CHARACTERIZATION OF GAMMA IRRADIATED TEA ACCESSIONS USING BIOCHEMICAL AND MORPHOLOGICAL MARKERS

BY

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DECLARATION

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Declaration by supervisors

This thesis is the candidate's original work and has been prepared with our guidance and assistance; it has been submitted with our approval as official supervisors.

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To my beloved wife Milka, daughter Grace, sons Fortunatus and Stephanas, parents, friends and relatives

ABSTRACT

Mutation breeding as an alternative method of crop improvement has widely been used to broaden the genetic variability of most cultivated plants except tea. A study was carried out to determine the effect of different gamma ray treatment (0, 50 and100Gy) on tea. The trial comprised of six open-pollinated seed stocks along with untreated controls totaling to 72 accessions. Data was collected for three seasons (dry - January to March, warm wet -September to December, and cold wet - April to August). Biochemical characterization indicated that gamma irradiation had significant effects on gallic acid (GA), epicatechin (EC) and total polyphenols (TP). Most of the traits were significantly ($p<0.001$) influenced by cultivar type. Compared to parental clones, progeny from clone TRFK 303/1199 registered an increase in GA (0.42%) whereas progenies from clones TRFCA SFS150 and EPK C12 registered an increase in EC at 2.00% and 2.56% respectively at 100 Gy. Cultivar means showed that progenies of clone TRFK 301/4 had the highest EC levels of 2.66%, whereas those of GW Ejulu had the lowest levels of 1.63%. 100 Gy treatment resulted in increased TP in progenies of clones TRFK 303/1199, TRFCA SFS150, and TRFK 301/1 that registered 0.6, 2.8 and 8.6% increase, respectively. Seasons affected all traits studied in tea ($p<0.05$) except GA% and C%. Twenty-one growth parameters were recorded for each treatment based on UPOV Tea Test Guidelines. Pattern of variations in measured characters were determined by Principal Component Analysis (PCA) using mean values of morphological observations. Evaluated germplasm were grouped by cluster analysis using the unweighted pair group method analysis (UPGMA) based on the similarity matrix of Euclidean distances of the morphological data. Morphological characterization revealed that pubescence, an important character of young shoots impacting quality of specialty teas such as white and orthodox teas was present in all the studied germplasm. Principal Component Analysis (PCA) using 17 descriptors showed that the first 8 principal components accounted for 78% of the total variance. PCA showed that 15 of the descriptors were informative and highly contributed to the phenotypic diversity among the gamma-treated progenies. Cluster analysis further revealed that characters of young shoot anthocyanin colouration at base of the petiole and leaf blade intensity were the most discriminating descriptors resulting to 4 phenotypically well-defined groups. Most traits showed significant correlation, indicating that they could be used for selection for tea improvement. This study has demonstrated that mutation breeding could be used to create genetic variability in tea for selection of novel varieties leading to enhanced foreign earnings and livelihoods for the rural population in tea growing regions of Kenya.

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LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AFLP-Amplified fragment length polymorphism

CTC-crush, tear, curl

DM% -Percentage dry matter

DNA-deoxyribonucleic acid

g- Grams

GW- George Williamson

Mm-Millimetre

nm-nanometers

PCA-principal component analysis

HPLC-high performance liquid chromatography

RPM-Revolutions per minute

RCBD-Randomized complete block design

cDNA -Complementary DNA

°C -Degree Celsius

min -Minute(s)

ml -Millilitre(s)

µg -Micro gram

v/v -Volume by volume

γ - Gamma

γ_1 -0Gy

γ_2 -50Gy

γ_3 -100Gy

γ_4 -Control

RAPDs-Random amplified polymorphic DNA

TBGI- Tea Breeding and Genetic Improvement

TRFK-Tea Research Foundation of Kenya

UV-Ultra violet

v/v- volume to volume ratio

Gy- The *gray* is the SI derived unit of absorbed ionizing dose

GA- garlic acid

EGC- epigallocatechin

C- catechin

EC- epicatechin

EGCG- epigallocatechin gallate

ECG- epicatechingallate

TP- total polyphenols

PCR -Polymerasechainreaction

% -Percent

SSR -Simple sequence repeat

pH- potential of hydrogen

ISO- International Standards Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background information

Tea (*Camellia sinensis* L. (O) Kuntze) is the second most consumed beverage after water (Wang *et al.,* 2013; Khan and Mukhtar, 2019; Lanting *et al.,* 2019). The crop emanated from China. (Kingdom-ward, 1950). It is a woody longstanding evergreen tree crop which when under cultivation is pruned horizontally to structure and keep a low spreading bush for comfortable harvesting of the young tender shoots used in processing the beverage. In Kenya, tea was first initiated by the Caine brothers in the early 1900s. They imported the dark-leafed 'Manipuri' hybrid seed from Assam and grew it in Limuru (Matheson, 1950). Its favourable founding led to spread of farming to the highlands east and west of the Great Rift Valley (Wachira *et al.,* 2002). These localities are defined by well-drained volcanic acidic soils of pH 4.0 - 5.8, well disbursed annual rainfall of above 1200 mm, altitude varying between 1500 and 2250m above sea level, and temperature range of 13-28^{o}C (Mukhopadhyay and Mondal, 2017).

The tea plant is heterogeneous with many converging morphological, biochemical and physiological traits (Purseglove, 1968; Banerjee, 1988; Gulati *et al.*,2009). Cultivated tea consists of three main taxa; *C. sinensis* var. *sinensis* (China type), *C. sinensis* var. *assamica* (Assam type) and *C. sinensis* var. *assamica* ssp *Lasiocalyx* (Cambod type). The three taxa can be distinguished by foliar, floral, growth attributes, biochemical and molecular closeness (Wachira *et al.,* 1995). Distinction of tea varieties using UV-Vis spectra and pattern recognition methods has also been demonstrated by Palacios-Morillo *et al*. (2013).

Assam germplasm is the most extensively grown variety in Kenya due to its fitness to manufacture black tea (Wight, 1959; Wambulwa *et al.,* 2017). Normally, leaf of Assam tea is thin, glossy with acuminate apex and distinct marginal veins. Leaf blade is usually predominantly elliptic, 8–20 cm long and 3.5–7.5 cm wide, base cuneate, margin obscurely denticulate to bluntly wide serrulate, and smooth or persistently pubescent on the midrib beneath (Wight, 1959; Tapan, 2014). China type are sluggish growing, dwarf and shrub-like plants with small, narrow, serrated and dark green leaves. The Cambod variety is speculated to be a middling between Assam and China types. Other recently described species include *bulsanensis*, *dehungensis*, *taliensis*, *furfucea*, *japonica and gymnogyma* (Ackerman, 1973; Chang and Bartholomew, 1984; Takeda, 1990).

Beverage tea products are categorised depending on the processing procedure. Green liquor tea is unfermented, while white, yellow/oolong teas are semi-fermented, and black/dark tea is entirely fermented (Hampton, 1992; Takeo, 1992; Zhang *et al*., 2019). The degree of fermentation influences the taste and colour of the products. Kenya is the third largest producer of tea after China and India, and the leading producer of oxidized black Cut, Tear and Curl (CTC) in the world (ITC, 2019). In the year 2018, Kenya produced 493,000 metric tonnes of made tea earning the country USD1.390 billion (ITC, 2019; KNBS, 2019). This accounted for over 27% of total foreign exchange income and over 4% of Gross Domestic Product (GDP). The industry is the largest agribusiness and export commodity from the country contributing significantly to the economy (KNBS, 2019). In addition, as the crop is mainly rural based, it contributes significantly towards poverty eradication, infrastructural growth, environmental protection through intensified water infiltration, weakened surface erosion and alleviation of global warming via carbon

sequestration (Kamunya *et al*., 2012; Chen *et al.,* 2013). Due to the labour intensive nature of operations in the industry, the sector supports more than 5 million livelihoods both directly and indirectly.

Tea improvement is attained through breeding and choosing of cultivars that are high yielding, have adequate cup quality for varied products, high functional components and toleration to biotic and abiotic pressures. Most tea breeding institutions have a strong foundation in conventional breeding. However, based on the associated limitations namely slow growth, allogamous characteristic and a long juvenile phase of the crop that hamper gains that can be realized and the rapid advancement in modern approaches, genetic tools have been developed and applied in tea improvement (Chen *et al*., 2007). Mutation breeding is an alternative method used to increase the genetic variability within crops. The method exposes seeds or other parts of plants to chemical or physical mutagens to change the genetic composition of a cell (Broertjes and Harten 1978; Oladosu *et al*., 2016). Contrasting conventional breeding approaches that involve the generation of new genetic mergers from so far existing parental genes, mutation breeding induces exclusively new gene combinations that are evaluated in order to identify elite genotypes (Piri *et al*., 2011).

1.2 Statement of the problem

Remarkable achievement in tea improvement is partly attributed to advances in conventional breeding approaches. This has been eased by the presence of diverse genetic collection, systematized vegetative propagation procedures, persistent germplasm enrichment through introduction of unique tea genetic resources from places of origin

through material exchange between research institutions and the relatively low costs implicated. However, strong self-incompatibility, slow growth, allogamous characteristic and a long juvenile phase of the tea hinder gains that can be realized. Further, due to its out-crossing nature the likelihood of obtaining superior progenies is low since the combination among parents is unknown. Mutation breeding offers an alternative approach to address these limitations in a relatively shorter time. Depending on doses applied or mutagens employed, mutations can be lethal. In order to deliver effective mutagenesis, choice of appropriate mutagen and dosage is a pre-requisite in achieving significant improvement in plants. Further, characterization of genetic resources is regarded as precedence in breeding programs since such data is critical in selection of material for their intergration into breeding activities. Screening for improved mutants with desired traits will help determine the most effective irradiation dose and identify superior cultivars.

1.3 Justification

Induced mutations have effectively broadened genetic variability and availability of novel commercial varieties in cultivated crops. Gamma radiation affects the growth and evolution of plants by creating cytologic, genetic, biochemical and physiological modifications in cells and tissues (Gunckel and Sparrow, 1961, Harwalkar *et al*., 1995; Oladosu *et al*., 2016). Previous work by Kamau *et al*. (2014) has demonstrated gamma irradiation as an effective method of inducing biochemical variations in tea. Notably, all mutated cultivars recorded lower caffeine content as compared to the parents, an indication of potential application in tea improvement programmes targeting tea. Further,

significant differences in total polyphenol content among the irradiated progenies has been observed (Kamau *et al*., 2014). Varying levels of polyphenols among different genotypes can be used for processing of diversified tea products tea. However, the results were based on one level of irradiation. In order to determine the optimum dose of radiation that would significantly affect genetic variability it is prudent to look into the effects of different levels of gamma doses thereby facilitating early development of superior cultivars. Further, mutation breeding has been recommended as alternative approach to widening the genetic base in plants. New promising lines identified with enhanced variability will be useful to enhance the ex-situ tea germplasm conservation. as well as use in the processing of specialty tea products.

1.4 Objectives

1.4.1 General Objective

To determine the effect of different levels of gamma irradiation on trait variation in tea.

1.4.2 Specific Objectives.

1. To determine the differences in biochemical profile in progenies arising from gamma-treated tea seeds.
2. To determine differences in morpho-physiological attributes in progenies arising from gamma-treated tea seeds.
3. To identify one dose of gamma rays with significant effect on trait variation of tea

1.5 Hypothesis

1. **H_o:** There is no significant variation in the phytochemicals in gamma-treated stocks in tea

   H_A: At least one level of gamma irradiation will result into significant phytochemical contents

2. **H_o:** Gamma treatment has no significant effect on morphotype of progenies arising from gamma-treated tea seed.

   H_A: At least one level of gamma irradiation will show significant effect on morphotype

3. **H_o:** Gamma treatment has no significant effect on tea traits to be observed ($\bar{y}_1 = \bar{y}_2 = \bar{y}_3 = \bar{y}_4$)

   H_A: At least one dose of gamma treatment will show significant variation ($\bar{y}_1 \neq \bar{y}_2 \neq \bar{y}_3 \neq \bar{y}_4$)

1.6 Scope of the study

This study was carried out in the KALRO-TRI, Kericho County, Kenya. The station is located within Timbilil Estate at 0^0 22´S, 35^0 21´E, altitude 2178 masl. This region is characterized by well-drained volcanic acidic soils of pH 4.0 - 5.8, well dispersed yearly rainfall of above 1200 mm and temperature range of 13-28^{o}C (Mukhopadhyay and Mondal, 2017).

CHAPTER TWO

LITERATURE REVIEW

2.1Tea Breeding

Tea, *Camellia sinensis* (L.) O. Kuntze, belongs to order *Theales* and family *Theaceae*. It is a dioecious insect-pollinated tree, which is spread through either sexual or vegetative propagation. Success in plant breeding can be judged by the magnitude of genetic gain.

Breeding programmes in tea is dictated by its very outbred kind, extended generation period from seed to flower and amenability of the crop to vegetative propagation. Early breeding work concentrated on production of adequate sowing material by mass multiplication or mass selection from random crosses and natural populations with little emphasis on yield and quality. Later however, emphasis was shifted to enhancing yields (Seurei, 1997). Target traits in tea improvement include elevated yields, acceptable processed tea cup quality, and toleration to abiotic and biotic pressures.

2.2Phases in Tea Improvement

Principally four processes contribute significantly to the progress in breeding and clonal selection (Figure 1), namely; production of hereditary variability, evaluation and selection of gainful genotypes from the generated offspring in progeny tests (PTs), comparative tests of selected genotypes to demonstrate their superiority in clonal field trials (CFTs) and multisite testing of elite clones in clonal adaptability trials (CATs).

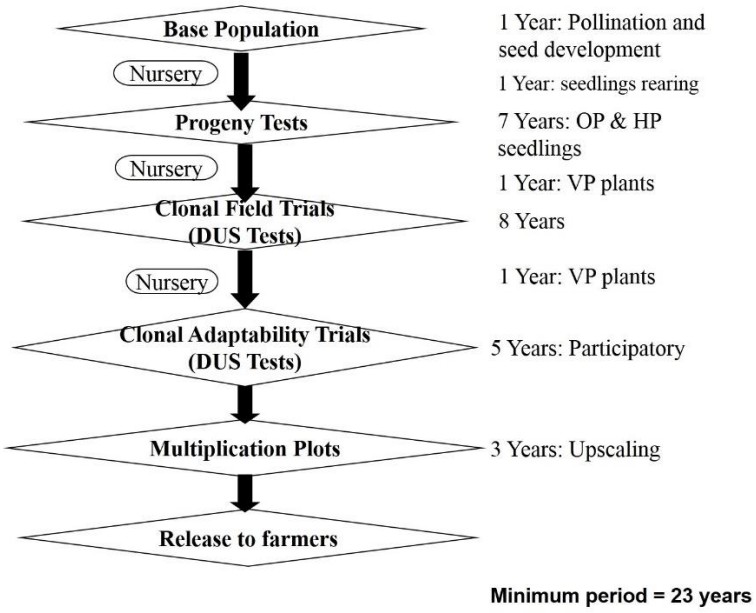

Figure 1: Phases of tea improvement at TRI Source: TRFK

Tea being out-crossing, its best genotypes are propagated asexually prior to release as clonal cultivars. Breeding of new high yielding and quality cultivars and their conservation remain to be significant for the viable cultivation of tea. Plant morphology is treated as the meticulous investigation of the physical form and external structure of plants which can be applied to carve up diversity into its systematic subunits (Kaplan, 2001). Clonal identification has traditionally been found on morphological descriptors like plant shape, leaf shape, young leaf type and fruit shape. Utilization of morphological traits is relatively cheap compared to the use of biochemical and molecular markers for initial depiction of large number of accessions to identify morphologically alike groups and for easy varietal discernment of phenotypically dissimilar cultivars (Martinez et al., 2003). The genetic differences between the genotypes can also be observed in the chemical make-up of the leaves. Variations in polyphenol oxidase action, individual

polyphenols, amino acids and chlorophyll contents have been used in biochemical classification of various genotypes (Sanderson, 1965; Owuor *et al*., 1987; Karori *et al*., 2014).

2.3 Health Benefits of Tea

The chemical make-up of tea is complex and includes polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, minerals, trace elements and other unknown compounds (Hilton, 1973; Karori *et al*., 2007). Polyphenols comprise the most fascinating group and are the major bioactive molecules in tea (He *et al*., 2015). The main polyphenolic compounds are the catechins (flavan-3-ols), which include: (-)-epicatechin (EC), (-)-epigallocatechin (EGC), (-)-epicatechingallate (ECG), (-)-epigallocatechin gallate (EGCG), (-)-gallocatechins (GC) and (-)-gallocatechingallate (GCG) (Figure 2) (Ferruzzi and Green, 2005; Ma *et al*., 2014). Catechins occur in large quantities in green tea accounting for 16% of the leaf dry weight with EGCG accounting 50% as the most dominant owing to its level of gallation and hydroxylation (Stewart *et al*., 2004; Peterson *et al*., 2005; Sang *et al*., 2011). Considering their chemical structure, catechins that accomodate three hydroxyl groups in the B-ring (positions 3', 4' and 5') are called gallocatechins while gallic acid replacement in location 3' of the ring is typical of catechin gallate (Pellilo *et al*., 2002). The auto-oxidation of leaf catechins in processing of both black and oolong teas results in complex polyphenol compounds; theaflavins (TFs) and thearubigins (TRs), that accord black tea its quality (Roberts and Smith, 1961; Obanda *et al*., 2001).

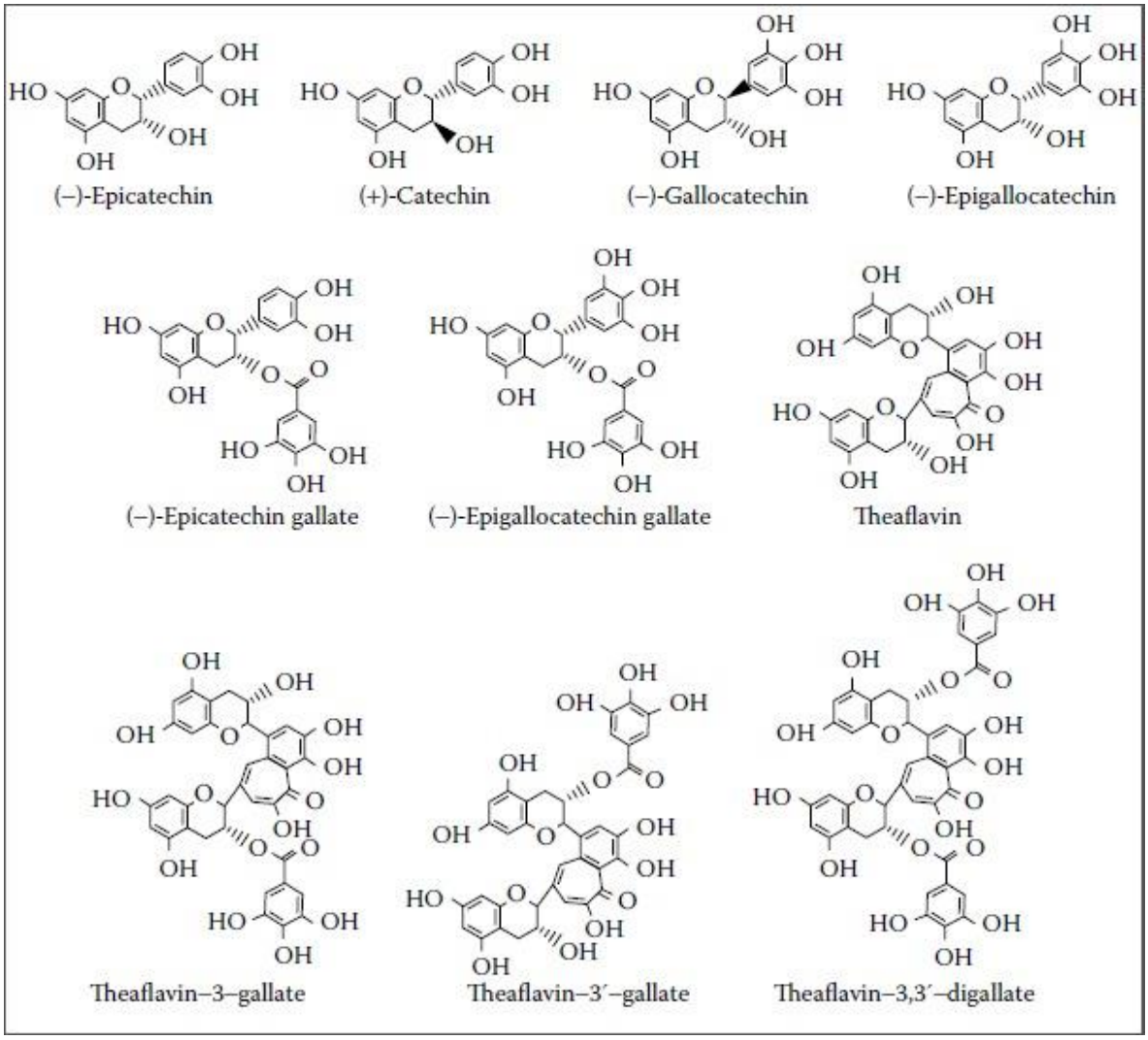

Figure 2: ***Structures of the flavan-3-ols of tea*** **Source: Wikipedia**

Leaf catechins are found in the cell sap in as much as oxidative enzymes are situated in the cell wall. In healthy leaves, these components are physically separated and therefore do not interact. When the leaf is processed, maceration of the leaf disrupts its structure extensively. This causes the catechins and the enzyme to mix and in the presence of oxygen (from the air) a series of biochemical reactions also called "fermentation", take place (Anon., 2002). These processes convert the catechins to theaflavins (TFs) and

thearubigins (TRs) granting black and oolong teas their habitual colours and flavours. Oolong tea is regarded as half fermented given that the leaves are shortly fired after rolling to stop the oxidation and then dried. Endogenous catechins (flavanols) are highly concentrated in the youngest two leaves and a bud, the amount decreasing as the leaves age (Anon., 2002). Assam tea that is predominant germplasm in Kenya is known to be more astringent attributed to higher polyphenol contents and thus fit for the production of black tea. However, in green tea, oxidative enzymes are deactivated by steaming the leaves before processing.

Research has showed the value of tea as a pharmacological agent (Magoma *et al.,* 2001; Naveed *et al.,* 2018). The major chemical components in the tea leaves are polyphenols, alkaloids (caffeine, theophylline and theobromine), amino acids, carbohydrates, proteins, chlorophyll, volatile compounds, fluoride, minerals and other unspecified compounds. Among these, polyphenols also known as catechins, are the most predominant pharmacologically active biochemicals (Wu *et al*., 2002; Jiang *et al*., 2019). The major polyphenolic aggregate includes (-) epicatechin (EC), (-) epigallocatechin (EGC), (-) epigallocatechin gallate (EGCG), (-) epicatechin gallate (ECG), (-) gallocatechin (GC), (-) gallocatechin gallate (GCG) (Zuo *et al.*, 2002; Kerio *et al.*, 2013). Research show that tea polyphenols have anti-aging, anti-fibrotic, anti-cancer, diabetic, anti-atherosclerotic, anti-stroke, anti-caries and anti-bacterial, anti-diarrheal, anti-parkinsonism, anti-inflammatory, and anti-oxidative properties (Miyoshi *et al*., 2015; Fujiki, 2017; Karori *et al*., 2008; Preedy, 2012). Epidemiological inspections have correlated the drinking of tea with a lesser risk of heart disease and anticancer activity in human beings (Cabrera *et al*.,2003; Vanessa and Williamson, 2004; Fujiki, 2017). Tea also has anti-allergic action

(Yamamoto *et al*., 2004; Kondo *et al*., 2015), potential anti-helmintic properties (Mukai *et al*., 2008), antidiabetic activity (Sabu *et al*., 2002) and anti-hyperglycaemic activity (Gomes *et al*., 1995). Green tea lowers hepaticsteatosis by lowering hepatic insulin resistance that is a critical mechanism in its pathological process (Gan *et al.*, 2015). Green tea has a capacity to raise immune system, reduce the genesis of reactive oxygen species (ROS), increase the activity of pro-oxidant enzyme such as glutathione peroxidase and superoxide dismutase via its antioxidant properties. In addition, green tea lowers diverse inflammatory chemokines and cytokines (Chacko *et al.,* 2010). Finally, green tea has anti-obesity (Sae-Tan *et al.,* 2011) and anti-hypertensive properties (Bogdanski *et al*., 2012). In tea, cultivar is one of the greatest factors affecting accumulation of phytochemicals, judicious breeding and selection are therefore very crucial.

2.4 Approaches in Tea Improvement

2.4.1 Conventional Breeding Approaches

Tea is cultivated either through seeds or cuttings. Being a cross pollinated species tea cultivars are developed by hybridization. Although the technique is more complex and costly, use of the seed gardens for production of commercial seeds or for controlled breeding purposes can be justified. Usually seeds are collected from orchard, stratified then sown in the nursery where it takes 12–18 months before transplanting to the field. Given the great genetic variations associated with seedling population, tea breeders have explored simpler methods for the establishment of large and uniform populations. In this case, vegetative cultivation of the elite variety using one leaf internode cuttings with an axillary bud are planted below shelter for 8 months accompanied by the transplanting of

rooted plants to the field. The technique is economical and time saving as it rapidly produced uniform stands of tea of consistent high yields and quality. Grafting as a substitute propagation approach has also gained substantial popularity. For numerous economically significant woody perennials generating nuts and fruits, current production structures are primarily relying on grafting of a cultivar on a rootstock that could be of the same or different species to regulate significant traits like; root-associated diseases, plant size and yield. These gains have recently been utilized to produce herbaceous dicots. The merging of genotypes in a single composite plant has led to a unique biological model that surpasses the acknowledged genetic patterns. The immediate physiological adjustment of desirable traits in the scion, that imitate genetic diversity, is resolved by various hypothesized mediums obtained from the rootstock. Trait advancement detours the reproductive sequence eradicating years of selection in breeding and is an outcome of cellular or genetic interplay in the somatic cells (Koepke and Dhingra, 2013).

Hybridization can be either natural or via controlled pollination. In natural hybridization, rooted on superior attributes, two parents are isolated in biclonal orchards to bear first filial (F_1) seeds. Alternatively, polyclonal seed orchards comprising more than two cultivars that aim to introduce more variability among the F1 seeds is adopted.

Although traditional tea breeding is entrenched and has contributed significantly towards tea furtherance over the ended decades, the process is sluggish owing to the longstanding nature of tea, long development periods (Grover *et al*., 2011), elevated inbreeding depression, self-incompatibility, lack of clear mutants of contrast biotic and abiotic stress

and clonal difference of flowering age and fruit bringing ability of several clones. These limitations have led to adoption of new approaches and modern techniques for accelerated tea breeding programs.

2.4.2 Mutation Breeding in Tea

Mutation is a sudden heritable change in an organism, affecting the structure of a gene through chemical or physical mutagens (Hugo de Vries, 1900; Altındal and Altindal, 2018). A breakthrough in genetics discovered that mutations can be artificially induced in organisms (Van Harten, 1998). Mutagens can either be physical or chemical (Lestari, 2012). Mutation breeding has been accepted as a valuable additive to conventional breeding in crop improvement (Mick *et al.,* 1985; Ashutosh *et al*., 2013; Suprasanna *et al*., 2015). Mutated genes are valuable resources to plant breeders and molecular biologists for understanding the function and isolation of genes (Hamideldin and Hussien, 2013; Suprasanna *et al*., 2015). The employment of induced mutations for the enhancement of crop plants has yielded a few mutants that have been utilized directly as new cultivars (Gottschalk and Wolf, 1983; Oladosu *et al*., 2016). In rice, seedlings treated at cumulative doses of 67 and 162 Gy showed the highest plant height with maximum tillering and highest number of filled grain respectively (Yasmine *et al*., 2019). In wheat, 200 Gy gamma radiation boosted germination, plant height, grains per plant, grain yields (Jamil and Khan, 2002) and disease resistance (FAO/IAEA, 2014). Chromosomal re-arrangements are frequently produced by both physical and chemical mutagens (Goyal and Verma, 2015, 1996; Kovacs and Kerezestes, 2002).

Mutations are phenotypically classified into two groups (Gaul, 1964): (i) Macro mutations that are easily detectable in individual plants, phenotypically visible and morphologically distinct that are qualitatively inherited as they occur in major genes or oligogenes;(ii) Micro mutations result in a small effect that can be detected only by the help of statistical methods and are quantitatively inherited that occur in minor genes or polygenes. (Toker *et al.,* 2007)

2.4.3 Chemical mutagens

The result of chemical mutagens on plant matter is normally considered more gentle (Acquaah, 2006). Notwithstanding the enormous number of mutagenic compounds, just a little number has been assessed in plants (Wani *et al*., 2014). Above 80% of the recorded new mutant plant diversity relayed in the International Atomic Energy Association (IAEA) database (IAEA, 2015) acquired through chemical mutagenesis were intigated by alkylating agents. Of these, three constituents are notable: 1-ethyl-1-nitrosourea, ethyl methane sulphonate (EMS) and 1-methyl-1-nitrosourea (Wani *et al*., 2014). At KALRO-TRI, mutagens have been used on *in vitro* cultured materials to expand the inside genetic variance of a crop followed by adoption criteria that strive to recognize important genotypes (those with higher yields, pest and disease tolerance and improved quality) (Anon. 2001).This approach is reported to have various advantages namely elevated shoot multiplication ratio whose aftermath is logical chimera dissociation, a radical cutback in time and space needs, ease of treatment with mutagens and successive removal of chemical mutagens, and voluntary facility for *in vitro* selection against assorted stresses. The technique has been used in tea for improvement of traits such as high yield and improved quality, multiple resistance to major insect pests and diseases, and

tolerance to abiotic stresses. The chemical mutagens used include; colchicine, sulfanilamide and hydroxyquinoline. The chemicals are incorporated in the artificial media used in plant tissue culture to be absorbed together with other nutrients (Anon. 2001).

2.4.4 Physical mutagens

Various forms of physical mutagens that include ultraviolet light, beta particles such as those from Phosphorus-32 and Sulfur- 35, Ion beams, neutrons and gamma radiation have been used widely in mutation programmes (Sharma and Sharma, 2014).

Gamma irradiation is one of the major physical mutagens in plant mutation breeding which can induce either beneficial or deleterious effects on the chromosomes of cultivated plants (Jayabalan and Rao, 1987; Viccini and Caravalho, 2001). In higher plants, chromosomal aberrations triggered by irradiation have been used for many years in classical genetic examination (McClintock, 1984) and as sources of initial material for gene segregation and mapping (Liharska *et al.,* 1997). Various researchers have recorded chromosomal variance obtained by gamma irradiation (Riera-Lizarazu *et al.,* 2000; Kumar and Rai, 2009; Minisi *et al.,* 2013). Generally, seeds are commonly used for radiation since they are manageable to handle and can be stored for long duration in a vacuum or under high pressure of oxygen/other gases (Siddiqui, 1994).

Gamma rays are part of ionizing radiation that associate with atoms or molecules to yield free radicals in cells. These radicals can destroy or reorganize important constituents of plant cells and are outlined to dissimilarly influence the morphology, anatomy, biochemistry and physiology of plants based on irradiation level (Jan *et al*., 2012; Minisi *et al*., 2013). Depending on dosage, such outcomes comprise alteration in the cellular

structure and metabolism of the plants namely; dilation of thylakoid membranes, changes in photosynthesis, regulation of the antioxidative organization and buildup of phenolic compounds (Wi *et al*., 2005; Marcu *et al*., 2013; Abou-Zeid and Abdel-Latif, 2014). Thus, there is need to ascertain the ultimate beneficial dose of gamma rays for enhancement of specific attributes in plants.

2.5 Methods for Characterization of Tea Germplasm

Plant genetic resources are limited and exposed to losses due to inception of new crop varieties in agriculture, growing urbanization, natural calamities and climatic evolvement (Upadhyaya *et al*., 2008). Therefore, assessment, characterization and evaluation of genetic resources are regarded as precedence in breeding programs since such data is critical in selection of material for their intergration into breeding activities (Getachew *et al*., 2013). Majority of the cultivated species are diploids (2n=2x=30) with the steadiness in chromosome numbers proposing the monophyletic origin of all tea species (Kondo, 1977; Banerjee, 1992). Nonetheless, stable polyploidy series of triploids (3n=45), tetraploids (4n=60), pentaploids (5n=75), and hexaploids (6n=90) have been singled out (Bezbaruah, 1975; Wachira and Kiplangat, 1991; Roy, 2006).

To assess plant genetic diversity, several descriptors have been developed to characterize agro-morphological variability. Theoritically such descriptors should neither be influenced by plant growth nor the growth environment.

2.5.1 Biochemical traits

Phytochemicals are chemical compounds that exist naturally in plants. They comprise secondary metabolites, most of which are manufactured for plant defenses and

adaptation to environmental strain (Mcclanahan 2012). Tea is recorded to contain about 4,000 biologically active compounds of which one-third is conferred by polyphenols (Tariq *et al*., 2010). Variation in biochemical compounds have been used in tea germplasm characterization (Magoma *et al*., 2003; Kottawa-Arachchi *et al*., 2013; Punyasiri *et al*., 2017). Catechins and catechin fractions, anthocyanins, caffeine, carotenoids, chlorophyll, theanine, tannins, total polyphenol and chlorogenic acid contents are commonly used for varietal identification and characterization in tea (Terahara *et al*., 2001; Wachira and Kamunya, 2005; Kerio *et al*., 2012; Kottawa-Arachchi *et al*., 2018). Some chemical parameters have been recognized as highly discriminative descriptors in black processed tea (IPGRI, 1997). These consist of theaflavin and thearubigin components and proportions, tea quality type and the terpene index (TI). Leaf terpenoids were utilized to distinguish closely linked Camellia species (Takeo, 1983; Nagata, 1986; Zhu *et al*., 2017). Owuor *et al*. (1987) used the terpene index (TI) to differentiate between Kenyan tea cultivars, while Magoma *et al*. (2000) used the ratio of dihydroxylated to trihydroxylated catechins to outline distinct varieties of tea. Low tannin-containing cultivars are sorted out in Japan for production of green tea. Using three NADP-linked dehydrogenase isozymes, Magoma *et al*. (2003) disclosed that tea clones could be precisely separated according to their phylogenetic roots.

2.5.2 Morphological traits

A list of standardized descriptors for the discrimination and classification of tea cultivars was first published in 1997 by the International Plant Genetic Resources Institute (IPGRI) and later in 2008 by the Union for Protection of Plant Varieties (UPOV). This provides an international format for demarcation of tea genetic resources. It involves the

systematic distinguishing of tea germplasm to assess their morphological diversity and to identify highly discriminating descriptors. The vegetative traits generally used to allocate taxonomic groups in tea comprise: leaf size (leaf length, leaf breadth), ratio of leaf length to breadth, internode length, length and girth of bud, petiole length, ratio of apical length, angle between leaf tip and axis, leaf margin and shoot density (Wachira *et al.,* 2013). Several studies to assess tea variation have been administered using morphological markers (Vo, 2006; Tran, 2009; Rajkumar *et al*., 2010). Utilization of morphological markers is relatively cheap compared to the use of biochemical and molecular parameters for initial differentiation of large number of accessions to identify morphologically comparable groups and for easy varietal recognition of phenotypically categorizable cultivars (Martinez et al., 2003). Among the multifold activities, the initial step for proper characterization of germplasm is to pinpoint the phenotypic variation abounding in the given germplasm. In order to attain this aim, the germplasm accessions require to be characterized using a standard set of descriptors.

Facts gathered can be utilized to understand patterns of genetic variation occuring in crop species (Perera and Fernando, 2000; Hagidimitriou *et al*., 2005) and to discriminate accessions with lofty genetic variability and to choose genetically nigh and far accessions (Anandappa, 1993). In tea, morphological characterization have been utilized to examine genetic variance (Chen *et al*., 2005; Toyao and Takeda, 1999), variation (Rajanna *et al*., 2011; Piyasundara et al., 2006; Su *et al*., 2007, phylogeny and grouping (Pi *et al*., 2009; Jiang *et al*., 2013). In addition, Visser (1969) supposed that a blending of slight pigmentation (anthocyanin) in the existence of pubescence impacted quality in teas. A scrutiny by Owuor and Obanda (1998) proposed the likelihood of using morphological

characteristics and assorted chemical components in screening for quality at single bush level. Multivariate statistical techniques such as principal component analysis and cluster analysis are the often-used methods for characterization and genetic variation analysis of perennial crops such as tea (Rajanna *et al*., 2011; Lv *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Test material

Viable open-pollinated tea seed stocks from six commercial cultivars TRFK 301/4, TRFK 301/1, EPK C12, TRFCA SFS150, TRFK 303/1199 and GW Ejulu-L (Appendix I) were sent to KALRO- Biotechnology Institute where the seeds were exposed to a Cobalt-60 source of gamma rays at doses of 0, 50, 100,150 and 200Gy (dose rate = 5.15 Gy min^{-1}). Seedlings were germinated at KALRO-TRI nursery, Kericho Centre, situated at 0° 22´S, 35° 21´E, altitude 2178 metres above sea level, before they were transplanted to Field 12C at the Centre in form of unreplicated progeny/single bush trial. Seedlings arising from 150 and 200Gy treatments did not survive and hence these treatments were discarded from the successive evaluations. Only one seedling survived at 100Gy across all the stocks.

3.1.1 Experimental design

The trial was planted on 15th June 2015 comprising segregating single bushes at the recommended spacing of 1.22 x 0.61m (i.e. 13,448 plants/ha). The experiment was conducted as a completely randomized design in a 6 X 5 factorial arrangement. A total of 360 seeds were planted. Six tea genotypes were used in this study where 20 seeds per genotype were treated with gamma radiation doses of 0, 50 and 100 Gy. From each treatment, five randomly selected plants were assayed. The untreated clonal parents were included as controls. Data was collected for three seasons (dry-December to March, warm wet- August to November and cold wet- April to July). Readings were taken in

replicates and means across the three seasons used to curb biasness linked to plasticity of the traits. Data collection began on 6th November 2017.

Model

$Yij = \mu + \tau i + \varepsilon ij$

where: Yij is the jth observation of the ith treatment, μ is the population mean, τi is the treatment effect of the ith treatment, and εij is the random error.

3.2.0 Biochemical analysis

3.2.1 Collection and processing of leaf samples

Approximately 500g of fresh shoots comprising two leaves and a bud were sampled from five randomly selected bushes of the treated progeny and their respective clonal control and kept in suitably labelled khaki bags. These were then taken into a cooler box containing ice packs and conveyed to the laboratory for further processing. Samples were taken to the laboratory and promptly microwave-dried for five minutes to deactivate polyphenol oxidase (an oxidizing enzyme). Thereafter, the shoots were dried in an oven for 24 hours at 100℃ to a persistent dry weight. The dry samples were then ground using a coffee miller and placed in appropriately labelled aluminium sachets and stored in a dark, dry environment for later analysis (Magoma *et al.,* 2000). The progenies were sampled in each of the three seasons.

3.2.2Total polyphenol extraction

Total polyphenol content was determined using Folin-Ciocalteu phenol reagent method following ISO (BS ISO 14502–1:2005 (E)) procedures for determination of substances characteristic of green and black tea and expressed as percent by mass on a dry matter

basis. Methanol (70% concentration) contained in a dispenser was preheated in a water bath at 70℃ for at least 30 min for the extraction mixture to equilibrate. Test sample weighing 0.2g was transferred into 15 ml falcon tube, 5.0 ml equilibrated methanol added and shaken to mix. This was heated in the water bath for 10 min, mixing on the vortex after 5 min and 10 min and thereafter, removed and allowed to cool to room temperature. After cooling, the tube was centrifuged at 3500 rpm for 10 min then the supernatant carefully decanted into a graduated tube of 10 ml capacity with 0.1 ml graduations. The process starting from addition of 5.0 ml equilibrated methanol till decantation of the supernatant was repeated on the sediment and the supernatant of the respective sample combined. The volume was topped up to 10ml with 70% cold methanol. One ml of the sample extract was then transferred into a one-mark 100ml volumetric flask and diluted to the mark with distilled water then thoroughly mixed. At the same time, 1.0ml of gallic acid standard solutions A, B, C, D and E was transferred in triplicate into graduated tubes using a pipette. These volumes corresponded to approximately 10 µg, 20 µg, 30 µg, 40 µg and 50 µg of anhydrous gallic acid, respectively. One ml of water in duplicate was also transferred into separate tubes to act as the reagent blank.

One ml of diluted sample extract was then transferred in triplicate into separate tubes and 5.0 ml of 10% Folin-Ciocalteu phenol reagent added into each tube. Within 3-8 min after the addition of Folin-Ciocalteu's phenol reagent, 4.0 ml of sodium carbonate solution was added into each tube. The tubes were then corked and contents mixed. The mixture was allowed to stand at room temperature for 1 hour and the optical densities measured against a blank on the spectrophotometer set at 765 nm. A calibration curve of standards versus concentration was plotted and used to determine the concentration of various

samples. The polyphenols were determined calorimetrically using Folin-Ciocalteu's phenol reagent. The reagent contains phospho-tungstic acids as oxidants, which on reduction by readily oxidized phenolic hydroxy groups yield a blue colour with a broad maximum absorption at 765nm. This is due to the formation of tungsten and molybdenum blues. The Folin-Ciocalteu's phenol reagent reacts with a wide range of polyphenol compounds and although the responses can vary with the individual components, selection of gallic acid as a calibration standard enables total polyphenol data to be obtained. The equation of the line was used to convert the sample absorbance into respective concentration considering the weight of the sample and dilution. Total polyphenols were obtained and expressed in percentage using the dry matter content. Calculation of anhydrous gallic acid of the standards A, B, C, D and E to the nearest 0.1µg was based on the formula:

$$M=\frac{m0 \times V \times wDM,\ \text{std} \times 10000}{100 \times 100}$$

Where:

m0 is the mass of Gallic monohydrate, in grams used to prepare the stock standard solution;

V is the volume of gallic stock standard solution, in milliliters, used to prepare the standard solutions A, B, C, D and E;

*w*DM, std is the dry matter content, expressed as a mass fraction, in percent of gallic acid.

A linear calibration graph was constructed from the mass of anhydrous gallic acid in standards A, B, C, D, and E against the gallic acid standard optical densities after subtracting the reagent blank optical density.

The total polyphenols content, *w*T, expressed as a percentage by mass on a sample dry matter basis, is given by the formula:

$$wT = \frac{(D\ \text{sample} - D\ \text{intercept}) \times V\ \text{sample} \times d \times 100}{S\ \text{std} \times m\ \text{sample} \times 10000 \times w\ \text{DM, sample}}$$

Where:

D sample is the optical density obtained for the sample test solution;

D intercept is the optical density at the point the best-fit linear calibration line intercepts the y-axis;

S std is the slope obtained from the best fit linear calibration;

m sample is the mass, in grams, of the sample test portion;

V sample is the sample extraction volume, in milliliters (10 ml for leaf tea);

d is the dilution factor used prior to the calorimetric determination (typically 1.0 ml to 100 ml, thus a dilution factor of 100);

*w*DM, sample is the dry matter content, expressed as a mass fraction in percent of the test sample.

3.2.3 Catechins analysis

The catechins content in green tea were determined using high-performance liquid chromatography (HPLC) following ISO 14502-2:2005(E) procedure. The HPLC system (Shimadzu LC 20AT, Kyoto, Japan) had an automatic injector and a C6 column (150 x 4.6 mm, 5 µ) (Phenomenex Inc. Torrance CA, USA) both placed in a column oven set at 35^{o}C, and a UV detector set at 278nm. The isolation of main catechins

was carried out using 9% acetic acid (eluent A) and 80% acetonitrile (eluent B). The gradient used was 0-10 min, 2% B; 10-25 min, 32%B; 25-32 min 32%B; 25-32 min 32%B; 33-43 min 0% B at the flow rate of 1.0 ml/min. The chromatographic peaks in the specimen were recognized by matching their retention times with calibration chemical standards (Appendix V).

The quantification of catechin content in the sample was carried out according to the equation below:

$$^{w}C = \frac{(A\text{sample} - A\text{intercept}) \times \text{Fstd} \times V\text{ sample} \times d \times 100}{S\text{ std} \times m\text{ sample} \times 10000 \times w\text{ DM, sample}}$$

Where:

A sample is the peak area of the individual component in the test sample;

A intercept is the peak area at the point the standard calibration line intercepts the y-axis;

S std is the standard calibration line;

F std is the relative response factor, measured with respect to caffeine for the individual component;

V sample is the sample extraction volume, in milliliters (10 ml for leaf tea);

d is the dilution factor used prior to the calorimetric determination (typically 5);

m sample is the mass, in grams, of the sample test portion;

*w*DM, sample is the dry matter content, expressed as a mass fraction in percent of the test sample.

3.3 Morphological characterization

3.3.1 Cultivar description

Comprehensive morphological characterization for distinctness of the novel cultivars from the mutants and their 6 control parents was carried out on 21 phenotypic characters. From each treatment, five randomly selected plants were assayed. The untreated clonal parents were included as controls. Data was collected for three seasons (dry-December to March, warm wet- August to November and cold wet- April to July). Readings were taken in replicates and means across the three seasons used to curb biasness linked to plasticity of the traits according to the International Union for the Protection of New Varieties of Plants guidelines for tea (UPOV, 2008). The guidelines give practical guidance for the harmonized identification of appropriate characteristics for the examination of distinctness, uniformity and stability (DUS) for variety description. Young shoot colour, immature leaf colour and mature leaf colour descriptors were measured using Royal Horticultural Society (RHS) colour chart for plant colour identification (Voss, 2002). Quantitative tea descriptors like leaf length, width of third leaf and internode length were determined using a ruler. Presence and density of pubescence of the bud was observed by use of hand lens.

Table 1: Morphological descriptors according to the International Union for the Protection of New Varieties of Plants (UPOV) tea guidelines

No.	Characteristic	Description
Qualitative traits		
1.	Plant vigor	Weak: 3; Medium: 5; Strong: 7
2.	Plant type	Shrubs:1; Semi-arbor: 3; Arbor: 5
3.	Plant growth habit	Upright:1; Semi-upright: 3; Spreading: 5
4.	Plant density of branches	Sparse: 3; Medium: 5; Dense: 7
5.	Plant branch zigzagging	Absent: 1; Present: 9
6.	Young shoot color of second leaf at 'two and a bud' stage	Whitish: 1; Yellow green: 2; Light green: 3; Medium green: 4; Purple green: 5
7.	Young shoot pubescence of bud	Absent: 1; present: 9
8.	Young shoot density pubescence of bud	Sparse: 3; Medium: 5; Dense: 7
9.	Young shoot anthocyanin coloration at base of petiole	Absent: 1; Present: 9
10.	Leaf blade attitude	Upwards: 1; Outwards: 3; Downwards: 5
11.	Leaf blade shape	Very narrow elliptic: 1; Narrow elliptic: 2; Medium elliptic: 2; Broad elliptic: 4
12.	Leaf blade intensity of green color	Light: 3; Medium: 5; Dark: 7
13.	Leaf blade shape in cross section	Folded upwards: 1; Flat: 2; Recurved: 3
14.	Leaf blade texture of upper surface	Smooth or weakly rugose: 1; Moderately rugose: 2; Strongly rugose:3
15.	Leaf blade shape of apex	Obtuse: 1; Acute: 2; Acuminate: 3
16.	Leaf blade undulation of margin	Absent or weak:1; Medium: 2; Strong:3
17.	Leaf blade serration of margin	Weak: 3; Medium: 5; Strong: 7
18.	Leaf blade shape of base	Acute: 1; Obtuse: 2; Truncate: 3
19.	Young shoot length of 'three and a bud'	Short: 3; Medium: 5; Long: 7
20.	Leaf blade length	Short: 3; Medium:5; Long: 7
21.	Leaf blade width	Narrow: 3; Medium: 5; Broad: 7

Source: UPOV, 2008

3.4.0 Data analysis

Biochemical data was subjected to analysis of variance (ANOVA) to test level of variability between the gamma-treated progenies. Morphological data across three seasons was subjected to Principal Component Analysis (PCA). Sinha and Mishra (2013) was selected to validate the cut-off check for the coefficients of considerable vectors. Based on this criterion, coefficients larger than 0.3 (nonetheless the direction positive or negative) have substantial effects to be considered in the overall variation observed. Morphological data were clustered using the unweighted pair group method analysis (UPGMA) based on the similarity matrix of Euclidean distances. Diversity and correlation analyses were also performed to test the degree of variation between mutants. Means were compared using the Duncan Multiple Range Test (Duncan, 1955). The statistical analyses were performed using Genstat software version 15.1 (VSN International Ltd, UK). Graphical presentations were done using Microsoft excel software.

CHAPTER FOUR

RESULTS

4.1 Biochemical characterization

4.2 Variation of Catechins and polyphenols

The tea biochemicals were tested in the following order Gallic Acid (GA), Epigallo catechin (EGC), Caffeine (Caff), Epicatechin (EC), Epigallocatechingalate (EGCG) and Epicatechin gallate (ECG). The parameters measured showed significant ($p<0.05$) variations attributed to cultivars, seasons or interactions among the various factors. A conspectus of catechin and total polyphenol contents of the various progeny and their parents is presented in Table 2. Percent biochemical deviation of gamma treated progeny as compared to their controls is found in Appendix III.

Table 2: Recapitulation of ANOVA table showing variation in measured parameters among all treatments and their interactions

		Mean Square (MS)								
Source of Variation	DF	GA	EGC	C	Caff	EC	EGCG	ECG	TC	TP
Cultivar (A)	5	0.113***	23.58***	0.172***	0.895**	7.907***	26.964***	15.312***	45.149***	60.132***
Gamma treatment (B)	3	0.026*	1.77	0.099	0.446	1.704*	2.045	1.639	14.594	19.244***
Season (C)	2	0.009	10.40	0.046	8.233***	6.423***	9.280*	12.776***	127.176***	106.983***
Cultivar*Gamma	15	0.015*	4.03*	0.078**	0.496	0.840	3.583	2.404***	60.018	8.456***
Cultivar*Season	10	0.025***	1.22	0.047	0.365	0.165	0.703	0.899	6338	13.730***
Gamma*Season	6	0.017*	0.73	0.045	0.445	0.405	1.396	0.904	5.742	4.367
A X B X C	30	0.016**	1.44	0.024	0.397	0.328	1.288	0.428	3.991	2.433
Error	144	0.008	1.566	0.037	0.290	0.546	2.370	0.761	7.317	3.046
%CV		28.345	29.273	33.164	18.331	37.508	29.23	31.255	18.173	8.5881

*Table notes: Asterisks *, **, and*** denote significance level at p < 0.05, p <0.01 and p<0.001 respectively. Gallic acid (GA), Epigallocatechin (EGC), catechin (C), Caffeine (Caff), Epicatechin (EC), Epigallocatechin gallate (EGCG), Epicatechin gallate (ECG), Total catechin (TC) and Total polyphenol (TP) respectively.*

Table 3: Total polyphenol and catechin mean contents of the test progenies and their controls

Progeny identity	Treatment	GA%	EGC%	C%	Caff%	EC%	EGCG%	ECG%	TC%	TP%
TRFCA SFS150	0Gy	0.30	3.42	0.67	2.97	1.48	5.24	2.87	13.69	21.1
TRFCA SFS150	50Gy	0.21	5.17	0.44	2.96	1.87	4.60	1.80	13.87	20.2
TRFCA SFS150	100Gy	0.18	5.98	0.41	2.95	2.00	6.04	1.96	16.39	21.9
TRFCA SFS150	**Control**	**0.38**	**4.68**	**0.47**	**3.37**	**1.70**	**5.99**	**1.91**	**14.75**	**21.3**
TRFK 303/1199	0Gy	0.28	5.40	0.46	2.82	1.71	6.94	2.48	16.99	20.4
TRFK 303/1199	50Gy	0.26	5.84	0.50	2.68	1.79	6.27	2.28	16.68	20.4
TRFK 303/1199	100Gy	0.42	5.31	0.57	3.47	1.32	8.92	2.75	18.87	22.6
TRFK 303/1199	**Control**	**0.30**	**5.18**	**0.45**	**3.02**	**1.62**	**7.18**	**2.48**	**16.91**	**22.5**
EPK C12	0Gy	0.24	4.30	0.72	2.62	1.57	4.61	2.11	13.29	18.2
EPK C12	50Gy	0.24	4.85	0.57	2.68	1.47	5.44	1.97	14.29	17.8
EPK C12	100Gy	0.23	5.25	0.63	3.19	2.56	4.81	2.60	15.85	19.4
EPK C12	**Control**	**0.25**	**5.36**	**0.61**	**2.38**	**1.92**	**6.09**	**2.48**	**16.44**	**21.1**
GW Ejulu-l	0Gy	0.31	4.26	0.58	2.89	1.74	5.58	3.25	15.41	22.0
GW Ejulu-l	50Gy	0.32	3.98	0.68	3.29	1.54	5.51	2.95	14.66	22.2
GW Ejulu-l	100Gy	0.30	4.21	0.53	2.61	1.34	5.58	1.78	13.44	17.7
GW Ejulu-l	**Control**	**0.35**	**2.85**	**1.01**	**3.30**	**1.82**	**5.00**	**4.68**	**15.36**	**25.2**
TRFK 301/1	0Gy	0.33	3.52	0.65	2.95	2.36	4.70	3.48	14.71	20.3
TRFK 301/1	50Gy	0.36	2.95	0.57	3.07	2.45	4.28	4.13	14.38	20.7
TRFK 301/1	100Gy	0.40	3.01	0.74	3.12	2.69	6.03	4.90	17.37	22.8
TRFK 301/1	**Control**	**0.40**	**5.23**	**0.83**	**3.78**	**3.00**	**4.87**	**3.20**	**17.12**	**21.0**
TRFK 301/4	0Gy	0.21	5.17	0.44	2.96	1.87	4.60	1.80	13.87	20.2
TRFK 301/4	50Gy	0.39	3.82	0.54	3.17	2.44	4.50	2.80	14.09	19.1
TRFK 301/4	100Gy	0.44	2.15	0.47	2.36	3.83	2.37	3.53	12.35	17.6
TRFK 301/4	**Control**	**0.50**	**2.77**	**0.61**	**2.94**	**3.91**	**4.00**	**4.39**	**15.69**	**21.0**
%CV		28.4	29.3	33.2	18.3	37.5	29.2	31.3	18.2	8.6

Table notes: P - significance at P<0.05, GA- garlic acid, EGC- epigallocatechin, C- catechin, Caff - caffeine, EC- epicatechin, EGCG- epigallocatechingallate, ECG- epicatechingallate, TC – total catechins , TP- total polyphenols

4.2.1 Variations in Gallic Acid (GA)

Gallic Acid content varied significantly ($p<0.05$) among the 66 progenies and their controls. Interaction between cultivar, gamma treatment and season was significant ($p<0.05$) (Table 2). The mean of Gallic acid did not vary significantly ($p<0.05$) across the three seasons evaluated. Moreover, means obtained across the gamma dosages differed significantly from clonal controls. Based on the overall means, clone TRFK 301/4 had the highest GA content at 0.50%, while 100 Gy-treated progeny of clone TRFCA SFS 150 had the least at 0.18% (Table 3). At 100 Gy, only progeny from clone TRFK 303/1199 registered an increase in GA at 0.42% translating to 40.0% more than the control, while progeny arising from clone TRFK 301/1 performed similar to their clonal control. All other progenies showed a decrease which ranged from 6.8% for clone TRFCA SFS150 to 53% for EPK C12. At 50 Gy and 0 Gy, all the progenies registered a decrease of up to 44.7% and 57.9%, respectively. Based on cultivar, progenies arising from clone 301/4 led with a mean of 0.38% (Table 4). Based on GA%, most of the progenies performed at par or lower than their control (Figure 3).

Table 4: Variation in GA as affected by cultivar, varying gamma dosage and season on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	**0GY**	**50GY**	**100GY**	**C**	**0GY**	**50GY**	**100GY**	**C**	**0GY**	**50GY**	**100GY**	**C**	**Means**
TRFK 303/1199	0.24	0.24	0.29	0.34	0.31	0.23	0.34	0.3	0.27	0.29	0.64	0.27	0.28e
EPK C12	0.23	0.20	0.22	0.27	0.20	0.26	0.27	0.26	0.29	0.27	0.20	0.21	0.24e
GW Ejulu-1	0.34	0.36	0.18	0.32	0.26	0.3	0.26	0.24	0.33	0.32	0.45	0.50	0.32bc
TRFK 301/1	0.30	0.37	0.29	0.35	0.37	0.36	0.53	0.56	0.3	0.35	0.37	0.28	0.35ab
TRFCA SFS150	0.27	0.15	0.10	0.75	0.26	0.29	0.30	0.23	0.37	0.20	0.12	0.17	0.26de
TRFK 301/4	0.41	0.51	0.46	0.55	0.28	0.29	0.31	0.51	0.34	0.38	0.56	0.44	0.38a
Means-gamma (all seasons)	0.30b	0.30b	0.33ab	0.36a									
Means-seasons		0.32a				0.31a					0.33a		
CV (%)	28.35%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

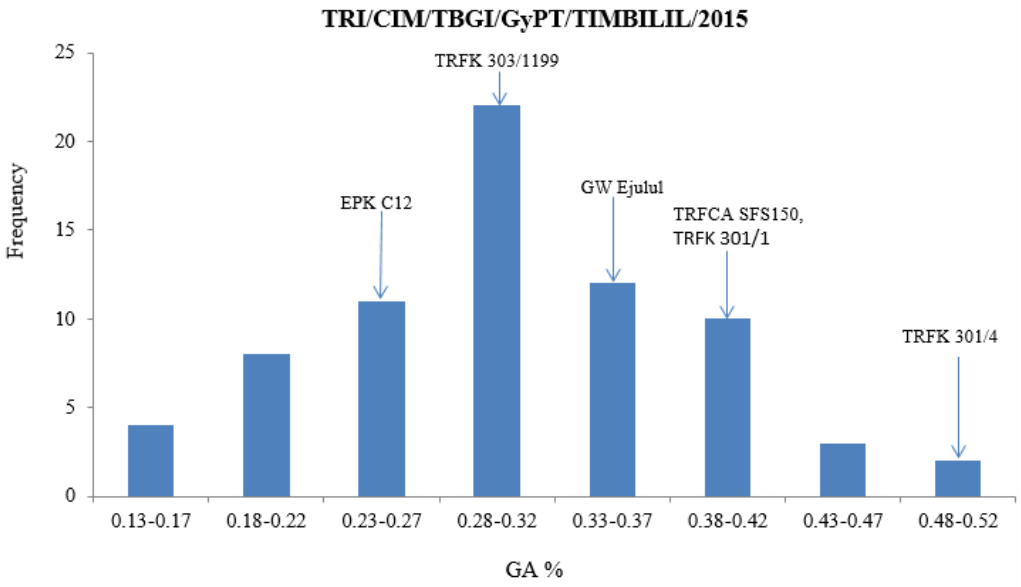

Figure 3: Variation in GA content across progenies of various cultivars under evaluation

Based on GA% the progeny test tended towards a bell-shaped curve with most of the progenies perfoming lesser than their control (Figure 3).

4.2.2 Variations in Epigallocatechin (EGC)

EGC content varied significantly between the cultivars ($p<0.001$), seasons ($p< 0.05$) and interaction between cultivar and gamma treatment (Table 2). However, there was no significant variation in Gamma treatment. Progenies of TRFK 303/1199 and TRFK 301/1 had the uppermost and lowest levels of EGC at 5.56% and 3.38%, respectively. Considering the overall means (Table 3), the 100Gy-treated progeny arising from TRCA SFS150 had the top EGC content at 5.98%, while those from TRFK 301/4 had the least at 2.15%. Moreover, progenies arising from GW Ejulu-l, TRFK 303/1199 and TRFCA SFS 150 had notably higher EGC content at 48%, 27.7% and 2.4%, respectively compared to control. Interestingly, the same trend was noted at 50 Gy treatment where these progenies showed an increase in EGC content of 39.8%, 12.6% and 10.3%, respectively. Surprisingly, the 0Gy-treated progenies of clones TRFK 301/4 and GW Ejulu-l had significantly high levels of EGC at 86.6% and 49.5% than their control, an indication that other factors apart from gamma treatment could be involved. Progenies of EPK C12 and TRFK 301/1 generally recorded decreased EGC at all levels of gamma treatment. The levels of EGC for EPK C12 relative to control seemed to decrease with increase in gamma treatment. The cool wet season had significantly ($p< 0.05$) higher EGC levels compared to the other seasons (Table 5).

Table 5: Variation in EGC as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
OP/Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	6.32	6.58	4.94	5.65	5.02	5.70	5.16	4.77	4.85	5.23	5.82	5.13	5.56a
EPK C12	4.85	5.58	6.14	4.82	3.68	4.62	4.50	5.26	4.37	4.35	5.10	5.99	4.70b
GW Ejulu-l	4.57	4.45	6.17	2.74	4.40	3.71	3.41	3.16	3.83	3.78	3.06	2.64	4.02cd
TRFK 301/1	3.45	3.20	2.64	8.56	3.72	1.80	3.46	2.63	3.40	3.84	2.92	4.49	3.38e
TRFCA SFS150	3.60	5.84	6.89	2.73	3.10	4.28	5.06	4.28	3.56	5.39	5.99	7.04	4.47bc
TRFK 301/4	3.60	4.20	2.33	2.63	3.69	3.67	2.13	2.74	3.70	3.58	2.00	2.94	3.53de
Means-gamma (all seasons)	4.09a	4.43a	4.32a	4.34a									
Means-seasons		4.69a				3.91b				4.29b			
CV (%)	29.27%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

4.2.3 Variations in Catechin (+C)

Catechin content varied significantly ($p<0.05$) between cultivars and interaction between cultivars and gamma treatment (Table 2). Overall, progenies from GW Ejulu-l and TRFK 303/1199 registered the highest and least means at 0.65% and 0.42%, respectively. (Table 6). Overall means ranged from 1.01% for control clone GW Ejulu-l to 0.41% for TRFCA SFS150 irradiated with 100 Gy (Table 3). All other clones whose seeds were exposed to similar dosage of irradiation, except those of EPK C12 and TRFK 303/1199, recorded a decline in C content. The two (i.e. progenies of EPK C12 and TRFK 303/1199) registered 0.63% and 0.57% catechin content translating to 3.3% and 26.7% increase, respectively when compared to clonal control. Only progeny of TRFK 303/1199 at 50Gy showed an increase of 11.2% catechin to the control. Notably,

progenies of TRFCA SFS150, EPK C12 and TRFK 303/1199 at OGy gave increased catechin content of 44.4%, 17.9% and 11.2%, respectively than their clonal controls, an indication of heterosis arising from natural crossing and/or that other factors apart from gamma treatment could be involved. Progenies of GW Ejulu-l, TRFK 301/1 and TRFK 301/4 recorded decreased C content at all levels of gamma treatment suggesting that they probably require a dose above 100Gy to express the trait.

Table 6: Variation in levels of Catechin (+C) as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	0.46	0.46	0.48	0.41	0.50	0.53	0.58	0.46	0.43	0.51	0.64	0.47	0.42c
EPK C12	0.71	0.57	0.66	0.58	0.75	0.60	0.59	0.63	0.69	0.53	0.63	0.61	0.64ab
GW Ejulu-l	0.60	0.81	0.49	1.77	0.52	0.53	0.43	0.54	0.61	0.69	0.66	0.72	0.65a
TRFK 301/1	0.57	0.52	0.77	1.04	0.69	0.56	0.69	0.62	0.70	0.62	0.76	0.82	0.64ab
TRFCA SFS150	0.71	0.51	0.44	0.63	0.59	0.41	0.42	0.38	0.73	0.39	0.36	0.39	0.54c
TRFK 301/4	0.59	0.57	0.52	0.64	0.55	0.52	0.43	0.67	0.56	0.52	0.45	0.53	0.55bc
Means-gamma (all seasons)	0.61a	0.55b	0.56b	0.66a									
Means-seasons		0.65a				0.55a				0.58a			
CV (%)	33.16%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

4.2.4 Variation in Caffeine

The level of caffeine varied significantly among cultivars ($p < 0.01$) and season ($p < 0.001$) (Table 2). Means obtained from the various levels of gamma did not differ significantly whereas cool wet season accumulated significantly lower levels compared to the other two seasons. Generally, the highest caffeine content of 3.09% were from progenies of

clone TRFK 301/1 (Table 7). Overaly, control clone TRFK 301/1 had the highest caffeine content at 3.78%, whereas TRFK 301/4 irradiated with 100 Gy had the least at 2.36% (Table 3). Most notably, several test progenies had lower caffeine levels as compared to their controls (Appendix IV and Figure 4). The dry season registered a significant ($p<0.05$) increase in caffeine based on the overall means across seasons and treatments.

Table 7: Variation in Caffeine as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	2.14	2.29	3.39	3.73	3.55	3.05	3.65	2.60	2.76	2.70	3.38	2.73	2.83bc
EPK C12	2.17	2.43	2.99	0.27	2.74	2.81	3.18	3.25	2.96	2.81	3.41	3.63	2.68c
GW Ejulu-l	2.45	3.05	2.37	3.18	3.18	3.32	2.07	3.07	3.05	3.52	3.39	3.65	3.07ab
TRFK 301/1	2.29	2.85	2.37	3.36	3.14	2.80	3.92	3.35	3.44	3.57	3.07	4.63	3.09a
TRFCA SFS150	2.75	2.54	2.50	3.49	2.96	3.03	2.81	2.74	3.19	3.30	2.53	3.89	2.99ab
TRFK 301/4	2.33	2.97	2.24	2.71	3.16	3.13	2.89	3.07	3.27	3.41	1.94	3.03	2.98ab
Means-gamma (all seasons)	2.861a	2.975a	2.950a	3.132a									
Means-seasons		2.62b				3.06a				3.22a			
CV (%)	18.33%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

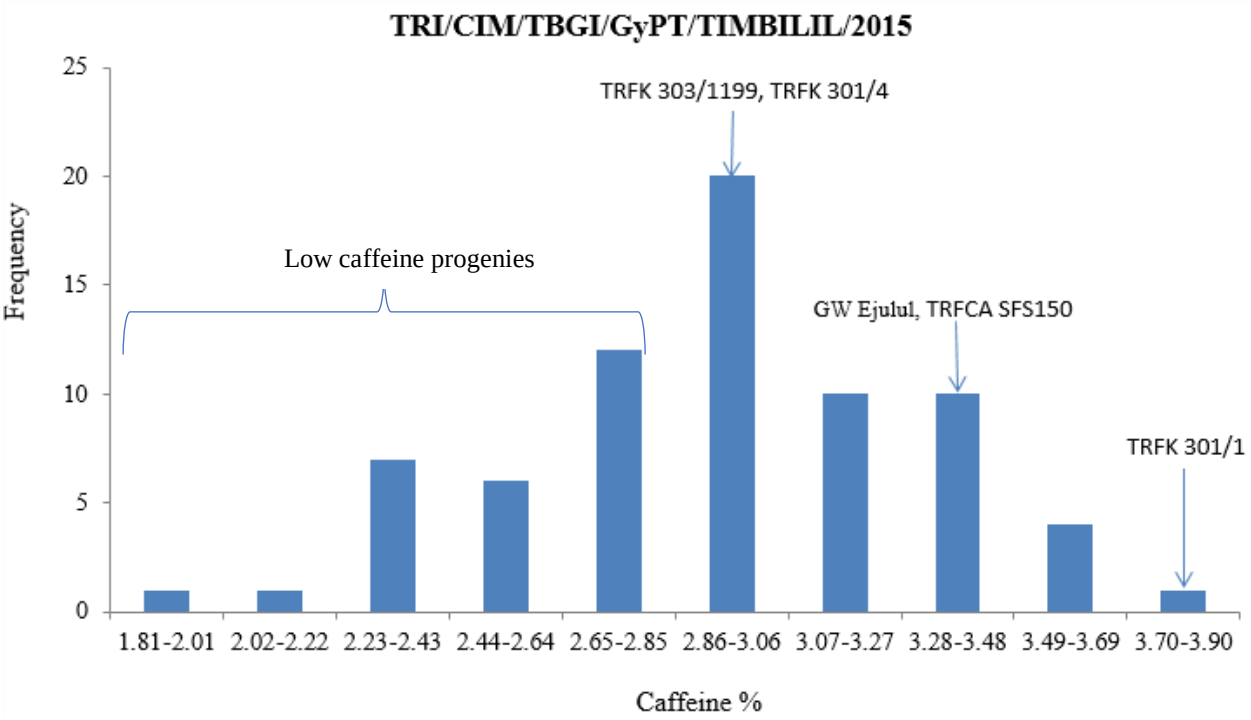

Figure 4: Caffeine content across progenies of various cultivars under evaluation

4.2.5 Variations in Epicatechin (EC)

There was a significant ($p < 0.05$) variation in EC content for all the three variables studied (Table 2). Overall, control TRFK 301/4 had the uppermost EC content at 3.91%, while 100Gy treated progenies arising from clone TRFK 303/1199 had the least at 1.32% (Table 3). At 100Gy, only progenies from clones TRFCA SFS150 and EPK C12 registered an increase in EC at 2.00% and 2.56%, translating to 17.50% and 33.70%, respectively compared to controls (Appendix III). The rest showed a decrease of 2.0-26.40%, with the highest and least being progenies arising from clone TRFK 301/4 and GW Ejulu-l, respectively. It is worth noting that progenies from clones TRFK 301/4, GW Ejulu-l, and TRFK 301/1 all registered decreased EC content at all levels of gamma treatment compared to their clonal controls. Cultivar means showed that progenies of

clone TRFK 301/4 had the highest EC levels of 2.66%, whereas those of GW Ejulu had the lowest levels of 1.63%. Seasonal EC variation was substantial ($p< 0.001$). The highest EC content (2.51%) was reported during the cool wet season (Table 8).

Table 8: Variation in EC as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	2.13	2.04	1.26	1.74	1.60	1.82	1.54	1.56	1.41	1.52	1.16	1.56	1.70b
EPK C12	1.74	1.71	3.46	2.00	1.55	1.52	2.22	2.05	1.42	1.19	2.01	1.00	1.64b
GW Ejulu-l	2.10	2.11	2.44	2.21	1.73	1.28	0.79	2.14	1.39	1.24	0.79	1.10	1.63b
TRFK 301/1	2.65	2.84	3.98	3.01	2.20	2.12	1.90	3.31	2.22	2.40	2.20	2.60	2.48a
TRFCA SFS150	1.59	2.35	2.33	1.92	1.50	1.56	1.43	1.55	1.35	1.71	2.23	1.63	1.70b
TRFK 301/4	2.72	2.58	4.46	4.84	2.12	2.84	2.39	2.66	2.35	1.89	4.63	4.24	2.66a
Means-gamma (all seasons)	1.875b	1.927ab	2.290ab	2.327a									
Means-seasons		2.51a				1.89b				1.89b			
CV (%)	37.51%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

4.2.6 Variations in Epigallocatechin gallate (EGCG).

EGCG content varied considerably among the cultivars ($p< 0.001$) and seasons ($p< 0.05$) (Table 2 and Figure 5). Cultivar means showed progenies of clone TRFK 303/1199 had the highest EGCG levels of 7.18%, whereas those of TRFK 301/4 had the lowest levels of 4.00% (Table 3). Notably, all gamma treatments of clone GW Ejulu-l exhibited positive increase in EGCG while those involving EPK C12 decreased. Cool wet season accumulated significantly ($p<0.05$) higher EGCG content compared to the dry season (Table 9). Individual treatment means indicated an increase of 24.2 and 23.8% for

progenies of TRFK 303/1199 and TRFK 301/4 irradiated with 100 Gy, respectively when compared to the controls. The highest decrease of 40.8% was observed on TRFK 301/4 treated with 100Gy. Substantial variation ($p< 0.05$) in seasons was noted with majority of the gamma-treated progenies including their control clones registering declined EGCG content during the dry season (Table 9).

Table 9: Variation in EGCG as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	6.99	6.68	9.70	9.84	7.67	6.59	8.95	5.33	6.17	5.54	8.12	6.37	6.85a
EPK C12	4.85	5.77	5.60	6.47	4.44	5.76	3.54	5.44	4.54	4.79	4.90	6.35	5.10bc
GW Ejulu-l	5.86	6.12	5.51	5.80	5.78	5.26	5.60	5.07	5.10	5.14	5.62	4.14	5.50b
TRFK 301/1	5.41	5.00	5.21	3.95	4.22	3.38	9.05	4.83	4.47	4.47	3.83	5.82	4.65c
TRFCA SFS150	5.63	4.13	6.36	7.01	5.48	4.58	7.16	4.92	4.62	4.50	4.60	6.04	5.11bc
TRFK 301/4	5.05	4.73	2.46	4.01	4.75	4.04	2.93	4.15	4.56	4.72	1.73	3.83	4.40c
Means-gamma (all seasons)	1.875b	1.927ab	2.290ab	2.327a									
Means-seasons		5.76a				5.37ab				5.00b			
CV (%)	29.23%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

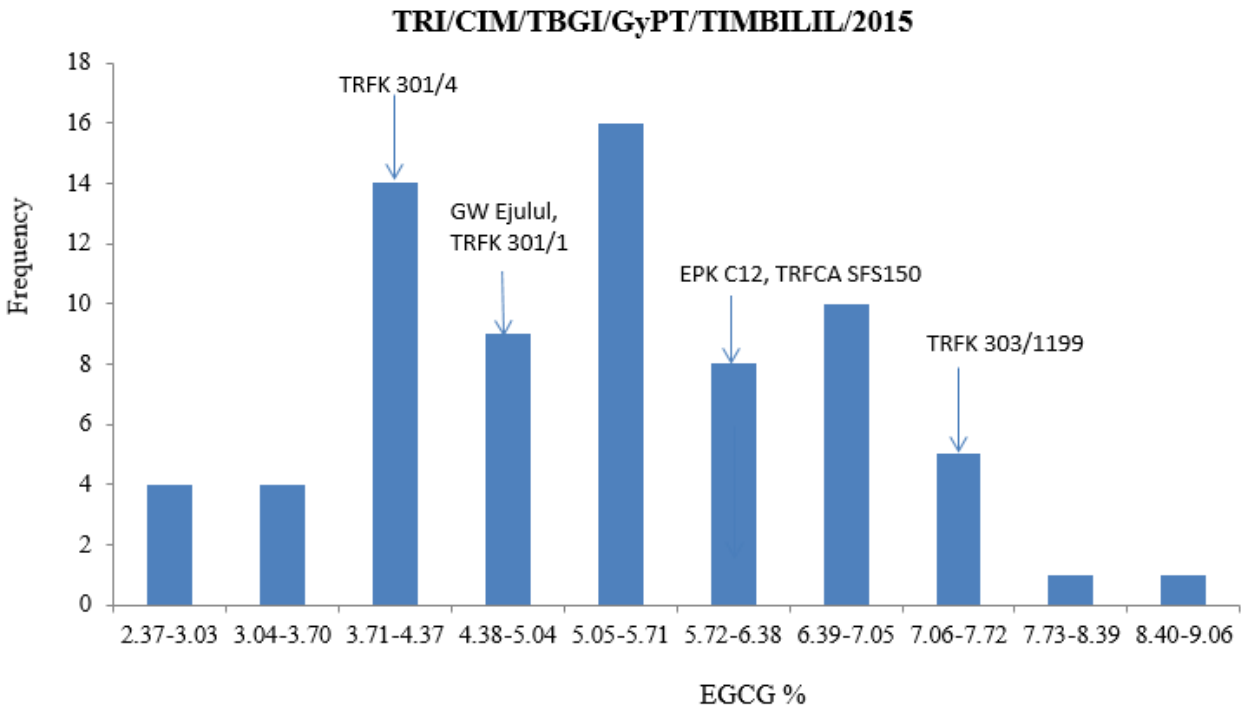

Figure 5: Histogram for EGCG content across progenies of various cultivars under evaluation

4.2.7 Variations in Epicatechin gallate (ECG)

There was significant variation ($p < 0.001$) in ECG content for cultivar, season and interaction between cultivars and gamma treatment (Tables 2 and 10). Cultivar GW Ejulu-l had the highest overall ECG levels at 4.68% while TRFCA SFS 150 had the least at 1.91% (Table 3). At 100Gy treated progenies of clones TRFK 301/1 and GW Ejulu-l had significantly highest and lowest ECG content at 4.90% and 1.78%, respectively. These represented 53.40% increase and 62.04% decrease compared to their parents, respectively (Appendix III). Progenies of TRFCA SFS 150, TRFK 303/1199, and EPK C12 registered increased levels, while the levels for TRFK 301/4 decreased. With the exception of TRFK 301/1 that recorded 29.1% increase in ECG levels at 50Gy, all other progenies recorded up to 37.0% (GW Ejulu-l) decreased in ECG content when compared with the control. At 0Gy, all progenies except those from clones TRFCA SFS150 and TRFK 301/1 registered reduced ECG. Progenies from clones GW Ejulu-l and TRFK 301/4 registered decreased ECG at all treatment levels. On the contrary, those from TRFK 301/1 responded positively with increasing in gamma treatment. Most of the gamma-treated progenies and their control clones had low ECG content in the dry season whereas the cold and wet season accumulated significantly ($p < 0.001$) high levels of ECG (Table 10).

Table 10: Variation in ECG as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	2.57	2.47	2.88	3.49	2.94	2.65	3.04	1.98	1.92	1.72	2.33	1.97	2.42c
EPK C12	2.26	2.12	3.75	2.79	2.16	2.19	2.15	2.60	1.89	1.58	1.89	2.05	2.12c
GW Ejulu-l	3.62	4.16	3.05	6.01	3.77	2.60	1.08	4.84	2.35	2.10	1.20	3.19	3.12b
TRFK 301/1	3.19	4.22	7.18	3.12	3.96	4.17	4.53	3.31	3.29	4.01	3.00	3.16	3.05a
TRFCA SFS150	3.29	2.01	2.29	2.56	3.13	1.85	2.16	1.65	2.19	1.44	1.44	1.51	2.27c
TRFK 301/4	2.80	3.01	3.46	4.47	2.99	2.88	3.83	4.5	2.46	2.51	3.3	4.21	2.97b
Means-gamma (all seasons)	2.82a	2.65a	2.92a	3.18a									
Means-seasons		3.37a				2.96b				2.36c			
CV (%)	31.25%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

4.2.8 Variations in Total Catechins (TC)

The TC content varied significantly ($p< 0.05$) for only cultivars and season (Tables 2 and 11). Cultivar means ranged from 14.75 to 17.12% for TRFCA SFS 150 and TRFK 301/1, respectively (Table 3). Variation in the accumulation of TC with gamma treatment at 100 Gy ranged from 18.81% for clone TRFK 303/1199 to 12.35% for clone TRFK 301/4. Clones TRFK 303/1199, TRFCA SFS150 and TRFK 301/1 registered increased TC levels of 11.1, 11.0 and 1.5%, respectively. A decrease of 21.3, 12.5 and 3.6% were recorded for TRFK 301/4, GW Ejulu-l and EPK C12 respectively. All progenies registered decreased TC content of between 1.4 for TRFK 303/1199 to 16.0% for TRFK 301/1 at 50 Gy. At 0Gy, only GW Ejulu-l and TRFK 303/1199 recorded marginal increase of below 1%. All other treatments showed a decline of up to 19.2%. Cool wet season recorded the highest TC content at 17% (Table 11).

Table 11: Variation in TC as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	18.47	18.20	19.25	21.10	17.73	17.30	19.27	14.10	14.77	14.52	18.08	15.50	17.01a
EPK C12	14.40	15.80	19.61	16.70	12.57	14.70	13.41	16.00	12.91	12.44	14.53	16.70	14.19b
GW Ejulu-l	16.74	17.70	17.66	18.50	16.20	13.40	11.31	15.80	13.28	12.96	11.34	11.80	14.93b
TRFK 301/1	15.26	15.80	19.78	19.70	14.79	12.00	19.63	14.70	14.08	15.32	12.71	17.00	15.00b
TRFCA SFS150	14.81	15.50	18.32	14.90	13.80	12.70	16.24	12.80	12.45	13.43	14.62	16.60	14.08b
TRFK 301/4	14.76	15.10	13.22	16.60	14.10	14.00	11.72	14.70	13.64	13.22	12.10	15.80	14.11b
Means-gamma (all seasons)	14.71a	14.66a	15.71a	16.05a									
Means-seasons		17.00a				14.71b				14.16b			
CV (%)	18.17%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

4.2.9 Variations of Total Polyphenols (TP)

The TP contents varied significantly ($p<0.001$) for cultivars, gamma treatments, seasons, and interactions between cultivar and Gamma and cultivar and season (Tables 2 and 12). Cultivar means ranged 25.2% for GW Ejulu-l to 21.0% for both Cambod cultivars TRFK 301/1 and TRFK 301/4 (Table 3). Progeny means ranged 16.08% for 50 Gy of EPK C12 during the dry season to 27.30% for control of GW Ejulu-l during the warm and wet season (Table 12). The mean of the TP for cool wet and warm wet seasons did not vary significantly ($p< 0.05$) except for dry season (Table 12). Progenies of TRFK 301/4 irradiated at 100 Gy had the least levels with a mean of 17.6%. There was a general decrease of TP levels resulting from 50 and 100Gy gamma treatment, this ranged from 1-29.8% with 100 Gy progenies of GW Ejulu-l registering the highest decline (29.8%) compared to the control clone. At both 0Gy and 50Gy, all progenies performed lower

than their clonal progenitors (Figure 6). However, 100Gy treatment resulted in increased TP in progenies of clones TRFK 303/1199, TRFCA SFS150, and TRFK 301/1 that registered 0.6, 2.8 and 8.6% increase, respectively.

Table 12: Variation in TP as affected by season and varying gamma dosage on OP populations

	Cool &wet season				Warm &wet season				Dry season				
Cultivar	0GY	50GY	100GY	C	0GY	50GY	100GY	C	0GY	50GY	100GY	C	Means
TRFK 303/1199	22.02	21.80	24.00	25.80	20.86	20.90	24.80	21.40	18.42	18.60	19.00	20.20	20.79b
EPK C12	18.66	18.40	20.90	23.20	18.50	18.90	19.30	19.00	17.32	16.08	18.00	21.20	18.39d
GW Ejulu-l	23.46	24.30	17.70	26.10	23.54	23.30	17.80	27.30	18.94	19.18	17.60	22.20	22.00a
TRFK 301/1	18.64	20.20	22.10	21.70	22.42	21.50	26.10	19.30	19.74	20.20	20.10	21.90	20.70b
TRFCA SFS150	22.62	20.70	24.00	24.60	21.20	20.20	21.60	18.30	19.46	19.74	20.20	21.00	20.83b
TRFK 301/4	19.20	18.40	16.20	21.60	20.38	20.50	18.30	21.90	18.36	18.48	18.30	19.50	19.23c
Means-gamma (all seasons)	20.21b	20.08b	20.33b	22.01a									
Means-seasons		21.51a				21.14a				19.32b			
CV (%)	8.59%												

NB: Values within the same column followed by the same letters are not significantly different, using Duncan's Multiple Range Test at 5% level. C represents control

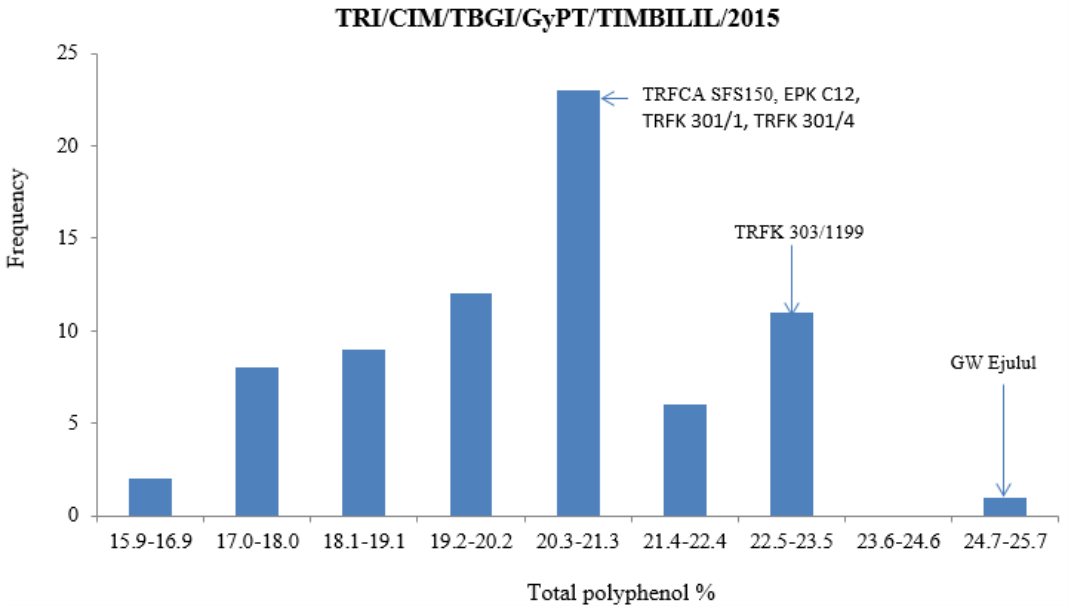

Figure 6: Histogram for total polyphenols based on individual progeny performance

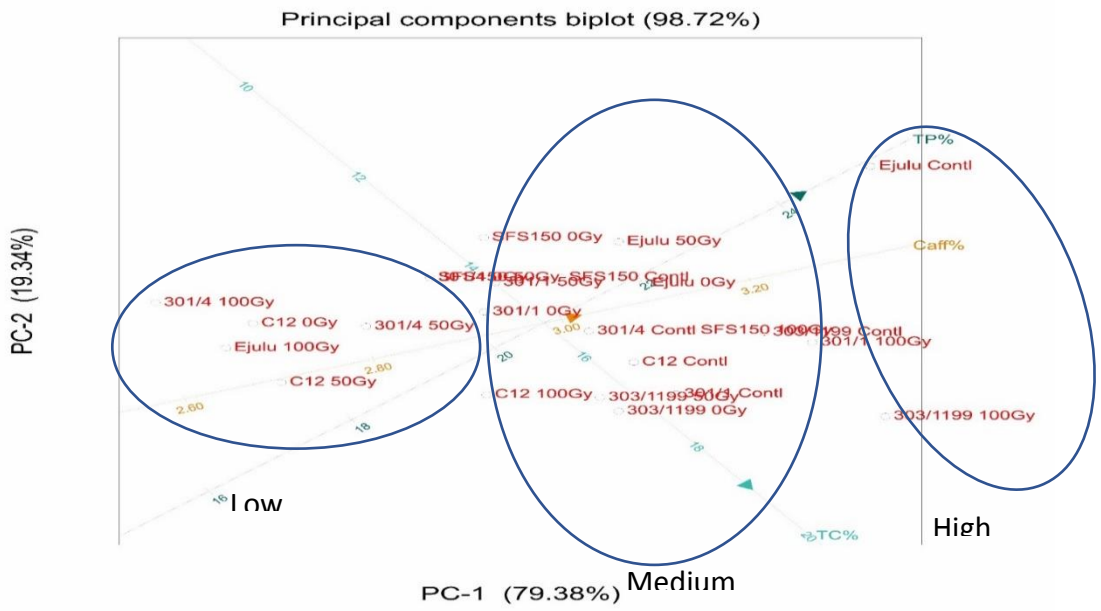

Figure 7: Principal component analysis using mean TC, TP and Caff data across three seasons.

The principal component analysis categorized the progenies into three distinct clusters (Figure 7). The first principal component attributed 79.38 % of the total variation, while the second principal component accounted for 19.34% variation, accruing to 98.72% variation.

4.3 Morphological characterization

Twenty one tea descriptors were utilized for this study to evaluate morphological traits among the sixty six progenies including their clonal controls. Upon further examination, it was found that four characters were monomorphic (not informative) and were thus eliminated from the analyses. A total of 17 descriptors were ultimately selected (Table 13).

Table 13: Plant morphological descriptors of tea (*Camellia sinensis*) used in for diversity analysis (UPOV 2008).

No.	Plant descriptor	Range /variation	Data type
1.	Plant vigor	Weak: 3; Medium: 5; Strong: 7	Quantitative
2.	Plant type	Shrubs:1; Semi-arbor: 3; Arbor: 5	Quantitative
3.	Plant growth habit	Upright:1; Semi-upright: 3; Spreading: 5	Quantitative
4.	Plant density of branches	Sparse: 3; Medium: 5; Dense: 7	Quantitative
5.	Young shoot density pubescence of bud	Sparse: 3; Medium: 5; Dense: 7	Quantitative
6.*	Leaf blade attitude	Upwards: 1; Outwards: 3; Downwards: 5	Quantitative
7.	Leaf blade shape	Very narrow elliptic: 1; Narrow elliptic: 2; Medium elliptic: 2; Broad elliptic: 4	Quantitative
8.	Leaf blade intensity of green color	Light: 3; Medium: 5; Dark: 7	Quantitative
9.*	Leaf blade shape of cross section	Folded upwards: 1; Flat: 2; Recurved: 3	Quantitative
10.	Leaf blade texture of upper surface	Smooth or weakly rugose: 1; Moderately rogues: 2; Strongly rugose:3	Quantitative
11.	Leaf blade undulation of margin	Absent or weak:1; Medium: 2; Strong:3	Quantitative
12.	Leaf blade serration of margin	Weak: 3; Medium: 5; Strong: 7	Quantitative
13.	Young shoot length of ‘three and a bud’	Short: 3; Medium: 5; Long: 7	Quantitative
14.	Leaf blade length	Short: 3; Medium:5; Long: 7	Quantitative
15.	Leaf blade width	Narrow: 3; Medium: 5; Broad: 7	Quantitative
16.*	Plant branch zigzagging	Absent: 1; Present: 9	Qualitative
17.*	Young shoot pubescence of bud	Absent: 1; present: 9	Qualitative
18.	Young shoot anthocyanin coloration at base of petiole	Absent: 1; Present: 9	Qualitative
19.	Young shoot color of second leaf at ‘two and a bud’ stage	Whitish: 1; Yellow green: 2; Light green: 3; Medium green: 4; Purple green: 5;	Pseudo-Quantitative
20.	Leaf blade shape of apex	Obtuse: 1; Acute: 2; Acuminate: 3	Pseudo-Quantitative
21.	Leaf blade shape of base	Acute: 1; Obtuse: 2; Truncate: 3	Pseudo-Quantitative

Key: *Trait was not informative

4.4 Morphological characterization results

Principal Component Analysis (PCA)

The eigen values of 17 morphological descriptors scored for 72 cultivars (comprising 66 progenies and 6 parental controls) are shown in Table 14. The first 8 PCs accounted for 78 % of the total variation present in the cultivars. Thus, only the first 8 PCs were retained for further analysis. Eigen vectors of each of the principal components (Table 15) revealed that only fifteen variables contributed substantially towards deciding the position of each PC. The first PC which attributed for 21% of the total variance was predominantly linked with young shoot anthocyanin coloration at base of the petiole, leaf blade intensity of green color, leaf blade shape of apex and young shoot color of second leaf at ‘two and bud’ stage. The second PC that accounted for 13 % of the total variance was related with leaf blade shape, leaf blade width and plant vigor. Density of branches, leaf blade shape and width, plant type and vigour were associated significantly with the third PC which accounted for 11% variation. The fourth and fifth PCs each accounted for 8% and were associated with young shoot anthocyanin coloration at base of the petiole, leaf blade length and young shoot length of ‘three and a bud’; and density of pubescence of bud, leaf blade serration of margin, young shoot color of second leaf at ‘two and bud’ stage respectively. The remaining 3 PCs, which accounted for 17 % of the total variation, were mainly associated with leaf blade width, plant type, leaf blade undulation of margin; leaf blade texture of upper surface and density of pubescence of bud (Table 15). Of the 17 descriptors scored, only 15 contributed significantly to the total variation present in the germplasm collection.

Table 14: Eigen values of the correlation matrix obtained from the principal component (PC) analysis of 17 morphological descriptors.

PC	Eigen value	Difference	Proportion	Cumulative	Cumulative %
1	3.633	1.393	214	214	21.4
2	2.240	0.438	132	345	34.5
3	1.802	0.470	106	451	45.1
4	1.332	0.015	78	530	53.0
5	1.317	0.263	77	607	60.7
6	1.054	0.028	62	669	66.9
7	1.026	0.156	60	730	73.0
8	0.870	0.170	51	781	78.1
9	0.700	0.042	41	822	82.2
10	0.658	0.051	39	861	86.1
11	0.607	0.095	36	896	89.6
12	0.512	0.049	30	926	92.6
13	0.463	0.201	27	954	95.4
14	0.262	0.010	15	969	96.9
15	0.252	0.111	15	984	98.4
16	0.141	0.009	8	992	99.2
17	0.132		8	1000	100.0

Table 15: Eigen vectors for first eight PCs of the 17 morphological descriptors

Descriptor	PC1	PC2	PC3	PC4	PC5	PC6	PC7	PC8
1	**0.3561**	0.21342	-0.009	**-0.3062**	0.01051	-0.1802	-0.1349	0.0074
2	-0.2761	0.26187	**0.3639**	0.17582	0.1114	**-0.3097**	-0.2102	0.18293
3	0.13637	0.15903	0.10756	-0.2065	**-0.528**	-0.1893	0.14225	**0.30116**
4	-0.2525	0.28099	0.25201	-0.1489	-0.0782	0.21945	0.15901	-0.1789
5	**0.3614**	0.18053	0.11753	-0.2964	0.26151	0.04146	0.1925	0.02362
6	0.27955	0.15768	-0.1742	**0.457**	0.02492	0.16341	-0.1156	-0.1031
7	-0.0668	**0.37605**	**-0.389**	0.08492	0.23668	-0.0246	0.27087	0.21569
8	-0.2983	0.11294	-0.1492	-0.1912	0.1139	0.21554	0.26915	-0.078
9	0.04314	0.24311	-0.1883	0.05236	**-0.602**	-0.184	-0.0083	0.05819
10	0.01659	**0.4743**	**-0.3034**	0.10538	-0.0602	**0.31952**	0.09144	-0.0308
11	-0.0377	0.19884	**0.4092**	-0.1152	-0.1956	**0.3595**	-0.0529	**-0.452**
12	-0.22	**0.3859**	**0.33731**	0.19137	0.19599	-0.2087	0.0258	0.21611
13	**0.3586**	0.02027	0.23801	0.149	-0.1127	0.10272	0.18725	-0.0264
14	-0.0069	0.20224	-0.1277	-0.1959	0.03063	0.26148	**-0.8**	0.14365
15	0.0776	-0.1688	0.2276	0.02888	-0.0211	**0.5512**	0.08767	**0.6993**
16	**0.4049**	0.15954	0.11205	-0.2071	**0.32773**	-0.138	0.00836	-0.0349
17	0.24873	0.04716	0.16834	**0.5497**	-0.0004	0.04095	-0.0338	-0.113

Bolded values represent variables which made significant contribution to total variance

Key:1Young shoot: anthocyanin coloration at base of the petiole
2. Density of Branches
3. density of pubescence of bud
4. Plant: Growth Habit
5. Leaf blade: intensity of green color
6. Leaf blade: length
7. Leaf blade: shape
8. Leaf blade: shape of base
9. Leaf blade: serration of margin
10. Leaf blade: width
11. Plant: Type
12. Plant: Vigor
13. Leaf blade: shape of apex
14. Leaf blade: texture of upper surface
15. Leaf blade: undulation of margin
16. Young shoot: color of second leaf at ‘two and bud’ stage
17. Young shoot: length of ‘three and a bud’

Cluster Analysis

Based on the first 8 principal components of the PCA, a dendrogram was generated (Figure 8), where 4 well defined clusters could be identified. Considering the clusters formed, 100Gy progenies arising from all clones were grouped in cluster 2 except those from TRFK 303/1199 and TRFK 301/4 that were in clusters 3 and 4, respectively. All the control controls were in cluster 2. Most of the gamma treated stocks with 50Gy were in cluster 3. Based on the fact that the progenies regardless of treatment could be found in any of the clusters and moreso not in the same cluster as their progenitors, indicated that other factors apart from gamma treatment could be involved, with particular emphasis on heterosis arising from crossing. The interpretation of the descriptors responsible for cluster divergence is presented in Table 16.

Table 16: The interpretation of the descriptors primarily responsible for cluster divergence

Cluster No.	**Morphological descriptors mainly responsible for cluster divergence**
Cluster 1	Young shoot: anthocyanin coloration at base of the petiole, Young shoot: length of 'three and a bud', Leaf blade: length, Leaf blade: intensity of green color
Cluster 2	Young shoot: color of second leaf at 'two and bud' stage, Density of pubescence of bud, Leaf blade: intensity of green color, Young shoot: anthocyanin coloration at base of the petiole
Cluster 3	Density of pubescence of bud, Leaf blade: shape of apex
Cluster 4	Plant: Vigor, Density Of Branches, Young shoot: anthocyanin coloration at base of the petiole

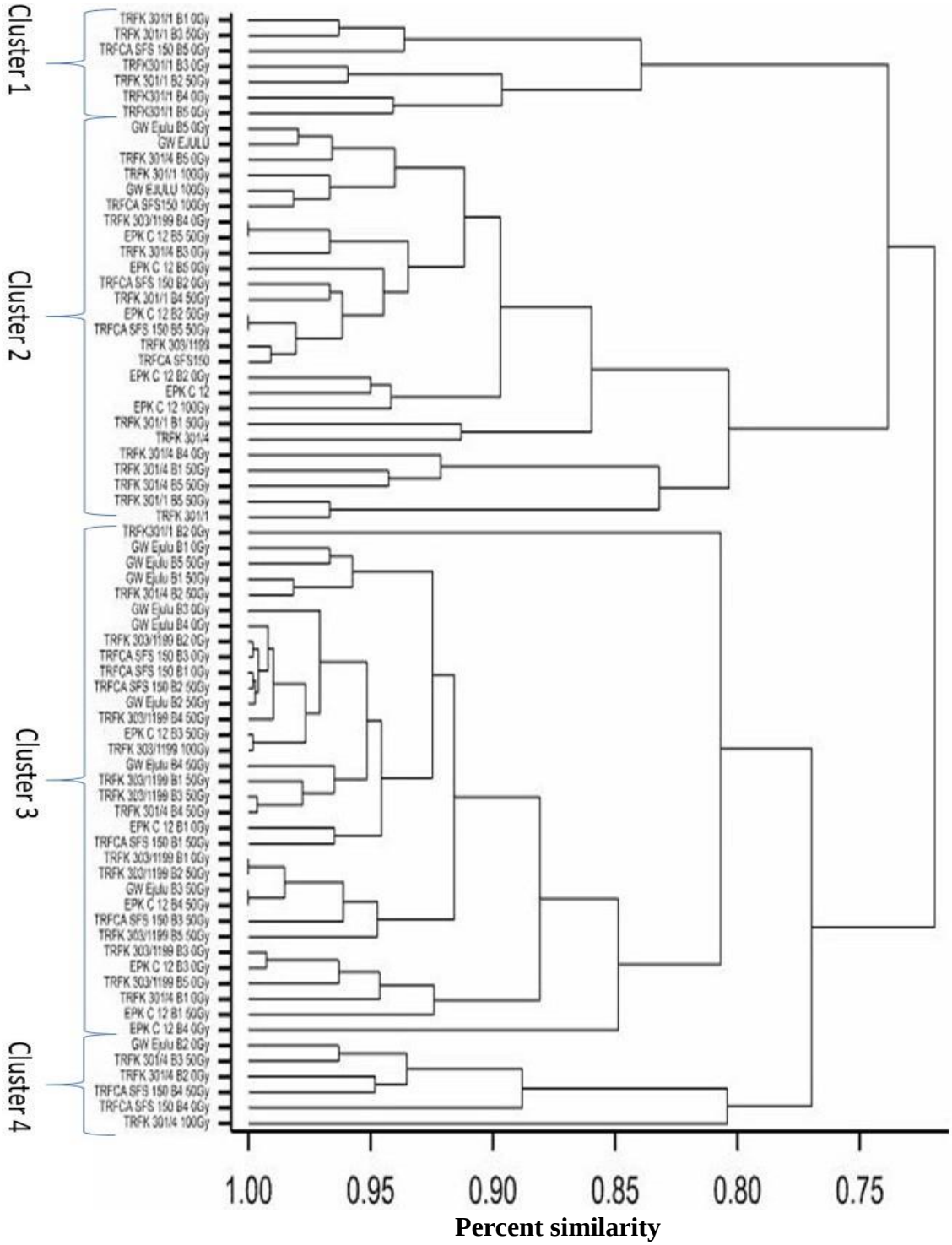

Figure 8: The Dendrogram obtained based on average linkage cluster analysis using 15 morphological descriptors of the 66 mutants and 6 parents

Frequency Distribution of Traits

The frequency distribution of the key morphological characters showed the presence of variation among the mutants studied (Table 17). Plant vigour trait varied among the gamma treated progenies with 61.1% of the plants studied having medium vigour. The semi arbor plant type formed the greatest portion of the plants at 98.6%. Plant density habits recorded from the genotypes were sparse (22.2%), medium (62.5%) and dense type (15.3%). In addition, the mutants with young shoot colour of second leaf at two and a bud stage were mainly yellow green (90.3%) with the remaining 9.7% being purple green. Density pubescence of bud also varied with sparse (58.3%) and intermediate at (41.7%). Anthocyanin colouration at base of petiole was only present in 18.1% of the population studied. Most of the plants (61.1%) had a short young shoot length at three and a bud stage. Leaf blade shape were mainly very narrow elliptic (23.6%), narrow elliptic and medium with proportion of 61.1% and 15.3%, respectively. Leaf blade intensity of green colour was mainly light (91.7%). The leaf blade texture of upper surface habit showed in the accessions were smooth or weakly rugose (59.7%), moderately and strongly rugose had 38.9% and 1.4% in that order. Leaf blade shape of apex observed in the tea mutants was acute (84.7%) and acuminate (15.3%). In addition, leaf blade undulation of margin was absent or weak (98.6%) and medium (1.4%). Leaf blade serration of margin observed in mutants was weak (94.4%) and medium (5.6%).

Shannon-Wiener Diversity Index (H')

The estimates of Shannon-Weaver diversity (H`) of the traits studied is presented in Table 17. Based on it**,** high diversity values (above 0.600) were obtained in eight of the fifteen traits examined. Considering all the traits, the minimum value of H` was 0.073 for

plant type and the maximum value was 0.929 for leaf blade shape. Traits such as leaf blade shape had greater value of H` (0.929), followed by plant density of branches (0.915), plant vigour (0.912), leaf blade length (0.799), leaf blade texture of upper surface (0.735) and leaf blade width (0.735) were more diverse compared to plant type, plant growth habit (0.463) and leaf blade serration of margin (0.215). The diversity indices of the eight traits further suggest the presence of adequate dissimilarity among the evaluated genotypes that is a potential for tea improvement through selection.

Table 17: Frequency distribution and Shannon-Wiener diversity indices (H') of fifteen traits of gamma-treated progenies

No.	Traits/habit	Range /Variation	Frequency %	H'
1	Plant vigor	Weak: 3	12.5	0.912
		Medium: 5	61.1	
		Strong: 7	26.4	
2	Plant type	Shrubs:1	1.4	0.073
		Semi-arbor: 3	98.6	
		Arbor: 5	0.0	
3	Plant density of branches	Sparse: 3	22.2	0.915
		Medium: 5	62.5	
		Dense: 7	15.3	
4	Young shoot color of second leaf at 'two and a bud' stage	Whitish: 1	0.0	0.319
		Yellow green: 2	90.3	
		Light green: 3	0.0	
		Medium green: 4	0.0	
		Purple green: 5	9.7	
5	Young shoot density pubescence of bud	Sparse: 3	58.3	0.679
		Medium: 5	41.7	
		Dense: 7	0.0	
6	Young shoot anthocyanin coloration at base of petiole	Absent: 1	81.9	0.472
		Present: 9	18.1	
7	Young shoot length of 'three and a bud'	Short: 3	61.1	0.728
		Medium: 5	37.5	
		Long: 7	1.4	
8	Leaf blade length	Short: 3	51.4	0.799
		Medium:5	45.8	
		Long: 7	2.8	
9	Leaf blade width	Narrow: 3	59.7	0.735
		Medium: 5	38.9	
		Broad: 7	1.4	
10	Leaf blade shape of base	Very narrow elliptic: 1	23.6	0.929
		Narrow elliptic: 2	61.1	
		Medium elliptic: 3	15.3	
		Broad elliptic: 4	0.0	
11	Leaf blade intensity of green color	Light: 3	91.7	0.340
		Medium: 5	5.6	
		Dark: 7	2.8	
12	Leaf blade texture of upper surface	Smooth or weakly rugose: 1	59.7	0.735
		Moderately rugose: 2	38.9	
		Strongly rugose:3	1.4	
13	Leaf blade shape of apex	Obtuse: 1	0.0	0.427
		Acute: 2	84.7	
		Acuminate: 3	15.3	
14	Leaf blade undulation of margin	Absent or weak:1	98.6	0.014
		Medium: 2	1.4	
		Strong:3	0.0	
15	Leaf blade serration of margin	Weak: 3	94.4	0.215
		Medium: 5	5.6	
		Strong: 7	0.0	

Table 18: Pearson similarity coefficient matrix utilizing fifteen key traits of the gamma-treated progenies

	Plant: Vigor	Plant: Type	Density Of Branches	Color of second leaf	Density of pubescence of bud	Anthocyanin coloration at base of the petiole	length of 'three and a bud'	Leaf blade: length	Leaf blade: width	Leaf blade: shape of base	Intensity of green colour	Texture of upper surface	Leaf blade: shape of apex	Undulation of margin	Serration of margin
1		.222*	.777**	-0.075	0.039	-0.167	-0.001	-0.088	0.168	.253*	-0.065	-0.139	-0.034	-0.027	-0.055
2			0.182	0.039	0.1	0.056	0.092	-0.104	0.095	-.208*	0.034	0.095	0.05	0.014	0.029
3				-0.194	-0.089	-0.184	-0.043	-.224*	-0.04	0.058	-.258*	-0.084	-0.142	0.014	0.028
4					0.103	.699**	.288**	.289**	0.008	0.044	.743**	0.098	.382**	-0.039	-0.08
5						.262*	0.05	0.03	0.081	-0.16	0.191	0.081	.268*	-0.1	.287**
6							0.123	.282**	0.11	0.005	.511**	.249*	.202*	-0.056	.201*
7								.441**	-0.004	-0.025	0.19	-0.107	.341**	0.137	0.046
8									.318**	0.085	0.185	0.125	.304**	-0.11	0.104
9										.497**	0.113	.231*	0.031	-0.095	.272*
10											0.095	-0.065	-0.193	-0.176	0.033
11												0.113	.470**	-0.034	-0.069
12													-0.043	-0.095	0.155
13														-0.05	0.066
14															-0.029
15															

Key: *. Correlation is significant at the 0.05 level **. Correlation is significant at the 0.01 level

1. Plant: Vigor
2. Plant: Type
3. Density Of Branches
4. Color of second leaf
5. Density of pubescence of bud
6. Anthocyanin coloration at base of the petiole
7. length of 'three and a bud'
8. Leaf blade: length
9. Leaf blade: width
10. Leaf blade: shape of base
11. Intensity of green colour
12. Texture of upper surface
13. Leaf blade: shape of apex
14. Undulation of margin
15. Serration of margin

Based on the correlation matrix (Table 18), most of the traits displayed great correlation with one another indicating that they could be chosen for variety improvement to save on time and labor. Plant vigour was found to be strongly positively correlated with density of branches (r=0.777) and plant type (r=0.222). Obviously, presence of plant vigour may go hand in hand with higher density of branches. Plant type was negatively correlated with leaf blade shape of base (r=-0.208), which indicates that accessions with semi arbor plant type may not necessarily produce narrow elliptic leaf blades. Density of branches had a negative relation with both leaf blade length (r=-0.224) and leaf blade intensity of green colour (r=-0.258). Young shoot colour at three and a bud stage positively correlated with the following traits namely; anthocyanin colour at base of petiole (r=0.699), length of three and a bud (r=0.288), leaf blade length (r=0.289), leaf blade intensity of green colour (r=0.743) and leaf blade shape of apex (r=0.382). Density of pubescence of bud correlated significantly with leaf blade serration of margin (r=0.287), anthocyanin colour at base of petiole(r=0.262) and leaf blade shape of apex (r=0.268). Interaction between anthocyanin colouration at base of petiole and leaf blade length, leaf blade intensity of green colour, texture of upper surface, leaf blade shape of apex and serration of margin was significant. In addition, highly significant correlations were obtained between the values recorded in young shoot length of three and a bud against leaf blade length (r=0.441) and shape of apex (r=0.341). Similarly, leaf blade width highly correlated with leaf blade shape of base (r=0.497), texture of upper surface (r=0.231) and leaf blade serration of margin (r=0.272). The interaction between leaf blade shape of apex and leaf blade intensity of green colour was also significant (r=0.470).

CHAPTER FIVE

DISCUSSION

5.1 Variation in biochemical profile in gamma treated stocks.

The results from biochemical analysis confirm that quality related components in tea are varietal dependent where each individual cultivar is unique in the level of biomolecules. Although, 0Gy and 50Gy decreased GA content in all the progenies, treatment with 100Gy pushed the levels to be at par with control. GA is known to have antioxidative, antimutagenic, anticarcinogenic, antiallergic, anti-inflammatory, antiviral, antibacterial and antiarteriosclerosis action (Imai *et al*., 1994; Choubey et al., 2015). Thus, a dose of at least 100Gy may be required for radio-stimulation of GA in tea seeds (Kamau *et al*., 2014).

Irradiation with 100 Gy resulted in high EGC levels for progeny of TRFCA SFS150, though the highest percentage increase of 47.7% was realized on GW Ejulu-l when compared with control. Interestingly, the same trend was noted at 50Gy treatment where the cultivar reported the highest increase in EGC content of 39.6%. Wani *et al*., (2018) reported enhanced leaf chlorophyl content in pongam oil tree after treatment with 100Gy treatment of seeds. In tea, chlorophyll plays a vital role in the regulation of EGC (Wei *et al*., 2011).

Catechin content varied significantly ($p< 0.001$) between cultivars but not seasons. The results obtained from this study corroborate with those of Cheruiyot *et al*., (2008), where changes in soil water levels did not affect the amount of catechin. Apart from influence by cultivar and gamma treatment interaction, the differences in the levels of catechin in the different cultivars could further be linked to the up regulation or down regulation of

the enzymes. Singh *et al*., (2008) reported that there was a positive correlation between the concentration of catechins and flavanone 3-hydroxylase (F_3H) gene expression in leaves at divergent growth stages. Whereas CsF_3H expression was down-regulated due to drought, abscisic acid and gibberellic acid were up-regulated as a result of wounding.

At 0Gy progenies of TRFCA SFS150, EPK C12 and TRFK 303/1199 showed increased catechin content indicating that other factors apart from gamma treatment could be involved, key among them being heterosis. Progenies of GW Ejulu-l, TRFK 301/1 and TRFK 301/4 however, recorded decreased catechin at all levels of gamma treatment suggesting that irradiation impacted catechin synthesis negatively. A higher dosage may be required for confirmation. The decrease in catechin levels among progenies arising from cambod variety TRFK 301/1 may be associated to the rise in regulation of the anthocyanin synthase in biosynthetic pathway for anthocyanins in place of the leucoanthocyanin reductase in catechin biosynthetic pathway likely due to environmental stimuli (Punyasiri *et al*., 2004). This could mean that this variety could be utilized in the production of purple tea that has been acknowledged as both medicinal and healthful beverage (Carmen *et al*., 2006). Pictorial variation of anthocyanin is found in Appendix VI.

Results obtained in the present study indicate that although caffeine accumulation was influenced by seasons and cultivar, it was not affected by gamma irradiation. Relatively high level of caffeine was observed during the dry season. ($p<0.05$). This may be attributed to the accumulation of various secondary metabolites that include caffeine by plants as a defense mechanism to drought, a mechanism that helps plants adapt to environmental stresses (Kirakosyan *et al*., 2004). Water stress is among the most

significant environmental aspects which can modulate plant growth and development, restrain plant production, and change the physiological and biochemical aspects of plants. Water stress is recognized to increase the quantity of secondary metabolites in plants (Zobayed *et al*., 2007). Caffeine content has also been recorded to be affected by genetic, agronomic and cultural factors (Cloughley, 1982).

High caffeine consumption by sensitive people can cause unfavourable outcome such as palpitations, gastrointestinal disturbances, anxiety, tremor, increased blood pressure, insomnia, reduction in bone mass and calcium absorption and birth deformity (Mohanpuria *et al*., 2011). The quantity of caffeine requisite to produce unfavourable effect varies from person to person based on their weight and responsiveness to caffeine. To prevent a significant health risk, a moderate consumption of 400 mg caffeine per day is recommended (Mohanpuria *et al*., 2011). Despite irradiation not influencing caffeine levels, progenies from clones TRFCA SFS150, GW Ejulu-l, and TRFK 301/1 registered decreased caffeine at all levels of gamma treatment as compared to their parents. Further, treatment of TRFK 301/4 and GW Ejulu-l with 100Gy registered reduction in caffeine levels as compared to their clonal controls. In order to give rise to low-caffeine tea, normal breeding may take more than 25 years to develop an elite cultivar whereas the industrial decaffeination process is expensive in addition to the effects on the taste of the product (Ashihara and Crozier, 2001; Ashihara *et al.,* 2008). Thus, mutation breeding could provide an alternative avenue to developing low caffeine teas. The EC content was influenced significantly by gamma treatment. EC plays a role in preventing stroke (Shah *et al*., 2010). Further, previous studies of the gastroprotective activity of plants have highlighted the importance of EC in the treatment of gastric ulcers (Rozza *et al*., 2012).

Based on the current study, EGCG levels were mainly affected by cultivar and seasonal variation. Generally, irradiation increased EGCG levels in TRFK 303/1199, TRFK 301/4 and GW Ejulu-l. EGCG is recognized for its anti-inflaming, hypoglycemic, hypocholesterlemic, anticancer, antihypertensive, antioxidant, antiviral, and pancreatic anticancer benefits (Yousaf *et al*., 2014). In addition, EGCG has been more associated with antioxidant activities than other type of catechin parameter (Dale *et al*., 2006), revealing considerable room for selection of elite clones for this trait especially with regard to high value tea extracts (Kerio *et al*., 2013).

Progenies including their progenitors registered declined ECG content during the dry season. There was significant ($p< 0.001$) variation in ECG content with respect to cultivar and season. Interaction between cultivars and gamma treatment was also significant ($p< 0.001$). Seasonal variation in ECG contradicts an earlier study by Cheruiyot *et al.*, (2008) but is in harmony with Wang *et al*., (2016) who reported that drought stress significantly reduces ECG content in tea leaves (Wang *et al*., 2016).

Total catechin levels varied significantly ($p< 0.001$) with respect to cultivars and season. Cool wet season recorded the highest TC content at 17%. Progenies from clone TRFCA SFS150 treated at 100Gy registered an increase in TC slightly above 11.0% demonstrating radio-stimulation on genes governing TC. Total catechin levels could be utilized to point out the quality potential of tea, with high levels being linked to high quality (Obanda *et al*., 1997).

Most of the progenies assayed exhibited high level of affiliation between the total polyphenols, gamma treatment and season. Lower polyphenols content in dry season

compared to both cool- wet and warm -wet seasons was also reported by Cheruiyot *et al* (2008). The phenomenon was attributed to the fact that water among the raw materials for photosynthesis which directly affects organic synthesis of primary and secondary metabolites in plants. The leaf is the main origin of photo assimilates that produces the major predecessors of secondary metabolism like malonyl- CoA and coumaroyl- CoA that require light and soil water content (Magoma *et al.,* 2000). Results obtained by Obanda *et al.* (1997), outlined that phenolics in green tea shoots differed between clones, while Cheruiyot *et al.* (2008) revealed positive correlation between water content and biochemical parameters namely TC, EC and EGC. Kottur *et al*. (2010) reported that polyphenol content in tea leaves were profoundly affected by season. On the other hand, 100Gy treatment resulted in increased TP in progenies of clones TRFK 303/1199, TRFCA SFS150, and TRFK 301/1. This may suggest that treatment with at least 100Gy is required to influence changes in TP levels though it is cultivar dependent. High levels of polyphenols have been correlated with high quality in aerated tea (Kerio *et al*., 2013). A previous study by Sung *et al*., (2013) showed that ionizing radiation can increase total polyphenol contents in several plants extracts. In addition, significant increase in total phenolic and flavonoid contents by gamma dosage have been recorded (Wani *et al*., 2018). Gamma irradiations encourage the action of phenylalanine ammonialyase and phenylalanine amount, that hikes the polyphenolic acids (Oufedjikh *et al*., 2000). Li *et al*. (2010) showed that the polyphenols in tea leaves could be utilized as chemotaxonomic markers. The study confirmed the genetic link among 89 wild, hybrid, and cultivated tea trees from China and Japan.

In general, the present study indicated the existence of polymorphism among the tested accessions for the major characters studied and there is considerable potential for tea improvement program in the future. The utilization of biochemical profiles could be a novel technique in locating genotypes for use in tea breeding programs. This method is advantageous as it is cheap and has high throughput. It can also be used to compliment the molecular techniques, which are difficult to adopt due to high costs.

5.2 Determination of differences in morpho-physiological attributes

The present study confirms that the morpho-physiological characterization can be used effectively for the determination of genetic diversity of different accessions of tea. Use of morphological characters is relatively cheap contrasted to the use of biochemical and molecular markers for initial assessment of a large number of accessions to place morphologically identical groups and for simple varietal recognition of phenotypically detectable cultivars (Martinez *et al*., 2003).

In tea, morphological traits have been utilized to study genetic diversity (Toyao and Takeda, 1999), variation (Rajanna *et al*., 2011; Piyasundara *et al*., 2006; Su *et al*., 2007, phylogeny and classification (Pi *et al*., 2009; Luna and Ochoterena. 2004). Owuor and Obanda (1998) proposed the probability of using morphological attributes and assorted chemical constituents in choosing for quality at single bush level. From the results obtained in the present study, key among the contributor descriptors for each principal component with agronomic importance were; anthocyanin coloration at base of the petiole, intensity of green color, color of second leaf and density of pubescence of bud. This finding is in harmony with Piyasundara *et al*. (2009) who reported these as the maximum contributors for morphological variation. The mutants with young shoot colour

of second leaf at two and a bud stage were mainly yellow green (90.3%). Weilian *et al.* (1987) expressed that made tea produced from the shoots with green or yellow-green colour is of good quality in most cases. Similar observations were noted by Venkataramani and Padmanabhan (1964) stating that the light green colour of the foliage and pubescence are practical basis in selecting for quality. Pubescence, is a key pointer of tea quality and hence is of significance in selection. Pubescent accessions produce better quality orthodox made tea than glabrous ones (Wight and Barua, 1954). From the results obtained in the present study, it was generally observed that plants irradiated with 100Gy gamma radiation had medium pubescence at par with non-irradiated controls whereas those irradiated at 50Gy had sparse pubescence depicting that a dose below 100Gy and specifically 50Gy dose, negatively influenced this trait. White tea is the minimum processed since it is only naturally (sun) withered and dried. Moreover, for white teas only buds that are covered with fine white hair (pubescent) and one or two very young leaves, are used (Balentine, 1992, Carloni *et al.*, 2013). Gamma irradiations are generally applied on plants to develop varieties that are agriculturally and economically significant and constitute high productivity and efficiency potential (Jain *et al.*, 1998; Sato *et al.*, 2006).

Anthocyanin pigmentation has been proven as a chemical marker in characterization of several tea cultivars with the advantage of being easily observable. Moreover, anthocyanins are powerful antioxidants (Joshi *et al*., 2015; Kerio *et al*., 2013). In addition, Visser (1969) believed that a combination of slight pigmentation (anthocyanin) in the presence of pubescence influences quality in teas. Young shoot characters such as pigmentation in young leaves has been used before for diversity analysis of tea (Smith

and Barua, 2011). Variation of pigment contents in tea has been attributed to environmental differences such as shade level, fertilizer application, (Mahanta and Hazarika, 1985) and cultivar differences (Kottawa-Arachchi *et al*., 2013). Results from the current study indicated that gamma irradiation negatively influenced anthocyanin pigmentation. Although anthocyanin was absent in clonal parent TRFK 301/1, the open pollinated progenies showed various intensities of reddish-brown pigmentation (Appendix VI).

The diversity indices of the eight traits further suggest the presence of adequate dissimilarity among the evaluated genotypes that is a potential for tea improvement through selection. A low H` indicates unbalanced frequency classes for a single trait and absence of diverseness for the trait (Hennink and Zeven, 1991). Considering that estimated genetic differences change with age, season and environment that the genetic tests are conducted (Zhang *et al.*, 2004; Wachira *et al.*, 2002), their utilization is restricted to the environment in which the study was carried out. Owuor *et al.*, (2010) evaluated 20 commercial genotypes under identical management in three locations in Kenya and reported significant variance in plain black tea quality parameters. Further, Nyabundi *et al*. (2016) revealed that production of tea under different elevation influenced the growth, productivity and quality of tea. Their outcome advocated that it is unlikely to produce tea of the similar quality even from the same cultivars if the production area is altered. The production of black tea from the same vegetatively grown cultivars in Kenya and Malawi has revealed dissimilarity in both chemical composition and quality (Owuor *et al.*, 2008). There is need, therefore, to replicate this study in additional locations with unlike environmental aspects to reinforce the authenticity of the

obtained genetic variation (Falconer, 1989). Information gathered can be used to help understand patterns of genetic variation existing in crop species (Perera and Fernando, 2000; Hagidimitriou *et al*., 2005) and to identify accessions with high genetic variability and also to select genetically close and distant accessions (Anandappa, 1993) that could be used as preliminary measure for identification of cultivars for further evaluation and testing in the latter stages of crop improvement program.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. Biochemical characterization showed significant variation in black tea quality parameters suggesting that gamma irradiation was effective in inducing biochemical variations in tea. Six out of nine traits studied were influenced by gamma treatment or interaction between gamma treatment and cultivar.

2. Out of the 17 standard morphological descriptors used, except plant growth habit and leaf blade shape of base, all other 15 descriptors could be used to distinguish the gamma-treated progenies into well-defined phenotypic groups.

3. Dosage as low as 50Gy was sufficient to alter quality characters in tea.

4. Reduction in caffeine levels among mutant progenies allude to future use of mutation breeding in developing low caffeine teas.

6.2 Recommendations

1. The utilization of biochemical profiles and morpho-physiological characterization can be fruitfully utilized to estimate the level of variation in gamma treated stocks and elucidate their genetic diversity for utilization in tea breeding programs and commercial ventures.
2. The overall results confirm influence of gamma treatment on GA, EC and TP parameters up to a dose of 100 Gy. Use of gamma irradiation as a derive could enable breeders to improve tea in a particular trait of choice within a low duration, contrasted to other techniques

3. There is need to evaluate the stability and adaptability of the promising gamma treated progenies in other tea growing environments.
4. This study should also be completed by molecular assessment for comprehensive understanding of genetic diversity in the gamma treated progenies

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APPENDICES

APPENDIX I: ATTRIBUTES OF THE SIX CLONAL PROGENITORS WHOSE IRRADIATED PROGENIES ARE UNDER INVESTIGATION

SN	Accession Name	Variety Type	Source of the material	Institute/ Breeder	Special attributes	Current Status
1	TRFK 301/4	Cambod	Reunion	TRFK	Acceptable black tea quality, moderate yielder	Commercial
2	TRFK 303/1199	Assam/chinary	OP of TRFK 6/8	TRFK	High black tea quality, high yielding	Commercial
3	TRFK 301/1	Cambod	Reunion	TRFK	Acceptable black tea quality, moderate yielder	Commercial
4	EPK C12	Chinary	Field selection(local)	EPK	High black tea quality, moderate yielder	Commercial
5	GW Ejulu	Chinary	GW	GW	High black tea quality	Commercial
6	TRFCA SFS150	Assam	Ex-Malawi	TRFCA	Drought tolerance	Commercial

APPENDIX II: MATERIALS AND APPARATUS

Analytical balance, capable of weighing to accuracy +/- 0.001g.

Water bath, capable of being maintained at 70^{o}C +/- 1^{o}C.

Dispenser, for methanol/water extraction mixture.

Centrifuge (capable of 3500 revolutions/minute)

Spectrophotometer set at 765 nm.

Pipette, able to cover the volume range for standard and sample extract dilutions.

One mark volumetric flasks, of capacities 100ml, 250ml, 500ml and 1litre.

Vortex mixer, for efficient mixing during extraction.

Stoppered 10 ml extraction tubes (withstand centrifuge)

Graduated tubes, of 10ml capacity with 0.1ml graduations.

Oven set at 103^{o}C

Microwave oven

Khaki bags

Cool box

Ice cubes

Aluminum sachets

HPLC

APPENDIX III: TABLE SHOWING % MEAN BIOCHEMICAL DEVIATION OF GAMMA TREATED PROGENY AS COMPARED TO CONTROLS

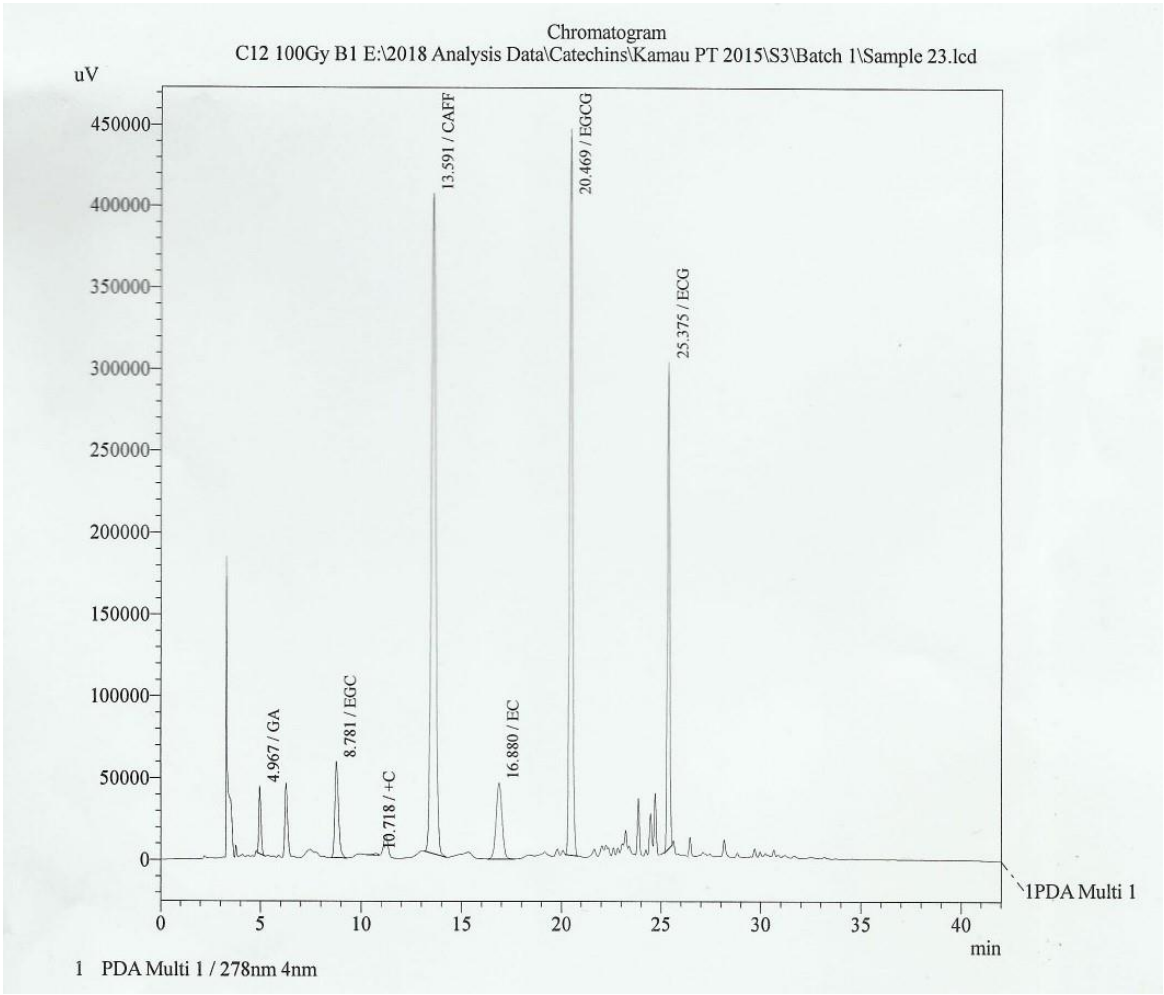
Progeny identity	Treatment	GA%	EGC%	C%	Caff%	EC%	EGCG%	ECG%	TC%	TP%
TRFCA SFS150	0Gy	0.30	3.42	0.67	2.97	1.48	5.24	2.87	13.69	21.1
	%Deviation from control	-21.04	-27.00	+44.43	-12.09	-12.90	-12.45	+50.56	-7.21	-1.1
TRFCA SFS150	50Gy	0.21	5.17	0.44	2.96	1.87	4.60	1.80	13.87	20.2
	%Deviation from control	-45.04	+10.33	-6.14	-12.37	+10.04	-23.15	-5.84	-5.95	-5.2
TRFCA SFS150	100Gy	0.18	5.98	0.41	2.95	2.00	6.04	1.96	16.39	21.9
	%Deviation from control	-53.04	+27.69	-12.86	-12.65	+17.45	+0.83	+2.97	+11.14	+2.8
TRFCA SFS150	Clonal Control	0.38	4.68	0.47	3.37	1.70	5.99	1.91	14.75	21.3
TRFK 303/1199	0Gy	0.28	5.40	0.46	2.82	1.71	6.94	2.48	16.99	20.4
	%Deviation from control	-15.82	+12.59	+11.19	-11.30	+10.58	-12.64	-8.04	-1.40	-9.0
TRFK 303/1199	50Gy	0.26	5.84	0.50	2.68	1.79	6.27	2.28	16.68	20.4
	%Deviation from control	-15.82	+12.59	+11.19	-11.30	+10.58	-12.64	-8.04	-1.40	-9.0
TRFK 303/1199	100Gy	0.42	5.31	0.57	3.47	1.32	8.92	2.75	18.87	22.6
	%Deviation from control	+39.56	+2.38	+26.87	+15.01	-18.52	+24.28	+10.89	+11.55	+0.6
TRFK 303/1199	Clonal Control	0.30	5.18	0.45	3.02	1.62	7.18	2.48	16.91	22.5
EPK C12	0Gy	0.24	4.30	0.72	2.62	1.57	4.61	2.11	13.29	18.2
	%Deviation from control	-2.16	-19.74	+17.91	+10.12	-18.16	-24.29	-15.11	-19.16	-13.8
EPK C12	50Gy	0.24	4.85	0.57	2.68	1.47	5.44	1.97	14.29	17.8
	%Deviation from control	-2.70	-9.45	-6.26	+12.61	-23.27	-10.61	-20.75	-13.07	-15.7
EPK C12	100Gy	0.23	5.25	0.63	3.19	2.56	4.81	2.60	15.85	19.4
	%Deviation from control	-6.76	-2.05	+3.30	+34.04	+33.74	-20.92	+4.70	-3.61	-8.2
EPK C12	Clonal Control	0.25	5.36	0.61	2.38	1.92	6.09	2.48	16.44	21.1
GW Ejulu-l	0Gy	0.31	4.26	0.58	2.89	1.74	5.58	3.25	15.41	22.0
	%Deviation from control	-12.26	+49.77	-42.71	-12.38	-4.29	+11.46	-30.56	+0.28	-12.8
GW Ejulu-l	50Gy	0.32	3.98	0.68	3.29	1.54	5.51	2.95	14.66	22.2
	%Deviation from control	-8.49	+39.79	-33.07	-0.16	-15.08	+10.07	-36.88	-4.56	-11.7
GW Ejulu-l	100Gy	0.30	4.21	0.53	2.61	1.34	5.58	1.78	13.44	17.7
	%Deviation from control	-16.04	+48.01	-47.85	-20.91	-26.24	+11.46	-62.04	-12.54	-29.8
GW Ejulu-l	Clonal Control	0.35	2.85	1.01	3.30	1.82	5.00	4.68	15.36	25.2
TRFK 301/1	0Gy	0.33	3.52	0.65	2.95	2.36	4.70	3.48	14.71	20.3
	%Deviation from control	-17.98	-32.59	-20.97	-21.89	-21.47	-3.44	+8.93	-14.05	-3.3
TRFK 301/1	50Gy	0.36	2.95	0.57	3.07	2.45	4.28	4.13	14.38	20.7
	%Deviation from control	-9.41	-43.65	-31.29	-18.68	-18.20	-12.00	+29.30	-15.98	-1.5
TRFK 301/1	100Gy	0.40	3.01	0.74	3.12	2.69	6.03	4.90	17.37	22.8
	%Deviation from control	0.00	-42.47	-10.48	-17.46	-10.22	+23.90	+53.39	+1.50	+8.6
TRFK 301/1	Clonal Control	0.40	5.23	0.83	3.78	3.00	4.87	3.20	17.12	21.0
TRFK 301/4	0Gy	0.21	5.17	0.44	2.96	1.87	4.60	1.80	13.87	20.2
	%Deviation from control	-57.87	+86.55	-28.59	+0.66	-52.20	+15.18	-59.14	-11.56	-3.7
TRFK 301/4	50Gy	0.39	3.82	0.54	3.17	2.44	4.50	2.80	14.09	19.1
	%Deviation from control	-21.07	+37.83	12.50	+7.85	37.77	+12.51	36.27	10.20	9.0
TRFK 301/4	100Gy	0.44	2.15	0.47	2.36	3.83	2.37	3.53	12.35	17.6
	%Deviation from control	-11.33	-22.26	-23.91	-19.75	-2.21	-40.62	-19.65	-21.29	-16.2
TRFK 301/4	Clonal Control	0.50	2.77	0.61	2.94	3.91	4.00	4.39	15.69	21.0

APPENDIX IV: TABLE SHOWING TOTAL POLYPHENOL AND CATECHIN CONTENT OF INDIVIDUAL IRRADIATED PROGENIES AND THEIR CONTROLS

Cultivar	Bush No.	Treatment	GA%	EGC%	C%	Caffeine%	EC%	EGCG%	ECG%	Total Cat%	TP%
303/1199	1	0Gy	0.23	5.78	0.56	3.10	1.93	7.03	3.00	18.31	20.4
303/1199	2	0Gy	0.30	5.00	0.39	2.76	1.65	6.88	2.28	16.19	19.5
303/1199	3	0Gy	0.25	5.44	0.46	2.48	1.57	7.07	2.06	16.61	21.8
303/1199	4	0Gy	0.30	4.56	0.45	2.81	1.56	6.83	2.68	16.08	20.1
303/1199	5	0Gy	0.30	6.21	0.44	2.94	1.85	6.90	2.35	17.75	20.5
303/1199	1	50Gy	0.14	5.43	0.57	2.29	2.28	3.72	1.63	13.63	19.0
303/1199	2	50Gy	0.28	4.55	0.40	3.10	1.40	7.17	2.98	16.49	20.9
303/1199	3	50Gy	0.32	5.97	0.53	2.86	1.74	7.38	2.61	18.23	21.2
303/1199	4	50Gy	0.36	7.19	0.56	2.76	1.88	7.04	2.15	18.81	20.4
303/1199	5	50Gy	0.18	6.04	0.43	2.38	1.66	6.05	2.03	16.22	20.7
303/1199		100Gy	0.42	5.31	0.57	3.47	1.32	8.92	2.75	18.87	22.6
303/1199		Control	0.30	5.18	0.45	3.02	1.62	7.18	2.48	16.91	22.5
C12	1	0Gy	0.13	5.64	0.47	2.28	1.45	3.75	0.97	12.27	15.9
C12	2	0Gy	0.20	5.28	0.54	3.08	2.48	4.18	2.56	15.04	18.6
C12	3	0Gy	0.26	2.75	1.03	3.05	1.50	4.39	3.18	12.83	19.9
C12	4	0Gy	0.35	2.68	1.06	1.81	0.95	4.18	1.89	10.75	18.0
C12	5	0Gy	0.26	5.15	0.48	2.91	1.47	6.54	1.93	15.57	18.7
C12	1	50Gy	0.28	5.60	0.46	3.04	1.90	6.99	2.69	17.64	18.8
C12	2	50Gy	0.29	3.13	0.90	2.76	1.26	4.08	1.75	11.12	17.8
C12	3	50Gy	0.21	5.95	0.55	2.86	1.87	5.20	1.94	15.50	17.4
C12	4	50Gy	0.22	5.33	0.54	2.61	1.30	5.45	1.74	14.36	18.9
C12	5	50Gy	0.19	4.24	0.40	2.14	1.01	5.48	1.71	12.86	16.2
C12		100Gy	0.23	5.25	0.63	3.19	2.56	4.81	2.60	15.85	19.4
C12		Control	0.25	5.36	0.61	2.38	1.92	6.09	2.48	16.44	21.1
Ejulu	1	0Gy	0.32	5.00	0.43	2.82	2.11	5.73	4.16	17.44	23.1
Ejulu	2	0Gy	0.41	3.26	0.57	2.98	1.49	8.00	4.34	17.66	23.0
Ejulu	3	0Gy	0.21	5.23	0.54	2.73	1.79	5.09	2.15	14.79	21.6
Ejulu	4	0Gy	0.32	3.87	0.67	3.10	1.50	4.82	2.96	13.81	21.6
Ejulu	5	0Gy	0.30	3.95	0.68	2.82	1.80	4.24	2.64	13.32	20.7
Ejulu	1	50Gy	0.39	3.09	0.71	3.32	1.26	5.58	2.88	13.51	21.5
Ejulu	2	50Gy	0.24	3.97	0.58	2.98	1.45	5.68	2.78	14.46	23.0
Ejulu	3	50Gy	0.33	4.09	0.49	3.52	2.05	5.44	3.00	15.07	21.4
Ejulu	4	50Gy	0.27	4.44	0.62	3.24	1.35	5.49	2.60	14.51	22.5
Ejulu	5	50Gy	0.39	4.31	0.98	3.41	1.60	5.35	3.51	15.75	22.9
Ejulu		100Gy	0.30	4.21	0.53	2.61	1.34	5.58	1.78	13.44	17.7
Ejulu		Control	0.35	2.85	1.01	3.30	1.82	5.00	4.68	15.36	25.2
301/1	1	0Gy	0.37	4.24	0.78	2.72	2.32	4.51	3.65	15.50	19.8
301/1	2	0Gy	0.31	3.77	0.68	3.48	2.57	5.02	3.61	15.65	20.7
301/1	3	0Gy	0.29	3.71	0.54	3.38	1.99	5.36	3.11	14.70	19.7
301/1	4	0Gy	0.36	3.22	0.61	2.83	2.52	4.30	4.11	14.76	20.5
301/1	5	0Gy	0.30	2.68	0.65	2.34	2.38	4.31	2.93	12.95	20.6
301/1	1	50Gy	0.30	3.96	0.70	2.81	3.07	3.81	2.60	14.14	21.3
301/1	2	50Gy	0.43	1.94	0.49	3.01	1.89	3.39	4.26	11.97	19.8
301/1	3	50Gy	0.36	1.64	0.52	2.64	2.46	2.81	4.91	12.34	18.8
301/1	4	50Gy	0.30	3.31	0.54	3.24	2.70	4.29	4.31	15.16	20.8

301/1	5	50Gy	0.40	3.87	0.59	3.67	2.14	7.12	4.58	18.29	22.6
301/1		100Gy	0.40	3.01	0.74	3.12	2.69	6.03	4.90	17.37	22.8
301/1		Control	0.40	5.23	0.83	3.78	3.00	4.87	3.20	17.12	21.0
SFS150	1	0Gy	0.25	3.61	0.96	3.03	1.46	5.30	2.24	13.57	21.2
SFS150	2	0Gy	0.26	3.57	0.78	3.05	1.27	5.82	2.58	14.02	22.6
SFS150	3	0Gy	0.31	3.54	0.47	3.62	1.86	6.44	4.67	16.97	23.1
SFS150	4	0Gy	0.30	3.47	0.40	2.86	1.45	5.36	2.72	13.40	19.9
SFS150	5	0Gy	0.40	2.91	0.76	2.26	1.36	3.30	2.14	10.47	18.6
SFS150	1	50Gy	0.25	4.23	0.43	3.02	1.62	5.78	2.34	14.39	19.0
SFS150	2	50Gy	0.14	5.37	0.45	3.23	2.25	3.24	1.64	12.95	19.8
SFS150	3	50Gy	0.28	4.26	0.38	2.49	1.11	4.72	1.48	11.96	20.9
SFS150	4	50Gy	0.21	5.89	0.41	3.35	2.26	4.98	1.95	15.48	20.4
SFS150	5	50Gy	0.17	6.09	0.52	2.70	2.12	4.29	1.57	14.58	21.1
SFS150		100Gy	0.18	5.98	0.41	2.95	2.00	6.04	1.96	16.39	21.9
SFS150		Control	0.38	4.68	0.47	3.37	1.70	5.99	1.91	14.75	21.3
301/4	1	0Gy	0.36	4.28	0.49	2.77	2.27	5.60	2.90	15.54	20.4
301/4	2	0Gy	0.34	4.59	0.51	3.47	2.16	6.40	2.99	16.64	21.3
301/4	3	0Gy	0.28	2.56	0.72	2.63	1.69	2.93	2.08	9.98	17.0
301/4	4	0Gy	0.34	2.76	0.65	2.86	4.44	3.30	3.50	14.66	19.9
301/4	5	0Gy	0.40	4.11	0.47	2.87	1.43	5.71	2.28	14.00	17.9
301/4	1	50Gy	0.35	3.90	0.47	2.92	1.17	6.61	1.89	14.02	18.7
301/4	2	50Gy	0.52	2.30	0.73	3.30	3.50	2.50	4.25	13.29	20.8
301/4	3	50Gy	0.32	4.61	0.48	3.56	3.58	3.90	3.09	15.66	19.5
301/4	4	50Gy	0.43	4.39	0.52	3.18	1.86	5.54	2.48	14.79	19.5
301/4	5	50Gy	0.35	3.89	0.48	2.87	2.07	3.94	2.28	12.67	17.1
301/4		100Gy	0.44	2.15	0.47	2.36	3.83	2.37	3.53	12.35	17.6
301/4		Control	0.50	2.77	0.61	2.94	3.91	4.00	4.39	15.69	21.0
Overall mean			0.307	4.275	0.583	2.939	1.969	5.266	2.791	14.884	20.32
%CV			28.35	29.27	33.16	18.33	37.51	29.23	31.26	18.173	8.588

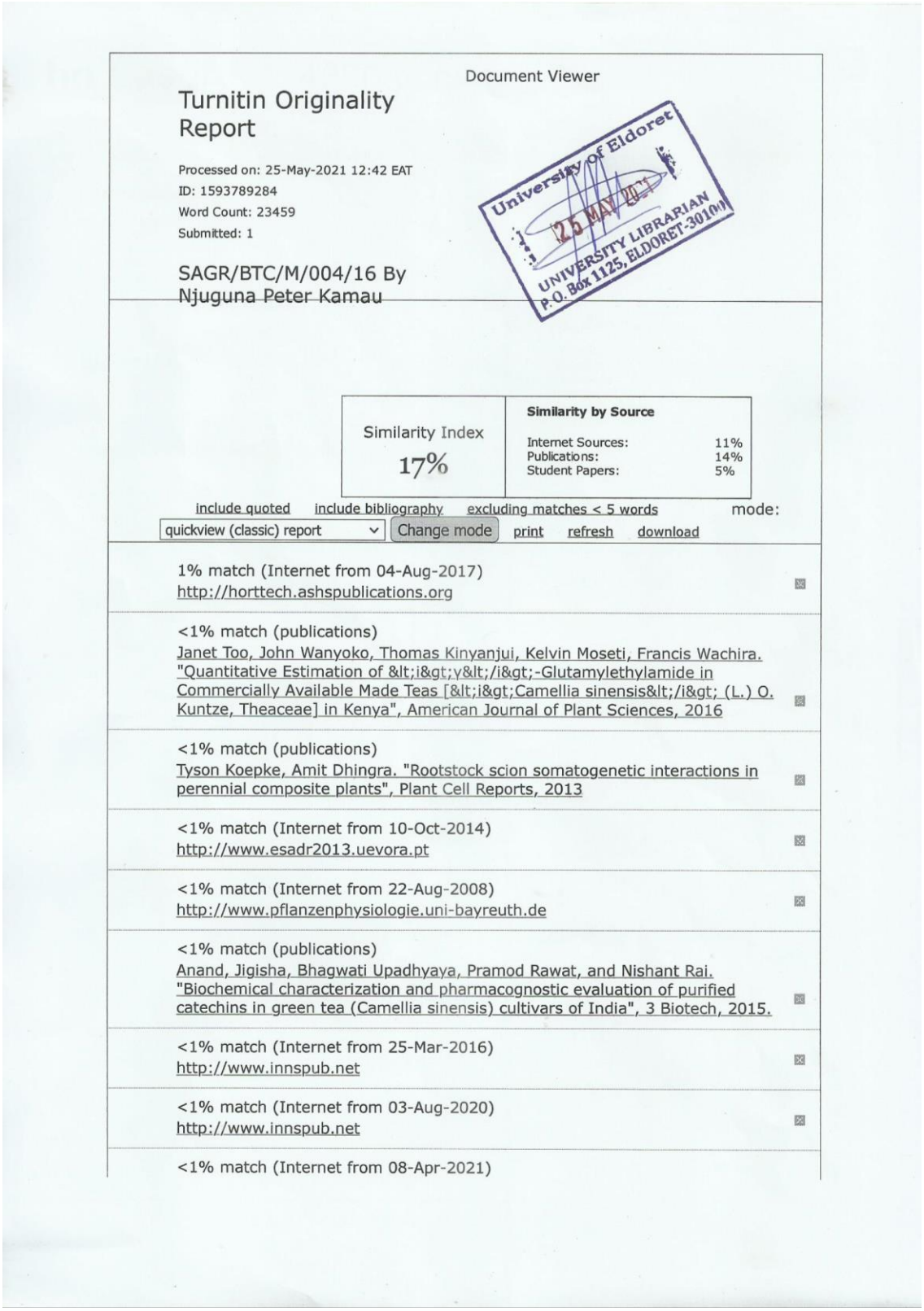
APPENDIX V: HPLC CHROMATOGRAM SHOWING CATECHIN FRACTIONS OF EPK C12 100GY OP



APPENDIX VI: VARIATION IN ANTHOCYANIN PIGMENT IN 0GY OP OF CLONE TRFK 301/1

(Source : Author, 2020)

APPENDIX VI: SIMILARITY REPORT



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