of black tea. These reports suggest that drought tolerance may be at the expense of high quality, since catechins are very important determinants of black tea quality. Synthesis and accumulation of compatible solutes such as proline increase under water stress in tea and other plants (Bary, 1997; Bruce *et al.*, 2002; Xiong *et al.*, 2002; Shao *et al.*, 2009). It has been reported that drought tolerant tea cultivars may accumulate more proline than susceptible tea cultivars during water stress (Handique & Manivel, 1990; Chakraborty *et al.*, 2002). Plant water stress also induces increased production of reactive oxygen species (ROS) such as superoxide, hydroxyl, hydroperoxide and alkoxyl radicals, and hydrogen peroxide (Upadhyaya *et al.*, 2012). The ROS can be formed as a result of reduced photosynthetic activity, which causes an imbalance between generation and use of electrons (Reddy *et al.*, 2004; Cavatte *et al.*, 2012). The ROS may also form when electrons from the chloroplast, mitochondria and plasma membrane of plant cell react with molecular oxygen during metabolism (Tripathi & Gaur, 2004). Increased ROS production causes oxidative stress, which leads to cell damage in plants (Upadhyaya & Panda, 2004). In order to counter oxidative stress, plants produce antioxidants. Upadhyaya *et al.* (2012) reported that the levels of antioxidants such as glutathione and ascorbate may decrease as water stress progresses, probably as a result of their use in countering the high levels of ROS. Drought causes cellular water deficit, resulting in increased cell solute concentration, lowering of cell volume and change in membrane shape, which leads to loss of turgor, disruption of membrane integrity and denaturation of proteins (Bary, 1997). Such cellular conditions are followed by regulatory processes that enable the plants to adjust cellular metabolism to levels suiting the new cellular conditions (Kumar *et al.*, 2012; Tuteja, 2007). These processes may be monitored in plants or cultivars that show differences in response to water stress and used as indices for selecting tolerant cultivars.

2.6.3.Low temperature tolerance

Temperature plays a significant role in growth of tea shoots or flushes that are the main components of tea yield. In tea, shoot growth rate depends on prevailing ambient temperature (Matthews & Stephens, 1998; Netto *et al.,* 2005), with ambient temperatures of between 18 and 25°C being ideal for normal growth and productivity of tea (Eden, 1976). Low temperature is one of the major abiotic stresses that limit productivity of crops (Cattivelli *et al.,* 2002). Some researchers have defined temperatures between 0 and 15°C as chilling but not freezing temperatures (Allen & Ort, 2001; Hendrickson *et al.*, 2004; Theocharis *et al*., 2012). The chilling temperatures are usually associated with cessation in active growth, depending on plant species and/or cultivars within the same species.

The average base temperature (T_b) for tea shoot growth is 12.5°C (Carr & Stephens, 1992), whereas that for tea shoot extension tends to vary from 7°C to about 15°C (Obaga *et al*., 1988; Stephens & Carr, 1990). Mean air temperatures below 13°C and above 30°C tend to reduce shoot growth through their adverse effects on various biosynthetic processes (Carr, 1972; Netto *et al.,* 2005). Studies in Malawi showed that low night temperatures (<12.5°C) can prevent shoot extension (increase in shoot length) and that shoot growth is linearly related to temperatures above 12.3°C up to 20.5°C (Tanton, 1982a). In Central Africa, minimum temperatures below 12.5°C routinely occur between April and July (Tanton, 1982a). Low temperature tolerant cultivars which are able to grow under these temperatures could have an extended and a more even cropping season and probably higher seasonal yields (Nyirenda *et al.,* 2009).

Geographical location of the area influences temperature, which affects tea shoot growth and extension. Tea shoots grow and develop actively all year round in areas near the equator and up to 16° north and south of the equator (Vyas & Kumar, 2005). This is mainly because these

areas experience minimum deviations from the optimum temperature for active shoot growth and productivity of tea. Optimal air temperature and availability of water are critical for initiation and extension of shoots and the production and partitioning of assimilates to shoots in tea (De Costa *et al*., 2007). Similarly, in red osier dogwood (*Cornus sericea* L.), the optimum temperature is also very important for initiating bud break (Svendsen *et al.*, 2007).

The genotype affects tea response to low temperature and different cultivars often show significant differences in shoot growth when exposed to low temperature (Tanton, 1982a; Vyas & Kumar 2005). Some tea cultivars grow actively at minimum ambient temperatures below the average base temperature of 12.5°C, while other cultivars become dormant (go banjhi) (Ellis & Nyirenda, 1995). The variation between cultivars suggests that shoot growth characteristics of tea cultivars are genetically controlled, which offers room for selection.

Physiological factors that control dormancy are not well understood in most crop plants (Fennimore *et al*., 1999). Barman (2002) reported that vegetative growth is significantly reduced when endogenous Indole-Acetic Acid (IAA) levels decline during winter. Similar to water stress, low temperature induces increased production of ROS, leading to oxidative stress. Oxidative stress retards shoot growth and affects some physiological processes that are critical for growth, such as photosynthesis. Plants grown under full light conditions are prone to oxidative stress when exposed to low temperature due to increased photo-inhibition (Vyas & Kumar 2005). For example, some plants accumulate secondary metabolites and low molecular weight solutes such as glycine, betaine and proline, in order to cope with the oxidative stress (Genga *et al.*, 2011; Hayat *et al.*, 2012). Plants use various enzymatic and non-enzymatic mechanisms to counteract high ROS levels. Response to the oxidative stress can be related to the degree of low temperature tolerance of the different crop cultivars.

The differences in response of tea cultivars to low temperature highlight the potential that exists in finding tea cultivars with lower base temperatures that will be adapted to growing in areas that experience low temperatures during winter months. There is thus a need to identify the biochemical, physiological and molecular characteristics that associate with low temperature tolerance. This will facilitate early and objective selection for low temperature tolerance in tea.

2.7 Conventional selection methods for desirable traits

Plant breeders use different methods to identify plant material that exhibit desirable traits. The identified plant materials are tested and promising genotypes are selected and eventually released as cultivars. In most cases, each trait is selected using specific characteristics, although in some rare cases a particular characteristic may be used to select for more than one desirable trait.

TRFCA follows conventional breeding and selection methods for developing new tea cultivars. The procedures followed when selecting for black tea quality, drought and low temperature tolerance have been reported previously (Appendix 2.1) (Nyirenda, 1993). These procedures are briefly described in this section.

Selection for high quality is a priority in most tea breeding programmes. Selections that are advanced to the preliminary observation plot (POP) and cultivar evaluation trial (CET) stages of selection are assessed for black tea quality. Young shoots are plucked from each selection and processed separately using an environmentally controlled miniature (ECM) manufacturing unit, following standard manufacturing procedures for black tea (TRFCA, 1990). The black tea samples are organoleptically and biochemically assessed for high black tea quality characteristics. In organoleptic assessment, an expert tea taster evaluates each tea

sample for leaf appearance and colour of infusion, liquor strength, briskness, brightness and milk-take, on a scale of 0 (for poor) to 10 (for best) and a total score for all the characteristics is calculated (TRFCA, 2009). Each sample is also given a nominal value (taster's valuation) that reflects the potential market price of the tea sample.

Biochemically, the samples are analysed for TF and TR content, liquor brightness (BR) and total colour (TC). These characteristics significantly correlate with high black tea quality (Ellis & Nyirenda, 1995; Apostolides *et al.,* 2006; Owuor *et al.,* 2006; TRFCA, 2009). The limitation associated with these methods is that each selection has to be planted out in the field and grown to a stage when it can produce sufficient amount of tea shoots for black tea manufacturing. This requires large inputs of land and time. Some biochemical assays are expensive and can only be repeated a few times due to budgetary constraints. These two methods are thus not suitable for use in the early stages of selection, which involve a large number of samples.

Tea beverage is also popularly known for its enormous health benefits. Selection for health benefit properties has introduced another dimension to breeding for quality in tea. For instance, in central Africa high black quality tea has been associated a high ratio of simple to gallated-catechins (Ellis & Nyirenda, 1995). This has indirectly biased selection towards high content of EGC, ECG and EC. In order to improve on health benefit properties, tea selections are now also assessed for high content of the gallated-catechins, such as EGCG (TRFCA, 2008).

Plants grown under rain-fed conditions are more susceptible to drought. The tea growing areas of central Africa have a long history of experiencing severe droughts. For instance, in Malawi, the first severe drought was in 1943 (Palmer, 1985) and the most recent one, which resulted in up to 70% vacancies in some young tea plantations, was in 2005 (Nyirenda, 2007).

Recurrent droughts are a major limitation to the use of new tea cultivars by growers, especially under rain-fed conditions where survival and establishment can be poor due to drought.

Some of the conventional methods for selecting drought tolerant cultivars are looking at leaf turgidity during the hot and dry months (September to November) and the number of surviving bushes in field plots of tea plants that have gone through several dry seasons. Rooting depth and root starch content have been suggested as potential characteristics that can be used to select drought tolerant tea cultivars (Nyirenda & Mphangwe, 2008; TRFCA, 2009). The major setback with some of these selection methods is that they are usually performed under uncontrolled stress conditions. In addition, there is lack of juvenile to mature phenotype relationship based on rooting characteristics, which renders this method not amenable to use in early stages of selection.

The tea growing areas of central Africa experience significant variations in temperature within a growing season. Significant cultivar differences in response to low temperature have been reported (Tanton, 1982a). For instance, cultivars SFS 150 and PC 198 exhibit active shoot growth under low minimum temperatures. The new selections are therefore assessed for active growth when low temperatures become a limiting factor to shoot extension and development. Visual assessment is done on mature tea bushes in the field during the cold season. Numbers of active and dormant shoots are counted on a bush, and the lengths of the developing shoots and leaves are measured in order to quantify tolerance to low temperature (TRFCA, 2009).

2.8 Marker assisted selection and breeding

Development of new cultivars with a good combination of desired traits such as high quality and drought tolerance is a priority in most tea breeding programmes (Ellis & Nyirenda, 1995; Kamunya & Wachira, 2005). However, selection of plant material that exhibits a good combination of the desirable agronomic traits and quality is challenged by lack of objective, reliable and time-effective criteria. This results in long and expensive selection cycles, mainly because most conventional selection criteria are time consuming (Hernández *et al.*, 2001). Some traits also show high levels of phenotypic plasticity. In addition, some selection methods involve expensive analytical assays and are thus of limited practical use in the early stages of selection (TRFCA, 2009). In some cases a lack of controlled-environment screening facilities compounds the problem.

Identification of genetic markers that associate with quality and desirable agronomic traits in crops offers enhanced possibilities and opportunities to improve precision and efficiency in selection and breeding. This is because the genetic markers are not affected by the environment and developmental stage of the plant material (Sorkheh *et al.*, 2009). Molecular markers can allow indirect screening for tolerance or resistance to a stressful condition, even in the absence of the stress factor (Henry, 1997). The marker assisted selection (MAS) is suitable for use in the early stages of selection when there are many clones.

Several types of markers have been identified and are used in breeding programmes of some crops, e.g. rice, maize, apples and eucalyptus. The markers can be broadly grouped into morphological, protein and isozyme and DNA markers. Molecular markers for most of the desired traits in tea are rare, probably because tea biotechnology is still at a nascent stage. The earliest biotechnological attempts in tea only date back to the mid-1990s and reports of practical use of MAS in tea are still rare (Tanaka, 1996; Gunasekare, 2009). Until recently,

published genetic information on tea was limited. However, good progress has been made in biotechnological advancement for the tea crop, for example, molecular markers have recently been used in studies of tea diversity (Wachira, 1997; Wachira *et al.*, 2001; Freeman *et al.*, 2004), and marker-trait association (Kamunya, 2010; Malebe, 2011; Mphangwe *et al.*, 2013). However, the molecular markers that have been identified in tea are probably still too few to saturate the tea genome that is estimated to be approximately four giga base pairs (Tanaka, 1996). Unsaturated genetic maps are of limited practical use in breeding and selection programmes because in some cases a marker that appears to be tightly linked to a gene of economic importance may be far away from the gene on the chromosome due to differences in recombination frequencies within the genome (Jones *et al.*, 1997; Kamel *et al.*, 2010). This necessitates the search for more molecular markers for different desired traits in order to saturate the genetic map. This would also facilitate integration of the genetic and chromosome maps and thereby allow cloning of the genes linked to the desired traits (Jones *et al.*, 1997)

2.8.1 Morphological markers

Use of morphological markers mainly relies on evaluation of phenotypic differences. For example, in tea, leaf pubescence has been suggested as an indicator of quality potential, where high pubescence is associated with high quality (Banerjee, 1992). However, morphological markers are heavily influenced by the environment, phenological stage of the plant and may lack sufficient levels of polymorphism (Chen & Yamaguchi, 2005; Ruan *et al.*, 2009). These factors render morphological markers non-reproducible and thus unreliable. In addition, some of the morphological markers may not fully reveal genetic differences among plant materials (Ruan *et al.*, 2009).

2.8.2 Isozyme markers

Isozyme markers are based on naturally-occurring enzymes that share a substrate but show differences in their mobility during electrophoresis. Polymorphism is based on the number and relative mobility of various enzyme products. These markers have a limited number of detectable loci and may be organ specific (Kaundun *et al.*, 2000). The variations between tissues or organs can therefore create sampling problems. These markers exhibit neutral effects on plant phenotypes and have co-dominant expression. The need for different protocols for each isozyme system is another setback that can limit the large-scale use of these markers in breeding programmes. Due to changes in analytical methods, some protein-based markers may still be practically useful.

2.8.3 DNA markers

DNA markers overcome some of the limitations associated with the morphological or protein markers. Several types of DNA-based molecular markers are being used in breeding programmes of various crops, e.g. rice and wheat (Ruan *et al.*, 2009). Some of the DNA molecular markers that have been extensively used are RFLP, RAPD, SSR and Sequence Characterized Amplified Region (SCAR) (Concado *et al.*, 2013).

RFLPs, which are produced by restriction enzyme digestion, were the first DNA markers to be used in genetic studies. RFLPs are based on the principle that restriction fragments from a given chromosome locus in different individuals will be different (Botstein *et al.*, 1980). RFLP markers are co-dominant, multi-allelic and may represent the entire genome. Use of RFLPs is limited by the need for specific probes and use of short-lived radioisotopes. In addition, the technique is slow, costly and requires large quantities of very high quality DNA that might be difficult to get in some plant species (Kaundun *et al.*, 2000; Mondal, 2002). The

need for prior information on the flanking region of the genome further limits the use of RFLPs in crops such as tea that are in the nascent stages of biotechnological development.

RAPD markers are based on the Polymerase Chain Reaction (PCR), where random DNA sequences are amplified using arbitrary primers (Williams *et al*., 1990). The RAPD technique does not require prior knowledge of the DNA sequence, is inexpensive and technically easy to develop (Ni *et al.,* 2008; Sorkheh *et al*., 2009). These attributes make the RAPD technique more attractive for use in genetic studies of crops with limited published genetic information such as tea. However, RAPDs are very sensitive to experimental conditions and this has in some cases resulted in poor reproducibility, especially between different laboratories (Freeman *et al.,* 2004; Ni *et al.,* 2008). In order to generate reliable data using RAPDs, it is important to adequately standardize experimental conditions and ensure that the amplification reactions are reproducible (Belaj *et al.*, 2003). Despite these limitations, RAPDs are still one of the most commonly used markers in various genetic investigations, such as diversity and fingerprinting and marker-trait association studies (Wachira *et al*., 2001; Kaundun & Park, 2002; Chen & Yamaguchi, 2005). RAPDs have also been used to investigate genetic relationships, genetic diversity, parentage analysis and genetic mapping of tea plants (Chen & Yamaguchi. 2005).

Microsatellites or SSRs are short tandem repeats of short sequences that may range from 2 – 8 base pairs. SSRs are highly polymorphic and give higher information content than AFLPs and RAPDs (Belaj *et al.,* 2003). The SSRs are co-dominant, simple and easy to analyse, reliable and hyper-variable at loci, probably because they originate from replication slippage that can occur more frequently than the other forms of variability that give rise to the other markers (Jacob *et al.,* 1991; Belaj *et al.,* 2003). One of the limitations of SSRs is high initial

development cost due to the need for cloning and sequencing (Mace & Godwin, 2002; Freeman *et al.*, 2004).

The SCAR markers are developed from a specific amplified region of the genome. They can be developed after identifying a fragment that amplifies a specific region on the genome. For instance, a region amplified by a RAPD marker can be converted to a SCAR marker in order to overcome some of the limitations associated with RAPDs. For example, a SCAR marker that is associated with drought tolerance in tea was developed (Malebe, 2011). In birch, SCAR markers that associate with fibre length have been developed (Ruan *et al.*, 2009). One of the challenges with development of SCAR markers has been the difficulties encountered in the cloning of the RAPD fragments in some plant species.

2.9 Summary

Tea improvement programmes face a number of challenges, which include lack of availability of effective and reliable criteria that can be used in the critical screening stages of cultivar development. In cases where some analytical criteria are available, financial limitations preclude their practical use. Some of these challenges can be addressed by finding selection methods that are more cost-effective, reliable and easy to implement in practical plant breeding. Use of molecular markers such as the RAPD technique, which is simple and relatively inexpensive, can help in improving precision and efficiency in selection of elite tea cultivars. The RAPD marker technique has very good potential to be easily incorporated in a conventional breeding programme and also help in establishing a marker-assisted-selection programme for tea.

Plants respond to different abiotic stresses in variety of ways, some of which can help to elucidate underlying mechanisms for tolerance to these stresses. Drought and low temperature stress trigger several biochemical and/or physiological changes in plants which have potential for use as indices for selecting tolerant cultivars. There is evidence of significant differences in response to these stresses among tea cultivars. This variation suggests that there is potential to use some of the biochemical and physiological characteristics observed in stressed plants in development of tolerant cultivars.

Appendix 2.1: Conventional breeding and selection cycle at the TRFCA in Malawi (Adopted from Nyirenda, 1993; Wium, 2009)

Stage	Years	Activities	Indicative Numbers
1	0	Cross pollinations between selected parental stocks	5,000
	1	Germination of progeny seeds, seedlings in the nursery	3,750
	2	Select on vigour	
	2	Line out selections in the field	2500
	3	Select on quality (chloroform test), leaf size, recovery from prune, vigour and tolerance to *Helopeltis*	
2	4	Vegetative propagation and select on rooting potential and nursery growth (30-50 cuttings/genotype)	350
	5	Plant out 16 bush plots for Preliminary Observations of field performance	150
3	6-8	Select on survival, regrowth, quality (biochemical and organoleptic), pest and drought tolerance , nursery performance	20
4	8-15	Prelease 15-20 promising selections to commercial growers Plant multi-site cultivar evaluation trials (25 – 30 plants per plot) of the promising line/selections Evaluate on all traits of agricultural importance	20
5	15+	Possible release of one or more new cultivar	1 - 3

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CHAPTER 3

IDENTIFICATION OF RAPD MARKERS ASSOCIATED WITH BLACK TEA QUALITY, DROUGHT AND LOW TEMPERATURE TOLERANCE[1]

3.1 Introduction

Selection of new cultivars that exhibit desirable traits is challenged by lack of objective, reliable and time-effective selection criteria. As a result, breeding and selection programmes are long and expensive (Hernández *et al*., 2001; Kamunya *et al.*, 2010). Most of the criteria that are currently in common use are time consuming, show continuous variation and high levels of phenotypic plasticity (Mewan *et al.*, 2005) and may involve expensive analytical assays (TRFCA, 2009). In some cases this is compounded by lack of appropriate screening facilities.

Identification of molecular markers that associate with quality and desirable agronomic traits in crops offers an opportunity for improving precision and efficiency in breeding. This is because molecular markers are not affected by environmental factors and developmental stage of the plant material (Sorkheh *et al.*, 2009). Molecular markers can allow indirect screening for tolerance or resistance to stress (Henry, 1997). This can also facilitate early identification of genotypes that possess desired traits which can be used in conventional crossing programmes. Genotyping of cultivars used as parents in a breeding programme can help to reduce costs because it is less expensive than phenotyping (Holland, 2004; Bernardo, 2008). For example, in soybean, use of markers in early generation selection of single plants speeded up the release of cultivars (Cahill & Schmidt, 2004). DNA markers can significantly

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aid selection for quantitative traits which are greatly affected by genotype-by-environment interactions.

Tea is not biotechnologically advanced and reports of practical use of marker-assisted-selection (MAS) are still rare (Tanaka, 1996; Gunasekare, 2009). Several factors may have contributed to the slow pace in tea biotechnology. Until recently, use of molecular markers that require prior knowledge of nucleotide sequences of the flanking locus, for example RFLPs, was considered less applicable to tea. This is mainly because there has been limited genetic information on tea available in the public domain. The high level of heterozygosity and the perennial nature makes genetic dissection of tea difficult (Rajapakse, 2003; Mondal *et al.,* 2004). However, progress has been made in use of molecular markers in tea diversity studies (Wachira, 1997; Wachira *et al.,* 2001; Freeman *et al.,* 2004) and identification of molecular markers associated with different agronomic traits. For instance, Mishra and Sen-Mandi (2004) identified a RAPD marker that associated with drought tolerance in tea cultivated in Darjeeling, India. This work was independently repeated and confirmed by Wium (2009) and Malebe (2011), using tea cultivars from Malawi; the work by Kamunya *et al.* (2010) identified quantitative trait loci (QTL) associated with yield at some sites in Kenya; identification of 112 novel tea unigene-derived microsatellites (Sharma *et al.,* 2011) and, the sequencing of the tea transcriptome that has revealed a number of uni-genes for tea that have increased the coverage over the tea genome (Shi *et al.*,2011). However, the molecular markers that have been identified in tea are probably still too few to saturate the large tea genome that is approximately four gigabase pairs (Tanaka *et al.,* 2006). Unsaturated genetic maps limit the practical use of the molecular markers in breeding and selection (Kamel *et al*., 2010). In addition, there are still a number of desired traits in tea for which no molecular markers have been identified. This necessitates the search for more molecular markers for different agronomic traits in crops. Use of molecular markers will be more

beneficial for traits where phenotypic selection takes a long time, is expensive or in some cases give inconsistent results (Holland, 2004; Bernardo, 2008).

RAPDs and other types of molecular markers such as RFLP, Amplified Fragment Length Polymorphism (AFLP), Inter-simple Sequence Repeats (ISSR), SSRs and Diversity Array Technology (DArT) have been associated with some important crop traits such as yield, quality, disease and pest resistance and tolerance to drought in many crop plants (Ruan, 2009). In wheat, a RAPD marker that is closely associated with a locus for flag leaf senescence under drought conditions has been identified and can be used in breeding for drought tolerance (Barakat *et al.*, 2013). RAPD markers associated with the determinate and indeterminate growth habit, tallness as well as sweetness characteristics in white Lupin have also been identified (Gilbert *et al.*, 1999). In olive, RAPD markers were successfully used to identify olive plants and in gene mapping that led to establishment of marker-trait associations (Cancado *et al.*, 2013). In maize (*Zea mays* L.), markers linked to genes that enhance production of abscisic acid and SNPs, which showed close association with changes in leaf water content and fluorescence (Fv/Fm) under drought conditions, were identified (Yu *et al.*, 2013). In tea, genes that are associated with synthesis of catechins, which are the major determinants of tea quality, have been identified (Kaundun & Matsumoto, 2003). These genes can therefore be used in marker-assisted breeding to improve quality. Other types of markers, such as Cleaved Amplified Polymorphisms (CAPs) (Kaundun & Matsumoto, 2003); RAPDs (Kaundun & Matsumoto, 2000) and SSRs (Ni *et al.*, 2008) have been associated with taste and quality in tea. Markers that associate with other complex traits like pest or disease resistance have been identified in several crops. For instance, AFLP markers linked to genes that confer resistance to black spot in roses (Von Malek *et al* 2000); RAPD markers for nematode resistance in soybeans (Cahill & Schmidt, 2004); anthracnose resistance in tea (Ni *et al.*, 2008) and sorghum (Rahman *et al.*, 2012); stem rust in barley (Henry, 1997) and mite

resistance in coconut (Shalin *et al.*, 2007). In wheat, an association has been established between DArT markers and grain yield (Yu *et al.*, 2012).

Molecular markers have also been associated with tolerance to abiotic stresses such as cold (low temperature) or salinity. For example, in sorghum, SSRs linked to genes for early cold tolerance were identified (Rahman *et al.*, 2012), while RAPD markers associated with cold tolerance have been identified in peaches (Dirlewanger & Arŭs, 2004). A single nucleotide polymorphism (SNP) marker for cold tolerance was also identified in rice (Rahman *et al.*, 2012). In addition to establishing direct marker-trait associations, molecular markers have also been deployed in genetic diversity studies, characterization of germplasm and development of genetic linkage maps (Shepherd & Jones, 2004; Zhao *et al.*, 2008; Ohsako *et al.*, 2008; Yu *et al.*, 2013). These endeavours have helped to establish marker-assisted breeding and better management and utilization of genetic resources that are available to the breeder. These reports are a manifest of the potential that exists in identifying molecular markers that associate with several desired traits in crops.

3.1.1 Objective

The objective of this study was to identify RAPD markers that associate with black tea quality, drought or low temperature tolerance in order to develop a marker assisted selection programme for these traits.

3.1.2 Null Hypothesis

Ho_1. There will be no statistically significant differences in the presence of specific RAPD band(s) that positively associate(s) with any of the traits 1-3, in the cultivars that have a particular trait and those that lack the trait.

Ho_2. There will be no statistically significant differences in the presence of specific RAPD band(s) that negatively associate(s) with any of the traits 1-3, in the cultivars that lack the particular trait and those that have the trait.

Traits: (1) Black tea quality, (2) Drought tolerance and (3) Low temperature tolerance

3.2 Materials and methods

3.2.1 Tea cultivars

Screening for marker - trait association requires proper and accurate phenotyping of the test cultivars, especially in the absence of a properly planned screening population (Henry, 1997). The tea cultivars used in this study were obtained from the breeding programme at the TRFCA in Malawi. The cultivars were chosen based on prior knowledge and information gathered from more than ten years of field observations, at multiple sites, on whether a specific trait was absent (-) or present (+) in a particular cultivar. Since these evaluations were done over several years, it was safe to assume that the problems of seasonal variations had been adequately addressed. In the preliminary screening of RAPD primers, 18 cultivars were chosen for each trait, of which nine cultivars had been classified to have the trait and the other nine cultivars were classified as lacking the desired trait. The cultivars that were used in this part of the study and their historical ranking are shown in Table 3.1.

Table 3.1: Cultivars used for preliminary screening of RAPD primers across three traits

Black tea quality		Drought tolerance		Low temperature tolerance	
Cultivar	**Rank for trait**	**Cultivar**	**Rank for trait**	**Cultivar**	**Rank for trait**
PC104	+	PC185	+	PC198	+
PC105	+	PC122	+	PC213	+
PC108	+	PC198	+	PC206	+
PC110	+	PC213	+	SFS150	+
PC168	+	PC175	+	15M-17	+
PC117	+	PC168	+	84/3-13	+
PC118	+	NVS10	+	94/6-13	+
SFS204	+	84/13-20	+	PC153	+
88/79-2	+	95/4-43	+	95/4-43	+
PC206	-	PC110	-	PC136	-
RC6	-	PC104	-	PC132	-
RC1	-	PC131	-	PC165	-
NVS10	-	PC118	-	PC80	-
88/50-8	-	PC119	-	PC81	-
SFS42	-	PC80	-	RC6	-
NKW30	-	PC105	-	RC1	-
NKW44	-	PC1	-	CL12	-
TOC	-	PC113	-	88/35-2	-

Note: (+) and (-) denote presence and absence of a trait, respectively.

After the preliminary screening of primers, cultivars were selected from the two sub-groups under each trait to form a large sample of 32 cultivars that was used for confirmatory experiments on association of the promising RAPD bands with the different traits. The 32 cultivars that were chosen for this part of the study and their historical classification for the three traits are presented in Table 3.2.

Table 3.2: Phenotypic ranking of cultivars used to screen promising RAPD primers

Number	Cultivar Name	Parentage	Black tea quality	Drought tolerance	Low temperature tolerance
1	PC168	PC1 x K6/8	+	+	+
2	PC198	SFS150 x MT12	-	+	+
3	SFS204	Open pollination	+	-	+
4	95/4-43	SFS150 x PC168	-	+	+
5	RC4	PC1 x SL9	-	+	+
6	PC81	O11 x CL12	-	+	-
7	PC153	SL5 x SFS150	-	+	+
8	PC122	PC1 x SFS204	+	+	-
9	PC80	C5 x CL12	+	-	-
10	RC6	PC1 x CL12	-	+	-
11	PC131	M9 x SFS204	+	-	-
12	RC1	Open Pollination	-	+	-
13	43/28-20	M9 x SFS204	?	?	?
14	SFS371	Open Pollination	+	?	?
15	PC136	Unknown	-	+	-
16	PC114	PC1 x SFS204	+	-	+
17	PC185	PC1 x MT12	-	+	-
18	PC175	CL12 x PC1	-	+	-
19	SFS150	Open Pollination	-	+	+
20	PC110	PC1 x SFS204	+	-	+
21	PC108	PC1 x SFS204	+	-	-
22	PC105	PC1 x SFS204	+	-	-
23	PC104	PC1 x SFS204	+	-	-
24	PC150	M9 x SL1	?	+	?
25	PC119	PC1 x SFS204	+	-	-
26	PC1	M9 x CL17	+	-	-
27	PC113	PC1 x SFS204	+	-	+
28	NKW30	Open Pollination	-	?	?
29	MT12	Open Pollination	+	?	?
30	K6/8	Open Pollination	+	?	?
31	CL12	Open Pollination	?	?	-
32	NVS10	Open Pollination	-	+	?

Note: (+) and (-) denote presence and absence of a trait, respectively. A (?) indicates that the cultivar had not been previously classified for presence or absence of the trait.

3.2.2 Collection of leaf samples and extraction of genomic DNA

Fresh leaf samples of each cultivar were collected from tea bushes growing in the field at the Mimosa Tea Research Station of the TRFCA in Malawi (16° 05´ S, 35° 35´ E, 630 m above mean sea level). The leaves were dried using silca gel and preserved in zip-lock plastic bags containing dry silica gel, following the procedure described by Malebe (2011). The dried leaf samples were taken to University of Pretoria in South Africa within a week after preparation where they were stored in a 4°C cold room prior to extraction of the DNA. This procedure of handling leaf samples has been used previously and the samples yielded acceptable quality of DNA for PCR (Malebe, 2011). Genomic DNA was extracted from each sample according to the procedure described in the DNEasy Plant mini kit Handbook (Qiagen, 2006). A sample of dried leaf (0.05 g) was put in a 2 mL tube with a screw cap in which a ceramic bead had been placed at the bottom. After putting in the sample, 2 mL of extraction buffer (AP1) was added and another bead was placed on top of the leaf sample. A cap was tightly screwed onto each tube. After preparing a set of 12 samples, the tubes were put into a Fast Prep instrument FP 120 (QBiogene, Carlsbad, CA, USA) to homogenize the samples by spinning the tubes twice at 4 m/s for 10 s. The homogenized samples were incubated for 10 min at 65°C in a water bath, mixing 2-3 times during incubation in order to help lyse the cells. This was followed by addition of 244 µL of a protein precipitating buffer (AP2) to each tube and incubating the tubes on ice for 5min and thereafter centrifuging for 5 min at 10,000 x g. A clean supernatant was pipetted out and put into a QIAshreder spin column placed in a 2 mL tube. The samples were centrifuged at 13,000 x g for 2 min. The flow-through was transferred to a new 1.5 mL tube and DNA binding buffer (AP3/E), 1.5 x (v/v) the volume of the flow-through, was added to each tube. From this mixture, 650 µL was pipetted out into a mini-spin column placed in a 2 mL tube, centrifuged for 60 s at 6,000 x g and the flow-through was discarded. The DNeasy column was transferred to a new 2 mL tube and 500 µL of wash buffer (AW) was added to

the column and centrifuged at 6,000 x g for 60 s. This step was repeated but during the repeat, the samples were centrifuged at 13,000 x g to dry the column membranes. The column was transferred to a new 1.5 mL tube and 75 µL of DNA elution buffer, preheated at 65°C was pipetted directly onto the column membrane, incubated at room temperature for 5 min and centrifuged at 6,000 x g for 60 s. This step was repeated in order to get 150 L eluted DNA for each sample. The eluted DNA samples were stored in a -20°C deep freezer until time of use in polymerase chain (PCR) reactions.

The quality and quantity of the extracted DNA for each sample were determined with a nanodrop (ND-1000) spectrophotometer (Nanodrop Technologies, USA). DNA quality was based on the 260 to 280 nm absorbance ratio. A ratio of 1.7 to 1.9 shows that the DNA is pure. If this ratio is less than 1.7, it shows that the DNA sample still has some proteins or phenols (Qiagen, 2006). All the DNA samples used in this study were of good quality as per this criterion. The stock solutions were diluted using AE buffer (10 mM Tris-HCl, 0.5 mM EDTA, pH 9.0) (Qiagen, 2006) to prepare DNA working solutions for the PCR reactions. The DNA quality was also checked on agarose gel prior to starting the PCR reactions. DNA that formed intact bands was used as this showed it was of good quality.

3.2.3 Choice and synthesis of RAPD primers

RAPD primers were chosen from the literature based on their previous use in similar studies in tea or other crop plants. The primer decamers were synthesized by Inqaba Biotech (Pretoria, South Africa) based on their sequences as reported in the literature or obtained from the website for Operon Technologies (http://www.operon.com). Sixty RAPD primers (Appendix 3.1 and 3.2) were initially screened for possible association with the three traits. Only those primers that yielded consistent results were chosen for the next stage.

3.2.4 Polymerase chain reaction (PCR)

The PCR reaction was conducted following the procedure of Williams *et al.* (1990) with some modifications. The reaction was done in a total volume of 13 µL comprising 15 ng of genomic DNA, 0.2 mM of each dNTP (Fermentas, Burlington, Canada), 1% (v/v) dimethyl Sulfoxide (DMSO) (Sigma Aldrich) and 1x S-T Exsel buffer (20 mM magnesium sulfate) (JMR Holdings, UK), 0.4 µM of the primer, 0.15 U of Exsel High Fidelity DNA Polymerase (JMR Holdings, UK) topped up with sterile triple distilled water.

The amplifications were done with a Bio-Rad My Cycler Thermal-Cycler (Bio-Rad Systems, Australia), programmed as follows: One cycle for initial denaturation at 94°C for 5 min, followed by 45 cycles for denaturing at 94°C for 1 min, annealing at 36°C for 1 min and extension at 68°C for 2 min and a final extension step at 68°C for 10 min. The Thermal-Cycler was programmed to end with a well holding temperature of 4°C at the end of the run. The amplifications for each primer were independently repeated at least twice to ascertain consistence and reproducibility of the RAPD bands.

3.2.5 Agarose gel electrophoresis

The RAPD amplification samples were diluted with 6x loading dye (0.025% w/v bromophenol blue, 30% (v/v) glycerol) in a 6:1 ratio and 10 µL of the diluted sample was loaded onto a 1.25% (w/v) agarose gel containing agarose (Sea Kem, Lonza, Rockland, USA), 1x TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8 and glacial acetic acid) and run at 100 volts for 1.5 h to separate the amplification products. The gels were stained with Gel Red 1/1000 in water (Biotium Inc, USA) which was pre-mixed with the loading dye (0.467% v/v) prior to diluting the PCR amplifications with the loading dye. A 1 Kb-Plus DNA ladder (Fermentas, Burlington, Canada) was used to estimate the sizes of the RAPD

bands. The gels were visualized and photographed under UV light using the Gel-Doc XR+ system (Bio-Rad Systems, Australia).

3.2.6 Scoring of RAPD bands on agarose gels

The RAPD profiles were scored for absence (0) or presence (1) of bands for each sample. It was assumed that each RAPD band represented a dominant allele at a unique genetic position (Kaundun *et al.*, 2000). Only the bands that were consistent and reproducible can be used as possible markers. The presence or absence of a specific band in different cultivars with or without a particular trait was used to determine whether a specific band was positively or negatively associated with a particular trait(s).

3.2.7 Discriminating ability of the identified RAPD markers

One of the characteristics of a good marker is the ability to detect differences or to discriminate between the variable materials being evaluated in a breeding or selection programme. In order to test this characteristic the potential markers were evaluated for ability to discriminate between the released (23) and the not-released (9) tea cultivars. For each marker, the cultivars that had the marker were counted and summed up in each sub-group of the cultivars. A ratio of the total number of cultivars that had the marker in the released cultivars to that in the not-released cultivars was calculated and used to determine the discrimination ability of the specific marker between the two groups of cultivars. RAPD markers that showed a ratio of 2.5 or above, which was the ratio of the released to not-released cultivars, were regarded to have good discriminating ability.

3.3 Results

A total of 60 RAPD primers were screened using a sample of 18 cultivars under each of the three traits. This led to identification of 10 primers that showed promising results (Table 3.3). The 10 primers were screened further, using a sample of 32 cultivars, chosen from the cultivars that were used during the preliminary screening of the RAPD primers under each trait. The 32 cultivars still represented the two extremes: absence or presence of each of the three traits.

Out of the 10 primers that were initially promising, only six primers generated specific RAPD bands that closely associated with either the absence or presence of the three traits (Table 3.4). The sizes of the RAPD bands ranged from 350 to 2200 base pairs (bp).

TABLE 3.3 RAPD primers that generated specific bands that associated with absence or presence of the three traits during the preliminary screening

Number	RAPD code	Approximate band size (bp)	Associated trait	Criterion
1	RAPD 11	1500	Low temperature tolerance	Absence
2	RAPD 15	2000	Low temperature tolerance	Presence
3	RAPD 16	2200	Black tea quality	Presence
4	RAPD 21	2000	Black tea quality	Presence
5	RAPD 27	800	Drought tolerance	Presence
6	RAPD 29	2000	Black tea quality	Absence
7	RAPD 32	900	Low temperature tolerance	Presence
8	RAPD 34	1400	Drought tolerance	Presence
9	RAPD 36	350	Low temperature tolerance	Presence
10	RAPD 44	1500	Drought tolerance	Presence

TABLE 3.4 RAPD bands that associated with black tea quality and drought and low temperature tolerance

RAPD Marker	Primer sequence (5´ - 3´)	Associated trait	Criterion
RAPD 16$_{(2200)}$	TTCATACGCG	Black tea quality	Presence
RAPD 21$_{(2000)}$	CCTGCTCATC	Black tea quality	Presence
RAPD 29$_{(2000)}$	GGTCCCTGAC	Black tea quality	Absence
RAPD 27$_{(800)}$	CAATCGCCGT	Drought tolerance	Presence
RAPD 44$_{(1500)}$	GAACCTGCGG	Drought tolerance	Presence
RAPD 36$_{(350)}$	TGTCTGGGTG	Low temperature	Presence

3.3.1 RAPD markers associated with black tea quality

The 32 cultivars used in this phase of the study had 16 cultivars that were previously classified as having the high black tea quality trait and the other sixteen cultivars lacked the trait (produced low black tea quality). Three RAPD primers generated specific bands that associated with black tea quality. Two of these, RAPD 16$_{(2200bp)}$ and RAPD 21$_{(2000bp)}$, showed positive association with black tea quality. RAPD 16$_{(2200bp)}$ was present in 12/16 (75.0 %) of the cultivars that are known to produce high black tea quality (Figure 3.1). However, the RAPD 16$_{(2200bp)}$ band was also present in cultivar RC 1 that produces low black tea quality. RAPD 21$_{(2000bp)}$, was present in 5/16 (31.3%) cultivars that produce high quality tea (Figure 3.2). The RAPD 21$_{(2000bp)}$ band was absent in all cultivars that produce low black tea quality. The two bands associated with high quality, if used as a panel would correctly select 13/16 (81.3%) of the high quality cultivars. This implied that these bands could be very good positive markers for selecting for high black tea quality.

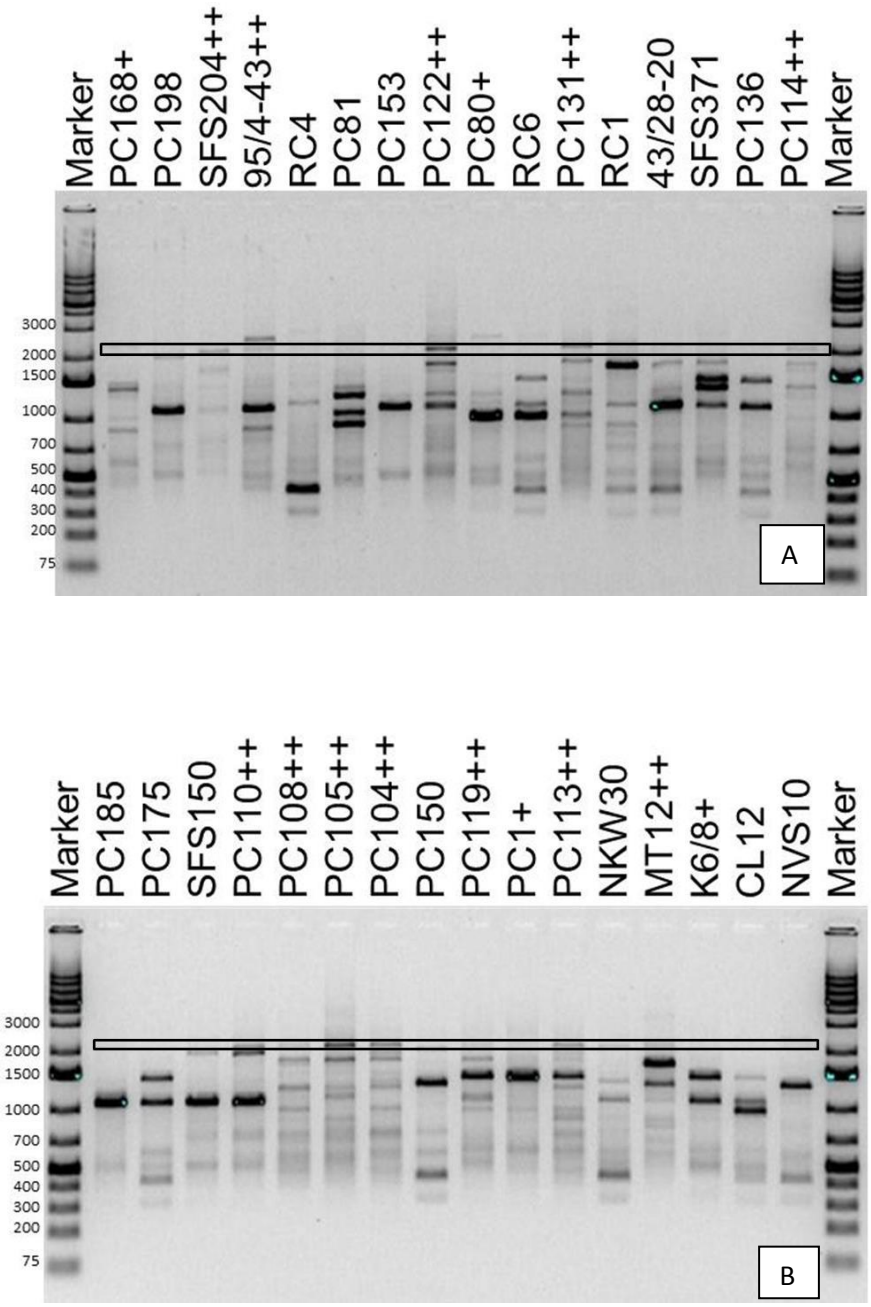

FIGURE 3.1: A gel picture for RAPD $16_{(2200bp)}$ **marker for high black tea quality. The symbol (+) denotes cultivars with the trait, whereas (++) denotes cultivars with the trait in which the marker was present. The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B).**

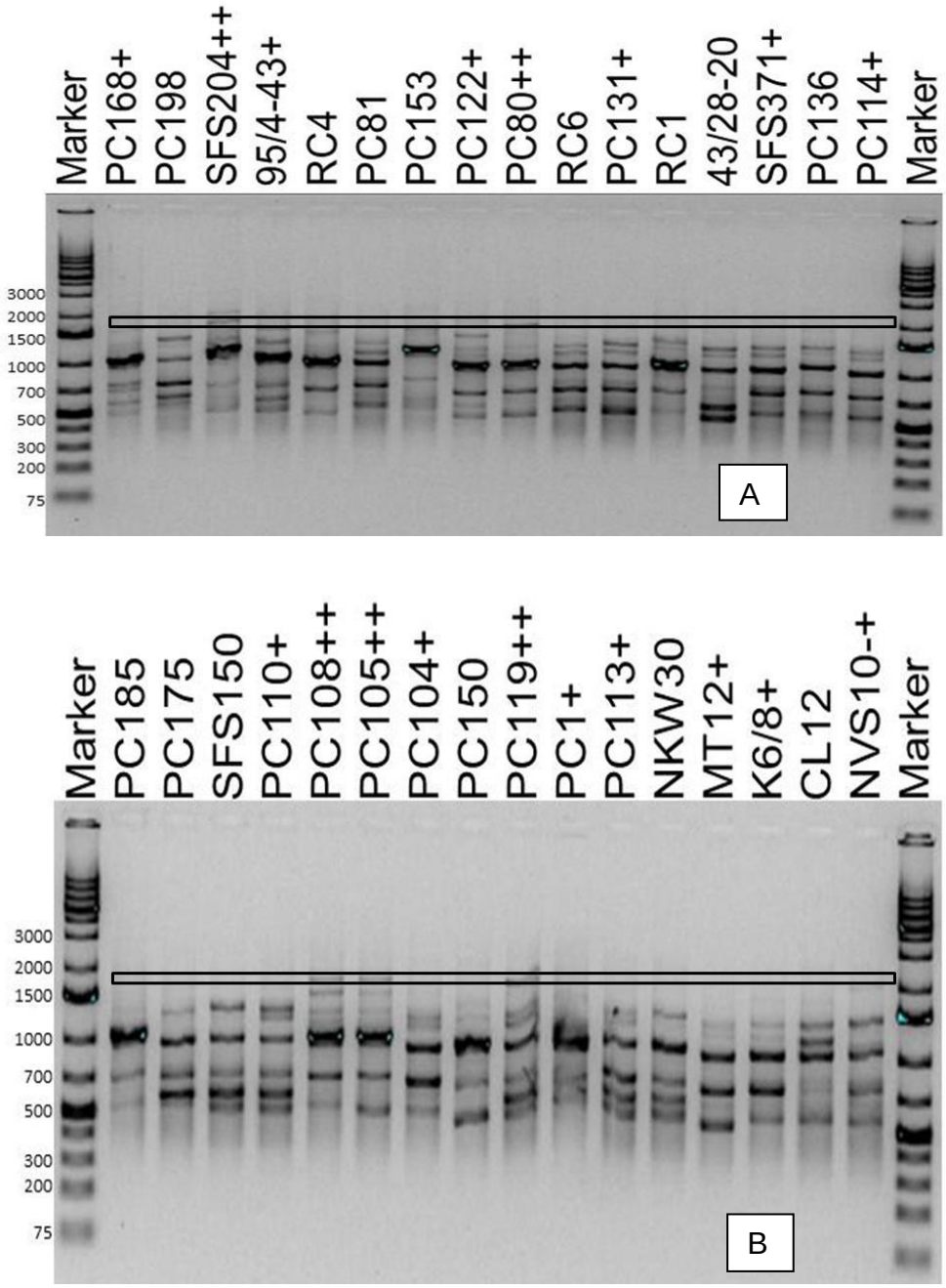

FIGURE 3.2: A gel picture for RAPD $21_{(2000bp)}$ **marker for high black tea quality. The symbol (+) denotes cultivars with the trait whereas (++) denotes cultivars with the trait in which the marker was present. The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B).**

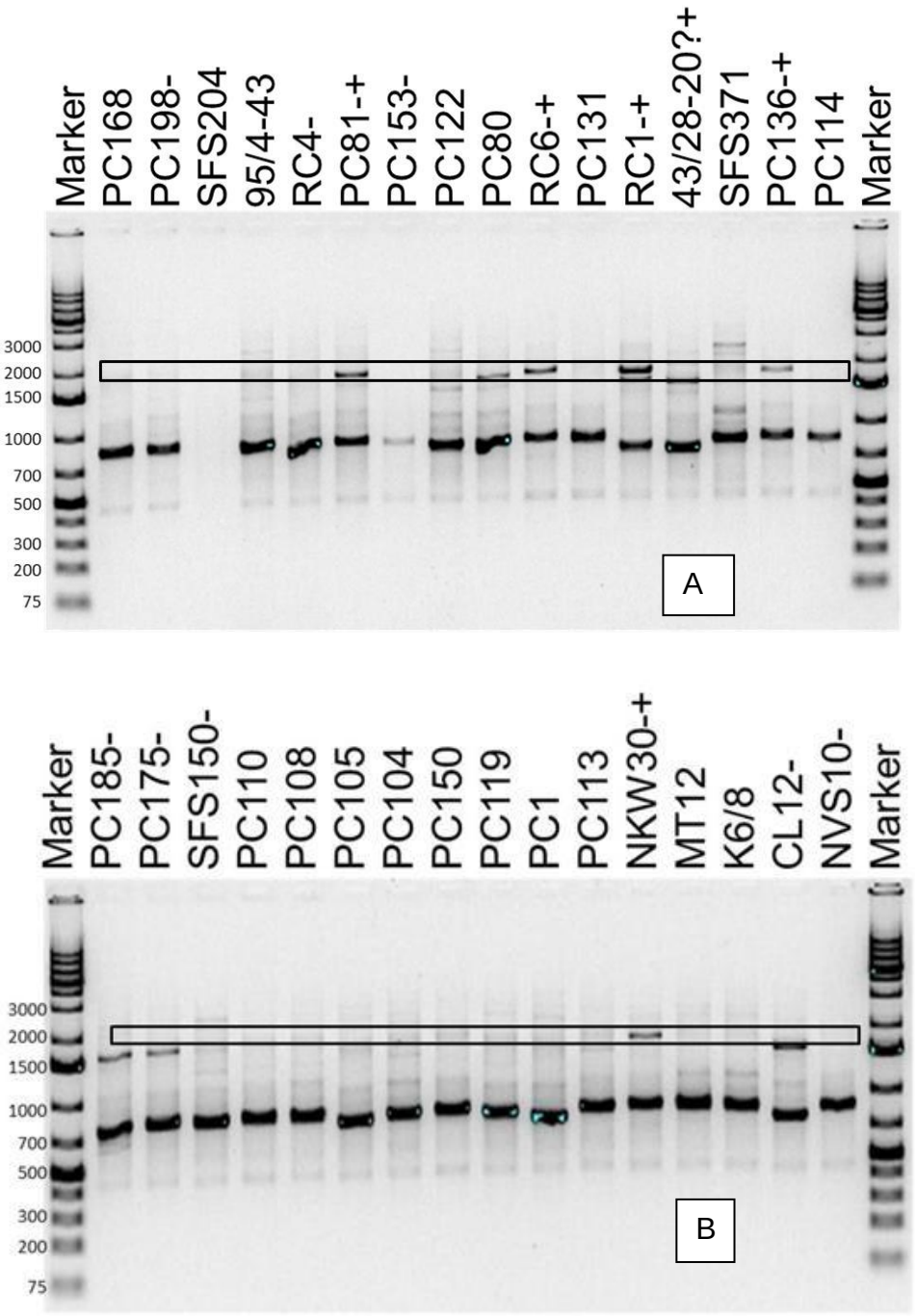

FIGURE 3.3: A gel picture for RAPD $29_{(2000bp)}$ **marker for low black tea quality. The symbol (-) denotes cultivars without the trait whereas (-+) denotes cultivars without the trait in which the marker was present. A (?) denotes a cultivar that was not previously classified as high or low quality. The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B).**

The RAPD $29_{(2000bp)}$ band showed negative association with black tea quality. It was present in 6/16 (37.5%) of the cultivars that produce low black tea quality (Figure 3.3). The band was absent in all the cultivars that produce high black tea quality, showing its strong negative association with the trait.

3.3.2 RAPD markers associated with drought tolerance

Two specific bands, RAPD $27_{(800bp)}$ and RAPD $44_{(1500bp),}$ showed positive association with drought tolerance. RAPD $27_{(800bp)}$ and RAPD $44_{(1500bp)}$ were, respectively, present in 9/16 (56.3%) (Figure 3.4) and in 7/16 (43.8%) of the drought tolerant cultivars (Figure 3.5). However, RAPD $27_{(800bp)}$ was also present in cultivar PC 131 whereas RAPD $44_{(1500bp),}$ was also present in PC 110 and PC 1, all of which succumb to severe drought stress. A panel of these two markers would correctly select all 16 drought tolerant cultivars present in the sample of cultivars used in the present study.

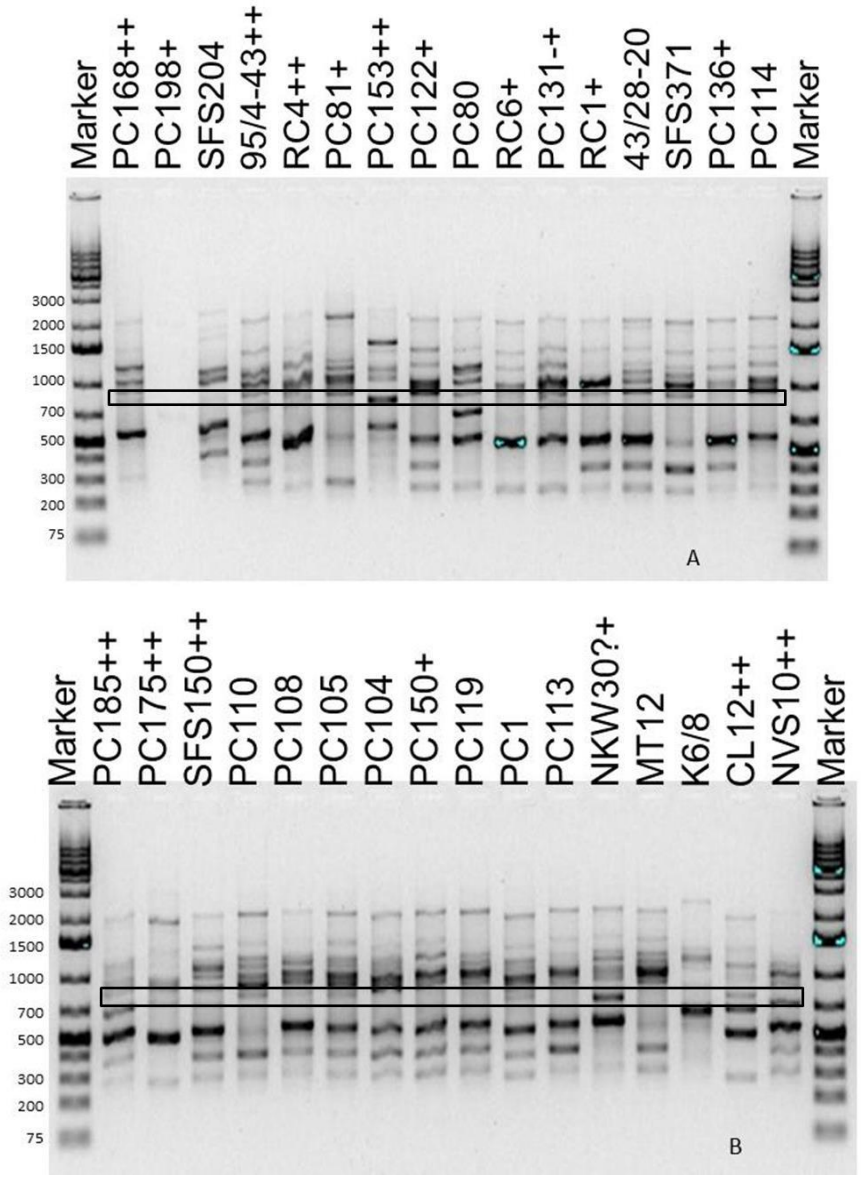

FIGURE 3.4: A gel picture for RAPD $27_{(800bp)}$ **marker for drought tolerance. The symbol (+) denotes cultivars with the trait whereas (++) denotes cultivars with the trait in which the marker was present. A (?) denotes a cultivar that had not been previously classified as tolerant or susceptible to drought.The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B).**

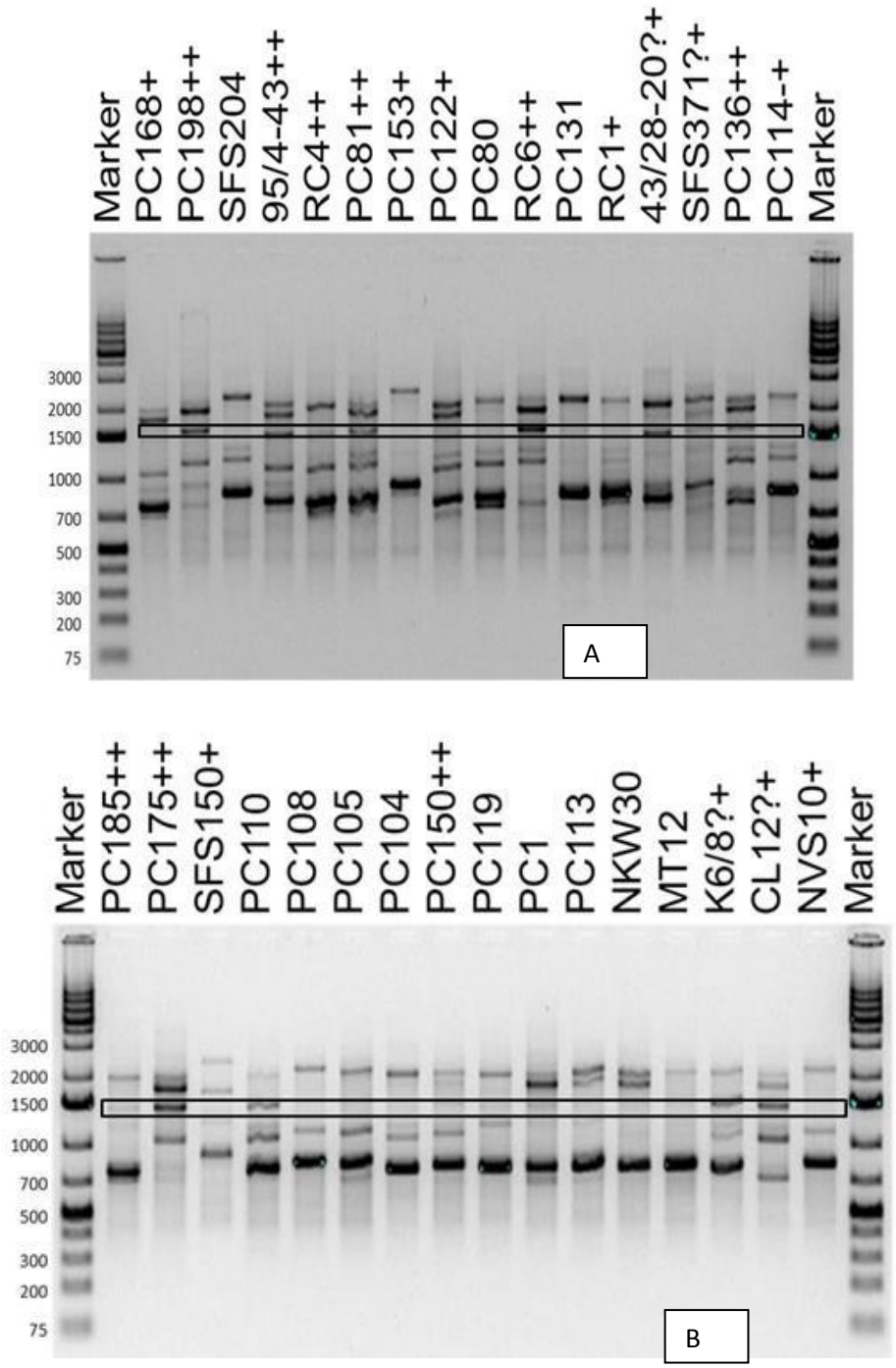

FIGURE 3.5: A gel picture for RAPD $44_{(1500bp)}$ **marker for drought tolerance. The symbol (+) denotes cultivars with the trait whereas (++) denotes cultivars with the trait in which the marker was present. The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B). A (?) denotes cultivars that had not been previously classified as tolerant or susceptible to drought.**

3.3.3 RAPD markers associated with low temperature growth

During the confirmatory screening of RAPD primers, only one band, RAPD $36_{(350bp)}$, was closely associated with tolerance to low temperature and was present in 4/10 (40.0%) of the cultivars that have this trait (Figure 3.6). The band was absent in all cultivars that are known to be sensitive to low temperature.

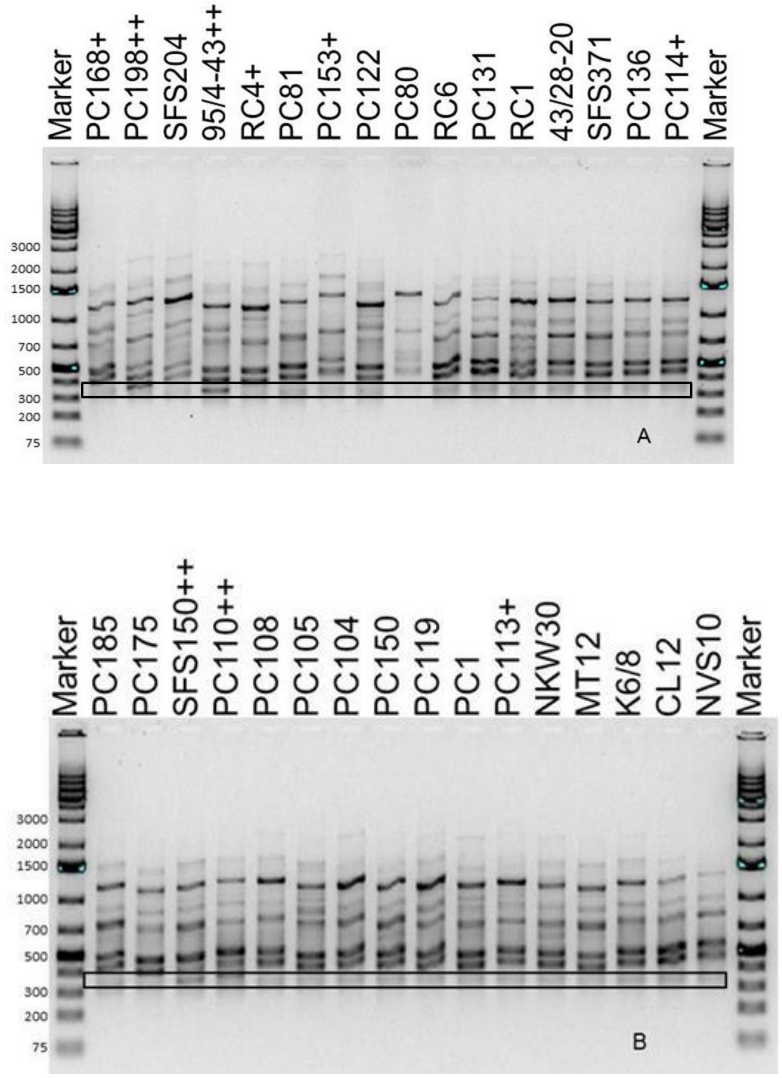

FIGURE 3.6: A gel picture for RAPD $36_{(350bp)}$ **marker for low temperature tolerance. The symbol (+) denotes cultivars with the trait whereas (++) denotes cultivars with the trait in which the marker was present. The lanes labelled 'marker' denote a 1kb DNA size ladder (Fermentas). Sixteen cultivars are shown in (A) and the other sixteen are in (B).**

3.3.4 Discriminating ability of the identified RAPD markers

The 32 cultivars that were used in the confirmatory studies were sub-divided into two groups: released and not-released. There were 23 released and 9 not-released cultivars, giving a ratio of 2.6. For each marker, cultivars that could be selected for a particular trait in the released and not-released sub-groups were counted and expressed as a ratio. If this ratio was ≥2.5, the marker was considered to have good discrimination ability. The results of this analysis are shown in Table 3.5. Based on this analysis, 4/6 (66.6%) of the markers showed good discrimination and 2/6(33.3%) showed poor discrimination ability between the two cultivar groups. The markers with good discrimination ability were associated with black tea quality ($RAPD16_{(2200bp)}$, $RAPD21_{(2000bp)}$, $RAPD29_{(2000bp)}$ and low temperature growth ($RAPD36_{(350bp)}$). Both markers that associated with drought tolerance showed poor discrimination between the released and not-released cultivars. This could probably be a result of having more cultivars in the not-released group that were classified as drought tolerant, which were correctly selected by the markers in this sub-group and hence a lower discrimination ratio.

For each cultivar, the difference between the total score for markers with good discrimination ability and those with poor discrimination ability was calculated to determine the net discrimination score for each cultivar if a panel of these markers was used. Taking a net discrimination score of each marker ≥0, and taking into account all the markers, 16/32 (50%) cultivars would be advanced to the next stage of selection. Out of these, 15/16 (93.8%) would be from the released cultivars and only 1/16 (6.2%) would be from the not-released cultivars. This implied that use of these markers would allow the breeder to reduce the number of selections that enter into elaborate field testing by 50%. In practice this would save a lot of resources that are used in preliminary field evaluations.

TABLE 3.5 RAPD band scores and net discrimination score in the released and not-released tea cultivars

	Cultivar	Cultivar sub-group	R16-2200	R21-2000	R27-800	R36-350	R44-1500	R29-2000	Total bands	Net score for discrimination
1	43/28-20	0	0	0	0	0	1	1	2	-2
2	95/4-43	0	0	0	1	1	1	0	3	-1
3	CL12	0	0	0	1	0	1	0	2	-2
4	K6/8	0	0	0	0	0	1	0	1	-1
5	NKW30	0	1	0	1	0	0	1	3	-1
6	NVS10	0	0	1	1	0	0	0	2	0
7	PC104	0	1	0	0	0	0	0	1	1
8	PC136	0	0	0	0	0	1	0	1	-1
9	PC150	0	0	0	0	0	1	0	1	-1
Total selected (A)		**9**	1	0.1	4	1	6	7		**2**
10	PC105	1	1	1	0	0	0	0	2	2
11	MT12	1	1	0	0	0	0	0	1	1
12	PC1	1	0	0	0	0	0	0	0	0
13	PC110	1	1	0	0	1	1	0	3	1
14	PC131	1	1	0	1	0	0	0	2	0
15	PC153	1	0	0	1	0	0	0	1	-1
16	PC80	1	0	1	0	0	0	0	1	1
17	RC1	1	0	0	0	0	0	1	1	-1
18	RC4	1	0	0	1	0	1	0	2	-2
19	RC6	1	0	0	0	0	1	1	2	-2
20	SFS371	1	0	0	0	0	1	0	1	-1
21	PC108	1	1	1	0	0	0	0	2	2
22	PC113	1	1	0	0	0	0	0	1	1
23	PC114	1	1	0	0	0	0	0	1	1
24	PC119	1	1	1	0	0	0	0	2	2
25	PC122	1	1	0	0	0	0	0	1	1
26	PC168	1	0	0	1	0	0	0	1	-1
27	PC175	1	0	0	1	0	1	0	2	-2
28	PC185	1	0	0	1	0	1	0	2	-2
29	PC198	1	0	0	0	1	1	0	2	0
30	PC81	1	0	0	0	0	1	1	2	-2
31	SFS150	1	0	0	1	1	0	0	2	0
32	SFS204	1	1	1	0	0	0	0	2	2
Total selected (B)		23	10	5	7	3	8	20		**14**
Ratio of B: A		2.56	5.00	50	1.75	3.00	1.33	2.86		

3.4 Discussion

Biotechnology has been steadily developing over the years. Subsequently there are many types of markers that are in use and/or under development. Each type of molecular marker has its own merits and demerits. It is therefore not possible to find a molecular marker technique that has all the desired properties. In most cases, a researcher's best option is to use a marker technique that combines at least some of the desired properties (Semagn *et al.*, 2006). RAPDs are technically simple, quick and easy to perform and require no prior sequence information of the genome (Patade *et al.*, 2006). The capital investment in RAPD marker technique is much lower than what is required for the more advanced types of molecular markers (Cancado *et al.*, 2013). The major drawback associated with RAPDs is poor repeatability, especially between different laboratories. However, previous research has suggested how to address some of the limitations of RAPDs. This has made RAPDs one of the most used PCR-based marker techniques (Byrne, 2007). These properties of RAPDs influenced the choice of this technique for use in the current study because RAPDs can easily be used in selection of progeny and would be easier to perform under the breeder's setting (Rajpakse, 2003). RAPDs can easily be incorporated in the breeding programme at TRFCA in Malawi, where there are no facilities for the more advanced marker techniques due to resource limitations. However, it will be very important to ensure that the identified RAPDs are consistently reproducible by optimizing the laboratory conditions in Malawi.

The current study considered black tea quality, drought- and low temperature-tolerance as traits of economic importance for teas produced in central Africa. For each trait, nine cultivars that had the trait and another nine that did not have the trait (classification based on previous assessments during the selection process), were screened with different RAPD primers. A number of RAPD bands were identified which showed polymorphism between the

two cultivar sub-groups. For each trait, some of the RAPD bands were specifically present or absent in cultivars with or without the trait. This suggested that the specific bands were closely associated with the traits and could be used as markers to select for the different traits (Langridge & Chalmers, 2004; Mohler & Singrun, 2004).

After screening 60 RAPD primers, it was possible to identify RAPD markers for each of the three traits. Most of the RAPD markers identified in this study showed good association with the various traits. The level of association varied with the RAPD bands and also among traits, which ranged from 31.3 to 75.0%. A combination of several markers could in some cases correctly select all (100%) of the cultivars that have the trait of interest. These results were consistent with the results reported in other crops (Kamel *et al.,* 2010).

Three RAPD markers were identified that associated with black tea quality and two markers for drought tolerance. Combined use of more than one RAPD marker improved the discrimination efficiency between the cultivars with and without the trait. This observation was consistent with the results reported in other studies. For example, Chen and Yamaguchi (2005) used four RAPD markers in order to completely discriminate 24 tea germplasms at inter-specific level, which was not possible with a single RAPD marker. Kaundun *et al.* (2000) reported that a minimum of three RAPD markers was required to completely discriminate 27 elite accessions of tea from Korea, Japan and Taiwan. In other crops, Shalini *et al.* (2007) who were screening for mite resistance in coconut, reported that a combination of three RAPD markers accounted for 83.86% of mite resistance, whereas the three markers used individually only accounted for 3.07%, 8.5% and 72.27% of the mite resistance. These results highlight the need to identify more markers for each trait in order to improve precision in selection.

The RAPD bands associated with drought tolerance showed some false-positive bands. Two possible causes for this were postulated. One of these is that possibly some of the cultivars were not accurately classified as tolerant or susceptible to drought in the historical field observations. This was more likely in this case since the classification was largely based on phenotypic differences that are heavily influenced by environmental factors. Accuracy of the historical, phenotypic classification is sometimes greatly compromised by the lack of control over severity and duration of drought under field conditions and the seasonal variations of the stress. Confounding effects of genes not related to the trait of interest, which can also affect the morphological markers, could also contribute to misclassification of cultivars, based on their phenotype (Ruan, 2010). The other possible cause may involve differences in the mechanisms used by the different cultivars in exhibiting the various traits. These problems were likely to occur and are probably unavoidable, especially where the test cultivars were not from a properly designed mapping population.

Reducing the number of selections that have to be advanced to the next stage of selection cycle is a crucial but challenging decision facing breeders at various stages of a selection programme. This becomes a daunting task in the early stages of selection, where a large number of selections are evaluated for many traits. Since molecular markers are amenable to use in the early stages of selection, the RAPD markers identified in this study would therefore help the breeder to objectively make such a decision at an early stage. Early rejection of non-promising materials would help to reduce costs, since only the few promising breeding lines would be subjected to the resource intensive long term, multi-location field evaluation. The markers would therefore help to improve selection precision and efficiency, because they are more reliable than phenotypic markers. The markers can also be used in selection of genotypes for use in breeding. Molecular-aided breeding can greatly increase the chance of improving on black tea quality, and tolerance to drought and low temperature, which are all

polygenic and difficult to select for using phenotypic markers. The markers can be used to select genetically diverse parental stocks for breeding and thereby increase the genetic variation in progeny from which selection can be done.

3.5 Conclusions

Most tea improvement programmes rely on use of conventional breeding and selection methods which have several limitations in accelerating genetic improvement of tea as well in improving precision of selection. This is mainly because there are still very few molecular markers that are closely associated with important traits in tea. In the current study, six RAPD markers were identified which associated well with black tea quality, tolerance to drought and low temperature. The identification of these markers is therefore an important contribution towards the goal of finding molecular markers that associate with important traits in tea for use in marker assisted selection (MAS). Use of these RAPD markers can help improve selection precision at different stages of the tea breeding and selection cycle. This can be achieved through targeted choice of parental breeding stocks and early identification of selections that possess the desirable traits. Use of more than one marker to select a particular trait improved effectiveness of selecting for the desirable traits. This highlighted the need to search for more markers that associate with a particular trait. The identified RAPD markers can easily be integrated into conventional tea breeding and selection to improve precision and efficiency. The markers can be used at the stage where parental stocks are chosen for hybridization and during evaluation of promising breeding lines in order to identify those that exhibit potential for the desired traits. This would have a positive impact on the success of the breeding or selection programme through increased number of released cultivars. In addition, these RAPD markers avail a good starting point for developing

sequence characterised amplified region (SCAR) markers for these traits. The level of association observed between the markers and the traits of interest suggested that specific RAPD bands can associate with some desirable traits either positively or negatively and such bands can be used as molecular markers for early selection of plants with great potential for the desirable traits. Since these RAPDs were only tested in one laboratory where they proved to be reproducible, it would be important to re-test this aspect when the technique is applied in a different laboratory in Malawi. Consistency of the markers should also be tested when screening a different group of cultivars, coming from a specific cross.

Appendix 3.1: RAPD primers 1–30 screened for possible association with different traits in tea

No.	Code	Sequence	Crop/plant	Growth habit	Trait	Referenxce
RAPD 1	UBC 162	AACTTACCGC	*Cornus sericea* -Red osier dogwood	Perennial	Low temperature induced dormancy	Svendsen et al., 2007
RAPD 2	474	AGGCGGGAAC	*Brassica napus* - seed rape	Annual	Winter survival	Asghari *et al*., 2008
RAPD 3	528	GGATCTATGC	*Brassica napus* - seed rape	Annual	Winter survival	Asghari *et al*., 2008
RAPD 4	430	ATGCGGCACC	*Brassica napus* - seed rape	Annual	Winter survival	Asghari *et al*., 2008
RAPD 5	UBC 218	CTCAGCCCAG	*Eucalyptus globus* - Eucalyptus	Perennial	Freezing resistance	Fernandez *et al.*, 2006
RAPD 6	UBC 237	CGACCAGAGC	*Eucalyptus globus* - Eucalyptus	Perennial	Freezing resistance	Fernandez *et al.*, 2006
RAPD 7	OPAH02	GAGACCAGAC	*Camellia sinensis* - tea	Perennial	Drought tolerance	Mishra & Sen-Mand, 2004
RAPD 8	Ope06	CCACGGGAAC	*Lens culinaris* Medikus - Lentil	Annual	Anthracnose resistance	Tullu *et al.*, 2003
RAPD 9	UBC 704	GGAAGGAGGG	*Lens culinaris* Medikus - Lentil	Annual	Anthracnose resistance	Taran *et al*, 2003
RAPD 10	P6-920	TCGGCGGTTC	*Tritcum aestivum* L. - wheat	Annual	Drought tolerance	Pakniyat &Tavakol, 2007
RAPD 11	P7	CTGCATCGTG	*Tritcum aestivum* L. - wheat	Annual	Drought tolerance	Pakniyat &Tavakol, 2007
RAPD 12	OPAB19	ACACCGATGG	*Glycine max* - soybean	Annual	Phomopsis resistance	Carvalho *et al*., 2002
RAPD 13	OPA10	GTGATCCCAG	*Malus spp* - Apple	Perennial	Dysaphis devecta resistance (insect)	Roche *et al.*, 1997
RAPD 14	OPC08	TGGACCGGTG	*Malus spp* - Apple	Perennial	Dysaphis devecta resistance (insect)	Roche *et al.*, 1997
RAPD 14	OPC-08	TGGACCGGTG	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010
RAPD 15	OPT09	CACCCCTGAG	*Malus spp* - Apple	Perennial	Dysaphis devecta resistance (insect)	Roche *et al.*, 1997
RAPD 16	3-1	TTCATACGCG	*Tritcum aestivum* L. - wheat	Annual	Mayetiola destructor resistance (Hessian fly)	Dweikat *et al*., 1997
RAPD 17	5-1	CGCATTTGCA	*Tritcum aestivum* L. - wheat	Annual	Mayetiola destructor resistance (Hessian fly)	Dweikat *et al*., 1997
RAPD 18	6-1	GTTTCGCTCC	*Tritcum aestivum* L. - wheat	Annual	Mayetiola destructor resistance (Hessian fly)	Dweikat *et al*., 1997
RAPD 19	OPA 04	AATCGGGCTG				?
RAPD 20	OPU 06	ACCTTTGCGG				?
RAPD 21	OPU 07	CCTGCTCATC				?
RAPD 22	S 12	CCTTGACGCA	*Camellia sinensis* - tea	Perennial	Albino tea cultivar identification	Wang, *et al* 2010
RAPD 23	OPP-05	CCCCGGTAAC	Barley	Annual	Stem rust resistance	Borovkova *et al*., 1995
RAPD 24	OPH-13	GACGCCACAC	Barley	Annual	Stem rust resistance	Borovkova *et al*., 1995
RAPD 25	OPH-15	AATGGCGCAG	Barley	Annual	Stem rust resistance	Borovkova *et al*., 1995
RAPD 26	OPH-01	GGTCGGAGAA	Hemp	Annual	Cultivar differentiation	Forapani, et al., 2001
RAPD 27	OPA-11	CAATCGCCGT	Hemp	Annual	Cultivar differentiation	Forapani, et al., 2001
RAPD 28	OPA-02	TGCCGAGCTG	?	?	?	
RAPD 29	OPA-06	GGTCCCTGAC	?	?	?	
RAPD 30	OPA-12	TCGGCGATAG	?	?	?	

Appendix 3.2: RAPD primers 31–60 screened for possible association with different traits in tea

No.	Code	Sequence	Crop/plant	Growth habit	Trait	Referenxce
RAPD 31	OPA-15	TTCCGAACCC	?	?	?	
RAPD 32	OPB-07	GGTGACGCAG	?	?	?	
RAPD 33	OPB-15	GGAGGGTGTT	?	?	?	
RAPD 34	OPB-19	ACCCCCGAAG	?	?	?	
RAPD 35	OPC-01	TTCGAGCCAG	?	?	?	
RAPD 36	OPC-10	TGTCTGGGTG	?	?	?	
RAPD 37	OPC-11	AAAGCTGCGG	?	?	?	
RAPD 38	OPC-12	TGTCATCCCC	?	?	?	
RAPD 39	OPC-19	GTTGCCAGCC	?	?	?	
RAPD 40	OPK-04	CCGCCCAAAC	?	?	?	
RAPD 41	OPAC-19	AGTCCGCCTG	?	?	?	
RAPD 42	OPE-04	GTGACATGCC	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 43	OPE-06	AAGACCCCTC	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 44	OPG-07	GAACCTGCGG	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 45	OPP-15	GGAAGCCAAC	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 46	OPP-16	CCAAGCTGCC	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 47	OPE-18	GGACTGCAGA	Coconut	Perennial	Mite resistance	Shalini *et al*,2007
RAPD 48	OPA-16	AGCCAGCGAA	*Lycopersicon esculentun* - Tomato	Annual	Hea tolerance	Kamel *et al.*, 2010
RAPD 49	OPB-16	TTTGCCCGGA	Barley	Annual	Drought tolerance	?
RAPD 50	OPD-15	CATCCGTGCT	*Pisium sativum* - Pea	Annual	Powderly mildew resistance (er1 gene)	Tonguc & Weeden, 2010
RAPD 51	OPB-11	GTAGACCCGT	*Pisium sativum* - Pea	Annual	Powderly mildew resistance (er1 gene)	Tonguc & Weeden, 2011
RAPD 52	BC-210	GCACCGAGAG	*Pisium sativum* - Pea	Annual	Powderly mildew resistance (er1 gene)	Tonguc & Weeden, 2012
RAPD 53	BC-483	GCACTAAGAC	*Pisium sativum* - Pea	Annual	Powderly mildew resistance (er1 gene)	Tonguc & Weeden, 2013
RAPD 54	BC-407	TGGTCCTGGC	*Pisium sativum* - Pea	Annual	Powderly mildew resistance (er1 gene)	Tonguc & Weeden, 2014
RAPD 55	OPZ-13	GACTAAGCCC	*Lycopersicon esculentun* - Tomato	Annual	Hea tolerance	Kamel *et al.*, 2010
RAPD 56	OPC-02	GTGAGGCGTC	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010
RAPD 57	OPC-03	GGGGGTCTTT	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010
RAPD 58	OPC-05	GATGACCGCC	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010
RAPD 59	OPC-14	TGCGTGCTTG	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010
RAPD 60	OPC-15	GACGGATCAG	*Lycopersicon esculentun* - Tomato	Annual	Heat susceptibility	Kamel *et al.*, 2010

Appendix 3.3: Scientific Article published based on some of the results obtained in Chapter 3

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Screening of Tea (*Camellia sinensis*) for Trait-Associated Molecular Markers

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Abstract This study was done to identify random amplified polymorphic DNA (RAPD) markers that may associate with seven important traits in tea. Sixty RAPD primers were first screened using 18 cultivars under each of the 7 traits, followed by confirmatory screening of 20 promising primers with 32 tea cultivars. Six RAPD primers generated a total of nine specific bands that associated with six desired traits: black tea quality and tolerance to drought, high temperature, low temperature, *Phomopsis theae*, and high yield. These markers would allow early identification of plant material with the desired traits that can be advanced to the next stage of selection and enhance targeted choice of breeding stocks with the desirable traits. The nine RAPD markers identified in this study could improve precision and efficiency in tea breeding and selection and are an important contribution towards the establishment of marker-assisted selection in tea breeding programmes.

Keywords Tea · RAPD · Trait · Marker · Selection

Introduction

Tea is a very important crop in many countries [1]. It plays a major role in the economies of some Asian and African countries such as India, China, Sri Lanka, Japan, Kenya and Malawi [2, 3]. Despite its widespread cultivation, some tea-producing areas experience sub-optimal growing conditions during several months of the year and this affects both quality and yield.

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The tea-producing areas in central Africa (Malawi and Zimbabwe) experience large variations in temperature, rainfall and incidences of pests and diseases within and between seasons that result in significant reductions in tea yield and affect quality [4–7]. All these factors impact negatively on sustainable tea production.

Development of new cultivars, through breeding and selection, that can tolerate low and very high temperatures, drought or severe water stress, major pests and diseases and produce high yield and quality processed tea is a priority for most tea breeding programmes, including the one at the Tea Research Foundation of Central Africa (TRFCA) [7, 8]. However, selection of new cultivars that exhibit most of these traits is challenged by lack of objective, reliable and time-effective criteria. This is complicated further by tea's long juvenile period of about 5 years and the long development time shown by most of the desirable traits [2]. This results in long and expensive selection cycles [2, 9], because most of the criteria that are in use now are time consuming, show high levels of phenotypic plasticity and sometimes involve expensive analytical assays [10, 29, 30]. In some cases, this is compounded by the lack of controlled environment screening facilities.

Use of molecular markers in breeding and selection for desirable agronomic traits in crops offers a number of enhanced possibilities and opportunities even for traits that are difficult to evaluate under uncontrolled conditions. This is because molecular markers are least affected by the variable environmental factors and developmental stage of the plant material [11] and they can allow screening for tolerance or resistance to a stressful condition even in the absence of the stress factor [12].

Several types of DNA-based molecular markers are being used in breeding programmes of various crops, e.g. rice, apples, eucalyptus and maize. However, tea is one of those crops that has not greatly benefited from biotechnological efforts in the past. The earliest biotechnological research on tea dates back to the mid-1990s [13] and reports of practical use of marker-assisted selection are still rare [14]. Until recently, use of some molecular markers, such as restriction fragment length polymorphism (RFLP) and microsatellites that require prior knowledge of the flanking locus nucleotide sequences, was considered less applicable to tea because of the limited genetic information that is available in the public domain. Good progress has, however, been made in identifying molecular markers for various purposes in tea that range from diversity studies [15–17] to the search for molecular markers associated with different agronomic traits. For instance, the work by Mishra and Sen-Mandi [18] on identification of a RAPD marker for drought tolerance in Darjeeling, India that was later independently repeated by Wium [19] and Malebe [20] using cultivars from Malawi; the work by Kamunya et al. [21] on identification of quantitative trait loci associated with yield; identification of 112 novel tea unigene-derived microsatellites [22] and sequencing of the tea transcriptome that has revealed a number of unigenes for tea and increased the coverage over the tea genome [23] are some of the recent developments in tea biotechnology. However, the molecular markers that have been identified in tea are probably still too few to saturate the big tea genome which is estimated to be about four gigabases [24]. Unsaturated genetic maps limit the practical deployment of the molecular markers in breeding and selection [25] and therefore necessitate more research work on molecular markers.

RAPD markers do not require prior knowledge of the DNA sequence as is required by other types of DNA-based molecular markers, such as RFLPs; they are also inexpensive and easy to develop [2, 11]. One of the limitations associated with RAPDs is their sensitivity to experimental conditions, which has in some cases been associated with poor reproducibility, especially between different laboratories [2, 17]. However, RAPDs still remain one of the most commonly used markers in various genetic investigations, such as diversity and fingerprinting, as well as marker–trait association studies [1, 16, 26]. RAPDs can still

generate reliable data, provided the experimental conditions are standardised and the amplification reactions are reproducible [27]. RAPDs and other types of molecular markers such as RFLP, amplified fragment length polymorphism and inter-simple sequence repeats have been used to identify markers that are associated with some important traits such as yield, quality, disease and pest resistance and tolerance to drought in many crop plants [28].

The objective of this study was to identify RAPD markers that may associate with seven important traits for tea produced in central Africa, including: (1) black tea quality, (2) drought tolerance, (3) *Helopeltis schoutedeni* (tea mosquito bug) tolerance, (4) high temperature tolerance, (5) low temperature tolerance, (6) *Phomopsis theae* (stem canker) tolerance, and (7) yield, in order to develop a marker-assisted selection programme for the various traits. It was hypothesised that each of these traits was under genetic control that could be correlated to presence or absence of specific RAPD bands and/or a panel of RAPD bands generated by several RAPD primers

Methods and Methods

Choice of Tea Cultivars

In screening for marker–trait associations, it is important to properly phenotype the test cultivars, especially in the absence of a properly planned screening population [12]. The test tea cultivars used in this study were obtained from the tea breeding programme at the TRFCA in Malawi. They were selected based on prior knowledge and information obtained over several years of field observation at multiple sites on whether a specific trait was present (+) or absent (−) in a particular cultivar. During the preliminary screening of the RAPD primers, two groups of cultivars were formed for each trait, one with cultivars that had the trait (nine cultivars) and the other comprising cultivars that lacked the trait (nine cultivars). After the preliminary screening, cultivars were selected from the two groups for each trait to form a large sample of cultivars that was used for confirmatory experiments on the association of the promising RAPD bands with the different traits. The 32 cultivars that were chosen for this part of the study and their historical classification on the various traits are presented in Table 1.

Extraction of Genomic DNA

Fresh leaf samples were collected from field-grown tea bushes at the Mimosa Tea Research Station of the TRFCA in Malawi (16°05′ S, 35°35′ E, 630 m above mean sea level). The leaves were preserved in zip lock plastic bags containing silica gel that had been dried in the oven at 70 °C for 48 h to enable it to absorb any surface moisture from the leaves, following the procedure described by Malebe [20]. The leaf samples were then sent to the University of Pretoria in South Africa where genomic DNA was extracted according to the procedure described in the DNEasy Plant mini kit manual (Qiagen, Germany) [35]. The quality and quantity of the extracted DNA for each sample was determined with a nanodrop (ND-1000) spectrophotometer (Inqaba Biotech, USA). The quality of the DNA was based on the ratio of the 260–280 nm absorbance which ranges between 1.7 and 1.9 for good quality DNA. All the DNA samples used in this study were of good quality as per this criterion. The extracted DNA stocks were stored in a −20 °C freezer. The stock solutions were diluted using AE buffer (10 mM Tris–HCl, 0.5 mM EDTA, pH 9.0) [35] to prepare working solutions for the polymerase chain reactions.

Table 1 Phenotypic ranking of cultivars used to screen promising RAPD primers

Number	Cultivar name	Black tea quality	Low-temperature tolerance	*Phomopsis* tolerance	High-temperature tolerance	Drought tolerance	Yield	*Helopeltis* tolerance
1	PC168	+	+	+	−	+	+	−
2	PC198	−	+	+	+	+	+	−
3	SFS204	+	+	+	−	−	−	+
4	95/4-43	+	+	?	+	+	+	+
5	RC4	−	+	+	+	+	+	+
6	PC81	−	−	−	−	+	+	+
7	PC153	−	+	+	+	+	+	−
8	PC122	+	−	−	+	+	+	−
9	PC80	+	−	−	?	−	−	−
10	RC6	−	−	+	+	+	+	−
11	PC131	+	−	+	?	−	−	?
12	RC1	−	−	−	?	+	+	?
13	43/28-20	?	?	?	?	?	?	−
14	SFS371	+	?	−	?	?	+	?
15	PC136	−	−	?	?	+	+	?
16	PC114	+	+	−	+	−	+	+
17	PC185	−	−	+	+	+	+	+
18	PC175	−	−	+	+	+	+	+
19	SFS150	−	+	−	+	+	+	+
20	PC110	+	+	+	?	−	+	+
21	PC108	+	−	+	?	−	+	−
22	PC105	+	−	−	?	−	+	+
23	PC104	+	−	?	?	−	+	?
24	PC150	?	?	+	−	+	+	?
25	PC119	+	−	−	+	−	−	?
26	PC1	+	−	−	?	−	−	−
27	PC113	+	+	−	+	−	+	?
28	NKW30	−	?	?	?	?	?	−
29	MT12	+	?	−	?	?	+	?
30	K6/8	+	?	?	?	?	+	?
31	CL12	?	−	−	?	?	−	?
32	NVS10	−	?	?	?	+	+	?

A (−) and (+) denotes the cultivar lacks and exhibits the trait of interest, respectively. For black tea quality, (+) represents high quality and (−) low quality. A high-quality cultivar would generally achieve a mean taster's total score for the various black tea organoleptic parameters of >20. For yield, (−) denotes low yielding, a mean yield of <2,500 kg made tea ha^{-1} and (+) denotes high yield an overall mean yield of >2,500 kg made tea ha^{-1}. A (?) denotes that the cultivar had not been previously classified for the trait

Choice of RAPD Primers

RAPD primers were chosen from the literature based on their previous use in similar studies in tea or other crop plants. The primer decamers were synthesised by Inqaba Biotech

(Pretoria, South Africa) based on sequences of the different primers as reported in the literature or obtained from the website for Operon Technologies (http://www.operon.com). Sixty RAPD primers were initially screened for possible association with the seven traits. Only those primers that yielded consistent results were chosen for the next stage.

Polymerase Chain Reaction

The polymerase chain reaction was conducted in a total volume of 13 μL comprising 15 ng of genomic DNA, 0.2 mM of each dNTP (Fermentas, Burlington, Canada), 1 % dimethyl sulphoxide (Sigma Aldrich) and 1× S-T Exsel buffer (20 mM magnesium sulphate; JMR Holdings, UK), 0.4 μM of the primer, 0.15 U of Exsel High Fidelity DNA Polymerase (JMR Holdings) topped up with sterile triple distilled water.

The amplifications were done with a Bio-Rad My Cycler Thermal-Cycler (Bio-Rad Systems, Australia), programmed as follows: one cycle for initial denaturation at 94 °C for 5 min, followed by 45 cycles for denaturing at 94 °C for 1 min, annealing at 36 °C for 1 min, and extension at 68 °C for 2 min and a final extension step at 68 °C for 10 min. The Thermal-Cycler was programmed to end with a well-holding temperature of 4 °C at the end of the run. The amplifications were independently repeated at least two times to ascertain consistence and reproducibility of the RAPD bands.

Gel Electrophoresis

The RAPD amplification samples were diluted with 6× loading dye (0.025 % *w*/*v* bromophenol blue, 30 % glycerol) in a 6:1 ratio and 10 μL of the each diluted sample was loaded onto a 1.25 % (*w*/*v*) agarose gel containing agarose (Sea Kem, Lonza, Rockland, USA), 1× TAE buffer (0.04 M Tris-acetate, 1 mM EDTA, pH 8 and glacial acetic acid) and run at 100 V for 1.5 h to separate the amplification products. The gels were stained with Gel Red 1/1000 in water (Biotium Inc, USA) which was pre-mixed with the loading dye (0.467 % *v*/*v*) prior to diluting the amplification samples with the loading dye. A 1Kb Plus DNA ladder (Fermentas) was used to estimate the sizes of the RAPD bands. The gels were visualised and photographed under UV light using the Gel-Doc XR+ system (Bio-Rad Systems).

Data Collection and Analysis

The RAPD bands were scored for absence (0) or presence (1) for each sample. It was assumed that each RAPD band represented a dominant allele at a unique genetic position [33]. Only consistent and reproducible bands could be used as possible markers. The presence of a specific band in different cultivars with or without a particular trait was used to determine whether a specific band was positively or negatively associated with a particular trait(s). For the RAPD bands that were negatively associated with a trait, absence of the band in a cultivar in which the trait is present was considered desirable and given a score of 1 when counting the number of desirable bands for each cultivar.

Results

A total of 60 RAPD primers were screened using a sample of 18 cultivars under each of the seven traits. This led to identification of 20 primers that showed promising results. The 20 primers were screened further using a sample of 32 cultivars, chosen from the cultivars that were

used during the preliminary screening of the RAPD primers under each trait. The 32 cultivars still represented the two extremes: presence and absence of each of the seven important traits.

Out of the 20 primers used in the second phase of screening with a larger sample of cultivars, six primers were identified that generated nine specific RAPD bands that closely associated with either the presence or absence of the different traits (Table 2). The sizes of the RAPD bands ranged from 350 to 2,200 base pairs (bp).

Markers Associated with the Specific Important Traits

Data was collected from agarose gel pictures, bands were scored as absent (0) or present (1) in each of the 32 cultivars, for all six RAPD primers. The scores for presence or absence of the nine RAPD bands are summarised in Table 3. An example of the agarose gel picture is shown in Fig. 1.

RAPD Bands Associated with Black Tea Quality

Three RAPD primers generated specific bands that associated with black tea quality. Two of these, RAPD 16 $_{(2,200\ bp)}$ and RAPD 21 $_{(2,000\ bp)}$, showed positive association with black tea quality and were, respectively, present in 12/16 (75 %) and 5/16 (31.3 %) of the cultivars that are known to produce high black tea quality. The two bands if used as a panel would correctly select 13/16 (81.3 %) of the high-quality cultivars. This implied that these bands could be very good positive markers for selecting for high black tea quality. The RAPD 21$_{(2,000\ bp)}$ band was absent in all cultivars that are known to produce low black tea quality, whereas RAPD 16 $_{(2,200\ bp)}$ was, however, also present in cultivar RC1 that produces low black tea quality.

The band generated by RAPD 29 $_{(2,000\ bp)}$ showed negative association with black tea quality and was present in 7/16 (43.8 %) of the cultivars that produce low black tea quality. The band was absent in all the cultivars that produce high black tea quality, showing its strong negative association with the trait.

RAPD Bands Associated with Drought Tolerance

Two specific bands, RAPD 27$_{(800\ bp)}$ and RAPD 44$_{(1,500\ bp)}$, were positively associated with drought tolerance. RAPD 27$_{(800\ bp)}$ and RAPD 44$_{(1,500\ bp)}$ were, respectively, present in

Table 2 Size of RAPD bands associated with absence or presence of some important traits

RAPD code and band size (bp)	Primer sequence (5′-3′)	Associated trait	Criterion
RAPD 16$_{(2,200)}$	TTCATACGCG	Black tea quality	Presence
RAPD 16$_{(1,500)}$		*Phomopsis* tolerance	Absence
RAPD 16$_{(1,200)}$		High temperature tolerance	Presence
RAPD 21$_{(2,000)}$	CCTGCTCATC	Black tea quality	Presence
RAPD 27$_{(800)}$	CAATCGCCGT	Drought tolerance	Presence
RAPD 44$_{(2,000)}$	GAACCTGCGG	Yield (biomass)	Presence
RAPD 44$_{(1,500)}$		Drought tolerance	Presence
RAPD 36$_{(350)}$	TGTCTGGGTG	Low-temperature tolerance	Presence
RAPD 29$_{(2,000)}$	GGTCCCTGAC	Black tea quality	Absence

Table 3 Absence (0) or presence (1) of RAPD bands in different tea cultivars

Cultivar	Released	R16-2200	R16-1200	R21-2000	R27-800	R36-350	R44-2000	R44-1500	R29-2000	R16-1500	Total bands	Net score for discrimination
43/28-20	0	0	1	0	0	0	0	1	1	1	4	−4
95/4-43	0	1	1	0	1	1	1	1	0	0	5	−2
CL12	0	0	1	0	1	0	0	1	0	1	4	−4
K6/8	0	0	1	0	0	0	1	1	0	1	4	−4
NKW30	0	0	1	0	1	0	1	0	0	0	3	−3
NVS10	0	0	1	0	1	0	1	0	0	0	3	−3
PC104	0	1	0	0	0	0	1	0	0	0	2	0
PC136	0	0	1	0	0	0	1	1	0	1	4	−4
PC150	0	0	0	0	0	0	1	1	0	0	2	−2
Total cultivars (A)	9	2	7	0	4	1	7	6	8	5		3
PC105	1	1	0	1	0	0	1	0	0	0	3	1
MT12	1	1	1	0	0	0	0	0	0	1	3	−1
PC1	1	0	0	0	0	0	1	1	0	1	3	−3
PC110	1	1	1	0	0	1	0	1	0	0	4	0
PC131	1	1	0	0	1	0	0	0	0	1	3	−1
PC153	1	0	1	0	1	0	0	0	0	0	2	−2
PC80	1	0	0	1	0	0	0	0	0	1	2	0
RC1	1	1	1	0	0	0	0	0	1	0	3	−1
RC4	1	0	0	0	1	0	0	1	0	0	2	−2
RC6	1	0	1	0	0	0	1	1	1	0	4	−4
SFS371	1	0	1	0	0	0	1	1	0	1	4	−4
PC108	1	0	0	1	0	0	0	0	0	0	1	1
PC113	1	1	0	0	0	0	1	0	0	1	3	−1
PC114	1	1	0	0	0	0	0	0	0	0	1	1
PC119	1	0	1	1	0	0	0	0	0	1	3	−1

Table 3 (continued)

Cultivar	Released	R16-2200	R16-1200	R21-2000	R27-800	R36-350	R44-2000	R44-1500	R29-2000	R16-1500	Total bands	Net score for discrimination
PC122	1	1	1	0	0	0	1	0	0	1	4	−2
PC168	1	1	0	0	1	0	1	0	0	1	4	−2
PC175	1	0	1	0	1	0	1	1	0	1	5	−5
PC185	1	0	1	0	1	0	1	1	0	0	4	−4
PC198	1	0	1	0	0	1	1	1	0	0	4	−2
PC81	1	0	0	0	0	0	1	1	1	1	4	−4
SFS150	1	0	1	0	1	1	0	0	0	0	3	−1
SFS204	1	1	0	1	0	0	0	0	0	0	2	2
Total cultivars (B)	23	10	12	5	7	3	11	9	20	11		17
Ratio of B/A	2.6	5.0	1.7	50.0	1.8	3.0	1.6	1.5	2.5	2.2		8.5

Total cultivars denote the number of cultivars that would be selected using a specific marker in the not-released (A) and the released (B) sub-groupings of the cultivars. A marker that recorded a lower total number of the not-released cultivars (A) and a higher total number of the released cultivars (B) was considered to have good discrimination. The net score for discrimination was therefore calculated as the difference in the total of scores between good and bad discriminating markers

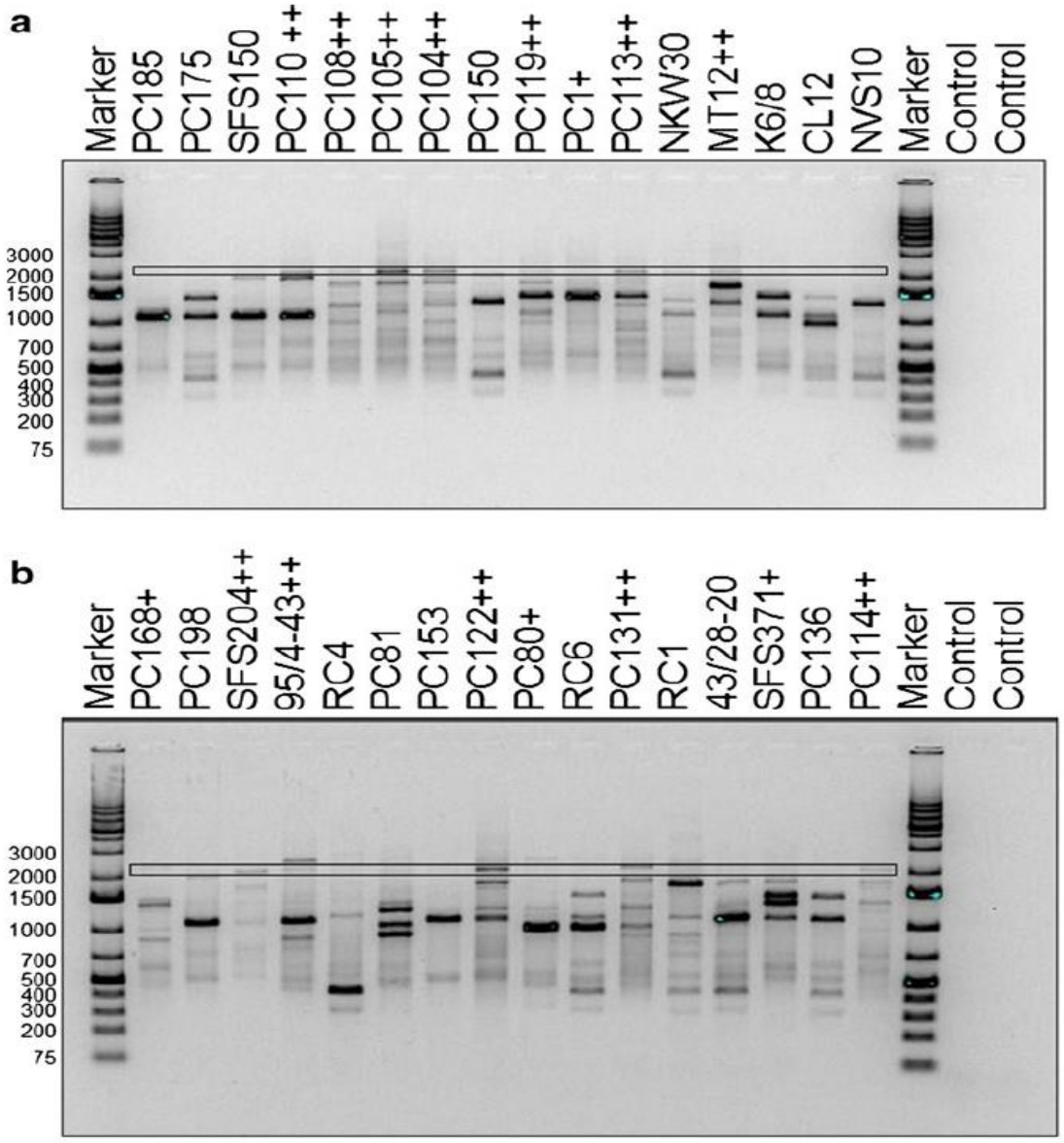

Fig. 1 Polymerase chain reaction (PCR) amplification products for 32 tea cultivars generated by RAPD 16. The RAPD $16_{(2,200\ bp)}$ band was associated with high black tea quality and was present in 12 of the 16 cultivars that are known to produce high black tea quality (trait is present). A 1 kb DNA molecular size ladder was used to estimate the size of the amplification products. The samples loaded in the control lanes of the gel had all the PCR reaction reagents except genomic DNA (negative control)

10/16 (62.5 %) and 13/16 (81.3 %) of the drought-tolerant cultivars. However, RAPD $27_{(800\ bp)}$ was also present in cultivar PC 131 and RAPD $44_{(1,500\ bp)}$, in PC 110 and PC 1, all of which

succumb to severe drought stress. A panel of these two markers would correctly select all 16 drought-tolerant cultivars present in the sample of cultivars used in the present study.

RAPD Bands Associated with High Temperature Tolerance

One specific band, RAPD $16_{(1,200\ bp)}$, was positively associated with high temperature (heat) tolerance. The band was present in 9/12 (75.0 %) of the cultivars that had previously been classified as tolerant to high temperature. The band was absent in all the cultivars that are sensitive to high temperature.

RAPD Bands Associated with Low Temperature Tolerance

One band, RAPD $36_{(350\ bp)}$, was more closely associated with tolerance to low temperature and was present in 4/10 (40.0 %) of the cultivars that have this trait. The band was absent in all cultivars that are known to be sensitive to low temperature.

RAPD Bands Associated with *P. theae* Tolerance

RAPD $16_{(1,500\ bp)}$ was negatively associated with tolerance to *P. theae* and was present in 10/14 (71.4 %) of the cultivars that were classified as susceptible. However, this band was also present in two cultivars, PC 175 and PC168, which showed intermediate tolerance to the disease during the field assessments done in 2011.

RAPD Bands Associated with High Yield

RAPD $44_{(2,000\ bp)}$ band was positively associated with high yield and was present in 13/24 (54.2 %) of the cultivars that were known to produce high yields. One cultivar PC1, which is classified as a medium-yielding cultivar, also had the band. The band was absent in all the six cultivars that produce low yields.

Discriminating Ability of the Identified RAPD Markers

One of the characteristics of a good marker is ability to detect differences or to discriminate between the variable materials being evaluated in a breeding or selection programme. In order to test how the markers identified in this study complied with this criterion, the 32 cultivars were sub-divided into released (23) and not-released (9) cultivars (Table 3). For each marker, the cultivars that had the marker were counted and summed up in each sub-group of the cultivars. A ratio of the total number of cultivars that had the marker in the released cultivars to that in the not-released cultivars was calculated to determine the discrimination ability of the specific marker between the two groups of cultivars. A ratio that was significantly greater than 2.6 (which is the ratio of the released to the not-released cultivars (23/9) in the sample of 32 cultivars) denoted good discrimination ability of the marker. Based on this analysis, 3/9 (33.3 %) of the markers showed good discrimination and 6/9(66.6 %) showed poor discrimination ability between the two cultivar groups. The markers with good discrimination ability were associated with black tea quality ($RAPD16_{(2,200\ bp)}$ and $RAPD21_{(2,000\ bp)}$) and low temperature growth ($RAPD36_{(350\ bp)}$).

For each cultivar, the difference between the total score for markers with good discrimination ability and those with poor discrimination ability was calculated to determine the net

score of discrimination for each cultivar if a panel of these markers was used. Using a net marker discrimination score of ≥−2, it would be possible to select a total of 20/32 (62.5 %) cultivars for advancement to the next selection stage of which 17/20 (85 %) would be from the released cultivars and 3/20 (15 %) from the not-released cultivars. This implies that only 2/3 of the selections would be advanced to later stages of selection and 85 % (17/19) of these would have a high likelihood of being finally released for commercial cultivation.

Discussion

The current study considered seven traits of agronomic importance for teas produced in central Africa. For each of the seven traits, nine cultivars that had the trait and another nine that did not have the trait were screened with different RAPD primers. A number of RAPD bands were identified which showed polymorphism between the different groupings. Some of these bands segregated with the absence or presence of the trait. This showed that the specific bands could be used as markers to select for the different traits [31, 32].

After screening a total of 60 RAPD primers, it was possible to identify RAPD markers for six of the seven traits that were considered. No specific RAPD bands were found that could be associated with tolerance to *H. schoutedeni* (tea mosquito bug). Wium [19] screened 18 RAPD primers but also failed to identify a marker associated with tolerance to *Helopeltis* in tea. It is probable that the mechanisms for tolerance to insect pests are more complex to be associated with a specific dominant RAPD marker. Most of the RAPD markers identified in this study showed high association with the various traits, which was consistent with the results reported in other crops [25].

In two of the six traits, black tea quality and drought tolerance, more than one RAPD marker was identified. Combined use of more than one RAPD marker improved the discrimination efficiency between the cultivars with and without the trait. This observation was consistent with the results reported in other studies. For example, Chen and Yamaguchi [26] used four RAPD markers in order to completely discriminate 24 tea germplasms at inter-specific level, which was not possible with a single RAPD marker; Kaundun et al., [33] reported that a minimum of three RAPD markers was required to completely discriminate 27 elite accessions of tea from Korea, Japan and Taiwan. In other crops, Shalini et al., [34], who were screening for mite resistance in coconut, reported that a combination of three RAPD markers accounted for 83.86 % of mite resistance whereas the three markers used individually only accounted for 3.07, 8.5 and 72.27 % of the mite resistance. These results highlight the need to identify more markers for each trait in order to improve precision in selection.

The RAPD bands associated with tolerance to drought and *Phomopsis* and yield showed some false-positive bands. Two possible causes of this were postulated. One of these is that of erroneous classification of some of the cultivars as to the absence or presence of a particular trait, since the classification was largely based on phenotypic differences that are heavily influenced by environmental factors. Confounding effects of genes not related to the trait of interest, but which also affect the morphological markers, could also contribute to misclassification of cultivar based on their phenotype [28]. The other possible cause may involve differences in the mechanisms used by the different cultivars in exhibiting the various traits. These problems were likely to occur and are probably unavoidable, especially where the test cultivars are not from a properly designed mapping population.

Reducing the number of selections that are advanced to the next stage of selection cycle is a crucial but challenging decision facing breeders at various stages of a selection programme. Since molecular markers are amenable to use in the early stages of selection, the

RAPD markers identified in this study would therefore help the breeder to make such a decision with a high degree of objectivity and at an early stage of selection. This would allow devoting of limited resources, for long term and multi-location field evaluation, to the screening of plant material with a very high potential of being eventually released for commercial use. The markers would therefore help to improve selection precision and efficiency because they are more reliable than phenotypic markers. This would have a positive impact on the success of the breeding or selection programme through increased number of released cultivars.

Conclusion and Future Work

There are still very few molecular markers that are closely associated with important traits in tea. The RAPD markers identified in this study are therefore an important contribution towards the identification of markers that can be used in marker-assisted selection. Use of these RAPD markers could possibly help shorten the long breeding and selection cycle for tea, through targeted choice of breeding stocks and early identification of selections that possess the desirable traits. These RAPD markers avail a good starting point for developing sequence characterised amplified region markers for these traits and this would offset some of the limitations associated with RAPD markers.

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CHAPTER 4

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS ASSOCIATED WITH TOLERANCE TO WATER STRESS IN TEA

4.1 Introduction

Tea is cultivated under very diverse environmental conditions around the world and is subjected to a number of abiotic stresses which affect its production and collectively cause crop losses of up to 65% (Waheed *et al.,* 2012). These abiotic stresses include low and high temperatures, drought, frost, high incident solar radiation, water logging and low nutrient status of the soil (Mahajan & Tuteja, 2005; Kumar *et al.*, 2012; Bansal *et al.*, 2014; Chemura *et al.,* 2014). Among these, drought (soil water deficit) is a widely experienced abiotic stress in many crops. Drought conditions can arise when there is insufficient precipitation, which results in lowering of soil water to levels where plants cannot easily extract enough water to sustain normal growth activities (Cavatte *et al.,* 2012). Approximately 45% of the world's arable land is exposed to continuous or frequent droughts (Waheed *et al.,* 2012).

Tolerance to water deficit is thus a desirable trait in the tea crop that is largely grown under rain-fed conditions in most areas (Upadhyaya & Panda, 2013). This is because severe and prolonged dry periods result in large losses in yield, ranging between 14 and 33% (Niranjana & Viswanath, 2008; Muoki *et al.*, 2012; Kumar *et al.,* 2012; Waheed *et al.,* 2012). In Malawi, a severe drought in 2005 reduced the national tea production by 24%, compared to the crop realized in the previous non-drought year (ITC, 2011). As a result, development of drought tolerant cultivars is an important objective in most tea breeding and selection programmes. Selection for drought tolerance under field conditions is usually not easy because the effects of drought are often exacerbated by other abiotic stresses such as extreme temperatures, high incident solar radiation and high vapour pressure deficits (Bansal *et al.*, 2014). This will

remain a major challenge for breeders because simultaneous occurrence of several stresses is also likely to increase with changes in climate (Chemura *et al.,* 2014). This raises the need to evaluate potential tea cultivars under conditions that can allow control over some of the other stress factors that are usually concurrent with drought.

Plant response to drought can vary depending on a number of factors, including the age of the plant, severity and duration of the drought, as well as the genetic make-up of the plant (Beltrano & Ronco, 2008; Carr & Lockwoods, 2011; Cavatte *et al.,* 2012). Since these factors interact in a variety of ways, it is often difficult to separate the effects of the individual factors, especially during field evaluations. These complex interactions complicate the process of selecting drought tolerant cultivars and thus limit the speed of genetic improvement for this trait (Blum, 2005). Tea genotypes show considerable variation in their responses to soil water stress (Carr & Stephens, 1992; Ellis & Nyirenda, 1995; Kumar *et al.,* 2012), which offers a chance to select tolerant cultivars. However, selection for drought tolerance is challenged by the complex nature of drought tolerance and lack of reliable and accurate selection criteria. These factors have contributed to the slow pace of developing drought tolerant tea cultivars. This creates a need to establish and understand the different mechanisms governing drought tolerance in different tea cultivars in order to devise appropriate selection methods.

Three physiological drought response mechanisms have been reported in plants: dehydration avoidance, dehydration tolerance, and ability to survive and recover rapidly after a severe stress (Kato *et al.*, 2008; Berger *et al.,* 2010). Dehydration avoidance occurs when plants develop appropriate phenology to complete the most sensitive stages of their life cycle before the onset of water stress (Courtois *et al.;* 2000; Upadhyaya & Panda, 2004). This is most common in annual crops. Dehydration avoidance occurs when plants maintain good water

status and turgor during stress (De Costa *et al.*, 2007). This can be achieved through development of a robust root system that maximizes the plant's ability to extract soil water in times of drought (Bruce *et al.*, 2002; Cattivelli *et al.*, 2002; Chemura *et al.*, 2014). Plants can also avoid dehydration through osmotic adjustment, by accumulating solutes (osmolytes) that help the plant to maintain turgor pressure and thereby sustain growth whilst meeting the demand for transpiration. Accumulation of osmolytes or osmo-protectants minimizes cell injury even when the leaf water potential is low (Da Matta, 2004). Dehydration tolerance occurs when the plant can sustain plant functions in a dehydrated state (Blum, 2005). This type of mechanism may be observed in plants that go through a dormant stage. Survival and rapid recovery from severe water stress is very important in tea and other perennial crops that go through several droughts in their life time (Chakraborty *et al.*, 2002). Slow recovery from water deficit can shorten the main tea harvesting period, thereby leading to reduced yield. This loss in yield can have a significant impact in tea growing areas that experience long dry periods every year (Wilkie, 1996).

Mechanisms of drought tolerance in tea and other crops are generally not sufficiently understood (Carr & Stephens, 1992; Shao *et al.*, 2009). This is partly because conventional selection relies heavily on plant survival counts and estimates of growth vigour under drought. Such an approach can identify tolerant plant material but fails to elucidate the underlying mechanisms of tolerance. If the assessments are done in a particular environment, the results are also not directly transferable to other environments. The situation worsens when tolerance is assessed under natural droughts with uncontrolled duration, intensity and timing. Prolonged and severe soil water stress can also result in many morphological, physiological, and biochemical changes in plants (Cellier *et al* 1998; Chakraborty *et al.*, 2002; Upadhyaya & Panda, 2004; Cavatte *et al.*, 2012; Gupta *et al.*, 2012), which need to be

properly described and understood in order to devise better strategies for breeding and selecting drought tolerant tea cultivars in future.

Plants will generally show some morphological changes when exposed to water stress, which may include shedding of leaves to minimize water loss and improve the water status in the remaining foliage (McDowell *et al.,* 2008); extending the root system to deeper soil levels (Collins *et al.,* 2008; Shao *et al.,* 2009) and, reducing the size of leaves in order to lower the total leaf area and minimize the evapotranspiration surface area (Berger *et al.,* 2010). Some of these changes are also exhibited by tea plants under drought. For example, tea cultivars may reduce the number of shoots per plant (Cheruiyot *et al.*, 2007) or show accelerated defoliation in order to reduce leaf surface and thus water loss through transpiration (Netto *et al.,* 2010). Root mass and vertical distribution of roots in the soil are also reported to be important for tolerance to drought of tea (Nagarajah & Ratnasuriya, 1981; Niranjana & Viswanath, 2008).

Similar morphological changes have also been observed in other crops, for example coffee, cocoa and wheat plants also shed leaves to reduce transpiration (Da Matta, 2004; (Beltrano & Ronco, 2008; Carr & Lockwoods, 2011). In wheat, accelerated defoliation may also be accompanied by stem die-back (Beltrano & Ronco, 2008). Increased rooting depth has also been reported in drought tolerant clones of coffee *(*Pinheiro *et al.,* 2005; Chemura *et al.,* 2014). In rice, rooting depth has been associated with drought avoidance (Kato *et al.,* 2008). Extensive root systems are reported to be common among woody perennial species and are used to access water and nutrients in the lower soil horizons (DaMatta, 2004). A deep rooting system is especially advantageous in short-duration water stress and could help plants to survive drought conditions (Courtois *et al.,* 2000).

Physiological changes caused by water stress in tea and other crop plants include lowering of the leaf relative water content (RWC) (Cheruiyot *et al.,* 2007; Beltrano & Ronco, 2008; Upadhyaya *et al.,* 2012); increased accumulation of osmotically active solutes (Blum, 2005); reduced rates of transpiration and photosynthesis (Squire, 1978; Hajra & Kumar, 1999; De Costa *et al.,* 2007) and changes in leaf diffusion resistance (Sandam *et al.,* 1981). Reduced leaf or shoot growth may be a result of reduced cell expansion due to loss of turgor pressure in the leaves under water stress (Shao *et al.,* 2009). In rice, relative growth rate under water stressed conditions has been identified as a reliable criterion for assessing drought tolerance during the vegetative growth stage of the plant (Kato *et al.,* 2008).

A reduction in RWC affects cellular solute concentration, which has a negative impact on cell homeostasis and physiological activities. Low RWC can lead to a reduction in photosynthesis as the plants may close the leaf stomata in order to minimize water loss and increase water use efficiency (Wang *et al.,* 2013). Stomatal closure inevitably reduces CO_2 diffusion into the leaf and thereby limits photosynthesis and growth. Plants that maintain high RWC under drought conditions can prevent cell desiccation. Such plants can use osmotic adjustment mechanisms to reduce water loss and keep leaf potentials high when subjected to water stress (Genga *et al.,* 2011). Maintaining high RWC during water stress has been associated with water stress tolerance among tea cultivars (Upadhyaya & Panda, 2013). In some plants it has also been demonstrated that reduction in RWC could be related to osmotic stress (Perez-Perez, *et al.,* 2009). For example, in almond, low RWC induced stomatal closure, which resulted in low CO_2 supply to the mesophyll cells, thereby reducing photosynthesis and causing changes in chlorophyll fluorescence parameters (Yadollahia *et al.,* 2011). In olive trees, low RWC was observed in leaves subjected to severe water stress (Boussadia *et al.,* 2008).

Water stress can also alter different biochemical processes in plants. For instance, synthesis and accumulation of compatible solutes such as proline may increase in plants subjected to water stress (Bary, 1997; Xiong *et al.,* 2002; Shao *et al.,* 2009). In tea, levels of proline were reportedly higher in drought tolerant than in drought susceptible cultivars under water stress and this was correlated to the degree of drought tolerance (Handique & Manivel, 1990; Chakraborty *et al.,* 2002). Phenolic content and activities of some enzymes e.g. phenylalanine-ammonia-lyase (PAL), polyphenoloxidase (PPO) and peroxidase in tea plants display an initial increase, but decrease if drought stress is prolonged (Chakraborty *et al.,* 2002; Upadhyaya *et al.,* 2012). This suggests that levels of polyphenols may be used to monitor the degree of drought tolerance amongst tea cultivars. As with many stresses, water stress induces production of reactive oxygen species (ROS) such as superoxide, hydroxyl, hydroperoxide and alkoxyl radicals, and hydrogen peroxide that cause oxidative cell damage (Upadhyaya & Panda, 2004). As a result, production of antioxidants that counter oxidative stress caused by increased levels of ROS can be used as a measure of tolerance to drought stress. Upadhyaya *et al.* (2012) reported that the levels of some antioxidants, e.g. glutathione and ascorbate decreased as water stress progressed. It was argued by these authors that the lowering of these antioxidants was due to their use in countering the high levels of ROS and thus reducing the oxidative stress on the plants. Reduced water uptake under soil water deficit causes cellular water deficit that in turn increases the cellular solute concentration and changes in cell volume and membrane shape (Bary, 1997).

Development of tolerant crop cultivars shall remain an important objective of most crop breeding and selection programmes. However, selection of tolerant cultivars is greatly hampered by lack of reliable and effective criteria, because some of the mechanisms of tolerance have not been fully established. Water stress causes a number of physiological and biochemical changes. Such changes show significant differences between cultivars, which

therefore offer an opportunity for developing indices that can be used in selection and also help to elucidate the mechanisms of tolerance. Recognizing the big influence of genotype on plant response to water stress, the current experiment investigated the effect of water stress on some of the physiological and biochemical changes that occur under drought in order to develop appropriate methods for selecting drought tolerant tea cultivars.

4.1.1 Null Hypothesis

There will be no statistically significant differences (P>0.05) in physiological and biochemical characteristics 1 – 6, between drought tolerant and drought susceptible tea cultivars when exposed to different levels of water stress.

1) *relative water content*
2) *rate of photosynthesis,*
3) *stomata conductance,*
4) *transpiration,*
5) *content of total polyphenols, and*
6) *antioxidants*

4.1.2 Objective

To establish and quantify physiological and/or biochemical characteristics that can associate with tolerance to soil water stress in tea cultivars, that were chosen based on historical information on their response to drought in the field, in order to devise better screening methods for drought tolerance in tea.

4.2 Materials and methods

4.2.1 Tea cultivars

Ten tea cultivars were selected for this study based on historical classification of drought response. Five of these cultivars were classified as having the drought tolerance trait (+) and the other five cultivars lacked the trait (-) (Table 4.1).

TABLE 4.1 Parentage and rankings of drought response of tea cultivars used in the drought tolerance study

Cultivar	Parentage	Rank for trait*
PC168	PC1 x K6/8	+
PC175	CL12 x PC1	+
PC185	MT12 x SFS150	+
PC268	PC1 x SFS150	+
NVS10	Open pollination	+
PC1	M9 x CL17	-
PC80	C5 x CL12	-
PC108	PC1 x SFS204	-
PC110	PC1 x SFS204	-
SFS204	Open pollination	-

***Note: (+) denotes presence and (-) absence of a trait**

Plants of each cultivar (Table 4.1) were raised from stem cuttings that were rooted in small plastic bags (with a volume of about 200 cm^3), filled with an acidic sub-soil [soil pH 4.5 in $CaCl_2$] with a loamy texture and low in organic matter, which is ideal for rooting of tea cuttings. The sub-soil was collected from a site that had previously been tested for soil pH and texture that is ideal for rooting of tea cuttings. The cuttings were kept under plastic tunnels that were constructed in a nursery with an overhead grass-thatch shade, which only allowed 20-25% of sunlight through until all the cuttings had developed roots. The rooted plants were hardened-off and kept in the open in a nursery that had a grass overhead shade that allowed about 60% of sunlight through. The hardened-off plants were later graded according to plant height and growth vigour and the best plants of each cultivar were transplanted into 20 L plastic bags filled with top soil of loamy texture. The plants were maintained in the nursery following recommended tea nursery management practices, which mainly included fertilizer application, watering and weeding (TRFCA, 1990). The plants were about two years old when they were transferred to the rain shelter where water stress (drought) treatments were imposed (Figure 4.1)

A: Rooted tea plants in small (0.2 L) plastic bags

B: Grown-up tea plants in big (20 L) plastic bags

FIGURE 4.1: Rooted and grown-up tea plants in small and big plastic bags.

4.2.2 Soil water stress treatments

All plants were watered to field capacity on the first day of the experiment by applying 2 L of water, which was established prior to the start of the experiment to be adequate for the size of the volume and type of soil used. The experiment had a split-plot design, arranged in randomised complete blocks. Water stress treatments (W 1 – W 3) were the main-plot factors and the tea cultivars (10 levels) were the sub-plot factors. Each treatment combination had three replications with a plot size of six plants for each cultivar in each replicate. The plot layout is shown in Figure 4.2

REP 1	W 2										W 1										W 3									
	PC 185	PC 268	PC 168	PC 110	PC 175	SFS 204	PC 80	PC 108	PC 1	NVS 10	PC 110	PC 168	NVS 10	PC 108	SFS 204	PC 268	PC 185	PC 80	PC 1	PC 175	PC 80	PC 168	PC 175	PC 268	PC 110	NVS 10	PC 185	PC 1	PC 108	SFS 204
REP 2	W 3										W 1										W 2									
	PC 168	PC 268	PC 80	SFS 204	PC 185	PC 1	PC 108	PC 175	PC 110	NVS 10	PC 268	PC 80	PC 185	NVS 10	SFS 204	PC 108	PC 175	PC 168	PC 1	PC 110	PC 80	PC 268	PC 168	SFS 204	PC 175	PC 110	PC 108	NVS 10	PC 185	PC 1
REP 3	W 1										W 2										W 3									
	PC 268	PC 108	PC 80	NVS 10	PC 110	PC 1	PC 168	PC 185	PC 175	SFS 204	PC 268	PC 108	PC 110	PC 80	SFS 204	PC 175	PC 185	PC 168	PC 1	NVS 10	PC 168	NVS 10	SFS 204	PC 1	PC 268	PC 110	PC 80	PC 108	PC 175	PC 185

FIGURE 4.2: Layout of plots in the drought tolerance experiment under a rain shelter at Mimosa Tea Research Station in Malawi. W1 - denotes plants that were watered regularly (not stressed), W2 and W3 denote plants that were water stressed for 4 and 8 days, respectively.

The water stress treatments were created by withholding water from the plants for set intervals as follows:

W1: plants were watered regularly to prevent any form of water stress (control plants)

W2: watering was withheld for 4 days

W3: watering was withheld for 8 days

Preliminary observations made on the same age of tea plants in the same pots and soil showed that water stress could start to show as early as four days after withholding water. It was then concluded that extending the stress period beyond 10 days could result in the death of some plants, particularly for the susceptible cultivars. Based on these observations, the water stress treatments could not be imposed for very long periods in addition to the very high temperatures that were experienced at the time of the experiment, which exacerbated the effects of water stress. It is likely that the high temperatures contributed to the quick drying of the soil in the relatively small plastic bags. At the end of each stress period, plants in groups W2 and W3 were re-watered by applying 2 L of water to each plant. Assessments for recovery from water stress were done between one and eight days after re-watering the plants.

4.2.3 Physiological assessments

Wilting: Plants under each water stress treatment were visually assessed for degree of loss of leaf turgidity (wilting) once every three days. Degree of wilting was quantified by using a score on a scale of 1 to 10. A wilting score of 1 was assigned to plants that showed no sign of wilting (leaves were fully turgid), whereas a score of 10 was assigned to plants that were severely wilted. Each of the six plants in a plot was assessed and an average score for each

plot was calculated which represented the wilting score for each treatment combination in each replicate. Although soil water content in individual pots was not measured, the use of an average of six plants in each replicate for the wilting assessment helped to even out the level of stress among the replicates. The wilting assessments were done between 14:00 and 15:00 local Malawi time, by two persons in order to reduce the levels of subjectivity in scoring. During the recovery period, wilting was assessed between 06:00 and 08:00 in order to identify plants that had reached the critical wilting point.

Leaf relative water content (RWC) was determined at 0, 4 and 8 days after withholding water following the procedure of Cheruiyot *et al.,* (2007). One leaf was taken from a randomly selected plant in each plot of the different treatment combinations in each of the three replicates, giving a total of three leaves per treatment. Samples of the third leaf below the apical growing point of the plant were collected and immediately weighed to determine the fresh mass (Fm). Each leaf was cut in half, floated in distilled water and placed in an environmentally controlled chamber set at 4°C for 24 hours. After the 24 hours, surface moisture was wiped off the leaf surface with a soft tissue paper and the leaves were weighed to determine the turgid mass (Tm). The turgid leaves were thereafter dried in an oven set at 70°C for 48 hours to determine the dry mass (Dm). RWC was calculated using the formula described by Cheruiyot, *et al* (2007) as shown in equation 4.1:

$$RWC = \frac{(Fm-Dm)}{(Tm-Dm)} * 100 \qquad (4.1)$$

Leaf gas exchange measurements: Photosynthesis rate (Ps), transpiration rate (Tr), and stomata conductance (g_s) were measured between 09:00 and 11:00 using a LI-6400XT

photosynthesis system (Li-Cor, Inc. Lincoln, Nebraska, USA). Measurements were made on the third leaf below the apical bud of one randomly selected plant in each plot in all three of the replicates.

Chlorophyll fluorescence parameters initial fluorescence (F_0), maximum fluorescence (F_m) and variable fluorescence (Fv), ($Fv = F_m - F_0$) were measured using a Plant Efficiency Analyser (Hansatech, Norfolk, UK), following the procedure described by Mphangwe and Nyirenda, (1997). The measurements were done on the third leaf below the apical bud on three plants that were randomly selected in each plot in all three the replicates (9 leaves per treatment per cultivar). The selected leaves were first dark-adapted for 60 minutes, using a leaf clip that was attached to the leaf before taking the measurements.

4.2.4 Biochemical assessments

One leaf was collected from each of the two randomly selected plants in each plot of the different cultivars under the three water stress treatments for use in biochemical analyses for total polyphenols and antioxidant capacity. The sampled leaves were packed in clearly labelled zip-lock plastic bags and frozen in liquid nitrogen within a few minutes after sampling. The frozen samples were packed in paper envelopes and transported to the College of Medicine in Blantyre, Malawi for temporary storage in a -80°C freezer until the time when they were taken to the University of Pretoria in a cooler box lined with ice-bricks that had been kept in a -80°C freezer for 48h in order to minimize thawing of the frozen samples. At University of Pretoria, the samples were kept at -20°C until the time of analysis. Each sample was analysed for total polyphenols and total antioxidant capacity as described below.

Total polyphenol content (TPC): The leaves in each sample packet were first crushed by hand into small pieces before weighing out a 20 mg sub-sample that was used to extract the

polyphenols. The initial crushing of the samples helped to homogenize the sample before taking a more representative sub-sample for extraction of polyphenols. Extraction of total polyphenol from each sample was done in 2 mL tubes with screw-on caps. A ceramic bead was put at the bottom of the tube and another one on top of the sample in the tube, following which samples were homogenized on a Fast Prep Instrument FP120 (QBiogene, Carlsbad, CA, USA) at a spinning speed of 4 ms^{-1} for 40 s. The beads were carefully rolled out of the tubes and 600 µl of aqueous methanol (70%, v/v) (Sigma-Aldrich, Germany) that had been incubated in a 70°C water bath was added to each tube, whereafter the tube was closed with a screw-on cap. The samples were briefly mixed on a vortex mixer and incubated in a water bath for 10 min at 70°C. This was followed by centrifugation at 7,500 x g for 10 min. The supernatant was carefully decanted into a 1.5 mL tube. Extraction was repeated by adding another 600 µL of aqueous methanol (70% v/v) to each sample and placing the tubes in the water bath for a further 10 min. The supernatant was again carefully decanted and mixed with the first supernatant. The volume of the supernatant was topped up to 1.2 mL using aqueous methanol. Total polyphenol content of the sample extracts was determined following the Folin-Ciocalteu method as described in International Standard Organization (ISO) discussion document for tea (ISO TC 34/SC8- ISO14502-1, 2003) with some modifications on mass of sample and volume of the extracting solvents. The reaction mixture was put in NUC 96-well flat bottom plates (NUC, Denmark). A total of 100 µL of the Folin-Ciocalteu Phenol reagent, diluted 10 times with triple distilled water, was first added to each well, followed by 20 µL of sample extract (diluted 100 times with triple distilled water). The plate was incubated for 10 min at room temperature before adding 80 µL of 7.5% (w/v) anhydrous sodium carbonate (Na_2CO_3) (Sigma-Aldrich, Germany). The plates were thereafter wrapped in aluminium foil and incubated at room temperature for 60 min. Gallic acid monohydrate (3,4,5-trihydroxybenzoic acid) was used as the standard phenolic

compound. Absorbance of the sample extracts was read on a Multiskan Ascent V1.24 Elisa plate reader (Amersham) at 690 nm. A calibration standard curve was generated using absorbance readings of Gallic acid standard concentrations of 0, 0.01, 0.02, 0.03, 0.04 and 0.05 µg/mL. Gallic acid was chosen as a standard because it is a simple phenol (three hydroxyl groups) that is pure, stable and not very expensive. Several previous studies using the Folin-Cioclateu assay have also used gallic acid as a standard and this would make it easy to compare results from the current study with those from previous investigations (Stratil, *et al.*, 2006). Percentage total polyphenol content (% TPC) was calculated as shown in equation 4.2:

$$\%TPC = \frac{(\mathrm{A}-\mathrm{I})*V*DF*100}{\mathrm{m}*\mathrm{M}*10000*\mathrm{DM}} \qquad (4.2)$$

Where:

A = average absorbance,

V = volume (mL) of extract used in the determination of TPC,

DF = dilution factor for the extract,

I = intercept from the standard curve,

m = is the slope,

M = mass (g) of the sample used in the extraction and

DM = dry matter content (%) of the sample as percentage of the mass

An example of the calibration curve for calculation of %TPC is presented in Appendix 4.1.

Total antioxidants (FRAP): A sample extract prepared following the same procedures as described for the TPC assay was used in this analysis. The FRAP method described by Griffin and Bhagooli (2004) was followed with modifications on sample mass, extraction

volume and wavelength for the absorbance readings. A total of 150 µL of the FRAP reagent (mixture of 300 mM sodium acetate, pH 3.6), 10 mM of 2,4,6 Tris-(hyrdoxymethyl)-aminomethane-2puridyl-s-triazine (TPTZ) (Sigma-Aldrich Germany) dissolved in 40 mM hydrochloric acid (HCl) and 20 mM iron(III) chloride ($FeCl_3$) in a 10:1:1 ratio v/v/v) was added to each well. An absorbance reading was taken at 595 nm on a Multiskan Ascent V1.24 micro plate reader (Amersham), which was followed by the addition of 20 µL sample extract or standard solution to each well. The plates were incubated for 30 min at 37°C and absorbance was read again at 595 nm. Change in absorbance, determined as the difference in absorbance between the first and second reading, was calculated for each sample as a FRAP value. A Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) calibration curve was constructed by plotting absorbance against concentration (µmol) using the following concentrations: 0, 50, 100, 200, 400 and 600 µmol L^{-1}. Trolox is commonly used as a standard for FRAP, which gives better results than other methods of determining antioxidant capacity. Trolox is not ideal for the Folin-Ciocalteu method because it is relatively less reactive and shows low absorbance values compared to Gallic acid (Stratil, *et al.*, 2006). An example of the calibration curve is shown in Appendix 4.1. The total FRAP for each sample was expressed as µmol Trolox g^{-1} dry mass of tea. Each sample had three replications (plate wells). The analysis was independently repeated at least two times.

4.2.5 Drought Susceptibility Index (DSI)

A drought susceptibility index (DSI) was calculated based on data of photosynthesis rate, stomatal conductance, transpiration and relative water content for non-stressed and stressed plants. The DSI calculations were performed using the equation of Fischer and Maurer (1978) as described by Damayanthi *et al.*, (2010) which is shown in equation 4.3:

$$DSI = \frac{[1-Y/Yp]}{[1-X/Xp]} \quad (4.3)$$

Where:

Yp = value of parameter (e.g. photosynthesis) under no stress,

Y = value of parameter (e.g. photosynthesis) under stress,

X = average of parameter over all cultivars under stressed conditions, and

Xp = average of a parameter over all cultivars under non-stressed conditions.

The lower the DSI value, the more tolerant the cultivar and *vice versa*

4.2.6 Data analysis

Data was first tested for normality and later subjected to uni-variate analysis of variance using the Genstat statistical software, 14th Edition. Comparison of means between the tolerant and susceptible tea cultivar classes was done using the unpaired, two tailed Student's t-test in order to detect whether there were significant differences between the tolerant and susceptible tea cultivar classes using the various parameters. Multi-variate analysis of variance, which involved principal component analysis (PCA), discriminant analysis and fitting of the Logit model, was performed with JMP software, 11th Edition (Sall *et al.*, 2012). A stepwise discriminant variable selection procedure of fitting variables into the model was followed in order to find a combination of factors that could be used to correctly distinguish the tolerant from the susceptible cultivars.

4.3 Results

4.3.1 Physiological parameters during stress

Wilting occurs as a result of loss of turgor in the leaves due to a drop in cell water potential and therefore the degree of wilting can be used to screen for tolerance to drought (Ellis & Nyirenda 1995). Assessments of wilting scores averaged over non-stressed and stressed

plants done at four days of water stress (4 DWS) showed significant (P = 0.049) differences between non-stressed (W 1) and stressed (W 2 and W 3) plants (data not shown). Non-stressed plants had a mean wilting score of 1.18 compared to 2.58 for plants that had been water-stressed for 4 days (4 DWS), representing a two-fold increase in wilting score. However, there were no statistically significant differences (p = 0.6234) in wilting scores between the drought tolerant and susceptible cultivars at 4 DWS (Table 4.2), although susceptible cultivars were expected to be more wilted than the tolerant cultivars.

TABLE 4.2: Mean wilting score for drought tolerant and susceptible cultivars after 4 days of water stress (W2) conditions

Class	Cultivar	Wilting score
Tolerant	PC 168	2.9
Tolerant	PC 175	4.1
Tolerant	PC 185	1.9
Tolerant	PC 268	3.0
Tolerant	NVS 10	1.7
Mean		*2.72*
Susceptible	PC 1	2.6
Susceptible	PC 80	3.1
Susceptible	PC 108	1.8
Susceptible	PC 110	2.9
Susceptible	SFS 204	1.9
Mean		*2.46*
T-test (P)		0.6234

Relative water content (RWC) averaged over non-stressed and stressed plants at four and eight days of water stress (W2 and W3) showed significant differences (P< 0.05) between the water stress treatments (Table 4.3). Plants that were not stressed (W1) registered higher average relative water contents than the stressed plants at four and eight days.

TABLE 4.3: Mean relative water content (RWC) for non-stressed and stressed plants of different tea cultivars at four and eight days of water stress (DWS)

Treatment	Non-stressed	Stressed	T-test (P)
%RWC at 4 DWS	81.5	78.1	0.0286
%RWC at 8 DWS	84.1	75.8	0.0014

RWC showed no significant differences between the tea cultivar classes, both at 4 DWS (p = 0.1296) and at 8 DWS (p = 0.8411) (Table 4.4).

TABLE 4.4: Mean relative water content (RWC) for drought tolerant and drought susceptible cultivars at four and eight days of water stress (W2 and W3)

Class	Cultivar	%RWC -W2	%RWC - W3
Tolerant	PC 168	84.7	72.1
Tolerant	PC 175	83.0	83.1
Tolerant	PC 185	81.3	81.2
Tolerant	PC 268	83.2	84.2
Tolerant	NVS 10	83.0	77.9
Mean		*83.0*	*79.7*
Susceptible	PC 1	79.1	83.0
Susceptible	PC 80	83.6	77.6
Susceptible	PC 108	81.1	81.3
Susceptible	PC 110	82.2	80.4
Susceptible	SFS 204	75.3	78.7
Mean		80.3	*80.2*
T-test (P)		0.1296	0.8411

Leaf gas exchange and fluorescence measurements

Photosynthesis measurements showed no significant interaction effects between water-stress and cultivar classes for all the parameters measured. There were also no significant differences between the tea cultivar classes in terms of stomatal conductance (g_s), rate of photosynthesis (Ps) and transpiration (Tr) at 4 DWS (Table 4.5).

TABLE 4.5: Leaf gas exchange parameters for different cultivars after four days of water stress (W2)

Class	Cultivar	Conductance *(mol H_2O m^{-2} s^{-1});*	Photosynthesis *(µmol CO_2 m^{-2} s^{-1})*	Transpiration *(mmol H_2O m^{-2} s^{-1})*
Tolerant	PC 168	0.068	4.64	4.17
Tolerant	PC 175	0.048	4.26	3.04
Tolerant	PC 185	0.068	5.43	4.11
Tolerant	PC 268	0.089	6.63	5.34
Tolerant	NVS 10	0.054	3.91	3.27
Mean		*0.0654*	*4.974*	*3.968*
Susceptible	PC 1	0.059	4.28	3.63
Susceptible	PC 80	0.041	3.82	2.75
Susceptible	PC 108	0.063	5.31	3.89
Susceptible	PC 110	0.091	6.30	5.23
Susceptible	SFS 204	0.098	6.58	5.61
Mean		*0.704*	*5.258*	*4.222*
T-test (P)		0.7061	0.7063	0.7327

The maximum quantum efficiency of PSII (Fv/F_m ratio) measured using the PEA showed no significant differences between tea cultivar classes at 4 DWS ($p = 0.882$) and at 8 DWS ($p = 0.6251$) (Table 4.6).

TABLE 4.6: Maximum quantum efficiency of PSII (Fv/F_m) for different cultivar classes after four (W2) and eight (W3) days of water stress

Class	Cultivar	Fv/F_m – W 2	Fv/F_m - W 3
Tolerant	PC 168	0.71	0.73
Tolerant	PC 175	0.74	0.78
Tolerant	PC 185	0.77	0.78
Tolerant	PC 268	0.75	0.78
Tolerant	NVS 10	0.75	0.77
Mean		*0.744*	*0.768*
Susceptible	PC 1	0.76	0.76
Susceptible	PC 80	0.71	0.74
Susceptible	PC 108	0.73	0.77
Susceptible	PC 110	0.76	0.76
Susceptible	SFS 204	0.75	0.78
Mean		*0.742*	*0.762*
T-test (P)		0.8882	0.6251

4.3.2 Physiological parameters during recovery from water stress

Recovery from drought was assessed at the end of the stress period, after the plants had been re-watered, by looking at some of the parameters that were assessed during the stress period. For the plants that had been stressed for 4 days (W2) and re-watered, stomatal conductance, photosynthesis and transpiration rate measured at eight days after re-watering the plants (8 DAR) showed no significant differences between tea cultivar classes (Table 4.7). There were, however, notable differences among cultivars within the two classes.

TABLE 4.7: Leaf gas exchange parameters measured on different cultivars at eight days after re-watering the plants (8 DAR) that were subjected to 4 DWS

Class	Cultivar	Conductance ($mol.H_2O.m^{-2}s^{-1}$)	Photosynthesis ($\mu mol.\ CO_2.\ m^{-2}\ s^{-1}$)	Transpiration ($mmol.H_2O.m^{-2}s^{-1}$)
Tolerant	PC 168	0.062	4.10	4.49
Tolerant	PC 175	0.057	3.94	4.18
Tolerant	PC 185	0.050	3.70	3.70
Tolerant	PC 268	0.075	4.99	5.16
Tolerant	NVS 10	0.040	3.21	3.04
Mean		*0.0568*	*3.988*	*4.11*
Susceptible	PC 1	0.050	3.79	3.76
Susceptible	PC 80	0.045	3.72	3.37
Susceptible	PC 108	0.047	4.37	3.43
Susceptible	PC 110	0.109	6.67	6.81
Susceptible	SFS 204	0.073	5.22	5.06
Mean		*0.0648*	*4.754*	*4.486*
T-test (P)		0.5755	0.2636	0.6319

Wilting score of plants that had been re-watered after eight days of water-stress, showed significant differences between the non-stressed (W1) and stressed (W3) plants at 3 days after re-watering the plants (3 DAR) (Figure 4.3). However, there were no significant differences between the tolerant and susceptible cultivar classes within the two water stress regimes (W1 and W3).

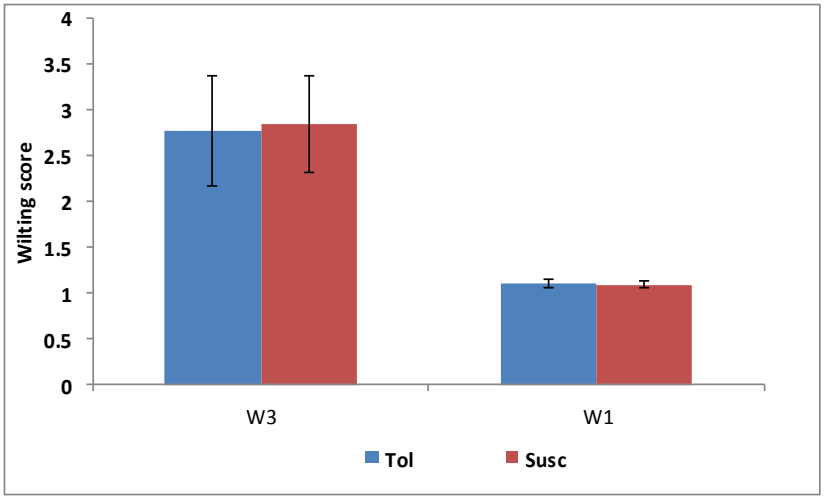

FIGURE 4.3: Mean wilting scores for drought tolerant (Tol) and susceptible (Susc) tea cultivars at three days after re-watering (3 DAR) for non-stressed (W 1) and plants that had been stressed for eight days (W 3). A wilting score of 1 was assigned to plants that showed no sign of wilting (leaves were fully turgid) whereas a score of 10 was assigned to plants that had severely wilted. The error bars represent standard error of the means, n = 5.

Chlorophyll measurements taken at 4 DAR on plants that had been subjected to 8 DWS showed no significant differences ($p = 0.9062$) between the tolerant and susceptible cultivars (Figure 4.4.). The mean chlorophyll content was 51.1 and 47.8 SPAD units for the tolerant and susceptible cultivars, respectively. The drought tolerant cultivars had relatively more chlorophyll per leaf than the drought susceptible cultivars at 4 DAR, but these differences were not statistically significant.

Relative water content (RWC) of plants that had been under water stress for eight days and then re-watered showed no significant differences between the tolerant and susceptible tea cultivars at five days after re-watering (5 DAR) (Figure 4.5). The RWC of the tolerant and

susceptible cultivars was very similar, both for the control plants (W1) and the plants that had been water stressed for eight days (W3).

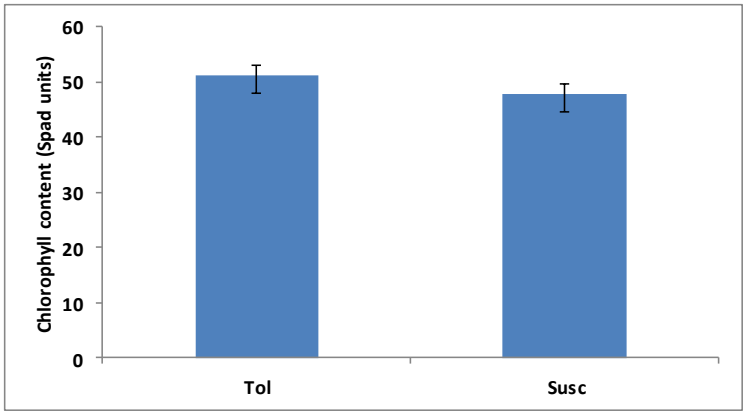

FIGURE 4.4: Mean chlorophyll content for drought tolerant (Tol) and susceptible (Susc) tea cultivars at four days after re-watering (4 DAR) of plants that had been water-stressed for eight days. The error bars represent standard error of the means, n = 5.

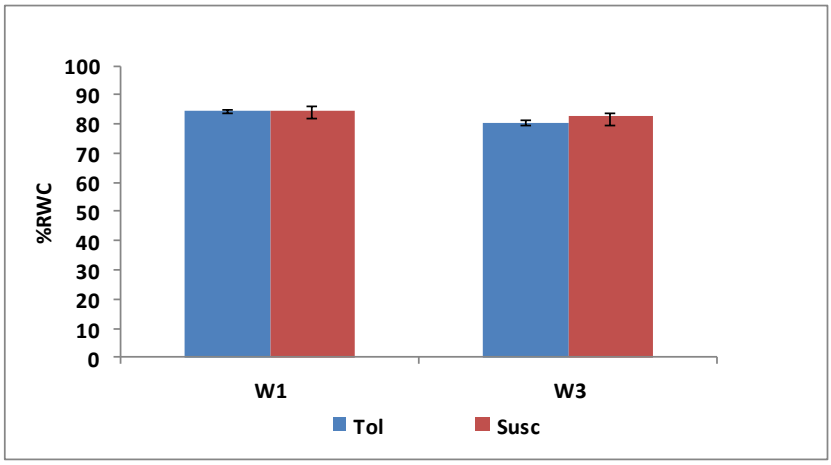

FIGURE 4.5: Relative water content (RWC) for tea leaves of drought tolerant (Tol) and susceptible (Susc) tea cultivars at 5 DAR for plants that were watered regularly (W1) and those that were water-stressed for eight days (W3). Error bars represent standard error of the means, n = 5.

4.3.3 Biochemical parameters during stress

Leaf chlorophyll content measured at the beginning and at the end of the experiment, using a Minolta SPAD 502 chlorophyll meter (Minolta, Japan), showed no significant differences ($p = 0.6385$) between the two tea cultivar classes at 4 DWS (Table 4.8).

TABLE 4.8: Mean leaf chlorophyll content (Spad units) for drought tolerant and susceptible tea cultivars after four days of water stress (W2)

Class	Cultivar	Spad units – W2
Tolerant	PC 168	47.63
Tolerant	PC 175	47.23
Tolerant	PC 185	50.36
Tolerant	PC 268	47.92
Tolerant	NVS 10	50.44
Mean		*48.716*
Susceptible	PC 1	45.99
Susceptible	PC 80	48.64
Susceptible	PC 108	51.16
Susceptible	PC 110	45.01
Susceptible	SFS 204	49.51
Mean		*48.062*
T-test (P)		0.6385

Total polyphenol content (TPC) at 4 DWS and 8 DWS showed no significant differences between the tea cultivar classes (Table 4.9).

Total antioxidant activity measured using the FRAP method showed no significant differences between the tea cultivar classes at 0 DWS and 4 DWS (Table 4.9). However, there was a general increase in antioxidant activity at 4 DWS in both classes of tea cultivars.

TABLE 4.9: Total polyphenol content (%TPC) and antioxidant activity (FRAP) for different tea cultivars at different periods of water stress

Class	Cultivar	%TPC at 4 DWS	%TPC at 8 DWS	FRAP 0 DWS	FRAP 4 DWS
Tolerant	PC 168	23.19	24.07	10257	10302
Tolerant	PC 175	20.62	20.40	8935	9178
Tolerant	PC 185	22.17	23.19	8459	10702
Tolerant	PC 268	20.41	18.89	9095	8127
Tolerant	NVS 10	20.33	19.09	7704	8485
Mean		*21.344*	*21.128*	*8890*	*9359*
Susceptible	PC 1	21.20	23.50	8789	9805
Susceptible	PC 80	19.52	21.06	9366	8832
Susceptible	PC 108	23.82	23.15	8079	9590
Susceptible	PC 110	19.81	18.87	8132	9484
Susceptible	SFS 204	21.40	21.26	7389	10402
Mean		*21.15*	*21.57*	*8351.00*	*9622.60*
T-test (P)		0.8442	0.7534	0.3465	0.6550

4.3.4 Drought Susceptibility Index

Some physiological or biochemical parameters can be used to develop indices for use in cultivar selection programmes. The data on photosynthesis rate that was recorded at 8 DAR was used to calculate the DSI for the cultivars used in the current study. The results showed no significant differences in DSI between the drought tolerant and susceptible tea cultivar classes (Table 4.10).

TABLE 4.10: Drought susceptibility Index (DSI) for different cultivars based on photosynthesis rate at eight days after re-watering

Class	Cultivar	Y	Yp	X	Xp	DSI
Tolerant	PC 168	4.36	3.16	4.25	3.90	0.92
Tolerant	PC 175	2.42	3.38	4.25	3.90	0.70
Tolerant	PC 185	2.86	4.90	4.25	3.90	0.78
Tolerant	PC 268	4.73	6.31	4.25	3.90	0.95
Tolerant	NVS 10	3.72	3.09	4.25	3.90	0.88
Mean		*3.618*	*4.168*	*4.25*	*3.90*	*0.846*
Susceptible	PC 1	4.02	5.02	4.25	3.90	0.90
Susceptible	PC 80	3.83	3.78	4.25	3.90	0.89
Susceptible	PC 108	2.37	3.74	4.25	3.90	0.69
Susceptible	PC 110	5.95	4.07	4.25	3.90	1.00
Susceptible	SFS 204	4.69	5.01	4.25	3.90	0.94
Mean		*4.172*	*4.324*	*4.25*	*3.90*	*0.884*
T-test (P)		0.4707	0.8297			0.6016

Note: *DSI = [1-Y/Yp]/[1-X/Xp] where Yp = the photosynthesis under no stress, Y = photosynthesis under stress, X = average photosynthesis over all cultivars under stressed conditions, and Xp = average photosynthesis over all cultivars under non-stressed conditions.*

4.3.4 Multivariate analysis

Data of various parameters that was collected at different time points during the stress and recovery periods was used in discriminant analysis in order to identify a parameter or a combination of parameters that can be used to distinguish the drought tolerant from the drought susceptible cultivars. This was done after the uni-variate analysis of variance showed

that none of the measured parameters on its own could be used to distinguish the tolerant and susceptible cultivars. However, trends of differences were observed within and between cultivar groups and this justified the need to conduct multivariate analysis.

At the start of the experiment (0 DWS), chlorophyll content (SPAD units), total polyphenols, (%TPC), antioxidant potential (FRAP) and fluorescence ratio (Fv/F_m) could not distinguish the drought tolerant from the susceptible cultivars with certainty, but chlorophyll content (SPAD units) showed a marginal relationship ($p = 0.0793$) with cultivar response to water stress. Discriminant analysis using a combination of all these parameters was able to correctly group 70% of the multivariate observations into the historical tolerant or susceptible classes (Figure 4.6). These initial measurements also showed the high level of inherent cultivar variation within the classes and overlaps between the cultivar classes.

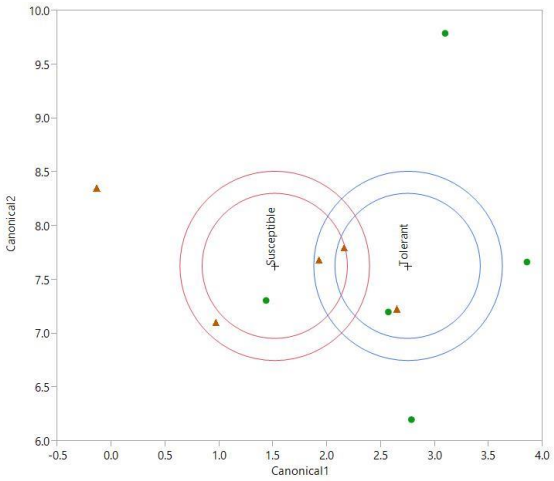

FIGURE 4.6: Canonical plots for drought tolerant and susceptible cultivars using mean values for tolerant and susceptible tea cultivars, based on chlorophyll content (SPAD units), total polyphenols, (%TPC), antioxidant potential (FRAP) and fluorescence ratio (Fv/F_m) measured at the start of the experiment. The green dots represent tolerant cultivars and brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

At 4 DWS, a combination of seven parameters (RWC, Fv/F_m, %TPC, FRAP, Ps, g_s, Tr and Ci/Ca), correctly grouped all the tested cultivars into their historical groups of tolerant or susceptible cultivars (Figure 4.7). When fitting the model with the five easy to measure parameters: RWC, %TPC, FRAP, Maximum quantum efficiency of PSII (Fv/F_m) and chlorophyll content (SPAD units), only 80% of the multivariate observations could be correctly associated with the historical tolerant or susceptible classes of the cultivars.

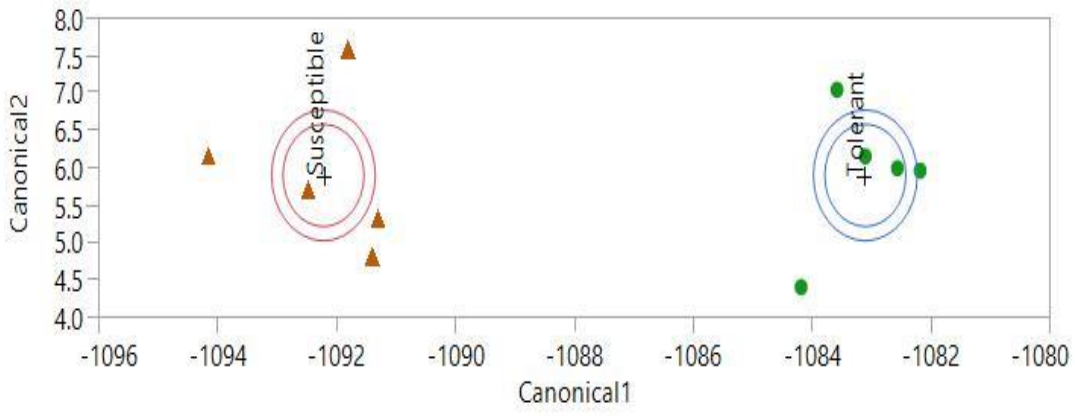

FIGURE 4.7: Canonical plots for drought tolerant and susceptible cultivars using mean values for cultivars on RWC, Fv/F_m, %TPC, FRAP, Ps, g_s, Tr and Ci/Ca) measured after four days of water stress. The green dots represent tolerant cultivars and brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

At 8 DWS, 90% of the multivariate observations of eight parameters were correctly grouped into tolerant or susceptible classes, (Figure 4.8).

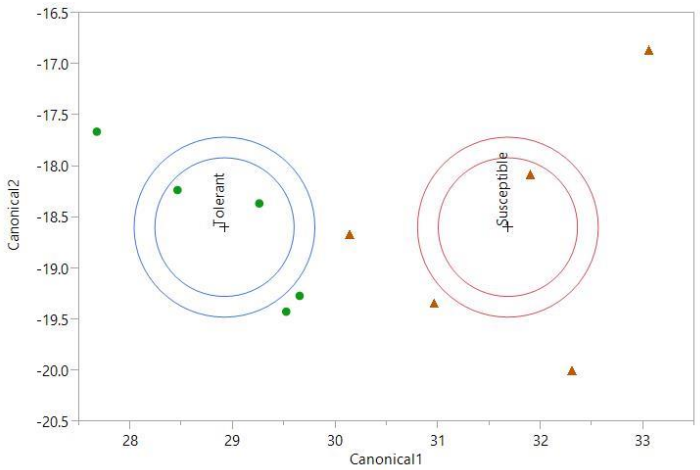

FIGURE 4.8: Canonical plots for distinguishing drought tolerant and susceptible cultivars by discriminant analysis using mean values for RWC, Fv/F_m, %TPC, FRAP, Ps, g_s, Tr and Ci/Ca) after eight days of water stress. The green dots represent tolerant cultivars and the brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

Six days after re-watering (6 DAR) the stressed plants, data collected on RWC, Fv/F_m, %TPC, FRAP, Ps, g_s, Tr and Ci/Ca) could still be used to correctly classify the cultivars into tolerant or susceptible groups, both for plants that had been subjected to 4 DWS (90%) and 8 DWS (100%) (Figure 4.9 A & B).

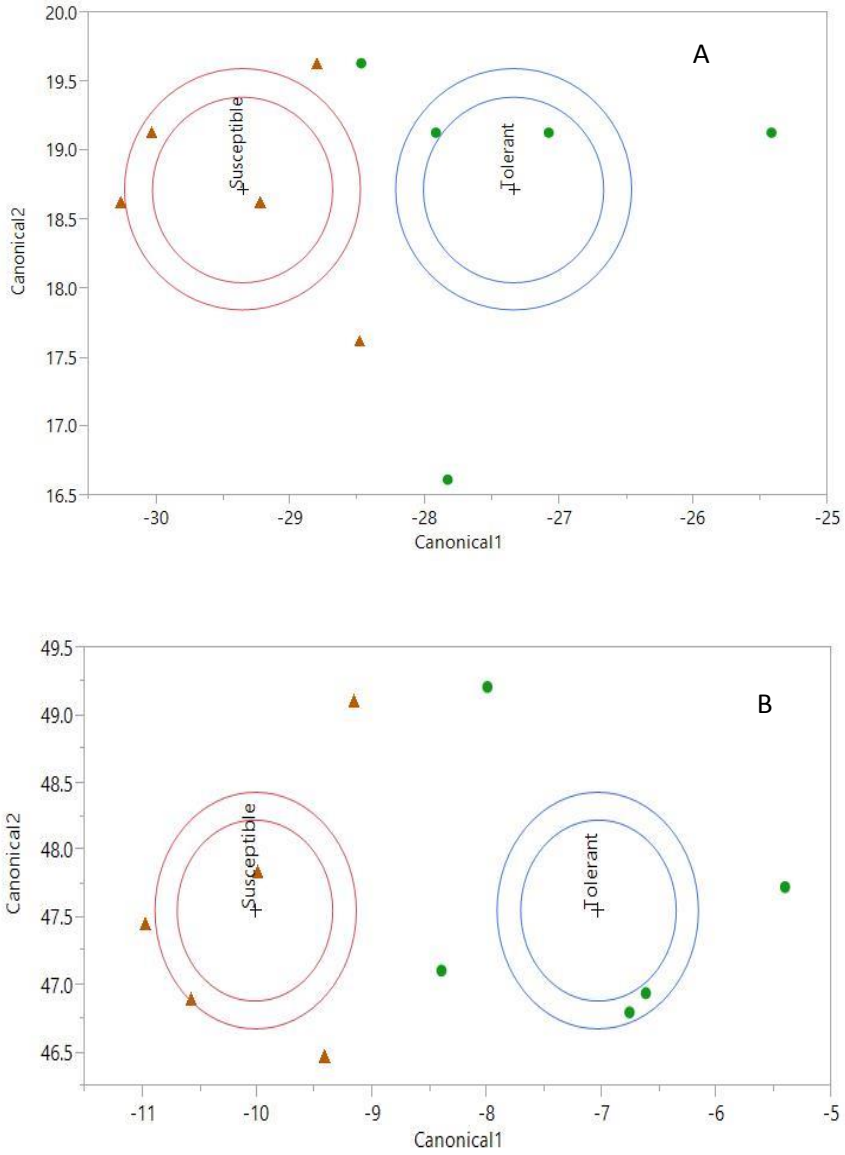

FIGURE 4.9: Canonical plots for drought tolerant and susceptible cultivars using mean values for RWC, Fv/F_m, %TPC, FRAP, Ps, g_s, Tr and Ci/Ca) at six days after re-watering plants that had been stressed for four days (A) and eight days (B). The green dots represent tolerant cultivars and the brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

Predicting the class of drought response of the tested cultivars

It would be desirable to find a single parameter that can correctly distinguish the drought tolerant from the susceptible cultivars. Howeve,r this is not always easy in practice because most traits, like drought, are under the influence of more than one parameter. It is therefore important to clearly define parameters that relate to a desired trait in order to develop reliable criteria for selection. Various parameters that were measured in the current experiment were assessed on how each parameter or a combination of several parameters could correctly predict the drought response of a cultivar using the Logit procedure.

At 4 DWS, a combination of all the tested parameters did not show a statistically significant prediction for cultivar response to water stress ($p = 0.1273$). Single factor Logit analysis only showed leaf relative water content (% RWC) as a parameter that could be used to accurately predict response to water stress ($p = 0.0531$, $R^2 = 0.27$). A combination of % RWC and total antioxidant potential (FRAP) provided the best combination for predicting whether a cultivar is tolerant or susceptible to water stress at 4 DWS ($p = 0.0406$, $R^2 = 0.46$). At 8 DWS, the best prediction for tolerance or susceptibility of a cultivar to water stress was obtained from a combination of stomatal conductance and rate of transpiration ($p = 0.0147$, R^2 0.61).

During recovery from the stress period, assessments done at three days after re-watering the plants (3 DAR) showed that none of the measured physiological parameters could be used to predict the recovery response of a cultivar from water stress. At 6 DAR, for plants that were exposed to 4 DWS, Fv/F_m, Ps, g_s, Tr and Ci/Ca provided a significantly ($p = 0.0165$) accurate prediction of tolerance or susceptibility of the different tea cultivars to water stress. However, none of these parameters could individually give an accurate prediction of the cultivar's response to water stress. For plants that had been subjected to 8 DWS, Fv/F_m and its

combination with Ps, g_s and Tr showed accurate prediction of tolerance or susceptibility of the tea cultivars to water stress ($p = 0.024$, $R^2 = 0.54$).

4.4 Discussion

In the current investigation, wilting, relative water content, leaf gaseous exchange parameters and chlorophyll fluorescence were considered as some of the physiological characteristics that could associate with soil water stress tolerance in tea. Results on the measurements for these parameters done during stress and recovery periods are discussed.

Wilting assessments done at 4 DWS showed no significant differences between the tea cultivar classes for drought tolerance. It has also been reported that wilting may appear much later than some physiological and biochemical responses to drought (Upadhyaya & Panda, 2013). This is probably because plants are able to maintain internal water balance through other means such as stomatal closure or osmotic adjustment that may occur prior to leaf wilting. Previous studies have also shown that some susceptible tea cultivars may show symptoms of wilting under drought stress later than tolerant cultivars (Gupta *et al.*, 2012). This can help to explain the low wilting scores that were observed on some of the drought susceptible cultivars used in the current study. These results suggest that although visual assessment for wilting may be a fast method of assessing the level of water stress in tea plants, degree of wilting cannot be reliably used as criterion for selecting water stress tolerance in tea. With the overlaps in degree of wilting between cultivars in the tolerant and susceptible classes, it could be difficult to identify cultivars with intermediate levels of susceptibility or tolerance to water stress based on wilting scores. However, due to its simplicity, this method can still be used to quickly show how different tea cultivars recover

from water stress and the method can thus probably complement other more robust screening techniques for drought tolerance.

The results on RWC showed that plants that had been well-watered had higher RWCs than plants that had been under water stress. This suggested that low RWC can be used to show the level of water stress in different tea cultivars. Plants can maintain high RWC if they have high cell wall elasticity that make them more sensitive to water loss and quickly close stomata to maintain an internal water balance for continued biochemical functionality (Sade, *et al.*, 2012). This could help such plants to be more productive under moderate water stress conditions. Drought tolerant cultivars maintained relatively higher RWC than the susceptible cultivars as the water stress progressed, but the differences were not statistically significant.

The intra-cellular- to ambient carbon (Ci/Ca) ratio, stomatal conductance (g_s), photosynthesis rate (Ps) and transpiration rate (Tr) of plants exposed to different water stress periods and after stress relief were assessed. At 4 DWS, there were overlaps in g_s, Ps, and Tr among cultivars in the drought tolerant and drought susceptible classes. These results showed that none of these three parameters could individually be used to separate the two groups of cultivars at 4 DWS. Since 4 DWS is a relatively short period of stress, the observed responses suggest that the level of stress was adequate to show differences among individual tea cultivars. However, these differences could probably relate more to how quickly the different cultivars perceived the stress. It is probable that cultivars that showed high values for g_s, Ps and Tr perceived the water stress late and therefore failed to evoke stress response mechanisms at 4 DWS. It was expected that g_s, Ps and Tr would decline more in the drought susceptible cultivars with progressing water stress, as has been reported in other studies (Reddy *et al.*, 2004; De Costa *et al*., 2007). On the other hand, it is also plausible that these cultivars might have used different stress coping mechanisms, e.g. osmotic adjustment, and

hence showed no decline in the three parameters that were measured. In some cases, reduced stomatal conductance can induce high Rubisco enzyme activity and a decrease in the electron transport in the thylakoids as a mechanism to cope with low conductance (Zingaretti *et al*., 2013). These responses may vary with species and/or genotypes (Wang *et al.*, 2013). Photosynthesis may decline under water stress due to photo-inhibition, especially for plants under high temperature (Wahid, 2007; Boussadia *et al.*, 2008). The effect of high temperature could directly relate to the results obtained in the current study because the ambient conditions were hot and dry during the period of experimentation (Appendix 4.2 and 4.3). These conditions could have affected the tea cultivars differently (Wahid, 2007) and hence contributed to the variations between the tolerant and susceptible sub-groups.

The chlorophyll fluorescence ratio, $Fv/_{Fm}$, is associated with stress tolerance because it directly relates to the physiological status of the leaves (Netto *et al.*, 2010). Fluorescence is one of the mechanisms that plants use to dissipate excess photochemical energy that cannot be used in photosynthesis. During the four and eight days of water stress, Fv/F_m was not significantly different between the two cultivar groups. These results were similar to those of Netto *et al.* (2010) who also found no significant differences in Fv/F_m between tea clones in the early stages of stress.

Water stress has been reported to result in various biochemical changes in stressed plants (Upadhyaya & Panda, 2012). Three biochemical parameters, chlorophyll content, total polyphenol content and total antioxidants, were considered in the current study. Water stress treatments and tea cultivar classes showed no significant differences in terms of leaf chlorophyll content at 4 DWS. Some previous studies have reported of a general decline in chlorophyll content under water stress (Gholami *et al.*, 2012). The lack of response observed in the current study could partly be due to the short duration of the stress period that was

imposed on the plants. Chlorophyll breakdown can take several days to manifest, especially in young and expanding leaves. In the current study, chlorophyll content was measured on the relatively young and expanding third leaf below the bud. It is possible that slight changes in chlorophyll content might have occurred over the duration of stress, but these were probably too small to be detected in the relatively young leaves that were measured.

Polyphenols are one of the several groups of secondary metabolites produced by tea plants. In the current study, total polyphenol content (TPC) did not closely correspond with the tea cultivars' known response to drought. Some previous studies have linked higher TPC to drought tolerance and lower TPC to drought sensitivity (Cheruiyot *et al.*, 2007; Upadhyaya & Panda, 2013). The results from the current study failed to show a significant link between TPC and drought response, probably due to the high level of overlaps in TPC between the tolerant and susceptible cultivar groups. The use of the cultivar group means which was done in this study other than using individual cultivar means as was done in the other studies could explain the differences in the results.

Water stress induces increased production of reactive oxygen species (ROS) that cause oxidative stress in plants (Reddy *et al.*, 2004). ROS may also result from impairment of the photosynthetic machinery or electron transport (Genga *et al.*, 2011; Hasanuzzaman *et al.*, 2013). Plants can respond to oxidative stress through enzymatic and non-enzymatic mechanisms. An increase in antioxidants under water stress has been reported in tea and other plant species (Reddy *et al.*, 2004; Upadhyaya *et al.*, 2012). In the current study, the level of total antioxidants (FRAP method) increased at 4 DWS in both the tolerant and susceptible groups of cultivars. The increase in antioxidant activity was more likely in response to accumulation of ROS in the stressed plants since the antioxidants reduce the ROS to non-reactive species. Reduction of the ROS by antioxidants helps to prevent oxidative cell damage. Antioxidants have also been implicated in stress signal transduction (Loicacono &

De Tullio, 2012). This creates a need to relate the time of measurement and the role that the ROS may play. It is probable that in the early stages of stress, the role of antioxidants could be more on the signalling of the stress than in the reaction to increased ROS. This was probably the cause of the marginal increases observed in the current study since the measurements were made at 4 DWS. Individual tea cultivar differences in stress tolerance could be due to differences in plant ability to perceive stress, signal transduction and how appropriate genes are expressed (Gupta *et al.,* 2012). There are sometimes big variations in antioxidant responses among woody plant species, ranging from no effects to decreases in certain antioxidant enzymes (Reddy *et al.,* 2004). FRAP can be lower as a response to low levels of ROS or alternatively, due to an increase in ROS that react with the antioxidants (Griffin & Bhagooli, 2004; Upadhyaya *et al.,* 2012).

The results of both physiological and biochemical characteristics showed some sub-group overlaps, probably suggesting that these characteristics exhibit continuous variation. Differences among cultivars in the same group as well as the close genetic relationships among some of the cultivars used in the study could also have contributed to the overlaps between the cultivar sub-groups for drought response. It should also be noted that the study used potted plants in a relatively small volume of soil, which resulted in drastic development of stress. The sample sizes for some of the measurements were also small. This probably influenced the level of variations within the cultivar groups and masked the differences between cultivar groups. This variation could also be partly attributed to the problem of subjectivity in historical field classification of the response of different cultivars to drought.

Indices of tolerance or susceptibility to a stress factor can be useful tools for effective selection of drought tolerant cultivars. In the current study a drought susceptibility index (DSI) was calculated based on the photosynthesis rate. There were no significant differences in DSI between tolerant and susceptible cultivars during the stress period. It was therefore not

possible to develop an index for tolerance to water stress based on the photosynthetic measurements that were done in this study.

Recovery from water stress was assessed by measuring the same physiological parameters that were used to assess water stress. Assessments on wilting made at three days after re-watering (3 DAR) and RWC of the non-stressed and stressed plants at 5 DAR suggested that these parameters cannot be used to differentiate the rate of recovery from water stress of the tolerant and susceptible cultivars. Although mere plant survival during stress may sometimes be given high priority, rate of recovery is very crucial for perennial crops like tea that go through prolonged dry periods. Tea cultivars that can quickly recover from water stress and come into production and plucking soon after the onset of the rains can produce higher yields, especially in areas like Malawi where the main harvesting season is shortened by other climatic limitations on tea shoot growth, for example, early cessation of the rains or low temperatures experienced during winter (Wilkie, 1996; Carr, 2010).

At 8 DAR, all the cultivars had not fully recovered from water stress but there were no significant treatment effects on g_s, Ps and Tr. The three gaseous exchange parameters also showed a very similar trend to what was observed at 4 DWS, which suggested a positive correlation between initial responses to water stress and how such cultivars would recover from stress. In practice this observation could further suggest that measurements taken during the early stages of stress can be used to predict the likely plant behaviour during the recovery period. This could be advantageous in a selection programmes as it would allow early selection and therefore improve selection efficiency.

Drought tolerant cultivars had relatively higher Fv/F_m ratio, reduced stomatal conductance and photosynthesis rate than the drought susceptible cultivars although the differences were not statistically significant. Reduced stomatal conductance and photosynthesis rate could

have caused in an increase in fluorescence due to an excess in absorbed energy. This probably suggested that the drought tolerant cultivars were able to dissipate excess photochemical energy that might have resulted from limitations on photosynthesis and other phytochemical processes due to water stress (Cavatte *et al.,* 2012). Cultivars that had lower Fv/F_m ratio were still stressed because this ratio usually decreases with increasing stress.

Although not statistically significant, drought tolerant cultivars had relatively higher chlorophyll content than the susceptible cultivars. Similar observations have been reported in barley (Li *et al.,* 2006). This can probably suggest that the tolerant cultivars showed less decrease in the leaf chlorophyll content than the susceptible cultivars. At the end of the stress period the tolerant cultivars were able to normalize their photosynthesis process much more quickly than the susceptible cultivars. This could be related to the differences in the rate of recovery from stress between the tolerant and the susceptible cultivars.

TPC values at 6 DAR were lower than the values that were recorded at 8 DWS. This suggested that the plants had probably not fully recovered from the water stress and that the polyphenols were still being used to counter oxidative stress caused by reactive oxygen species that usually accumulate under water stress. During recovery, the TPC is expected to increase as the oxidative stress lessens (Upadhyaya & Panda, 2013). Differences between the current and reported results could be due to several factors, such as level and duration of stress, type of plant material, as well as other stress factors that prevailed during the period of experimentation, for instance, the hot and dry ambient conditions which could have reduced the activities of the enzymes that enhance synthesis of polyphenols.

Photosynthesis measured during the recovery period was used to calculate a Drought Susceptibility Index (DSI). The results showed similar DSI values between the drought tolerant and susceptible cultivars. In other drought screening studies in tea, low DSI values

were associated with tolerance to water stress (Damayanthi *et al.*, 2010). The DSI values in the current study were lower than those reported in study by Damayanthi *et al.* (2010), probably because the current results were based on Ps of re-watered plants that were recovering from stress. Since the DSI was based on photosynthesis, which is also influenced by many factors, difference in results from the two studies was probably not surprising.

In breeding and selection, it would be desirable to identify a single parameter that can clearly distinguish genotypes with or without a particular trait. Univariate analysis of the results showed than none of the parameters could individually separate the tolerant and susceptible cultivars. As a result, multivariate analysis was done. Using the linear discriminant analysis procedure, at 4 DWS, it was possible to correctly classify the different cultivars into the tolerant and susceptible classes using a combination of RWC, TPC, antioxidant activity, Fv/F_m and gaseous exchange parameters. However, a backward selection of variables fitted into the model showed that a combination of leaf relative water content (% RWC) and a combination of % RWC and total antioxidant potential (FRAP) measured at four days of water stress, provided the best combination for predicting the response to water stress of the tested cultivars. However, when water stress was prolonged to eight days, stomatal conductance together with rate of transpiration gave the best prediction of tolerance or susceptibility of a cultivar to water stress. This showed that a combination of RWC and total antioxidant potential can potentially be used to select drought tolerant plants that were exposed to even a short period of water stress. These analyses probably helped to unmask the observed differences between cultivars that could probably not show up in the univariate analysis. The fact that there were differences between the cultivar groups after four days of water stress suggested that RWC and total antioxidant potential can allow early selection of new genotypes and thereby result in some savings in time and costs of selection.

4.5 Conclusions

This study was carried out to identify physiological and/or biochemical characteristics that could associate with tolerance to water stress in tea in order to develop selection criteria. The results showed that none of the measured parameters could individually be used to fully separate the tolerant from the susceptible tea cultivars. However, relative water content (RWC) in combination with antioxidant activity (FRAP) was found to be potentially useful in predicting the response of a cultivar to water stress. These two parameters can serve as useful indicators of the water stress tolerance in tea and can be used in selection programmes. RWC and FRAP are relatively easy and inexpensive to measure and could easily be incorporated in routine selection programmes for drought tolerance. This finding is of practical significance and will greatly help in developing effective and better methods for selecting tea cultivars that are drought tolerant for use in drought prone tea growing areas, such as Malawi.

Water stress tolerant cultivars generally maintain high relative water content and reduce Ps, Tr and g_s in addition to having high antioxidant activity under stress in order to optimize on water use. There were overlaps for some parameters between the drought susceptible and drought tolerant cultivars, partly due to nature of the parameters assessed as well the close genetic relationship among the tested cultivars. It is also probable that some of the tested cultivars show intermediate tolerance to drought, which could have contributed to the overlaps. The changes in some of the measured characteristics during the stress and recovery periods exhibited a similar trend. This suggested that assessments made during the early stages of stress could be used to predict some of the changes that might take place during the recovery period. This could be of practical advantage in helping to improve selection efficiency. The limitations of sample size should be addressed in future studies in order to get a true reflection of drought response to the parameters that were measured in the current study.

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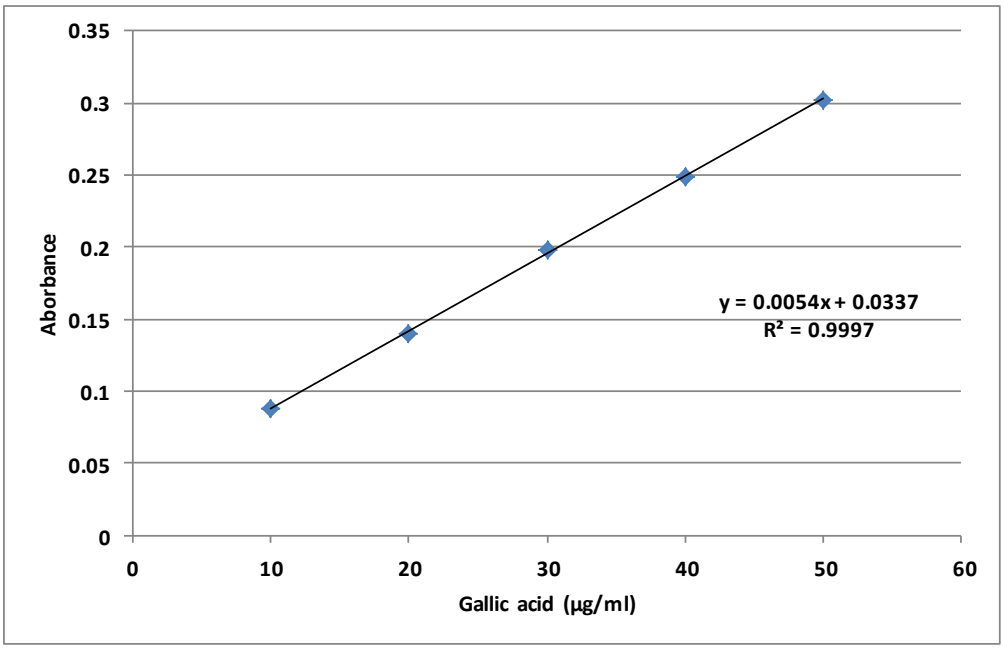
Appendix 4.1: Sample of a calibration curve for determination of percent total polyphenols



Sample calculation for Total Polyphenol content (TPC)

$$\%TPC = \frac{(\mathrm{A} - \mathrm{I}) * V * DF * 100}{\mathrm{m} * \mathrm{M} * 10000 * \mathrm{DM}}$$

A = average absorbance = say 0.2

V = volume (ml) of extract used in the determination of TPC, say = 1.2

DF = dilution factor for the extract, say = 100

I = intercept from the standard curve, = 0.0337 (from the calibration equation)

m = is the slope, = 0.0054 (from the calibration equation)

M = mass (g) of the sample used in the extraction, say = 0.02g

DM = dry matter content (%) of the sample as per cent of the mass, say = 98.5

$$\%TPC = \frac{(0.2-0.0337)*1.2*100*100}{0.0054*0.02*10000*98.5}$$

%TPC = 18.76

Appendix 4.2: Some weather parameters for Mimosa Research Station (October 2012)

Date	Temperature (°C)			Wind	Radiation	Rainfall	Evaporation		Relative Humidity	
	Max	Min	Mean	km/PD	Joules	(mm)	A Black	B White	08.00	14.00
1	30.8	19.3	25.1	2.2	162.4	0.0	7.7	E	83.0	43.0
2	30.8	18.9	24.9	1.5	155.6	0.0	7.1	E	74.0	40.0
3	34.1	19.8	27.0	1.2	167.8	0.0	7.1	E	69.0	30.0
4	33.2	16.7	25.0	0.9	137.1	0.0	6.1	E	54.0	36.0
5	34.9	18.1	26.5	1.5	70.8	0.0	7.7	E	51.0	24.0
6	34.3	16.3	25.3	1.6	169.3	0.0	8.7	E	57.0	24.0
7	35.1	17.3	26.2	1.3	175.3	0.0	9.2	E	54.0	26.0
8	37.6	16.2	26.9	1.9	179.4	0.0	10.7	E	43.0	18.0
9	35.8	18.4	27.1	2.1	174.3	0.0	10.7	E	54.0	19.0
10	36.3	15.1	25.7	1.4	173.8	0.0	8.7	E	65.0	20.0
11	37.0	17.8	27.4	2.0	166.8	TR	8.2	E	58.0	22.0
12	33.7	20.4	27.1	2.9	171.8	0.0	8.7	E	79.0	41.0
13	33.8	19.4	26.6	2.1	171.3	0.0	8.2	E	65.0	26.0
14	31.5	19.4	25.5	1.7	152.6	0.0	6.6	E	69.0	E
15	33.0	19.1	26.1	1.4	147.3	0.0	7.1	E	69.0	45.0
16	35.9	17.7	26.8	1.3	167.2	0.0	7.1	E	65.0	23.0
17	36.0	17.4	26.7	1.2	175.3	0.0	9.2	E	48.0	23.0
18	37.7	16.8	27.3	1.1	178.4	0.0	9.7	E	37.0	21.0
19	39.8	18.0	28.9	1.2	178.9	0.0	11.2	E	29.0	18.0
20	38.8	21.0	29.9	1.6	182.3	0.0	11.2	E	38.0	21.0
21	37.7	21.0	29.4	1.6	173.8	0.0	8.7	E	62.0	E
22	37.0	18.6	27.8	0.8	176.5	0.0	E	E	62.0	22.0
23	38.2	18.7	28.5	2.1	172.3	0.0	8.7	E	48.0	24.0
24	31.7	19.7	25.7	2.5	139.5	7.0	4.9	E	65.0	43.0
25	26.0	18.7	22.4	1.5	120.0	0.5	3.1	E	78.0	74.0
26	31.3	16.9	24.1	1.1	185.3	0.0	7.1	E	69.0	35.0
27	33.4	15.5	24.5	1.9	171.8	0.0	8.7	E	54.0	29.0
28	30.8	15.4	23.1	1.0	148.7	0.0	6.6	E	65.0	40.0
29	30.0	17.8	23.9	1.6	149.2	0.0	6.1	E	84.0	45.0
30	33.9	16.6	25.3	1.2	162.8	0.0	7.7	E	65.0	38.0
31	36.9	17.7	27.3	1.6	154.1	4.6	8.7	E	48.0	34.0
Mean	**34.4**	**18.1**	**25.4**	**1.6**	**161.7**	**3 Days**	**7.8**	**E**	**60.0**	**31.2**

Appendix 4.3: Some weather parameters for Mimosa Research Station (November 2012)

Date	Temperatures (°C)			Wind	Radiation	Rainfall	Evaporation		Relative Humidity	
	Max	Min	Mean	km/PD	Joules	(mm)	A Black	B White	08.00	14.00
1	24.7	19.4	22.1	1.7	100.6	8.2	0.2	0.0	88.0	94.0
2	24.0	16.4	20.2	1.6	108.9	6.2	0.1	0.0	83.0	64.0
3	26.9	15.5	21.2	1.3	152.1	0.0	0.5	0.0	64.0	41.0
4	30.2	11.5	20.9	1.0	190.6	0.0	0.7	0.0	64.0	E
5	34.7	12.7	23.7	0.9	185.8	0.0	0.8	0.0	57.0	25.0
6	37.1	15.3	26.2	1.1	179.9	0.0	0.9	0.0	54.0	21.0
7	38.8	18.8	28.8	1.8	177.4	0.0	1.0	0.0	48.0	18.0
8	35.6	19.8	27.7	1.8	140.5	0.0	0.6	0.0	70.0	55.0
9	32.3	19.8	26.1	2.2	173,8	0.0	0.9	0.0	74.0	38.0
10	35.7	17.9	26.8	2.0	176,3	0.0	E	0.0	69.0	39.0
11	33.4	20.2	26.8	2.0	147,8	0.0	E	0.0	74.0	43.0
12	34.8	17.6	26.2	1.2	182,8	0.0	E	0.0	65.0	36.0
13	37.0	19.1	28.1	1.3	169,8	0.0	0.8	0.0	69.0	27.0
14	37.4	19.7	28.6	2.7	153,6	0.0	1.0	0.0	52.0	26.0
15	35.6	22.0	28.8	2.4	187,2	0.0	0.9	0.0	55.0	36.0
16	36.8	20.3	28.6	1.2	164,8	2.1	0.8	0.0	62.0	39.0
17	39.1	20.5	29.8	1.2	167,8	0.6	0.9	0.0	62.0	45.0
18	36.8	18.0	27.4	1.3	169,3	66.2	E	0.0	52.0	E
19	32.7	18.0	25.4	1.2	182,8	0.0	0.6	0.0	88.0	46.0
20	34.9	20.0	27.5	1.6	189,6	0.0	0.9	0.0	74.0	37.0
21	36.0	18.7	27.4	1.4	190,1	0.0	0.8	0.0	69.0	34.0
22	32.7	19.4	26.1	1.6	166,8	0.0	0.7	0.0	69.0	49.0
23	31.8	17.5	24.7	1.0	155,1	0.0	0.5	0.0	84.0	52.0
24	36.3	17.8	27.1	1.1	186,7	0.0	0.9	0.0	69.0	37.0
25	37.4	20.7	29.1	1.2	156,1	0.0	0.9	0.0	46.0	E
26	28.9	23.3	26.1	1.6	126,4	24.4	0.5	0.0	79.0	74.0
27	27.6	20.0	23.8	2.2	130,3	3.1	0.4	0.0	94.0	84.0
28	27.8	18.3	23.1	1.8	156,1	TR	0.5	0.0	79.0	51.0
29	31.3	15.6	23.5	1.1	197,9	0.0	0.9	0.0	54.0	33.0
30	33.4	14.3	23.9	1.7	189,6	0.0	E	0.0	61.0	36.0
Mean	**33.4**	**18.3**	**25.8**	**1.5**	**165,2**	**0.0**	**0.7**	**0.0**	**67.6**	**43.7**

Note: ***E*** - denote data was not recorded

CHAPTER 5

PHYSIOLOGICAL AND BIOCHEMICAL CHARACTERISTICS ASSOCIATED WITH LOW TEMPERATURE TOLERANCE IN TEA

5.1 Introduction

Low temperature stress limits growth and productivity of crops (Cattivelli *et al.*, 2002). Response to low temperatures can vary with plant species because different plants have different optimum temperatures for their normal growth. Tea, for example, exhibits normal growth and productivity at ambient temperatures of between 18°C and 25°C (Carr, 1972). Mean air temperatures below 13°C and above 30°C tend to reduce shoot growth and hence tea yields (Carr, 1972; Tanton, 1982a; Carr & Stephens, 1992; Barman, 2002). It can therefore be expected that tea plants will suffer some form of stress when exposed to temperatures below 13°C and above 30°C.

A low temperature tolerant tea cultivar produces shoots that attain pluckable size of two to three leaves and a bud within a reasonable period under low temperature conditions. Rates of shoot growth are drastically reduced under low temperature. For instance, in Central Africa, shoots can take up to 84 instead of 42 days to attain the ideal pluckable size during the cold months (TRFCA, 1990). Slow growth of shoots significantly increases the interval between plucking days and therefore greatly reduces the number of times when tea can be plucked during the cold season. This subsequently results in low tea yields per year. However, tea cultivars show significant differences in response to low temperature (Tanton, 1982a).

Low temperature causes a progressive cessation of active tea shoot growth until the growing buds become dormant. However, there are varied opinions on the phenomenon of bud dormancy in tea. Omae and Takeda (2003) contended that tea bud dormancy encompasses a stage when new leaves stop to expand at the “banjhi” (dormant) stage and the apical buds fail

to flush or elongate even under optimal growth conditions ("wangi" dormancy). More recently, Thirugnanasambantham, *et al.* (2013) reported that winter dormancy is due to climatic conditions and, banjhi or wangi dormancy may be caused by mechanical stress such as mechanical plucking. These reports probably highlight the fact that bud dormancy in tea is complex and that cessation of active growth of tea shoots is influenced by many factors, including low temperature. De Costa *et al.* (2007) reported that initiation, extension and expansion of tea shoots or leaves are all influenced by air temperature. It is therefore reasonable to assert that temperature plays a vital role in growth of tea shoots. Ambient temperature below a plant's base temperature for growth results in complete cessation of active growth. For tea, 12.5°C is generally regarded as the base temperature (T_b) for shoot extension (Tanton, 1982a). However, other reports indicate that this can vary between 7°C and 15°C, depending on a cultivar (Obaga *et al*., 1988; Stephens & Carr, 1990; Carr & Stephens, 1992). This variation in response to temperature among cultivars can be exploited in selection programmes for low temperature tolerance.

Inherent cultivar characteristics may be largely responsible for the differences in base temperature for shoot extension. Genetic factors that control dormancy in most crop plants are diverse and not well understood (Fennimore *et al*., 1999). These factors need to be identified and clearly understood as a pre-requisite for developing objective selection criteria for low temperature tolerance (Tanton, 1982a; Cattivelli *et al.*, 2002; Vyas & Kumar 2005; Allinne *et al.*, 2009).

Plants exposed to sub-optimal temperatures show significant changes in many physiological processes, for example, photosynthesis (Zhang *et al.*, 2012). Photosynthesis, which is very sensitive to changes in temperature, can be used to assess the level of temperature stress. Processes or characteristics that are closely associated with photosynthesis, e.g. stomatal

conductance (g_s) have been associated with low temperature responses among tea cultivars (Joshi & Palni, 1998).

Change in chlorophyll fluorescence has also been correlated with tolerance to environmental stresses and can be used to indicate the extent of damage to the photosystem apparatus (Strand & Lundmark, 1987; Maxwell & Johnson, 2000). For instance, high fluorescence may suggest a reduction in the amount of the absorbed light energy that is used in photochemical reactions. In sunflower, chlorophyll fluorescence has been associated with cold tolerance (Allinne, *et al.*, 2009).

Low temperature predisposes plants to oxidative stress due to increased production of reactive oxygen species (ROS), especially under high radiation (Vyas & Kumar, 2005). Oxidative stress can directly affect shoot initiation and expansion processes (Jaleel *et al.*, 2009; Bocian *et al.*, 2011). This shows that changes in temperature will directly affect several physiological processes in tea plants, which will have an impact on shoot growth and development. Plants can tolerate oxidative stress by accumulating biochemical compounds that counter the ROS, for instance, polyphenols that can directly react with ROS by scavenging them. This is used as defence mechanism against oxidative stress. The same mechanism is used when plants produce small molecules that scavenge on the oxidants that are produced due to low temperature stress.

In the TRFCA breeding programme, low temperature tolerant cultivars are identified based on visual assessments for active shoot growth on field grown tea bushes and counting of flushing and dormant (banjhi) shoots during the cold months (Nyirenda, 1993). However, visual assessments are usually subjective, time consuming and less precise. In addition, it is very difficult to pick up small but significant differences in the response of different cultivars to abiotic stress factors such as low temperature using visual assessments. Mechanisms that

regulate response to low temperature in different cultivars may therefore remain obscure if selection is based solely on visual field observations. It might also be difficult to accurately separate the effects of several convergent stresses that occur under field conditions.

The mechanisms that underlie cultivar differences need to be well understood in order to identify characteristics that can be used as objective and reliable selection criteria for low temperature tolerance. However, most of the environmental and genetic factors that affect tea shoot growth are poorly understood (Tanton, 1982a; Barros *et al.*, 1997). These factors need to be unravelled through research and the current study was done to establish some of the characteristics that contribute to low temperature tolerance of tea cultivars.

5.1.1 Null Hypothesis

H_{o1} There will be no statistically significant differences in the growth rate between low temperature tolerant and susceptible cultivars under cold stress conditions at the 95% level of confidence.

H_{02} There will be no statistically significant difference in various physiological parameters between low temperature tolerant and susceptible cultivars under cold stress conditions at the 95% level of confidence.

H_{03} There will be no statistically significant difference in the polyphenol content and antioxidant capacity between low temperature tolerant and susceptible cultivars under cold stress conditions at the 95% level of confidence.

5.1.2 Objectives

1. To establish the effects of low ambient temperature on the rate of shoot development and extension in different tea cultivars

2. To identify physiological and biochemical characteristics that different cultivars use to tolerate low temperature in order to develop better methods for selecting low temperature tolerant tea cultivars

5.2 Materials and methods

The study had two components: one component was done in temperature-controlled growth chambers at the Hatfield Experimental Farm of the University of Pretoria in South Africa. The other component was done on field-grown tea bushes under rain-fed conditions at Mimosa Tea Research Station (MTRS) in Malawi during the cold season (May to August 2012).

5.2.1 Growth chamber experiment

Six tea cultivars were used in the experiment and three of these had been classified as low temperature tolerant: PC 153, PC 198 and PC 168, while the other three cultivars: PC 165, CL 12, and RC 6 had been classified as susceptible to low temperature, based on field observations. Each cultivar was propagated vegetatively and the rooted cuttings were then grown in polythene bags containing a growing medium (Earth 2 Earth, South Africa). The plants were managed following practices recommended by the TRFCA in terms of fertilizer application, watering and pests and disease control. The plants were about two years old at the start of the experiment and well-established plants for each cultivar were used in the experiment.

The temperature treatments in the growth chambers were devised to simulate temperatures that are usually experienced during the cold season at MTRS in Mulanje, Malawi. Long-term temperature data (1961 to 2013) for MTRS between May and August showed that the

average minimum and maximum temperature ranged from 9.2°C to 15.8°C and 21.5°C to 28.8°C, respectively. The long-term daily mean temperature [(Minimum + Maximum)/2] ranged from 15.3°C to 22.8°C. This information was used to determine the temperature regimes that were imposed on the plants. Once set, the temperature in each chamber was kept constant during the day and night for two weeks, whereafter it was adjusted downward by 2.5°C (Table 5.1). Two adjustments were made and this resulted in a total decline of 5°C over the whole duration of the experiment. The day and night period was maintained at 12h each, based on previous studies which showed that tea shoot extension may only be negatively affected when day length is shorter than 11h (Tanton, 1982b).

TABLE 5.1 Temperature (°C) settings in three growth chambers at Hatfield Experimental Farm, University of Pretoria, South Africa

Period	Chamber 1	Chamber 2	Chamber 3
Initial temperature	15.0	20.0	25.0
First change (day 15)	12.5	17.5	22.5
Second change (day 28)	10.0	15.0	20.5

The experiment followed a split-plot design with temperature as the main plot factor and the tea cultivars as sub-plot factors. The experimental units were completely randomized in each chamber and each treatment had four single-plant replicates. The one plant per plot approach was adopted in order to minimize plant-to-plant variations as well as to fit the trial within the limited space that was available in each growth chamber.

5.2.2 Shoot growth and extension

Shoot growth was monitored by plucking off the top one leaf and a bud from a shoot in order to remove apical dominance and the axillary bud below the plucked point was tagged on each of the four plants of each cultivar in each growth chamber. One axillary bud was tagged on each of the four separate plants of each cultivar in each temperature regime (chamber). The tagged bud was immediately measured using a ruler to determine initial bud length. In order to ensure that the measuring ruler is placed on the same starting point when taking the measurements, a reference point was clearly marked with a black permanent marker at the base of the axil of each tagged bud. Bud lengths were measured every seven days up to day 21 after which the lengths of the tagged buds were measured every three days until the end of the experiment. These measurements were used to calculate shoot extension rate (r).

For shoot extension measurements, an already developed shoot was tagged on each plant and the shoot was measured to determine the initial length. The tagged shoots were measured every seven days from the day of tagging until the end of the experiment. The initial and final shoot lengths were used to calculate shoot extension rate for the individual cultivars. This set of shoots was included in order to monitor how temperature would affect shoots that had already gone past the bud stage.

Physiological parameters: Photosynthesis rate (Ps), transpiration rate (Tr), and stomatal conductance (g_s) were measured using an LI-6400XT photosynthesis system (Li-Cor, Inc. Lincoln, Nebraska, USA). Measurements were made on the third leaf below the apical bud on each plant in all the three replicates.

Biochemical analyses for total polyphenols and antioxidant activity were done on leaf samples that were collected from each treatment at the start of the experiment and at each

point when the temperature in the chambers was adjusted. One leaf was collected from each plant in all three of the replicates in each chamber. The sampled leaves were packed in clearly labelled zip-loc plastic bags, put in a cooler box and taken to the laboratory where they were frozen in liquid nitrogen. The frozen samples were packed in paper envelopes and stored at -20°C prior to analysis. Each sample was analysed for total polyphenols and total antioxidant capacity as described in Chapter 4, sections 4.2.4.

5.2.3 Field experiment conducted at Mimosa Research Station in Malawi

The weather conditions in the tea growing areas of Malawi are characterized by a hot, wet summer (December to April), followed by a cold, dry winter (May to August) and a hot, dry spring (September to November) (Wright, 2002). This experiment was conducted during the winter period (May to August) in 2012 in Field 9 at Mimosa Tea Research Station, Malawi, using the same six tea cultivars that were used in the growth chamber experiment. All these cultivars were of the same age, growing in one field and received the same management inputs. The plants of the different cultivars were therefore also exposed to the same environmental conditions throughout the duration of the experiment. The daily minimum and maximum temperatures collected from Mimosa Meteorological station, situated about 500 m from the field where the experiment was done, are shown in Figure 5.1.

Shoot growth measurements were done following the procedure reported by Burgess and Carr (1997), with some modifications. Four tea bushes were chosen randomly in a 50 bush field plot of each cultivar. Five shoots comprising two or three leaves and a terminal bud (2+Bud or 3+Bud) were also chosen randomly on each of the four bushes and plucked to remove apical dominance. The axillary buds were tagged and measured in the same way as

was done in the growth chamber experiment. The shoot lengths were used to calculate the shoot extension rate (r) for the different cultivars.

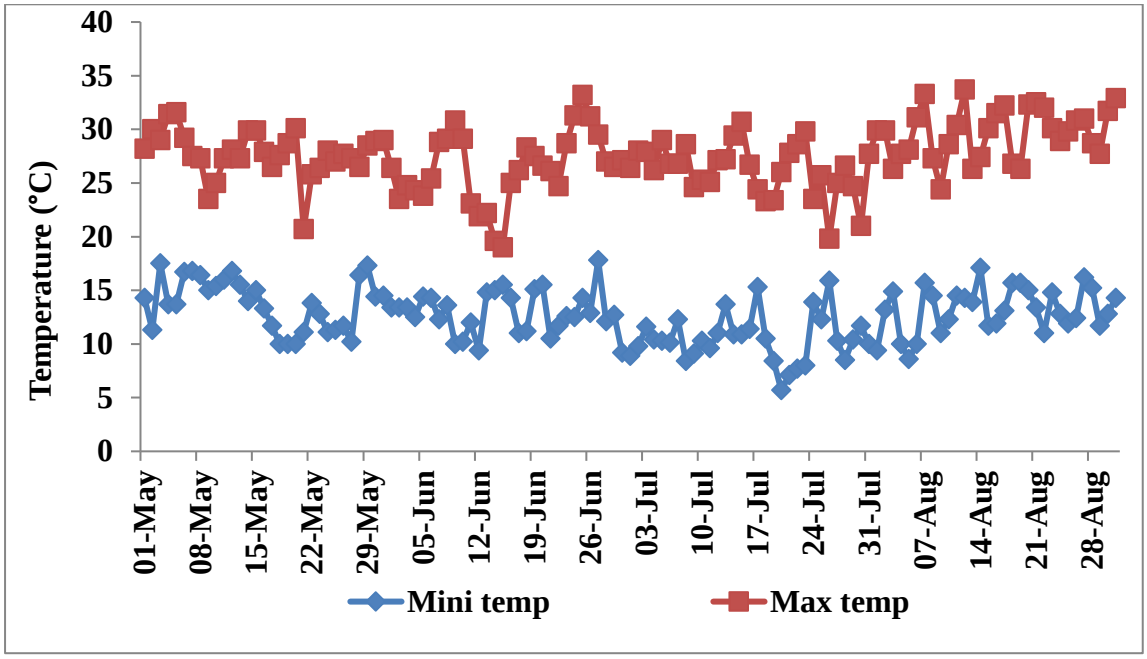


FIGURE 5.1: Daily minimum and maximum temperatures recorded at Mimosa Research Station during the period of experimentation (May – August 2012). The meteorological station was located about 500 m from the field where shoot measurements were conducted.

5.2.4 Data analysis

Data collected on various parameters was first tested for normality and later subjected to uni-variate analysis of variance using the Genstat statistical software, 14th Edition. Comparison of means between the low temperature tolerant and susceptible tea cultivar classes was done using the unpaired, two tailed Student's t-test in order to detect whether there were significant differences between the tolerant and susceptible tea cultivar classes for the various

parameters. This was followed by multi-variate analysis of variance, which involved principal component analysis (PCA), discriminant analysis and fitting of the Logit model, using JMP software, 11th Edition (Sall *et al.*, 2012). These analyses were done in order to find a combination of factors that could be used to correctly distinguish the cold tolerant from the susceptible cultivars.

5.3 Results

5.3.1 Growth chamber experiment

5.3.1.1 Shoot growth

Growth of tea shoots was monitored in temperature controlled growth chambers by looking at the rate of progression of shoot length for each cultivar in the three growth chambers. There were two groups of shoots, one group where an axillary bud was tagged below a plucked point and another group where shoots that had already started growing were tagged. For the first group of shoots, results on the initial bud lengths and subsequent shoot lengths taken from day 7 to day 50 in the three growth chambers are presented in Figure 5.2. In all the three chambers there were no significant differences ($p > 0.05$) in shoot lengths between cultivars that were pre-classified as low temperature tolerant and susceptible, according to earlier field observations (Fig. 5.2A, Fig. 5.2B & Fig 5.2C).

A different set of shoots that had already gone past the bud stage was tagged and monitored for rate of extension under different temperature regimes. The results from day 1 to day 52 are presented in Figure 5.3. In the chamber where temperature was adjusted from 15 to 10°C (Fig.5.3A), low temperature tolerant cultivars had significantly ($p< 0.05$) longer shoots than the susceptible cultivars over the whole duration of the experiment. The difference in shoot lengths was significantly bigger between days 20 and 52. In the second and third chambers where temperature was adjusted from 20 to 15°C (Fig.5.3B) and from 25 to 20°C (Fig.5.3C), respectively, low temperature tolerant cultivars also had significantly ($p< 0.05$) longer shoots than the susceptible cultivars between days 7 and 17, but the difference in shoot length became very marginal towards the end of the experiment when temperature had been adjusted to 15°C and 20°C, respectively.

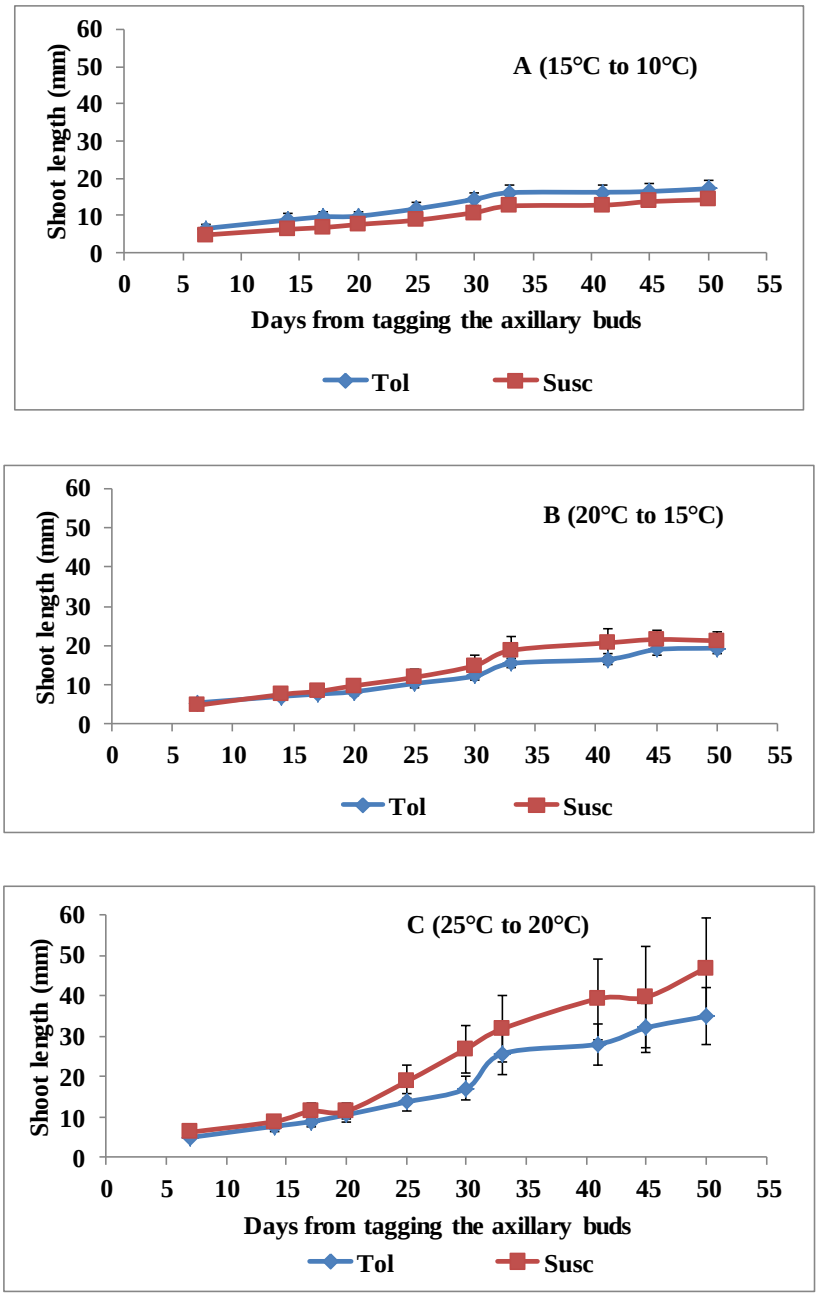


FIGURE 5.2: Mean shoot length (mm) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars from day 7 to day 50 after tagging the axillary buds in three growth chambers. The axillary buds had just been released from apical dominance by plucking the top shoot at the time of tagging. Temperatures from day 1 to Day 14, Day 15 to Day 28 and Day 29- Day 42 were 15°C, 12.5°C and 10.0°C in chamber 1 (A), 20°C, 17.5°C and 15°C in chamber 2 (B) and 25°C°, 22.5°C and 20.0°C in chamber3 (C). Error bars are of the standard error of the means, n = 12.

5.3.1.2 Physiological measurements

Intracellular to ambient (Ci/Ca) carbon ratio, stomatal conductance (g_s), photosynthesis rate (Ps) and transpiration rate (Tr) were measured at one day and 14 days after setting or adjusting the growth chamber temperatures.

Data collected one day after setting the temperature showed no significant differences between the low temperature tolerant and susceptible tea cultivars in terms of Ps, g_s and Tr in all three chambers (Figure 5.4). After 14 days of exposure to different temperatures, there were no significant differences ($p > 0.05$) between the low temperature tolerant and susceptible cultivars for photosynthesis, stomatal conductance and transpiration at the different temperatures (Figure 5.5). As expected, these parameters were generally higher under high temperature conditions.

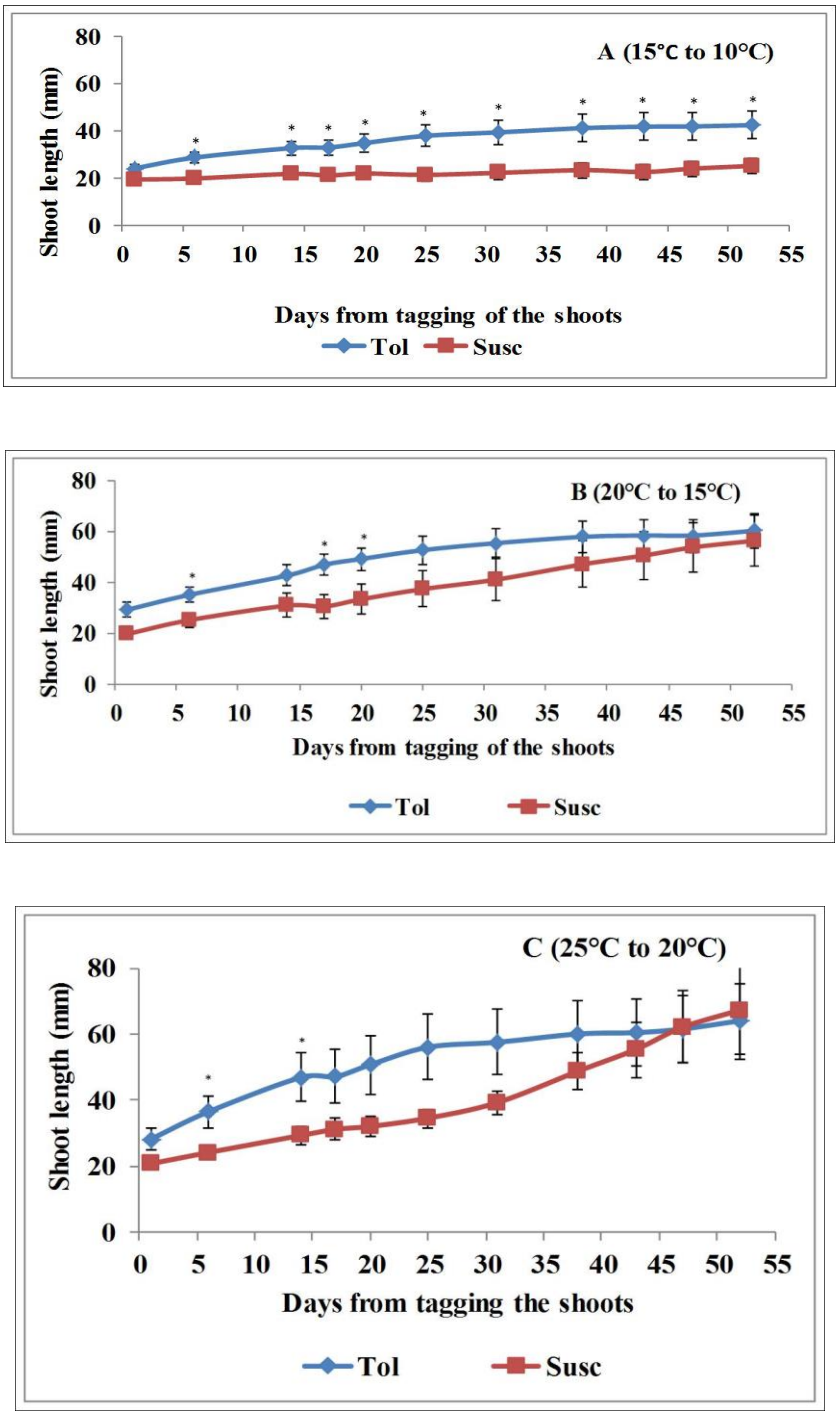


FIGURE 5.3: Mean shoot length (mm) of low temperature tolerant (Tol) and susceptible (Susc) tea cultivars from the day of tagging (Day 1) to the end of experiment (day 52) in three growth chambers. The shoots were already actively growing at the time of tagging. Temperatures from day 1 to 14, Day 15 to 28 and Day 29 to 42 were 15°C, 12.5°C and 10°C in chamber 1 (A), 20°C, 17.5°C and 15°C in chamber 2 (B) and 25°C°, 22.5°C and 20°C in chamber3 (C). Error bars are of the standard error of the means, n = 12. A (*) denotes significant differences at 95% level of confidence.

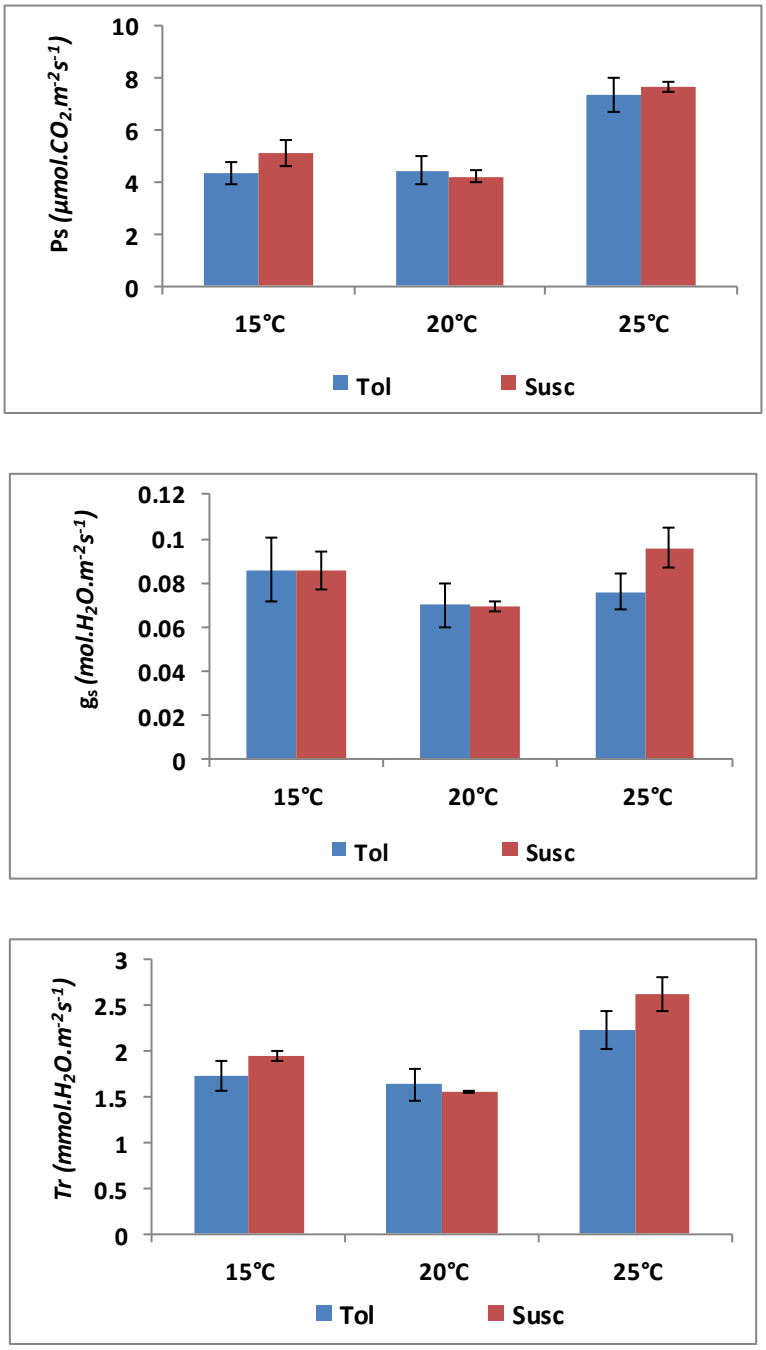


FIGURE 5.4: Mean photosynthesis, stomatal conductance and transpiration for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars under different temperatures measured at the start of the experiment. The error bars represent the standard errors of the means, n = 4.

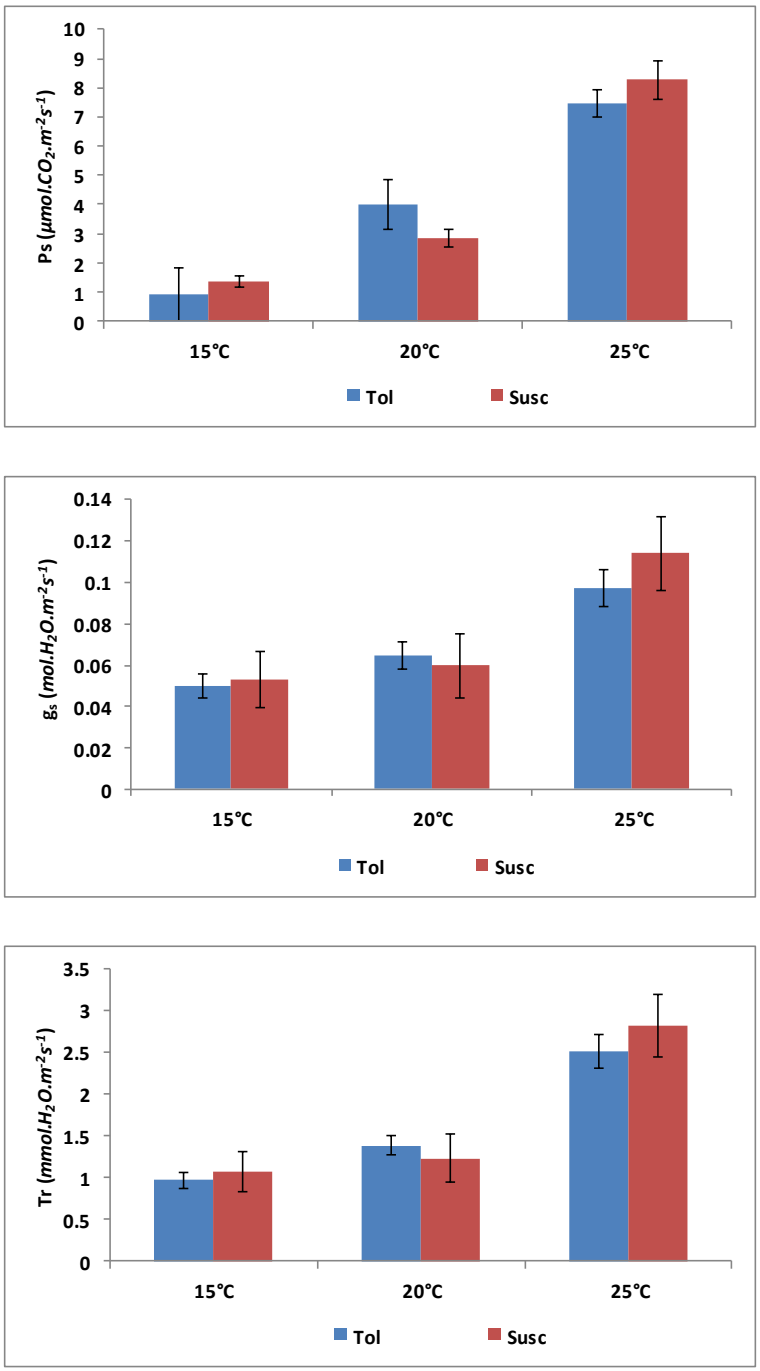


FIGURE 5.5: Mean photosynthesis (Ps) (µmol.CO_2.m^{-2}.s^{-1}) stomatal conductance (g_s) (mol.H_2O.m^{-2} s^{-1}) and transpiration (Tr) (mmol.H_2O.$m^{-2}s^{-1}$) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars after 14 days of exposure to different temperatures. Error bars are of the standard errors of the means, n = 4

At 14 days after the temperature in each of the chambers had been adjusted downwards by 2.5°C, there were still no significant differences in photosynthesis, stomatal conductance and transpiration rate between the low temperature tolerant and susceptible tea cultivars (Figure 5.6). Photosynthesis was more affected by low temperature than the other two parameters.

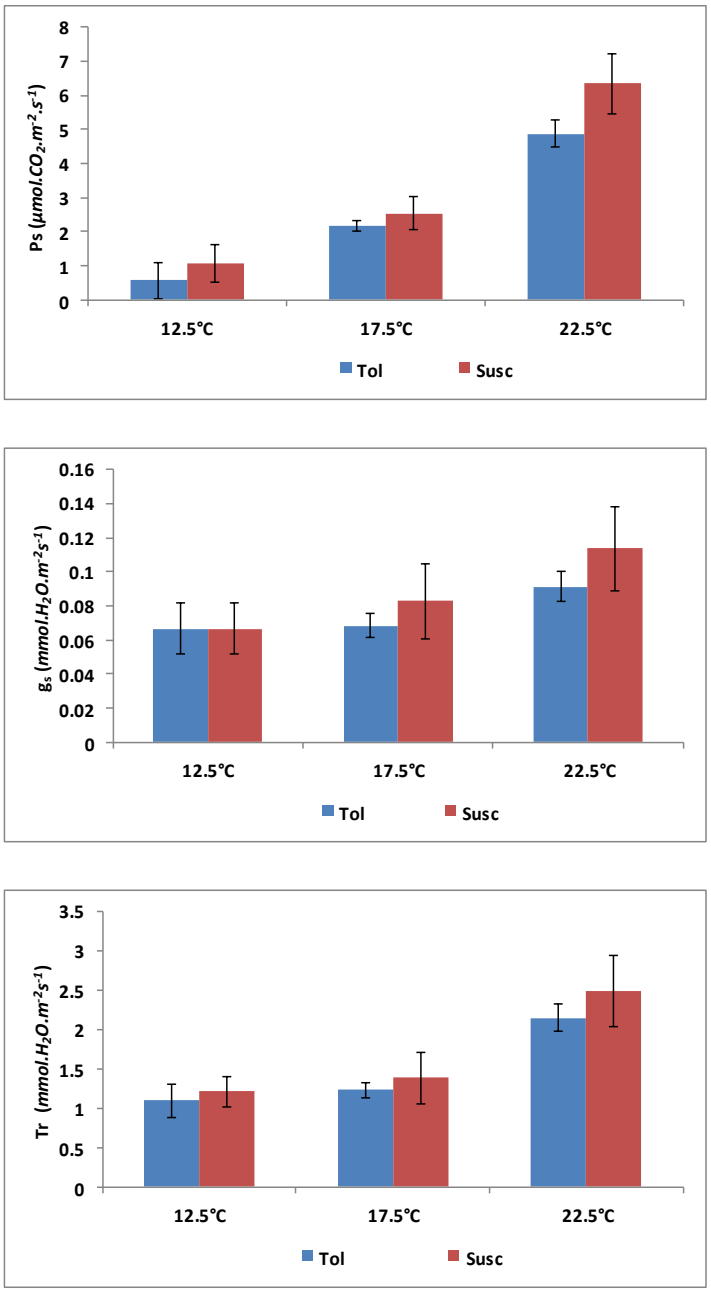


FIGURE 5.6: Mean photosynthesis (μmol.CO_2.m^{-2}.s^{-1}), stomatal conductance (g_s) (mmol.H_2O.$m^{-2}s^{-1}$) and transpiration (Tr) (mmol.H_2O. $m^{-2}s^{-1}$) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars after 14 days of exposure to new temperature settings in the three growth chambers. The error bars represent standard errors of the means, n = 4.

After the second downward adjustment in temperature by 2.5°C in all three chambers, photosynthesis (Ps) was significantly higher ($p = 0.0211$) in the low temperature susceptible than tolerant cultivars in the chambers where temperatures had been decreased to 15°C and 20°C (Figure 5.7). This trend was not as expected. The Ci/Ca ratio was however, significantly higher ($p = 0.0288$) in the tolerant than the susceptible cultivars in the chambers where temperatures had been adjusted to 10°C and to 15°C (Figure 5.7). However, Ps, g_s and Tr. for the low temperature tolerant and susceptible cultivars were not significantly different under the other temperatures.

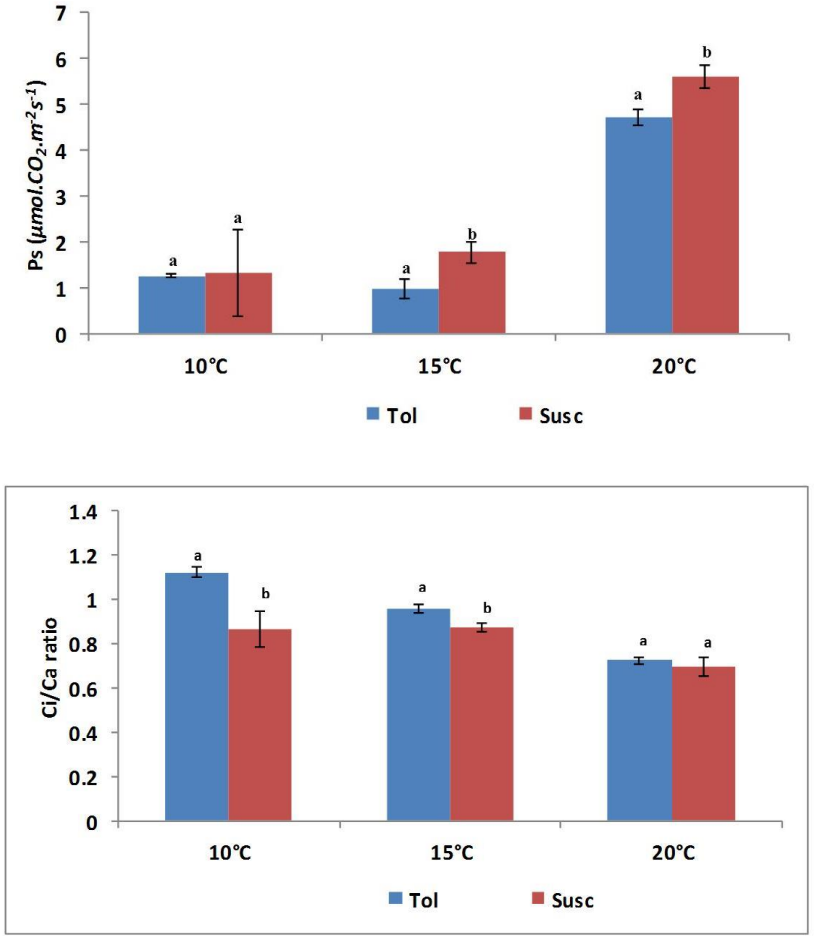


FIGURE 5.7: Mean photosynthesis (μmol.CO_2.$m^{-2}s^{-1}$), and Ci/Ca ratio for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars after 14 days of exposure to different temperatures in three growth chambers. The error bars represent standard errors of the means, n = 4. At each temperature, different letters (a) or (b) above the bar denotes significant differences at 95% level of confidence.

5.3.1.3 Biochemical measurements

Two biochemical parameters, total polyphenol content (TPC) and total antioxidant potential using the FRAP assay, were assessed. Assessments done at one day after effecting the temperature treatments showed no significant differences between the low temperature tolerant and susceptible cultivars in all three growth chambers (see Day 1, Figure 5.8). In the first growth chamber where temperature was adjusted from 15 to 10°C, there were no statistically significant differences in TPC between the tolerant and susceptible cultivars (Figure 5.8A). In the second growth chamber where temperature was adjusted from 20 to 15°C, TPC was significantly higher in low temperature tolerant than in susceptible cultivars at 20°C ($p = 0.0226$), 17.5°C ($p = 0.0101$) and at 15°C ($p = 0.0393$) (Figure 5.8B). Similarly, in the third chamber, TPC was significantly higher in the cold tolerant than in the susceptible cultivars at 25°C ($p = 0.0225$) and 20°C ($p = 0.0511$). However, there were no significant differences between the tolerant and susceptible cultivars at 22.5°C ($p = 0.8875$).

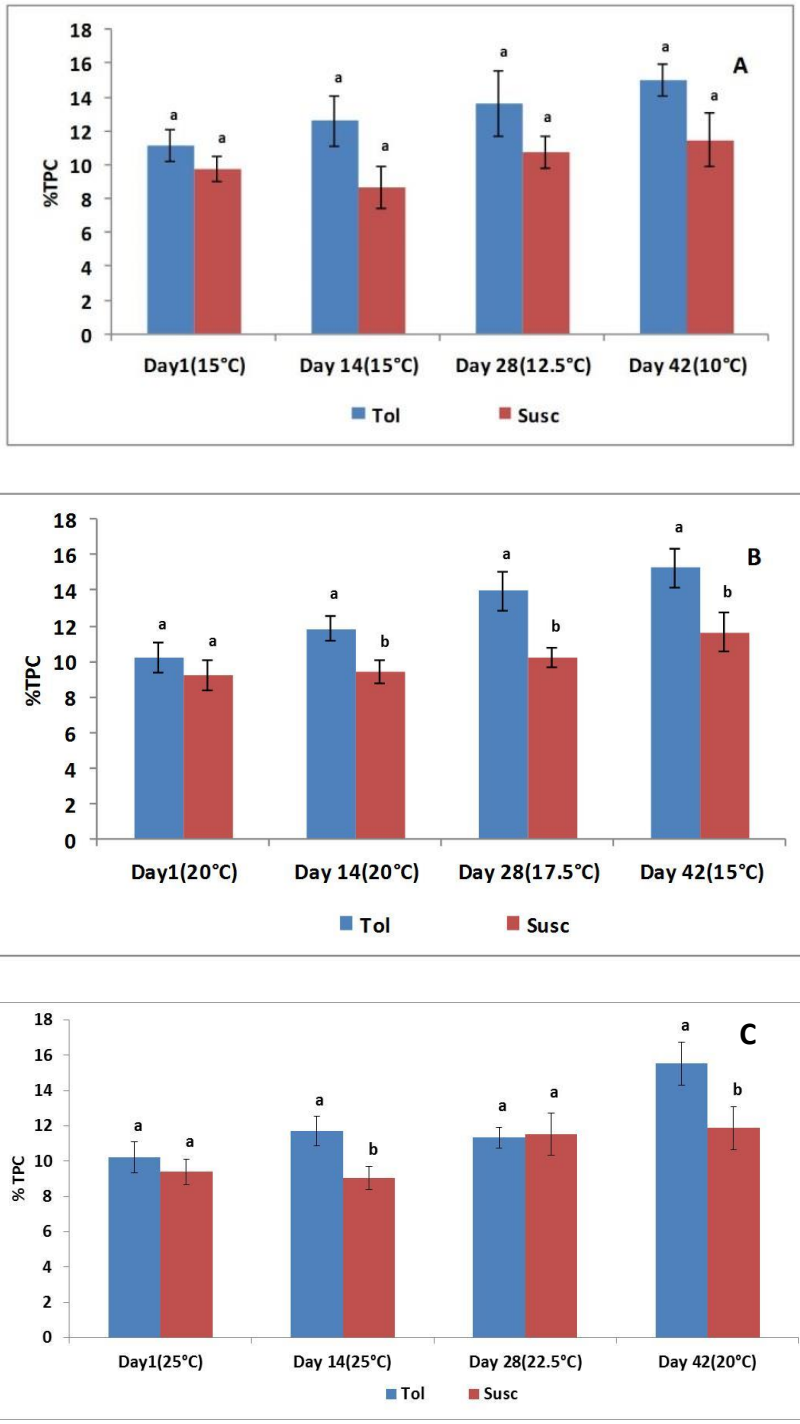


FIGURE 5.8: Mean total polyphenol content (%) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars exposed to different temperatures. Temperature were adjusted from 15°C to 10°C in the first chamber (A), from 20°C to 15°C in the second chamber (B) and from 25°C to 20°C in the third chamber (C). The error bars represent the standard errors of the means, n = 4. At each temperature, different letters (a) or (b) above the bar denotes significant differences at 95% level of confidence.

Results on total antioxidant activity estimated using the Ferric Reducing Antioxidant Power (FRAP) assay are presented in Figure 5.9. There were no significant differences ($p > 0.05$) between the low temperature tolerant and susceptible cultivars in antioxidant activity at the start of the experiment (Day 1 on Figure 5.9A, B & C).

Antioxidant activity was also measured at 14 days after implementing the three temperature regimes. At the first temperature regime (15°C to 10°C), antioxidant activity was significantly higher ($p = 0.0083$) in low temperature tolerant cultivars than in the susceptible cultivars when the temperature was set to 10°C (Figure 5.9A). However, there were no statistically significant differences between the two cultivar groups at 15°C and 12.5°C. In the second temperature regime (20°C to 15°C), the antioxidant activity in the low temperature tolerant cultivars was significantly higher than in the susceptible cultivars at 20°C ($p = 0.0093$), 17.5°C ($p = 0.004$) and at 15°C ($p = 0.0284$) (Figure 5.9B). Under the third temperature regime (25°C to 20°C), antioxidant activity was significantly higher ($p = 0.0155$) in the tolerant than in the susceptible cultivars only at 25°C (Figure 5.9C).

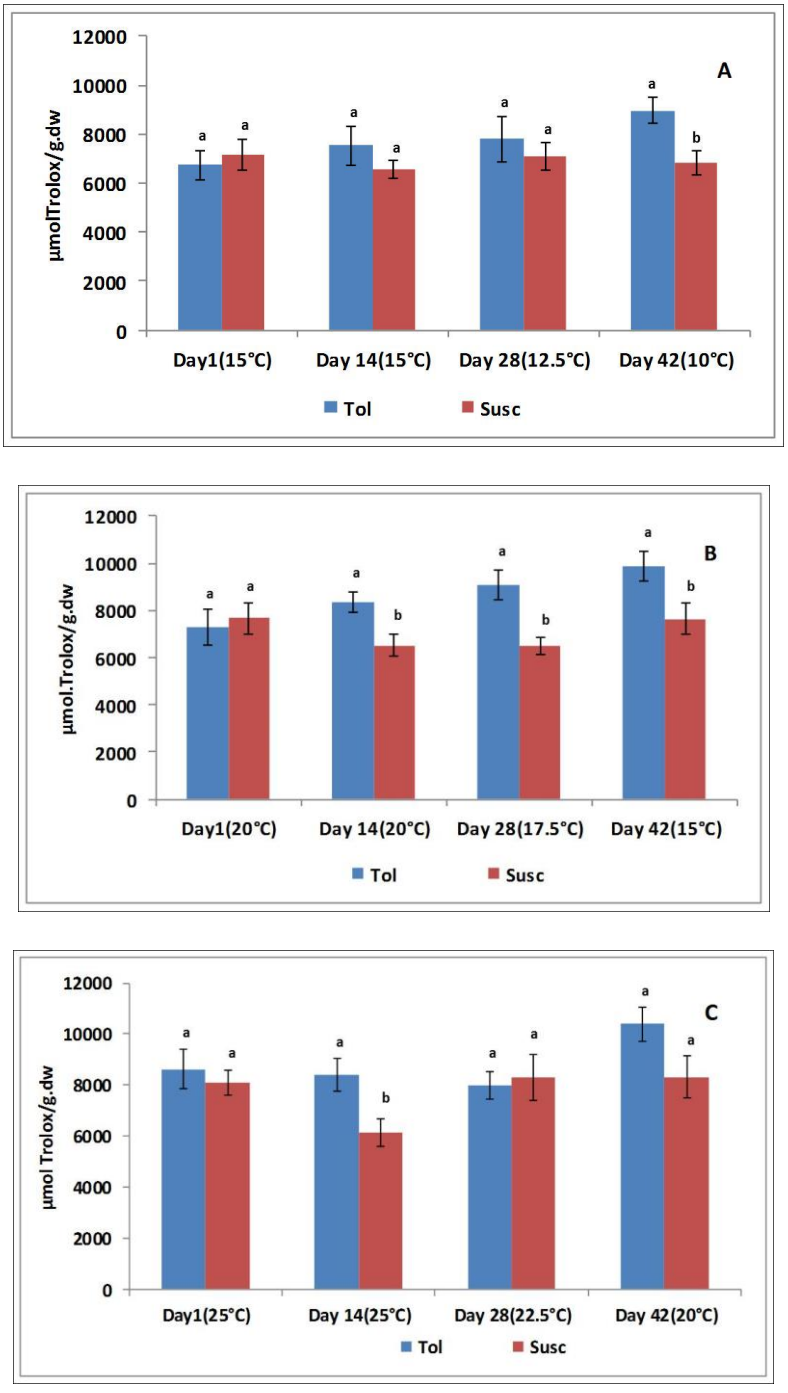


FIGURE 5.9: Mean antioxidant activity (µmol.Trolox.g^{-1}.dw) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars under different temperatures after fourteen days of exposure. Temperature were adjusted from 15°C to 10°C in the first chamber (A), from 20°C to 15°C in the second chamber (B) and from 25°C to 20°C in the third chamber (C). Error bars are of the standard error of the means, n = 4. At each temperature, different letters (a) or (b) above the bar denotes significant differences at 95% level of confidence.

The mean values for TPC and FRAP of tolerant and susceptible cultivars subjected to different temperature regimes over the whole duration of the experiment are summarised in Table 5.2. The mean values for TPC and FRAP of the low temperature tolerant cultivars were significantly higher than those of the susceptible cultivars over the entire period of the experiment under temperature regimes 20°**C** to 15°**C**. The increase in TPC and FRAP was also higher in the tolerant than in the susceptible group.

TABLE 5.2: Mean total polyphenol content (%TPC) and antioxidant activity (FRAP) for low temperature tolerant (Tol) and susceptible (Susc) cultivars under different temperature regimes

	Temperature regime 1 (15 to 10°C)			**Temperature regime 2 (20 to 15°C)**			**Temperature regime 3 (25 to 20°C)**		
Parameter	**Tol**	**Susc**	**t-test p-value**	**Tol**	**Susc**	**t-test p-value**	**Tol**	**Susc**	**t-test p-value**
%TPC (Day 1)	11.1	9.8	0.2761	10.2	9.2	0.4107	10.2	9.2	0.4752
%TPC (Day 14)	**12.6**	**8.7**	**0.0593**	**11.8**	**9.4**	**0.0226**	**11.7**	**9.4**	**0.0225**
%TPC (Day 28)	13.6	10.7	0.2123	**14.0**	**10.2**	**0.0101**	11.5	10.2	0.8875
%TPC (Day 42)	**15.0**	**11.5**	**0.0783**	**15.2**	**11.6**	**0.0393**	15.5	11.6	0.0511
FRAP^{+} (Day 1)	6754.3	7166.1	0.6451	7273.5	7676.3	0.6942	8622.3	8077.4	0.5624
FRAP (Day 14)	7527.8	6582.8	0.2982	**8343.4**	**6513.3**	**0.0093**	**8417.9**	**6143.6**	**0.0155**
FRAP (Day 28)	7791.6	7084.9	0.5345	**9048.7**	**6489.5**	**0.0040**	8291.8	7999.9	0.7829
FRAP (Day 42)	**8973.0**	**6844.1**	**0.0083**	**9882.8**	**7651.7**	**0.0284**	**10388.8**	**8326.6**	**0.0643**

$^{+}$*FRAP units are µmolTrolox.g^{-1}.dw of tea*

5.3.1.4 Discriminant analysis and Logit model application

Data on total polyphenol content (TPC), total antioxidant potential (FRAP), stomatal conductance (g_s), net photosynthesis (Ps) and transpiration (Tr) that was collected at different

time points was used in discriminant analysis in order to identify parameters that can be used to distinguish the low temperature tolerant from the low temperature susceptible cultivars that were subjected to low temperature stress.

The canonical plots for data collected after 14 days of exposing the plants to 15, 20 and 25°C in three different growth chambers showed that 88.9% (16/18) of the observations were correctly associated with the multivariate means for tolerance or susceptibility characteristics of the tested tea cultivars (Figure 5.10).

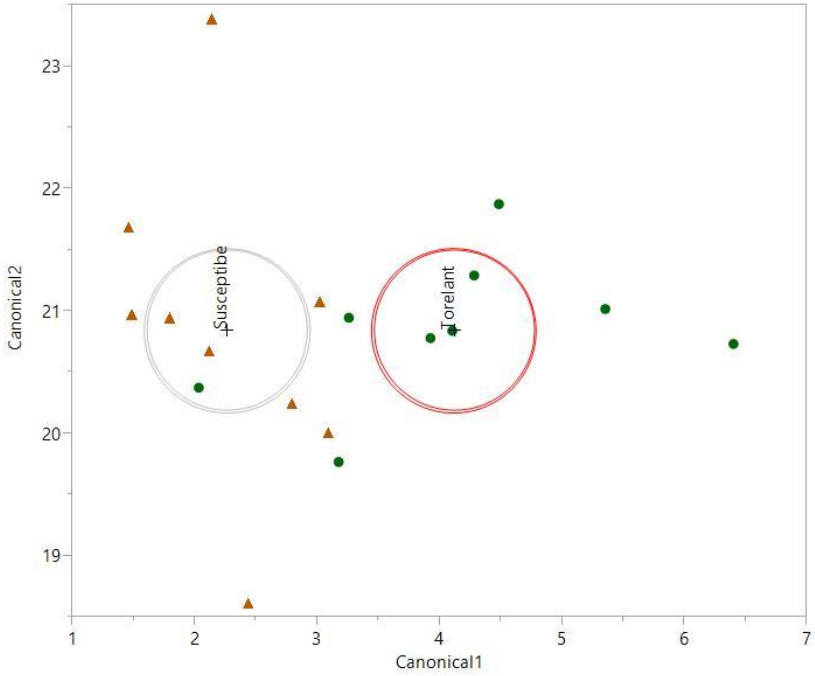


FIGURE 5.10: Canonical plots for low temperature tolerant and susceptible cultivars using mean values for total polyphenol content (TPC), total antioxidant potential (FRAP), stomatal conductance (g_s), net photosynthesis (Ps) and transpiration (Tr) at fourteen days after imposing the temperature treatments (15, 20 and 25°C). The green dots represent tolerant cultivars and the brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

Similar results were obtained when the same procedure was followed using data collected after 14 days of exposure to 12.5, 17.5 and 22.5°C (Figure 5.11). At this time point, 94.4% (17/18) of the multivariate observations correctly associated with the historical low temperature response groups of the tested cultivars.

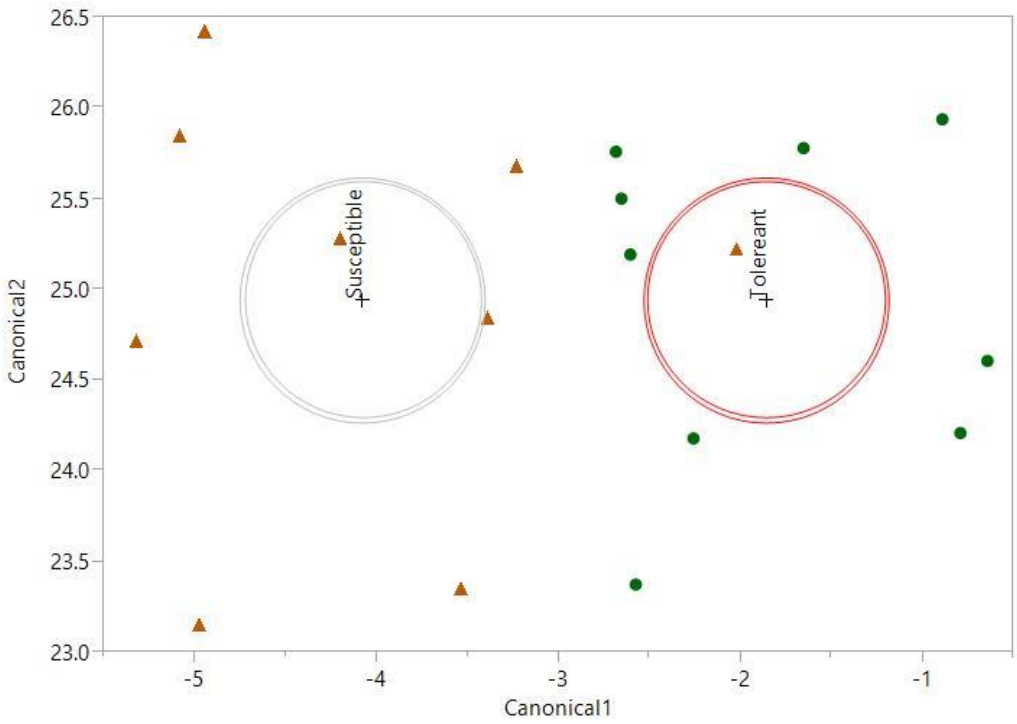


FIGURE 5.11: Canonical plots for low temperature tolerant and susceptible cultivars using mean values for total polyphenol content (TPC), total antioxidant potential (FRAP), stomatal conductance (g_s), net photosynthesis (Ps) and transpiration (Tr) at fourteen days after the first adjustment of temperature treatments (12.5, 17.5 and 22.5°C). The green dots represent tolerant cultivars and the brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

Using the results obtained after exposing the plants to the lowest temperatures in each of the three chambers (10, 15 and 22°C), discriminant analysis showed that all the multivariate observations (18/18) were correctly associated with the historical groups of the cultivars (Figure 5.12).The observations in the tolerant and susceptible groups were much closer to the 95% confidence ellipses of the multivariate means for each group.

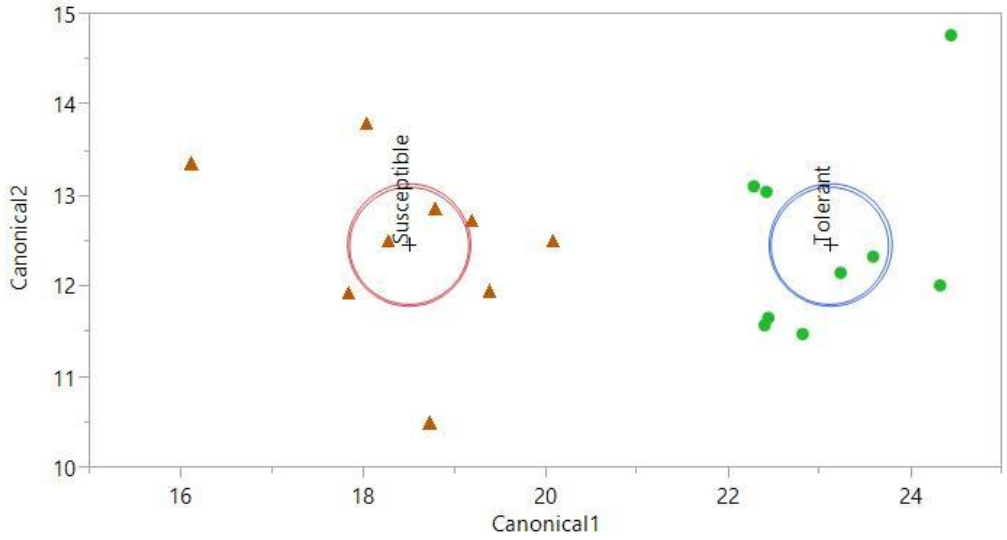


FIGURE 5.12: Canonical plots for tolerant and susceptible cultivars using mean values for total polyphenol content (TPC), total antioxidant potential (FRAP), stomatal conductance (g_s), net photosynthesis (Ps) and transpiration (Tr) at fourteen days after the second adjustment of temperature treatments (10, 15 and 20°C). The green dots represent tolerant cultivars and the brown triangles represent susceptible cultivars. The circles represent the 95% confidence ellipse of the multivariate mean for each group.

5.3.2 Tea shoot extension experiment under field conditions

This component of the trial represented the conventional method for assessing low temperature tolerance as it is done at TRFCA. Differences in shoot extension between the low temperature tolerant and susceptible cultivars started to emerge 39 days after the bud had been released from apical dominance (Figure 5.13). After day 39, the low temperature tolerant cultivars exhibited rapid extension rates, whereas shoots of the susceptible cultivars showed much slower extension after day 42.

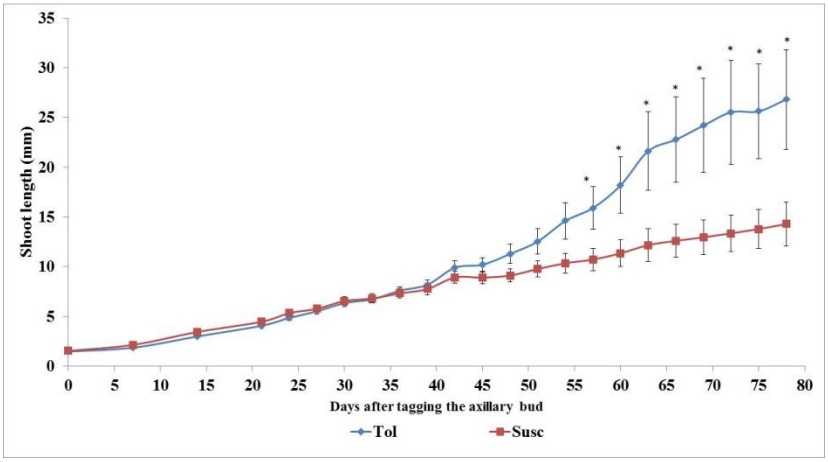


FIGURE 5.13: Mean shoot length (mm) for low temperature tolerant (Tol) and susceptible (Susc) tea cultivars from day 0 to day 78 after tagging the axillary buds at Mimosa research station in Malawi. The values are means for three cultivars in each group. For each cultivar 20 shoots were tagged on four bushes (five shoots on each bush). Error bars are of the standard error of the means, n = 12. A (*) denotes significant differences at 95% level of confidence.

The final shoot lengths at 78 days after removing apical dominance were used to calculate the change in shoot length per day (mm day^{-1}). The total change in shoot length for the low temperature tolerant cultivars was 25.9 mm compared to 12.8 mm for the susceptible cultivars (Table 5.3). Average shoot extension rate for the tolerant and susceptible cultivars was 0.33 and 0.16 mm day^{-1}, respectively. These results revealed significant differences (p =

0.0236) in extension rate between low temperature tolerant and susceptible cultivars. Shoots of low temperature tolerant cultivars extended at about twice the rate of the low temperature susceptible cultivars.

TABLE 5.3: Total change in shoot length and average shoot extension rate per day for different cultivars under field conditions during the cold season (May – August) at Mimosa in Malawi

Cultivar class	Total change (mm)	Shoot extension rate (mm per day)
Tolerant	25.9	0.33
Susceptible	12.8	0.16
Mean	19.3	0.24
P(0.05)	0.0236	0.0236

Daily minimum ambient temperature collected from a nearby Mimosa meteorological station linearly correlated reasonably well with average shoot length for the tolerant and susceptible cultivars over the same period (Figure 5.14). The regression line for the tolerant cultivars had a steeper slope (Fig. 5.14A) than the susceptible cultivars (Fig. 5.14B).

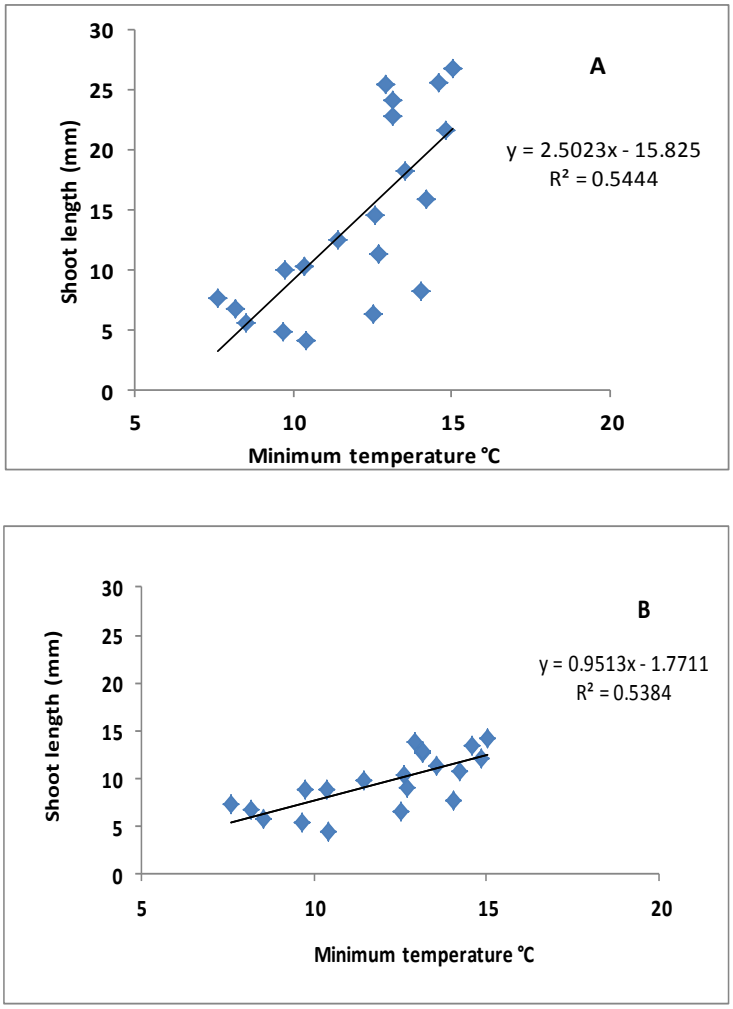


FIGURE 5.14: Correlation between shoot length (mm) and daily mean ambient temperature for low temperature tolerant (A) and susceptible (B) cultivars.

5.4 Discussion

Temperature affected shoot growth, as measured by increases in length. There were big differences between low temperature tolerant and susceptible tea cultivars when temperatures were ≤15°C, but these differences were less pronounced at temperatures between 20°C and 25°C. This was expected since it has been previously established that the average base temperature for shoot growth of seedling tea shoots in Malawi is about 12.5°C (Tanton, 1982a). However, this varies with cultivars and previous studies in other areas have suggested that the base temperature for tea can vary from 8°C to 13°C, depending on the cultivar (Carr, 2010). This implies that the base temperature of a tea cultivar can be lower or higher than 12.5°C. Field observations in Malawi also revealed variations among tea cultivars in terms of shoot growth during the cold season when minimum temperatures were below 12.5°C (Nyirenda & Mphangwe, 2001). Tea cultivars that exhibit active growth during the cold season are thus regarded as tolerant to low temperature.

In the growth chamber experiment, cultivars that had previously been classified as tolerant to low temperature (field observations) showed higher shoot extension rates than the susceptible cultivars at temperatures below 20°C. The growth chamber experiment results therefore confirmed the historical grouping of these two cultivar groups. These results further confirmed previous observations, which showed that tea cultivars respond differently to low temperature conditions (Carr, 2010; Upadhyaya, 2012). Shoot extension that was also monitored on shoots that were already growing (not buds) at the time of tagging also showed significant differences between the low temperature tolerant and susceptible cultivars. The results showed that temperature plays a crucial role in tea shoot extension. Again, the variations between the tolerant and susceptible cultivars were more conspicuous on plants that had been exposed to the lower temperature regimes (20 to 10°C). The results on shoot

growth clearly showed that tolerant cultivars had higher extension rates than susceptible cultivars under cold stress conditions. Thus Ho_1 is rejected based on these results. This implies that there was a statistically significant difference in the growth rates between the tolerant and susceptible cultivars under cold stress conditions. Such cold chamber experiments may be used to select cold tolerant cultivars in the future.

Changes in temperature affected the physiological performance of the tea plants. This varied among temperature regimes and cultivar groups. The physiological parameters Ci/Ca ratio, photosynthesis (Ps), stomatal conductance (g_s) and transpiration rate (Tr) were all influenced by temperature in the growth chambers. Rate of photosynthesis (Ps) appeared to be more sensitive to low temperature than the other parameters, an observation that has also been reported previously in tea and other crops (Theocharis *et al.*, 2012). For example, a study in *Coffee arabica*, showed similar results, where photosynthesis dropped by over 50% in plants that had been kept at 16°C / 20°C day / night temperatures for 10 days (Bauer *et al.*, 1990). The low temperature tolerant cultivars generally showed low g_s and Ps, although these differences were not statistically significant. This trend was not expected but probably suggested early stress signal perception and transduction in the tolerant cultivars. Reduction in Ps could be due to reduced assimilation of CO_2 as a result of reduced g_s due stomatal closure following a chill, or an indirect response to high internal leaf CO_2 concentration (*c*i) caused by a chill-induced loss of Rubisco activity (Allen & Ort, 2001). Squire (1978) studied the behaviour of stomata in tea and concluded that there might be a variable relationship between g_s and Ps, depending on the time of the day and season. Since the temperatures in the current study were kept constant during the day and night, changes in g_s due to variations in temperature at different times of the day were assumed to be minimal. There was an increase in Ci/Ca at low temperature. This suggests reduced assimilation of carbon-dioxide due to reduced Rubisco enzyme activity that is also very sensitive to temperature and disruption of

the major components of photosynthesis (Allen & Ort, 2001). In grapevine, chilling temperatures limited photosynthesis through stomatal closure or by inhibiting the electron transport in the thylakoids (Hendrickson *et al.*, 2004). Transpiration rates (Tr) were minimal at low temperatures, probably reflecting a decrease in the ambient evaporative demand due to low ambient temperature as well as stomatal closure that was evidenced by low g_s.

None of the physiological parameters measured in the current study could individually be used to distinguish tolerant and susceptible cultivars because the results consistently showed no significant differences between the two groups of tea cultivars. These parameters can therefore not be reliably used to select for low temperature tolerance in tea. This was in agreement with what had been hypothesized, viz. that physiological changes under low temperature could not be used to discriminate tolerant and susceptible cultivars. Based on these results, Ho_2 was accepted, implying that cold stress conditions resulted in no significant differences in the physiological parameters between the cold tolerant and susceptible cultivars. However, data on individual parameters showed some differences, which probably did not translate into differences between cultivar groups. This could have been a result of the limited number of plants that were used due to space constraints in the growth chambers. This must be taken into consideration when designing future experiments.

The biochemical analyses revealed significant variations in the response to low temperature of the tolerant and susceptible tea cultivars. Low temperature can cause oxidative stress in plants when the plants are exposed to chilling, but non-freezing temperatures (Upadhyaya, 2012). Oxidative stress is caused by increased production of reactive oxygen species due to low temperature stress (Griffin & Bhagooli, 2004; Jaleel *et al.*, 2009). In response, plants increase accumulation of phenolic compounds and antioxidants as a non-enzymatic defence mechanism against oxidants (Jaleel *et al.*, 2009; Hue *et al.*, 2012; Upadhyaya, 2012). In the

present study, cultivars that are known to be tolerant to low temperature had higher total polyphenol contents (TPC) than cultivars that are classified as susceptible to low temperature. It was therefore postulated that these tolerant cultivars had higher total polyphenol content in order to use the polyphenols to counter the oxidative stress caused by low temperature. In a similar study, Upadhyaya (2012) found that low temperature tolerant tea cultivars had higher TPC at low temperature (20°C) than at high temperature (30°C), even though the exposure period was shorter than the one tested in the current study. Plants can use polyphenols to quench some of the free radicals by acting as hydrogen ion (H^+) donors or by forming intra-molecular bonds (Benzie & Szeto, 1999; Jeyasekera *et al.,* 2011; Hue *et al.,* 2012). The trend observed with TPC was similar to that of antioxidants whereby low temperature tolerant cultivars showed higher antioxidant activity than the susceptible cultivars. Previous studies have also shown that antioxidant activity can increase in response to high ROS that are formed due to low temperature stress (Griffin & Bhagooli, 2004; Upadhyaya, 2012). The similarity in the trend between the TPC and antioxidant activity (FRAP) observed in the current study corroborated well with several other studies which showed a very good correlation between TPC and FRAP (Benzie & Szeto, 1999; Wang & Zheng, 2001; Jaleel *et al.,* 2009; Jeyasekera *et al.,* 2011). This close relationship between the two parameters can be used to minimize costs in screening for low temperature tolerance as either TPC or FRAP could be used as a surrogate measure of the other. The choice between the two assays could also depend on the complexity of analytical procedures and availability of the necessary screening facilities. These results did not agree with the hypothesis that low temperature tolerant and susceptible cultivars would produce the same levels of antioxidants under cold stress condition. Thus, Ho_3 is rejected, implying that low temperature tolerant cultivars would produce more antioxidants than the susceptible cultivars under cold stress. The tolerant

cultivars use this non-enzymatic mechanism to counter oxidative stress caused by low temperature.

Discriminant analysis showed that the tolerant and susceptible cultivars can be separated based on measurements of total polyphenol content (TPC), total antioxidant potential (FRAP), stomatal conductance (g_s), net photosynthesis (Ps) and transpiration (Tr). Assessments done at 14 days after every temperature setting showed that differences between the tolerant and susceptible cultivars widened as the temperatures in the chambers decreased. At the initial temperature settings, there were slight overlaps between the two cultivar groups but these were much less at the lowest set temperatures. The wide gap between the 95% confidence ellipses of the multivariate means of the tolerant and susceptible cultivars and the increasing density of the observations around each of the group means revealed the difference in response to low temperature between the two cultivars groups. This supported the view that a combination of the measured parameters can be used in screening for low temperature tolerance in tea.

Monitoring of shoot extension under field conditions is currently used to identify cultivars that exhibit active growth during the cold season. An average increase in shoot length per day could be used to distinguish low temperature tolerant and susceptible cultivars. The results from the current study showed that low temperature tolerant cultivars registered an average extension rate that was about twice the extension rate recorded by the susceptible cultivars over the 78 day-period. Although developing tea shoots do not show a constant rate of extension in all of their developmental phases (TRFCA, 1990), it can be justifiable to base cultivar differences in response to low temperature on an overall average extension rate calculated over the whole duration of the shoot development cycle, as was done in this experiment. In fact, some tea shoot growth models assume that the extension rate is constant

(Burgess, 1992). It was interesting to note that differences in shoot extension rate between the tolerant and susceptible cultivars were more conspicuous over a period when the ambient minimum temperature for the area was below the accepted base temperature of tea (12.5°C). The estimated extension rate for both the low temperature tolerant and susceptible cultivars showed a good correlation with ambient minimum daily temperature. The correlation line for the tolerant cultivars had a steeper slope than that of the susceptible cultivars. This implied that for every increase in minimum temperature there was more increase in the shoot length of the tolerant cultivars than the susceptible cultivars. The response of the tea cultivar groups to low temperature in the field was similar to the observations in the growth chamber experiment, especially for the growth chamber where temperature was adjusted downward from 15 to 10°C. However, this correlation should be taken cautiously since the temperatures in the growth chambers were kept constant for fourteen days, unlike in the field where both the minimum and maximum temperatures vary every day. In addition, the number of shoots tagged was limited since we had only one plant per plot and the plants did not have many branches. These observations further confirmed the important role of temperature in development of tea shoots. Under field conditions, the method of using shoot extension rates to differentiate tolerant and susceptible cultivars can, however, probably be improved by measuring the rate of extension at the growth stage of the shoot where cultivar differences are more conspicuous.

5.5 Conclusions

The response to low temperature stress differed between the tolerant and susceptible tea cultivars. The low temperature tolerant cultivars maintained active shoot growth under low temperature conditions, whereas the susceptible cultivars showed minimal active growth.

These differences can be monitored through shoot growth measurements and physiological and biochemical assessments.

Photosynthesis and stomatal conductance are physiological parameters that appeared to be more sensitive to low temperature than transpiration rate and Ci/Ca ratio. Photosynthesis and stomatal conductance can therefore be used as indicators of low temperature stress. However, average values of these two parameters for the tolerant and susceptible tea cultivars used in the current study could not be used to reliably distinguish between the two tea cultivar groups.

Total polyphenol content and antioxidant activity associated well with response to low temperature exposure of the tested cultivars. The low temperature tolerant cultivars accumulated more polyphenols and showed higher antioxidant activity than the susceptible cultivars. Whilst there were some overlaps between classification groups for low temperature tolerance of the cultivars, a trend emerged that suggested a good relationship between tolerance to low temperature and levels of polyphenol and antioxidant activity. The overall averages of TPC and FRAP for the tolerant and susceptible cultivars clearly demonstrated that these parameters can be used to distinguish the two groups. It was therefore concluded that tea cultivars can tolerate low temperature stress due to higher levels of total polyphenols and antioxidants that reduce oxidative stress. This is a non-enzymatic defence mechanism that has also been observed in other plant species (Jaleel *et al.*, 2009; Hue *et al.*, 2012).

Determination of shoot extension can also be used in selection for low temperature tolerance, particularly in the advanced stages of a selection programme where only a few selections (genotypes) are evaluated, since this method is time consuming and laborious. At that advanced stage of selection, use of growth chambers where temperature changes can be controlled would be more ideal than field screening, since the results from the current study

showed that cultivar differences in shoot extension rates were more conspicuous at temperatures that are much lower than the daily mean temperatures that would prevail in the field.

The results from this work have demonstrated that tea cultivars minimize the effects of oxidative stress caused by low temperature through increased accumulation of polyphenols and/or increasing antioxidant capacity, which are used to quench oxidants. This characteristic significantly varied with cultivars and enabled the tolerant cultivars to continue to show active shoot growth and/or extension under low temperature conditions. This was in agreement with what had been hypothesized at the start of the research, namely that these parameters significantly vary between the tolerant and susceptible cultivars and can therefore be potentially used as criteria for selecting low temperature tolerant tea cultivars in future breeding and selection programmes when temperature controlled chambers are available.

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Appendix 5.1: Plot layout* for the experiment on low temperature tolerance in growth chambers at Hatfield Experimental Farm, University of Pretoria

Chamber 1: (Temperature regime: 15, 12.5 and 10°C)

6(2)	1(3)	6(4)	2(4)	3(3)	6(3)
1(2)	5(4)	2(1)	3(1)	3(4)	6(1)
1(1)	1(4)	2(2)	2(3)	3(2)	4(4)
4(1)	5(2)	4(2)	5(3)	4(3)	5(1)

Chamber 2: (Temperature regime: 20, 17.5 and 15°C)

4(4)	4(2)	2(1)	6(1)	6(3)	5(3)
3(3)	1(4)	5(4)	4(3)	2(4)	3(1)
5(2)	5(1)	3(2)	1(1)	6(2)	2(3)
3(4)	1(3)	4(1)	1(2)	2(2)	6(4)

Chamber 3: (Temperature regime: 25, 22.5 and 20°C)

1(1)	1(3)	1(2)	6(2)	2(1)	2(2)
4(4)	3(3)	6(1)	2(3)	4(3)	5(3)
3(4)	6(4)	3(1)	4(1)	5(4)	2(4)
6(3)	1(4)	4(2)	3(2)	5(1)	5(2)

*Notes: Cultivars: **1** = PC 153; **2** = PC 198; **3** = PC168; **4** = PC 165; **5** = CL 12 and **6** = RC6. The numbers in brackets represent the replicate number. Temperatures were adjusted downward by 2.5°C every 14 days. The internal size of each chamber is 2.4 m long and 1.2 m wide.

CHAPTER 6

CONCLUDING DISCUSSION AND RECOMMENDATIONS

6.1 Concluding Discussion

Tea is an important crop worldwide. Since most tea producing countries export a large percentage of their processed tea, the crop is a major source of foreign exchange earnings for these countries. The tea industry offers direct employment opportunities for many people who work on tea farms and, indirectly, to those who work in other industries associated with the tea beverage. In Malawi, for example, tea is the third biggest cash crop and contributes significantly to the economy of the country as well as the social development of the people around the tea plantations. Malawi is the second largest exporter of black tea in Africa and commands about 4% share of the global black tea market. With this contribution and impact, it is always desirable to have a sustainable tea industry.

Tea production in Malawi, like in many other areas, is faced with several challenges, which include the need for high quality tea that satisfies consumer needs and preferences on the international market, and the need to grow tea cultivars that can produce high yields under constraining biotic and abiotic stresses. Some of the major environmental challenges to sustainable tea production in Malawi are recurrent droughts and low temperatures. These render the Malawi tea industry to be a seasonal producer whereby about 80% of the annual crop is harvested in about five months. In order to cope with some of these challenges, the Malawi tea industry invests in research by funding the Tea Research Foundation of Central Africa (TRFCA), which conducts research in all aspects of tea production. One major area of the TRFCA research is the breeding programme. Improving quality of black tea and tolerance to biotic and abiotic stresses is a primary objective of many tea breeding programmes. In central Africa, black tea quality and tolerance to water stress (drought) and low temperature

are economically important traits. The tea breeding programme at TRFCA endeavours to improve on these traits in cultivars that are recommended for commercial production through breeding and selection. Tea quality and tolerance to drought and low temperature are complex traits that are difficult to select for. This is partly due to lack of objective and reliable criteria, high cost and the need for expensive equipment required to carry out some of the available assays, particularly those that can be used at an early stage of selection. As a result, most tea breeding and selection programmes are long and expensive as they can take more than 15 years before an improved cultivar is produced and made available to growers. It also takes a breeder a long time to characterize some base materials from which parents for a breeding programme can be chosen.

Some of the conventional screening methods used are less efficient and do little to add to the knowledge regarding the plant characteristics that govern the desired traits. For instance, plant survival can be a good measure of tolerance to water stress in the field but the technique fails to elucidate why some cultivars survive water stress better than others. Advances in plant biotechnology have unveiled opportunities for improving the speed and accuracy of selection of superior crop cultivars. Use of molecular markers in breeding and selection can allow indirect selection of plants with desired traits in addition to speeding up the identification of plant materials that can be used as parents in hybridization. For example, identification of markers that associate with tolerance to drought or low temperature tolerance can allow screening of tea germplasm for these traits, even where the plants are not physically exposed to the stress. The use of biotechnology in tea breeding has been relatively slow. As a result, there are still very few markers that are closely associated with desired agronomic traits. This is partly due to the natural characteristics of the tea plant. Tea has a large genome which would require a lot of markers to be saturated. The tea plant also has a

long generation cycle, which delays the development of some characteristics that are associated with some desired traits.

Notwithstanding the progress made in tea biotechnology over the past few decades, there is still need to search for markers that associate with traits of agronomic importance. It must also be noted that the importance of traits varies with regions of tea cultivation and this in turn impacts on the priorities of different tea research programmes. The traits that were considered in this study are of great importance to tea growers in Malawi and possibly in other tea growing countries that face similar tea production challenges.

The main purpose of the current study was to identify tea plant characteristics that can be used in devising better selection methods for black tea quality, drought and low temperature tolerance. This would help to improve precision and probably speed of developing improved tea cultivars. Such cultivars would help the tea industry in Malawi to remain viable.

One of the objectives of the current study was to identify RAPD markers that associate with black tea quality and tolerance to drought and low temperature. The RAPD technique is not necessarily new and there newer and probably more advanced molecular markers that are proclaimed to be better than RAPD. The RAPD technique was chosen because it is easy to perform and uses inexpensive equipment. In addition, RAPD can be used on crops where genetic information is limited, as is the case with tea, because it does not require prior knowledge of the DNA sequence. It was also considered that these merits of the RAPD technique would make it easy to incorporate the RAPD method in the conventional selection methods used at TRFCA in order to enhance precision and efficiency of selection. This part of the study started with the screening of RAPD primers in order to identify those that could give consistent and reliable results. This exploratory part of the study used 18 cultivars that

had been characterized for presence (9 cultivars) or absence (9 cultivars) of each of the three traits: black tea quality, drought tolerance and low temperature tolerance. The cultivars were selected from the breeding programme because it was not possible to get a segregating population of plants for each of the traits. A total of 60 primers were screened and 20 of these gave satisfactory results and were subjected to confirmatory tests. This was a very important step because RAPDs can sometimes show poor repeatability. After the preliminary screening, the 20 most promising RAPD primers were used on a group of 32 cultivars, most which had a known history in terms of absence or presence of the three traits of interest.

From this work, a total of six putative RAPD markers were identified, two of which associated with high black tea quality, one marker associated with low tea quality, two markers associated with drought tolerance and one other marker associated with low temperature tolerance. The level of association between the marker and the trait of interest was estimated by looking at how many of the test cultivars that are known to have the trait carried the RAPD band (for markers that showed positive association). Markers which showed negative association were expected to be present only in tea cultivars that lacked the trait. This analysis showed that the level of association for the six markers ranged from 37.5 to 81%. Out of the six markers, three showed no false positives for the specific trait with which they associated whereas the other three showed some false positive results, which was estimated at 6.3% for two of them and 12.5% for the third marker. Two of the three markers which showed false positives were associated with drought. Since drought is not very easy to accurately score using qualitative conventional methods, these results could be a reflection of the difficulty in accurate scoring under field conditions. Association between an individual marker and a trait of interest varied with the trait and ranged from 31.3 to 81.3%. Where more than one marker had been identified, using the markers as a panel increased the level of

association to between 81.3 and 100%. These results indicated the need to search for more markers for all traits.

The identification of the six RAPD markers is an important contribution towards the search for trait-associated markers in tea and will have a positive impact on successful tea breeding once the technique is incorporated in the selection programme. These RAPD markers can be incorporated in the breeding programme in order to improve precision and efficiency in selection. The markers can be used to timely identify parental breeding stocks that have very good potential for quality and drought and low temperature tolerance. The selected parental stocks could then be deployed in a deliberate crossing (hybridization) programme in order to create populations from which to select plants that possess the desired traits. This can help to concentrate alleles that are associated with traits of interest and thereby increase the chances of developing more heterotic progenies. There is also potential to use these RAPD markers in the early stages of selection, for example, at the preliminary observation plot stage. The relative technical ease and low expense associated with RAPDs would make their use more feasible, especially in situations where access to highly advanced marker technologies would either be difficult or hampered by lack of funding. Incorporating use of RAPD markers at the stages of conventional selection programme mentioned above can help to rationalize use of resources (land, time and personnel) that are deployed in preliminary field evaluations. This can also help to ease the problem of inadequate resources for research, which is a common bottle-neck at most institutions. In addition, early selection of plant lines that have high potential for the traits of interest can help to speed up the development of new cultivars by quickly advancing potential selections to the confirmatory stages of field experimentation. The shortlisted selections can easily be subjected to the more elaborate but relatively expensive screening assays that cannot be used in the early stages of selection due to high costs.

Since the ultimate aim of tea breeding and selection is to release new cultivars to growers for commercial use, the RAPD markers identified in this study were tested for their discriminating ability between 23 released and nine (9) not-released cultivars. Two RAPD markers that associated with black tea quality and one marker associated with low temperature tolerance showed good discrimination ability for the two sub-groups of cultivars. Using a panel of all six the markers, it may be possible to screen out about 50% of the base material with poor potential for the desired trait and allow the remaining 50% to be advanced to the next stage of the selection programme. This analysis showed that the identified markers could greatly help the breeder to screen out a large proportion of the breeding lines that lack potential for the different traits of interest at an early stage. This would allow the breeder to only continue with materials with good potential and are likely to be released for commercial use. Incorporation of the identified markers in the conventional breeding programme can therefore help to improve the success of the breeding programme through increased number of cultivars that are recommended for commercial use.

One of the technical problems associated with RAPD markers is low levels of reproducibility. In the present study, the screening conditions were optimized and the results were repeatable. This showed that it should be practically possible to optimize conditions for RAPD and incorporate them in a conventional breeding and selection programme.

Selecting for complex traits such as drought and low temperature is usually difficult, especially when using conventional selection criteria which cannot be directly correlated to specific plant characteristics that influence cultivar performance under stressful conditions. As a result, mechanisms associated with tolerance to abiotic stresses such as drought or low temperature remain obscure in many crops, including tea. This dearth in knowledge of

mechanisms that influence expression of a trait presents a practical challenge in devising appropriate objective and reliable selection criteria.

Drought tolerance was studied using potted tea plants that were about two years old at the start of the experiment. There were ten cultivars, which were grouped in tolerant (5) and susceptible (5) groups, based on historical field observations. Drought (water stress) was induced by withholding water for four and eight days. It was hypothesized that water stress would cause a number of physiological and biochemical changes within the plant which could be used to distinguish the two groups of cultivars. The experiment looked at wilting, leaf relative water content, stomatal conductance, photosynthesis and transpiration rate, chlorophyll content, maximum quantum efficiency of photosystem II, total polyphenol content and antioxidant activity as possible characteristics that could be associated with tolerance to drought of different tea cultivars. Most of these characteristics showed significant differences between the stressed and non-stressed plants after both four and eight days of water stress. This suggested that the stress was high enough to cause some changes in these parameters. However, uni-variate statistical analysis showed that none of these parameters could individually show significant differences between the drought tolerant and drought susceptible cultivars. For some parameters, e.g. relative water content (RWC), the general trend was that drought tolerant cultivars maintained higher RWC than the susceptible cultivars as the water stress progressed, but the differences were mostly not significant. Large differences were observed among individual cultivars for most of these parameters, which suggest that they can be used to indicate level of stress within the plant. Since the stress period was not very long, it is possible that the responses observed, particularly at four days of stress, could relate more to differences in the ability of the individual cultivars to perceive the stress.

In multivariate linear discriminant analysis using data of the various characteristics showed that a combination of several characteristics could be used to correctly distinguish the tolerant from the susceptible cultivars. While measuring several parameters could be cumbersome and expensive, the results from the current study suggested that a combination of relative water content and total antioxidant activity could be used to correctly predict the response of individual cultivars to water stress as early as four days after withholding water. It was therefore concluded that these two characteristics can be used as criteria for selecting drought tolerant cultivars under short duration of water stress.

From the point of view of feasibility of using the different parameters in a selection programme, RWC and antioxidant activity can easily be incorporated in the selection programme and be used routinely due to their low cost of analysis and need for simple equipment. However, when water stress was prolonged to eight days, stomatal conductance together with rate of transpiration provided the best prediction of tolerance or susceptibility of a cultivar to water stress in multivariate analysis. This suggested that these parameters could be used under prolonged water stress, although accurate measurements of most leaf gaseous exchange parameters require use of expensive equipment and a lot of time to prepare the plant materials prior to taking the measurements. This may therefore make the gas exchange parameters not to be amenable to use in early stages of selection programmes, where there is a large number of lines to be evaluated.

There were some overlaps between the drought tolerant and susceptible cultivars in terms of these physiological and biochemical characteristics. This could be partly attributed to differences in tolerance mechanisms to water stress used by the different cultivars within the tolerant or susceptible cultivar groups. In addition, this might be a reflection of some inadequacies in the method that was used to classify the cultivars for drought tolerance and some level of inter-plant variation within a cultivar. In practice, it is very difficult to

differentiate a tolerant cultivar from another that shows intermediate response to a complex trait such a drought. The problem of inter-plant variation within a cultivar can be addressed by measuring soil water content in individual plant pots which was not done in the current study due to lack of necessary equipment for non-destructive measurement of soil water content.

Recovery from water stress was assessed by measuring some of the characteristics measured during the stress period after the plants had been re-watered. It was interesting to note that the differences between the tolerant and susceptible tea cultivars in total polyphenol content and antioxidant activity observed during the stress period showed a similar trend during the recovery period. This observation was thought to be important because it revealed the possibility that plant behaviour during the recovery period could be inferred from measurements taken during the stress period. In practice, this would enable tea growers to take necessary measures to ensure good recovery based on what they see on stressed plants. The importance of quick recovery of tea plants from drought cannot be over-emphasized, particularly in Malawi, where extended periods of water stress are usually experienced and about 80% of the tea area is not irrigated. For rain-fed tea plantations, quick recovery from water stress would ensure higher yield during the main harvesting season.

It must be noted, however, that physiological characteristics can vary with plant age and growing conditions. For example, other convergent stress factors to drought like high temperatures and vapour pressure deficit can also influence plant response to drought and these should be considered when describing how different cultivars respond to the drought stress. Sometimes there is an inordinate relationship between plant age and response to drought. Under field conditions, young tea plants are more vulnerable to drought than mature plants, more likely because the roots have not grown very deep. In addition, the drought

stress imposed on young potted plants is usually quick and drastic, which may not be the case in the field where drought may take a long time to manifest.

Tea polyphenols are routinely analysed to determine black tea quality potential of individual cultivars. It has also been reported that total polyphenol content positively correlates with total antioxidant activity and that each of these parameters can be a surrogate measure of the other. The good association between drought tolerance and total antioxidant activity that was observed in this study may, therefore, possibly suggest a link between quality and drought tolerance. This can possibly allow concurrent selection for these two important traits in tea. However, the nature of relationship between quality and drought tolerance will more likely vary among cultivars. This has been observed in some of TRFCA tea cultivars, for example, PC 168 shows a very good combination of drought tolerance and quality of made tea, but cultivar SFS 204, which also has high quality, shows poor drought tolerance.

A drought susceptibility index (DSI), based on the photosynthesis rate of stressed and non-stressed plants, was determined for the ten cultivars tested. DSI computed using photosynthesis and other physiological parameters collected during stress showed no significant differences between tolerant and susceptible cultivars. It was therefore not possible to develop an index for tolerance to water stress based on the gaseous exchange measurements that were done in this study.

Phenotypic assessment for low temperature tolerance can be done by looking at the rate of growth or extension of shoots when exposed to cold stress. In the present study, low temperature tolerance was assessed on potted plants that were put in growth chambers and on mature tea plants that were growing in the field. Shoot lengths and several physiological and biochemical parameters were assessed in the growth chamber experiment, whereas in the field experiment, only shoot lengths were measured during the cold months.

Results from the growth chamber experiment showed no significant differences in terms of shoot development from tagged axillary buds. In the two growth chambers where temperatures ranged between 15°C and 25°C, low temperature susceptible cultivars had relatively longer shoots than the tolerant cultivars, though not significant. This was not expected, but was probably a result of plants being exposed to a mean temperature that was generally above 12.5°C (the base temperature for tea shoot growth), since the temperatures in the growth chambers were kept constant during the day and night. The observed differences among cultivars in terms of shoot length in these chambers were probably more related to differences between genotypes than the effect of the temperatures set in these two chambers.

For a population of shoots that were tagged after they had already gone past the bud stage, there were significant differences in shoot lengths between the low temperature tolerant and susceptible cultivars in the chamber where temperatures were adjusted from 15 to 10°C. These results highlight both the critical role that temperature plays in shoot extension and the differences in response to low temperature exhibited by tea cultivars.. However, in the two chambers where temperatures were above 15°C, the differences in shoot length became marginal as temperatures approached 15°C and 20°C. The differences in the shoot length between the two groups of cultivars can partly be attributed to differences in base temperature for shoot growth and shoot extension in tea. The base temperature for extension has been reported to be about 3°C higher than that for shoot growth (Burgess, 1992).

Photosynthesis (Ps), stomatal conductance (g_s) and transpiration (Tr) showed no significant differences between the low temperature tolerant and susceptible cultivars. However, the rates of all three the parameters were generally reduced under lower temperature, with photosynthesis being the most affected parameter. There were statistically significant differences between the low temperature tolerant and susceptible tea cultivars for Ps and

Ci/Ca in the growth chamber where temperatures had been adjusted from 17.5 to 15°C. Low temperature susceptible cultivars showed higher rates of photosynthesis than the tolerant cultivars. This was not expected, but was probably due to high variation within the two cultivar groups. These results, nevertheless, suggest that changes in photosynthesis (Ps) and stomatal conductance (g_s) can be used to indicate the level of low temperature stress in tea plants, but they can probably not be used as reliable criteria to select for low temperature tolerance.

Total polyphenol content and antioxidant activity varied significantly between the low temperature tolerant and susceptible cultivars at temperatures between 20°C and 15°C. The low temperature tolerant cultivars registered higher total polyphenol content than the susceptible cultivars. However, the differences between the tolerant and susceptible cultivars in TPC were not very consistent at the other temperature regimes. This was not expected but could probably be due to big variations among individual cultivars within the tolerant and susceptible cultivar groups. The antioxidant activity (FRAP) in the low temperature tolerant cultivars was significantly higher than in the susceptible under temperatures between 20°C and 15°C. Similar to TPC, FRAP only showed significant differences at 10°C in the first chamber and at 25°C in the third chamber. The reasons for this trend in antioxidant activity could be the same as was reported for TPC. Low temperature causes oxidative stress in plants through increased levels of reactive oxygen species. Since both polyphenols and antioxidants can be used to counter oxidative stress, it was plausible to conclude that the low temperature tolerant cultivars accumulate higher levels of polyphenol and antioxidants as a mechanism to cope with low temperature stress. There was a good correlation between the observed trends in total polyphenol content and antioxidant activity (FRAP) under different temperatures. As has been suggested above, measuring one of these two parameters could suffice and the cost of analysis, and consequently of selection, could be reduced.

It is interesting to note that the FRAP which associated with drought tolerance, also showed good association with low temperature tolerance. This observation can probably be due to a cross-talk that might exist in the signal perception and transduction for drought and low temperature stresses. In addition, this also demonstrated that drought and low temperature may have similar effects on plants. Although TPC had shown some association with quality, drought and low temperature, it would be difficult to selectively use TPC when considering all the traits because the relationship between these traits can be positive or negative, depending on the individual cultivars.

The rate of shoot extension of plants that are growing in the field during the cold season can be a useful criterion for identifying low temperature tolerant cultivars. The low temperature tolerant cultivars had higher shoot extension rate than the susceptible cultivars when ambient conditions were generally cold (minimum temperatures below 12.5°C). However, it would be necessary to use a large population of shoots in order to reduce error due to shoot to shoot variation. Probably the number of cultivars in each tolerance group should be more than three in order to reduce the chances of one cultivar off-setting the group average. Since shoot growth measurement is time consuming, use of this method could probably be more applicable in the advanced stages of cultivar selection where there is a small number of plant lines to be evaluated. However a large population of shoots per cultivar must be used for shoot extension rate assessments.

6.2 Recommendations

Based on the results from the current study, the following recommendations are made for future research:

- The identified RAPD markers should be incorporated in the TRFCA breeding programme in order to improve selection efficiency and precision through early identification of parental stocks for the crossing programme in order to accelerate genetic improvement of the tea plant on the most important traits.
- There is a need to continue with the search for more molecular markers for each of the three traits considered in this study in order to increase the level of marker-trait association and thereby improve the effectiveness of marker-assisted selection.
- A deliberate move should be taken to create a population of plants through a properly planned crossing programme that should be adequately phenotyped for various traits for use in the development of new markers for the desired traits. Such a population would allow an elaborate genetic analysis of the results in order to establish the extent of co-segregation between the markers and the traits of interest.
- There is a need to incorporate analysis of leaf relative water content and antioxidant capacity in the selection programme for drought tolerance in tea in order to provide better insights into the mechanisms involved in the expression of the traits of interest.
- Future work on drought should aim to adequately quantify the intensity of drought, which was not done in the current study due to lack of appropriate equipment for non-destructive measurement of soil water content in the pots. The duration of water stress should also be extended because response to drought is affected by both its intensity and duration.

- The total polyphenol content and antioxidant activities should be part of the criteria for selecting low temperature tolerant tea cultivars.
- Shoot extension measurements in the field should target a growth phase of tea shoots that shows maximum variations among tea cultivars and measurements should be done on a large number of shoots for each cultivar.
- The number of cultivars used in the contrasting groups should be at least four to six in order to reduce chances of including cultivars that show intermediate response to drought or low temperature. This would help to address the problem of variations among cultivars within the same group as well as overlaps between sub-groups.
- Results on some parameters seemed to have been affected by small sample sizes. This limitation should be considered in future in order to avoid big variations that can mask significant differences between cultivar groups.