

**THE IMPACT OF INDUCED MUTATIONS ON KEY NUTRITIONAL AND  
AGRONOMIC TRAITS OF SORGHUM**

---

**ZODWA MBAMBO**

Submitted in fulfilment of the academic requirements for the degree of Doctor of Philosophy (PhD) in the Discipline of Microbiology, School of Life Sciences, University of KwaZulu-Natal, Durban.

As the candidate's supervisor/co-supervisor I have approved this thesis for submission.

**Signed:** \_\_\_\_\_ **Name:** Prof. Johnson Lin **Date:** \_\_\_\_\_

**Signed:** \_\_\_\_\_ **Name:** Dr. Luke Mehlo **Date:** \_\_\_\_\_

## **PREFACE**

The experimental work described in this dissertation was carried out at the Council for Scientific and industrial Research (CSIR), Pretoria and at the School of Life Sciences, University of KwaZulu-Natal, Westville, from June 2011 to June 2013, under the supervision of Professor Johnson Lin and Dr Luke Mehlo.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been made of the work of others it is duly acknowledged in the text.

---

Zodwa Mbambo (200200246)

October 2013

## FACULTY OF SCIENCE AND AGRICULTURE

### DECLARATION 1 – PLAGIARISM

I, Zodwa Mbambo declare that;

1. The research reported in this dissertation, except where otherwise indicated is my original research.
2. This dissertation may not be submitted for any degree or examination at any other university.
3. This dissertation does not contain any other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
4. This dissertation does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
  - a. Their words have been re-written but the general information attributed to them has been referenced.
  - b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.
5. This dissertation does not contain text, graphics or tables copied and pasted from the internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the References section.

Signed.....

FACULTY OF SCIENCE AND AGRICULTURE

DECLARATION 2 – PUBLICATIONS

DETAILS OF CONTRIBUTION TO PUBLICATIONS that form part and/or include research presented in this dissertation (include publications in preparation, submitted, *in press* and published)

Publication 1 (published)

**Mehlo, L., Mbambo, Z., Bado, S., Lin, J., Moagi, S., Buthelezi, S., Stoychev, S and R. Chikwamba.** 2013. Induced protein polymorphisms and nutritional quality of gamma irradiation mutants of sorghum. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis.*749(1-2):66-72.

Publication 2 (published)

**Grootboom, A.W., Mkhonza, N.L., Mbambo, Z., O’Kennedy, M.M., da Silvab, L.S., Taylor, J., Taylor, J.R.N., Chikwamba, R. and L. Mehlo.** 2013. Co-suppression of synthesis of major  $\alpha$ -kafirin sub-class together with  $\gamma$ -kafirin-1 and -2 required for substantially improved protein digestibility in transgenic sorghum. *Plant cell Reports.*33:521-537

Publication 3 (published)

**Mbambo, Z., Minnis-Ndimba, R., Pineda, C., Ndimba, B., Bado, S., Lin, J., Chikwamba, R and L. Mehlo.** 2014. Proton induced X-ray emission (PIXE) and electron microscopy analysis of seeds of gamma irradiation induced mutants of sorghum. Tomlekova, N., I. Kozgar and R. Wani. Editors. *Mutagenesis: Exploring Novel Genes and Pathways.* Wageningen Academic Publishers, The Netherlands. ISBN: 978-90-8686-234-4

Publication 4 (published)

**Mehlo, L., Chikwamba, R and Z. Mbambo.** 2014. All set for Sorghum to take the center stage. Wambuku F. and D. Kamanga. Editors. *Biotechnology in Africa: Emergence, Initiatives and Future.* Science Policy Reports. ISBN 978-3-319-04000-4

Signed.....

## TABLE OF CONTENTS

	PAGE
<b><u>ACKNOWLEDGEMENTS</u></b> .....	i
<b><u>ABSTRACT</u></b> .....	ii
<b><u>LIST OF FIGURES</u></b> .....	iv
<b><u>LIST OF TABLES</u></b> .....	viii
<b><u>CHAPTER 1</u>     <b>INTRODUCTION AND LITERATURE REVIEW</b>.....</b>	<b>1</b>
<b>1.1</b> <b>SORGHUM NUTRITION</b> .....	<b>7</b>
<b>1.1.1. Carbohydrates</b> .....	<b>9</b>
<b>1.1.2. Proteins</b> .....	<b>11</b>
<b>1.1.3. Fat</b> .....	<b>12</b>
<b>1.2</b> <b>IMPROVEMENT OF NUTRITIONAL CONTENT OF SORGHUM</b> .....	<b>13</b>
<b>1.2.1. Conventional breeding</b> .....	<b>13</b>
<b>1.2.2. Biolistic transformation</b> .....	<b>13</b>
<b>1.2.3. <i>Agrobacterium</i>-mediated transformation</b> .....	<b>15</b>
<b>1.3</b> <b>PLANT MUTAGENESIS AND CROP IMPROVEMENT</b> .....	<b>16</b>
<b>1.3.1. Chemical mutagenesis</b> .....	<b>19</b>
<b>1.3.2. Physical mutagenesis</b> .....	<b>24</b>

<b>1.4</b>	<b>PLANT MUTAGENESIS AND FUNCTIONAL GENOMICS</b> .....	<b>26</b>
<b>1.5</b>	<b>SCOPE OF STUDY</b> .....	<b>28</b>
<b>1.5.1.</b>	<b>Hypothesis</b> .....	<b>29</b>
<b>1.5.2</b>	<b>Objectives</b> .....	<b>29</b>
<b>1.5.3</b>	<b>Experimental design</b> .....	<b>30</b>
<b>1.6</b>	<b>REFERENCES</b> .....	<b>32</b>

**CHAPTER 2**      **EFFECT OF GAMMA IRRADIATION**  
**ON ANTIOXIDANT PROPERTIES OF**  
**SORGHUM *BICOLOR* L. MOENCH**

<b>2.1</b>	<b>INTRODUCTION</b> .....	<b>48</b>
<b>2.2</b>	<b>MATERIALS AND METHODS</b> .....	<b>50</b>
<b>2.2.1</b>	<b>Plant material irradiation</b> .....	<b>50</b>
<b>2.2.2</b>	<b>Bleach/Chlorox test</b> .....	<b>51</b>
<b>2.2.3</b>	<b>Grain hardness evaluation</b> .....	<b>51</b>
<b>2.2.4</b>	<b>Grain colour determination</b> .....	<b>51</b>
<b>2.2.5</b>	<b>Moisture content determination</b> .....	<b>52</b>
<b>2.2.6</b>	<b>Crude protein content determination</b> .....	<b>52</b>
<b>2.2.7</b>	<b>Quantification of total polyphenols</b> .....	<b>52</b>

2.2.8	Determination of condensed tannins.....	53
2.2.9	Morphology study of sorghum endosperm using scanning electron microscope (SEM).....	53
2.2.10	Antioxidant activity assay .....	53
2.2.11	Statistical analysis .....	54
2.3	RESULTS .....	54
2.3.1	Effect of induced mutation on grain quality.....	54
2.3.2	Effect of induced mutation on tannin content.....	60
2.3.3	Effect of induced mutation on antioxidant capacity .....	60
2.3.4	Effect of induced mutations on total polyphenols.....	60
2.3.5	Scanning electron microscopy .....	62
2.4	DISCUSSION .....	64
2.5	REFERENCES.....	68

**CHAPTER 3    INDUCED PROTEIN POLYMORPHISMS AND ITS NUTRITIONAL IMPLICATIONS ON GAMMA IRRADIATED MUTANTS OF SORGHUM**

3.1	INTRODUCTION.....	75
3.2	MATERIALS AND METHODS .....	76

3.2.1	Plant material irradiation .....	76
3.2.2	Bran protein extraction .....	76
3.2.3	Fractionated protein extraction.....	78
3.2.4	SDS-PAGE patterns.....	79
3.2.5	Peptide sequencing for the identification of isolated protein bands.....	79
3.2.6	Amino acid concentration .....	80
3.3	RESULTS .....	81
3.3.1	Induced protein polymorphism in sorghum lines .....	81
3.3.2	Protein variation and amino acid accumulation .....	82
3.3.3	Compensatory synthesis of proteins in mutant sorghum lines with suppressed accumulation of key kafirins.....	82
3.4	DISCUSSION .....	91
3.5	REFERENCES.....	95

**CHAPTER 4      EFFECT OF GAMMA IRRADIATION ON  
NUTRITIONAL PROFILE OF SORGHUM BICOLOR  
L. MOENCH**

4.1	INTRODUCTION.....	99
4.2	MATERIALS AND METHOD.....	103

4.2.1	Protein digestibility .....	103
4.2.2	Total protein extraction .....	103
4.2.3	Fractionated protein extraction .....	104
4.2.4	Transmission electron microscopy analysis.....	104
4.2.5	Elemental distribution .....	104
4.2.6	Statistical analysis .....	105
4.3	RESULTS.....	106
4.3.1	Protein digestibility .....	106
4.3.2	Transmission electron microscopy .....	106
4.3.3	Elemental distribution.....	107
4.4	DISCUSSION .....	114
4.5	REFERENCES.....	119

## **CHAPTER 5    GENERAL DISCUSSION**

5.1	RESEARCH IN PESPERSCTIVE.....	126
5.2	POTENTIAL FOR FUTURE DEVELOPMENT.....	130
5.3	REFERENCES.....	132
	APPENDIX A .....	135

**APPENDIX B**.....141

**APPENDIX C**.....142

## ACKNOWLEDGEMENTS

### **The author wishes to express her gratitude to:**

Prof. J. Lin for his supervision and encouragement during this study.

Dr Luke Mehlo for his supervision, support and patience.

My mother Mavis Mbambo for her unwavering love and encouragement.

My sisters Slindile, Nomusa, Phindile and Andile for always being there.

My Fiancé, Gordon Tebo for his love, encouragement and support.

My extended family Mzi and Pam for believing in me.

Priscilla Dikiso, laboratory assistant at the CSIR, for her assistance.

My friends and Colleagues at the University of Silesia, Poland for their hospitality and kindness.

Council for Scientific and Industrial Research (CSIR) for funding and the platform to excel.

The International Atomic Energy Agency (IAEA) for funding and for a scientific visit/fellowship at the University of Silesia, Poland.

The University of KwaZulu Natal for the PhD Scholarship and conference funding.

Embrapa, The Brazilian Agricultural Research Corporation for funding and hosting me for my research.

Staff and post-graduate students at both CSIR and UKZN for their kindness and assistance.

## **ABSTRACT**

Climate change, shrinking arable land, burgeoning population and malnutrition have made all aspects of crop improvement a critical issue. Of these, nutritional quality of crops is perhaps one of the most important aspects. Most cereals consumed in marginal agro-ecological zones of Africa, for example sorghum and maize are impoverished nutritionally. Given therefore the sole reliance on and the levels of consumption per day of such staples (up to 450 g/day), it is clear that most people cannot obtain the recommended daily allowance (RDA) for many nutrients including fibre, edible oil, protein, vitamins and mineral elements. In this thesis, the development of a sorghum mutant population using gamma irradiation and the subsequent employment of various analytical techniques to unravel multiple mutant traits with a significant positive impact on nutritional enhancement in sorghum is described. Protein analysis revealed a mutant designated SY accumulating (at the time) the highest ever reported amount of free lysine (21.6 g/100g) and other essential amino acids and that these changes were associated with induced protein polymorphisms. Adaptation of proton induced x-ray emission (PIXE) for the spatial profiling of the distribution of 9 elements in sorghum seed tissue allowed for the discovery of mutants with variations in the concentrations and distribution of these elements. The observed changes included enhanced or diminished accumulation of elements in preferential accumulation tissues and entire changes in cellular localisation. The locations within a cell and the quantities of an element are often critical determinants of bioavailability. The accumulation of multiple mutations affecting multiple nutritional traits in individual mutant sorghum clearly indicates the versatility of gamma irradiation induced mutations in addressing multiple nutritional challenges of sorghum. This desirable phenomenon was further demonstrated by electron microscopic analysis of starch granules and protein bodies across the mutants. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) revealed changes in size, shape, ultra-structure and packed cell volumes of seed protein- and starch bodies. Induced mutation had a major effect

on the protein body structure which in turn resulted in changes to protein digestibility. High digestibility mutants had a unique dense protein matrix with dark inclusions. However, improved protein quality traits were also associated with floury endosperm texture. Since endosperm texture is an important grain quality attribute and plays a major agronomic role, it is important to ensure that future work focuses on improving grain hardness. The mutants obtained in this study are therefore a valuable germplasm source for sorghum breeding and present real opportunities for addressing nutritional challenges of sorghum.

## LIST OF FIGURES

	<b>PAGE</b>
<b>Figure 1.1</b>	<b>Three-year moving average for sorghum area, production, yield; and number of released varieties (3-year total) based on ICRISAT-bred material globally).....2</b>
<b>Figure 1.2</b>	<b>Three-year moving average for sorghum area, production, yield; and number of released varieties based on ICRISAT-bred material in West and Central Africa. ....2</b>
<b>Figure 1.3</b>	<b>Phenotypic diversity: seed size, shape, yield and colour.....4</b>
<b>Figure 1.4</b>	<b>IAEA mutant database from plant breeding programs as of 2010 (adapted from Shu <i>et al.</i>, 2012) A total of 2700 plant mutants were accounted for.....17</b>
<b>Figure 1.5</b>	<b>Molecular structures of commonly used alkylating agents in plant mutagenesis (Adapted from Shu <i>et al.</i>, 2012).....22</b>
<b>Figure 1.6</b>	<b>The S<sub>N</sub>1 and S<sub>N</sub>2 mechanisms of alkylation. RX represents the alkylating agent with its alkyl group (R) and a halogen atom (X), usually chlorine. Adapted from Puyo <i>et al.</i>, 2012 .....23</b>
<b>Figure 1.7</b>	<b>Comparative usage of different types of mutagens. The majority of mutant varieties are developed through irradiation (e.g. fast neutrons, X-rays, with more than 64% through gamma irradiation) .....26</b>
<b>Figure 2.1</b>	<b>Bleach/Chlorox analysis. a= Macia b= T120: white mutant sorghum, c= BR: brown mutant sorghum, d= DWARF mutant, e= BIO: biomass mutant sorghum, f= SY: soft yellow mutant sorghum, g= P898012 and h=RED: red mutant sorghum .....56</b>
<b>Figure 2.2</b>	<b>Scanning electron microscope analysis of grain sorghum endosperm. a= Parental P898012, b= BIO, c= RED d= BR e= T120, f= SY.....58</b>
<b>Figure 2.3</b>	<b>Relationship between antioxidant activity and total phenol content of all sorghum tested. Correlation is significant at p &lt; 0.05.....62</b>

**Figure 2.4** Scanning electron micrograph of grain sorghum endosperm, a=P898012; b= BIO; c= RED; d=BR; e= T120; f= SY; PB = Protein bodies; S = Starch granule. A lower resolution of the same image is shown in Appendix B.

.....63

**Figure 3.1** Stabilised M<sub>4</sub> mutant sorghum seeds used for analysis. (P898012) is chalky- white type II tannin sorghum used as a parental line for gamma irradiation. (BIO) is a mutant sorghum with enhanced biomass of multiple fertile tillers, and yielding at least five times more seed than the parental P898012. (T120) is a lemon yellow hard endosperm non-tannin mutant sorghum of P898012. (SY) is a lemon yellow mutant sorghum of P898012 with a floury-soft endosperm, low seed density and low yielding. (RED) is a mutant sorghum of P898012 with a red pericarp. (BR) is a mutant sorghum of P898012 with a brown pericarp. The top photograph shows a mutant red seed in a panicle containing chalky white seeds of the parental P898012 sorghum line.....77

**Figure 3.2** Representative protein polymorphisms of parental sorghum P898012 and its mutant lines. A: Kaf rin proteins extracted from sorghum endosperms using 60% t-butanol with a reducing agent. B: Kaf rin proteins extracted from sorghum endosperms using 60% t-butanol without a reducing agent. M = monomers; D = dimers; O = oligomers. Black arrow shows high band intensity. Red arrow shows decreased band intensity. Lane M is a PageRuler™ Prestained Protein Ladder (170 kDa) purchased from Thermo Scientific, cat. No. SM0671..... 84

**Figure 3.3** Representative protein polymorphisms of germ extracts of parental sorghum and mutant lines. Numbers and arrows indicate the relative position and size of variant protein profiles in seeds of mutant sorghums. Numbers (1–4) in the mutant SY and number (6) in the mutant BR represent unusual accumulation of kaf rins in the germ of mutant seeds. These proteins included kaf rin monomers (arrow 4), dimers (arrow 3), trimers (arrow 2) and polymers (arrow 1) of β-, and α-A1 kaf rins. The protein band represented by the arrow (5) across all sorghum lines

	analysed is a sorghum oleosin protein. Lane M is a PageRuler™ Plus– Prestained Protein Ladder (10–170 kDa) purchased from Thermo Scientific, cat. No. 26619 .....	85
<b>Figure 3.4</b>	<b>Protein bound amino acids of parental sorghum P898012 and mutant lines, measured in mg/100g of sorghum flour. Each value is an average of three replicates of the samples .....</b>	<b>88</b>
<b>Figure 3.5</b>	<b>Variations in the expression pattern of albumins A and globulins B in mutant P898012 sorghum seeds. Red arrows indicate relative positions of unique down-regulation and black arrows show up-regulated proteins. Lane M is a Page Ruler™ Prestained Protein Ladder (10–170 kDa) purchased from Thermo Scientific, cat. No.SM0671 .....</b>	<b>89</b>
<b>Figure 3.6</b>	<b>Variations in expression patterns of proteins extracted from the pericarp of mutant P898012 sorghum seeds. Arrows indicate the relative positions where variant proteins were observed. The mutants, RED, BR, T120 and SY had particularly elevated synthesis and accumulation of proteins in the region indicated by arrow (4). (M) is a PageRuler™ Plus – Prestained Protein Ladder (10–170 kDa) purchased from Thermo Scientific, cat. No. 26619.....</b>	<b>90</b>
<b>Figure 4.1</b>	<b>Representative SDS-PAGE gels for digestibility assay analysis. A: undigested and digested proteins of P898012 (P), BR and RED mutants, B: undigested and digested proteins of P898012, BIO and T120, C: undigested and digested proteins of P898012, YEL, SY. The controls are parental line P898012 and YEL. Tracks: M: Molecular weight marker, Page Ruler™ Prestained Protein Ladder (10–170kDa) purchased from Thermo Scientific,cat. No.SM0671.....</b>	<b>109</b>
<b>Figure 4.2</b>	<b>Representative TEM images of sorghum protein bodies preparations a) P898012; b) YEL; c) BIO; d) RED; e) BR; f) T120; g) SY; h) HD; cw = cell wall; p = protein body, arrows show invaginations.....</b>	<b>111</b>

**Figure 4.3** Elemental distribution maps for Fe and Zn showing the germ area. Control = P898012; Mutant-5 = SY; Mutant-4 = T120; Mutant-3 = RED; Mutant-2 = BR; Mutant-1 = BIO .....112

**Figure 4.4** Elemental distribution maps for K and P showing the germ area. Control = P898012; Mutant-5 = SY; Mutant-4 = T120; Mutant-3 = RED; Mutant-2 = BR; Mutant-1 = BIO .....113

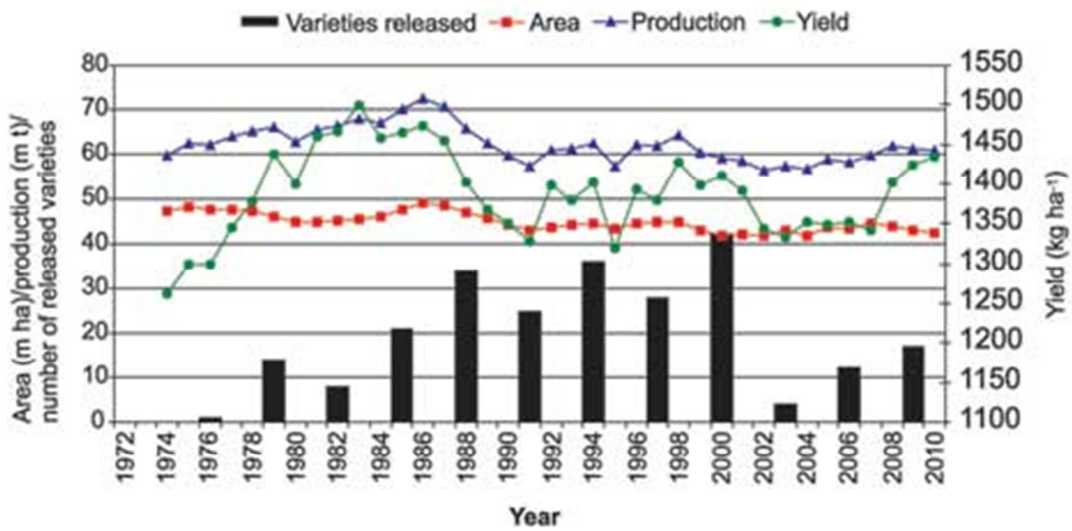
## LIST OF TABLES

	<b>PAGE</b>
<b>Table 1.1</b>	<b>Examples of the mutant crop varieties cultivated in some countries. (Forster and Shu, 2012).....18</b>
<b>Table 2.1</b>	<b>Grain qualities of parental strain P898012, yellow low-tannin sorghum (YEL), Macia (MC) and 5 mutant strains at the M<sub>4</sub> generation after Gamma ray induced mutation .....57</b>
<b>Table 2.2</b>	<b>Moisture, protein and ash contents of parental, macia, yellow low-tannin sorghum strains and 5 tested mutant sorghums.....59</b>
<b>Table 2.3</b>	<b>Tannin, phenol content and antioxidant activity of mutant sorghum.....61</b>
<b>Table 3.1</b>	<b>Free amino acid: Proximate amino acid composition for sorghum proteins (g/100g) .....86</b>
<b>Table 3.2</b>	<b>Protein bound amino acids: Proximate amino acid composition of sorghum proteins (g/100g) .....87</b>
<b>Table 4.1</b>	<b>Pepsin digestibility percentages for P898012, YEL and mutant sorghum proteins .....110</b>

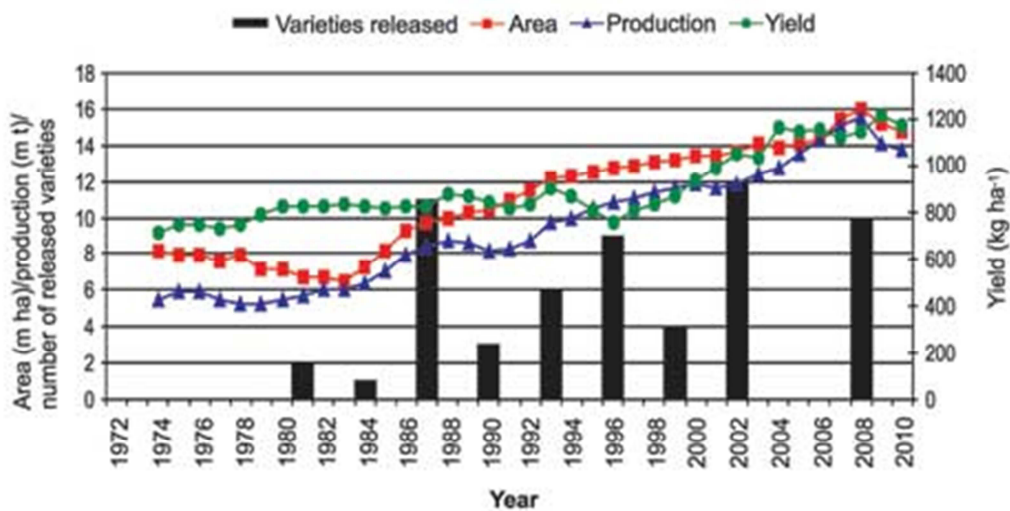
## **CHAPTER 1 INTRODUCTION AND LITERATURE REVIEW**

Sorghum (*S. bicolor* L. Moench) is the second most important cereal crop after maize in the Sub-Saharan Africa (SSA), with production of 21 million metric tonnes (MT) per year in SSA alone (Taylor, 2005). While global production has levelled (Figure 1.1) over the last 50 years and stabilised at 60 million MT (FAO, 2005) sorghum production is slowly increasing in Africa (Figure 1.2). Nigeria is the largest sorghum producer on the African continent with a 6.5 million MT average per year. The sorghum market is mainly divided into three segments: biofuels, the food and the feed industry. On average the USA produces 8.5 million MT a year (Index mundi, 2003). One-third of this amount is used for biofuels production while the remainder is used for feed and food. Sorghum grain is an excellent crop for sustainable ethanol production because it produces the same amount of ethanol per gallon as similar feed grains while using up to one-third less water in the plant growth process (Jia *et al.*, 2013). The grain, stalks and leaves are all animal feeding products. In addition, more than a billion people in the semi-arid regions of the world rely on sorghum to provide dietary calories and protein because other crops like maize, for example, are not as well adapted to the prevailing marginal agro-ecological conditions (Belton and Taylor, 2004).

Sorghum therefore is considered a major staple food for the poorest people on the African continent and is the principle source for energy, protein, vitamins and minerals (ICRISAT, 2009). The grain contains about 11% protein, which is similar to other major cereals. However, the protein quality, including lysine, methionine, tryptophan and other essential amino acids, and protein digestibility is inferior compared to other cereals (Taylor, 2005).



**Figure 1.1** Three-year moving average for sorghum area, production, yield; and number of released varieties (3-year total) based on ICRISAT-bred material globally



**Figure 1.2** Three-year moving average for sorghum area, production, yield; and number of released varieties based on ICRISAT-bred material in West and Central Africa.

The grain is also deficient in vitamin A and its iron and zinc content has limited bioavailability because these two elements are bound by phytate (Gibbon and Larkins, 2005). As a result, a diet solely based on sorghum as a staple food would most likely lead to malnutrition. This factor has a huge impact on communities where sorghum is a major staple.

Therefore, efforts to improve the nutritional quality of sorghum are underway. Strategies for sorghum nutrient fortification include: breeding of high nutrient cultivars, biofortification through genetic engineering and improved agronomic practices. Biofortification can be defined as the development of micronutrient-dense staple crops using the best traditional breeding practices and modern biotechnology tools (Nestel *et al.*, 2006). Effective biofortification of sorghum could contribute to improved nutritional quality of sorghum based foods which in turn could alleviate some of the malnutrition, especially macronutrient deficiencies in poorer communities (Henley *et al.*, 2010). The African Biofortified Sorghum Project (ABS), for example, took on an ambitious project, under the Bill and Malinda Gates Challenge (No.9) to create a full range of optimal bioavailable nutrients in a single staple (Lipkie *et al.*, 2013). The specific aims of the project were to i) increase iron and zinc bioavailability, ii) improve lysine content by 80-100%, iii) improve vitamin A levels up to 20 mg/kg, iv) increase tryptophan and threonine by 20%, and v) improve protein digestibility to approximately 60 – 80 %. These levels were based on the average quantities currently found in sorghum. In this work, gamma ( $\gamma$ ) irradiation was used to induce genetically heritable mutations which could provide an effective way of achieving trait variations in crops.

In order to breed for traits of enhanced nutrition, resistance and adaptation into crops, breeders need sources of genetic diversity to draw upon. Genetic variability is the most essential prerequisite for any successful crop improvement programme as it provides a wide range of variants for effective selection and development of new varieties with improved characteristics (Shu *et al.*, 2012).

This diversity is generally referred to as ‘genetic resources’. Genetic resources cannot be created artificially except to a limited extent through mutation breeding and genetic engineering (Shu *et al.*, 2012). Induced mutation is one of the best alternatives for the

regeneration and restoration of genetic resources, which is generally lost in the process of adaptation to various stresses.

Large germplasm collections aimed at gathering a maximum number of the existing plant biodiversity have been deposited in gene banks around the world. In addition, there is ongoing research work on the assembly of genetically diverse core collections for specific crops (Van Hintum and Van Soest, 1997; McKhann *et al.*, 2004; Gouesnard *et al.*, 2005; Murray *et al.*, 2009). The examples of sorghum phenotypic diversity for seed size, shape, yield and colour, all from one parental line are illustrated In Figure 1.2.



**Figure 1.3** Phenotypic diversity: seed size, shape, yield and colour (Mehlo *et al.*, 2013)

Genetic variability has been exhausted in certain crops due to natural selection and hence conventional breeding methods are no longer fruitful (Wani and Anis, 2001). Legumes generally loose different alleles for high productivity, seed quality, pest and disease resistance during the processes of adaptation to environmental stress. Owing to this, new sources of genetic diversity can be explored in order to compensate for the loss of variability caused by decades of domestication and breeding primarily focused on phenotypic selection (Magill, 2013). However, selection solely based on visual observations only works well for traits

regulated by one, or a few genes, but does not account for the genetic complexity of important agronomical traits e.g. yield or total soluble solids (Miedaner *et al.*, 2012).

In plant breeding programs, assessment of genetic relationships can be carried out in order to determine the uniqueness of a phenotype, genetic constitution of the organism and selection of parents for hybridisation (Ramu *et al.*, 2013). DNA markers are used to evaluate genetic diversity in different crop species (Gupta *et al.*, 2013). They are unlimited in number and are not affected by environmental factors and/or the developmental stage of the plant (Ramu *et al.*, 2013).

Molecular markers used for fingerprinting include, amplified fragment length polymorphism (AFLP) (Vos *et al.*, 1995), restriction fragment length polymorphism (RFLP) (Dubreuil and Charcosset, 1998), random amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990) and microsatellites (Smith *et al.*, 2000). RAPD is a modification of the PCR in which a single, short and arbitrary oligonucleotide primer, able to anneal and prime at multiple locations throughout the genome, can produce a spectrum of amplification products that are characteristics of the template DNA. The enormous attraction of RAPDs is that there is no requirement for DNA probes, or for any sequence information for the design of specific primers. The procedure involves no blotting or hybridising steps (Mphangwe *et al.*, 2013). RAPD markers offer many other advantages such as higher frequency of polymorphism, rapidity, technical simplicity, requirement of a few nanograms of DNA, no requirement of prior information of the DNA sequence and feasibility of automation (Choudhary *et al.*, 2013).

The AFLP technique is based on the detection of genomic restriction fragments by PCR amplification, and can be used for DNAs of any origin or complexity. Fingerprints are produced using a limited set of generic primers and the number of fragments detected in a single reaction can be modified by selection of specific primer sets. The AFLP technique is

robust and reliable because stringent reaction conditions are used for primer annealing (Ley and Hardy, 2013). There are many advantages to AFLP when compared to other marker technologies including randomly amplified polymorphic DNA (RAPD), restriction fragment length polymorphism (RFLP), and microsatellites. AFLP not only has higher reproducibility, resolution, and sensitivity at the whole genome level compared to other techniques (Silva *et al.*, 2013), but it also has the capability to amplify between 50 and 100 fragments at one time (Ley and Hardy, 2013).

Biotechnology, with the advent of the molecular markers, has rendered possible the exploration of diversity based on the analysis of genetic loci and thus allowed the discovery of beneficial alleles in accessions that would have never been phenotypically evident, for example in low yielding wild sorghum relatives. The use of wild relatives of sorghum has potential to increase yields in the crop (Smith and Frederiksen, 2000). Success in hybridisation between *Sorghum macrospermum* and *S. bicolor* promises to help introduce several pest and disease resistance traits to the cultivars (Price *et al.*, 2005). *Sorghum arundinaceum* Roem. & Schult. and other wild sorghums have proven to be sources of useful genes for improving grain yield in hybrid grain sorghum (Jordan *et al.*, 2004). In addition to demonstrating the usefulness of wild relatives for sorghum improvement, these studies also showed that the erosion of genetic diversity in the domesticated sorghum germplasm is an important issue to address.

One of the aims of the research described in this thesis was, firstly, to develop a gamma irradiation sorghum mutant population and then develop an efficient mutation screening criteria for the identification of mutant traits useful for nutritional enhancement in sorghum. Gamma sources have been used to irradiate a wide range of plant materials, like seeds, whole plants, plant parts, flowers, anthers, pollen grains and single cell cultures or protoplasts (Shu *et al.*, 2012). The lower doses/concentrations of the mutagenic treatments enhance

biochemical components, which are used for improved economic characters (Muthusamy *et al.*, 2003). Gamma radiation can induce useful as well as harmful effects on crops so there is need to experimentally determine the most beneficial dose for improvement of specific traits of crop plants (Jamil and Khan, 2002).

The development of sorghum mutant populations, M3 and M4, originating from seeds treated with gamma irradiation at a dose of 500 Gy using a Cobalt-60 source at the Seibersdorf, Austria Laboratories of the International Atomic Energy Agency is described in Chapter 2 along with the sorghum grain quality tests. In Chapter 3, physical and biochemical analysis of protein polymorphisms in seed storage proteins of a mutant population of sorghum is carried out. Notable changes in storage proteins accompanied by high level accumulation of free lysine and other essential amino acids in the endosperm were observed. Using a nuclear microprobe in Chapter 4, the distribution of elements within the grain is revealed along with protein digestibility and TEM images. In the general discussion (Chapter 5), the results are discussed in a broader context and the possible future research explored.

## **1.1. SORGHUM NUTRITION**

Sorghum grain quality is a culmination of many determinants: including visual appearance (colour, texture), nutritional composition (whole grain, protein and starch digestibility; nutrient bio-availability), the content of anti-nutritional factors such as tannins, and other characteristics including processing, cooking attributes and consumer acceptability (Hulse *et al.*, 1980). Most important cereal species, such as wheat, maize and sorghum contain inadequate amounts of some essential amino acids, particularly lysine, threonine, tryptophan and methionine. This is despite the importance of sorghum in the diets of the people in the semi-arid tropics. A wide range of variability has also been observed in the essential amino acid composition of the sorghum protein as a result of the crop being grown under diverse

agro-climatic conditions which affect its nutritional composition (FAO, 1995). The lysine content of sorghum is amongst the lowest in cereal grains (Hulse *et al.*, 1980).

On a dry weight basis, a mature sorghum kernel is composed of the embryo (10%), the pericarp (8%) and an endosperm (80%). The relative proportions may vary with genetic background, environment and degree of maturity (Da Silva *et al.*, 2012). The embryo is rich in protein, lipids, minerals and vitamin B; thus removal of the outer pericarp increases the protein and reduces the cellulose, lipid and mineral content of the grain (Mehlo *et al.*, 2013). The endosperm determines grain quality and is composed of two types of starch cells: amylose and amylopectin. Contents of amylose and amylopectin are important characteristics of starch for the selection of sorghum genotypes (Dicko *et al.*, 2006). Since amylose has a higher gelatinisation temperature than amylopectin (Whistler *et al.*, 1984), sorghum with low amylose content could be targeted for industrial brewing (Dicko *et al.*, 2006).

Sorghum minerals are located mainly in the pericarp, the aleurone layer and the germ. The grain is a good source of K and contains an adequate source of Mg, Fe, Zn, and Cu, though Fe and Zn are unavailable due to the binding influences of phytic acid (Smith and Frederiksen, 2000). Grain sorghum is also rich in Ca and P (Hulse *et al.*, 1980). The mineral composition of sorghum grain is highly variable largely due to conditions prevailing in the growing environment (FAO, 1995). The grain is also a good source of vitamins, especially the B vitamins (thiamin, riboflavin, pyridoxine), and the liposoluble vitamins A, D, E and K (Dicko *et al.*, 2006). Among the B group vitamins, concentrations of thiamin, riboflavin and niacin in sorghum are comparable to those in maize. Wide variations have been observed in the values reported, particularly for niacin (Hulse *et al.*, 1980).

Sorghum product quality is determined by endosperm texture and endosperm type which are important characteristics of the grain (Pushpamma and Vogel, 1982). Endosperm type is referred to as either corneous (vitreous or hard) or floury/soft (Dewar *et al.*, 1993).

Endosperm texture or hardness is the proportion of the corneous fraction of the endosperm with respect to floury, soft endosperm (Anglani, 1998). Seed weight and size are also important parameters for assessment of sorghum grain quality (House, 1985). In addition, grain size, shape, lustre and colour are the important grain quality traits that contribute to consumer preferences and acceptability. Grain size has a positive correlation with grain yield (Audilakshmi and Aruna, 2005) and the stay-green trait in sorghum (Borrell *et al.*, 2003).

In composition, sorghum is similar to maize. Starch is the major component in both, followed by protein, fat and fiber. However, sorghum generally contains at least one per cent less fat and more waxes than maize. The kernel consists of about 70% carbohydrates (maize, 70%), 12% protein (maize 8.1), 3% fat (maize, 4.8), 2% fiber (maize, 1.1) and 1.5% ash (maize, 1.3%) (Dicko *et al.*, 2006).

### **1.1.1 Carbohydrates**

Carbohydrates are defined as large biomolecule or macromolecules consisting of carbon, hydrogen, and oxygen (sugars, starches, and cellulose), usually with twice as many hydrogen atoms as carbon or oxygen atoms. They are produced in green plants by photosynthesis and serve as a major energy source in animal diets (Waniska and Rooney, 2002). Carbohydrates present in sorghum are a significantly important quality parameter that can influence consumer acceptance of the end product (Pushpamma and Vogel, 1982). Starch is the most abundant chemical component (60 to 80%), while soluble sugars and crude fibre are low (Waniska and Rooney, 2002). Starch is also the main constituent of the endosperm and deposited as granules in the endosperm cells. Regardless of source, starch is structurally composed of two high molecular weight homopolysaccharides known as amylose (a straight chain) and amylopectin (a branched chain polymer of glucose) which are held together by hydrogen bonds and are arranged radially in spherical granules (Rooney and Pflugfelder, 1986).

Amylose content in sorghum grain is genotype-dependent. Waxy sorghums contain very low levels of amylose (< 1%) while in normal sorghums it ranges from 10 to 17% (w/w, fresh weight), constituting approximately 20-30% of starch (Wu *et al.*, 2013). Amylose is composed of homogenous linear units of  $\alpha$ -(1 $\rightarrow$ 4)-D-glucopyranose, which can form helicoidal structures in solution (Manners, 1974; Jarvis and Walker, 1993; Dicko *et al.*, 2006). The interior of the helix is hydrophobic, allowing amylose to form a complex with free fatty acids and iodine. Dicko *et al.*, 2006 reported a significant inter-varietal difference of content of amylose among sorghum varieties.

Amylopectin is comprised of short chains of  $\alpha$ -(1 $\rightarrow$  4)-D-glucopyranose (majority 10-20 units in sorghum starch) branched to  $\alpha$ -(1 $\rightarrow$  6)-D-glucopyranoses to form a highly branched structure (Blennow *et al.*, 2001). Amylopectin content is also variety dependent and ranges from 45 to 54% (w/w, fresh weight) (Dicko *et al.*, 2006). Both the contents of amylose and amylopectin are important characteristics of starch as they influence starch digestibility (Dicko *et al.*, 2006). Amylose is more susceptible to retrogradation (a reaction that takes place in gelatinised starch when the amylose and amylopectin chains realign themselves, causing the liquid to gel) than amylopectin and as a result waxy sorghum is less viscous than normal sorghum (FAO, 1995; Dicko *et al.*, 2006). Low amylose-containing sorghum varieties are preferred for extrusion-cooking since they give better functional characteristics of the extrudates, such as enzyme susceptibility and solubility (Dicko *et al.*, 2006; Wu *et al.*, 2013).

In its properties, sorghum starch resembles maize starch and the two can be used interchangeably in many industrial and feed applications. When boiled with water, the starch forms an opaque paste of medium viscosity. On cooling, this paste sets to a rigid, non-reversible gel. The gelatinisation temperature ranges from 68°C to 80°C (Sweat *et al.*, 1984).

The remaining carbohydrates are largely sugars, which can be quite high in certain rare varieties of sorghum grains. The primary sugars present in sorghum grain are fructose,

glucose, raffinose, sucrose and maltose. According to Murty *et al.* (1985), soluble sugar content of the caryopsis changes during development but the maximum can be 5.2%. At maturity, the average soluble sugar content ranges from 0.8 to 4.2% with sucrose being 75% of the sugars (Subramanian *et al.*, 1980). Mature caryopsis contains 2.2 to 3.8% soluble sugars, 0.9 to 2.5% free reducing sugars, and 1.3 to 1.4% non-reducing sugars (Bhatia *et al.*, 1972). Glucose concentration ranges from 0.6 to 1.8% and fructose from 0.3 to 0.7%. High lysine and sugary cultivars contain more soluble sugars than normal sorghums (Subramanian *et al.*, 1980; Murty *et al.*, 1985).

On the second day of germination, sugars accumulate in the sorghum endosperm with maximum concentration occurring after eight days (Newton *et al.*, 1980; Waniska and Rooney, 2002). The major soluble carbohydrate in the caryopsis changes from sucrose to glucose and fructose after two days. The monosaccharides are located in the endosperm and the scutellum, however, sucrose is localised in the scutellum and is the highest on the fourth day after germination (Waniska and Rooney, 2002). Rooney and Pflugfelder (1986) reported that the soluble sugar content, free glucose and maltose, increases during germination.

Sorghum carbohydrates are also affected by temperature, time of day, maturity, cultivar, culm section, spacing and fertilisation (Anglani, 1998). Plants respond physiologically to short term changes in environmental conditions (Lechtenberg *et al.*, 1973), as a result during cold nights the amount of starch synthesised is reduced. In sorghum cultivars, a diurnal fluctuation of carbohydrate content has been reported (Wu *et al.*, 2013). Sucrose accumulation varies widely with temperature, whereas glucose and fructose show little variation.

### **1.1.2 Protein**

The protein content of sorghum is more variable than that of maize and can range from 7 to 15 per cent (maize, 8%) (Jordan *et al.*, 2004). Sorghum proteins are located in the endosperm (80%), germ (16%), and pericarp (3%) with kafirins or prolamins being the major fractions

(Taylor and Schussler, 1986). These fractions are located primarily within the protein bodies and protein matrix of the endosperm.

The protein quality of sorghum is based on the concept that the nutritive value of a protein depends primarily on its content of essential amino acids. A closer examination of the essential amino acid composition of sorghum grain, and its comparison to monogastric nutritional requirements, reveals that lysine is deficient and that there is great excess of leucine (Mehlo *et al.*, 2013). The methionine content of sorghum is also low, but considering the cysteine content of 1.5%, the overall sulphur amino acid content approaches the required quantities. The tryptophan content of sorghum is adequate in contrast to the low tryptophan content in normal maize (Dendy, 1995).

After screening more than 9 000 accessions in the world germplasm collection, Singh and Axtell (1973) identified two sorghum lines of Ethiopian origin, IS 11758 and IS 11167 that had significantly high lysine at relatively high levels of protein. The average lysine content of the whole kernel of IS 11758 and IS 11167 were 3.13 g and 3.33 g per 100 g protein respectively. The percentage of total protein content of the kernel was 17.2% and 15.7% respectively. Normal sorghum grown under similar conditions contained 12% of the total protein content and 2.1 g lysine per 100 g protein.

Protein quality is critically important in developing countries where the human diet consists mainly of cereal grains. In several cereal grains, including sorghum, an inverse correlation has been observed between grain yield and protein content (Doudu *et al.*, 2003). Moreover, the protein content of the grain is significantly and inversely correlated with its weight and starch content (FAO, 1995). Likewise, the ash content and protein content of the sorghum grain are positively correlated (Subramanian and Jambunathan, 1982). Inheritance studies suggested that the increased amount of lysine in each line is controlled by a single recessive gene that could be easily transferred by plant breeding procedures (Singh and Axtell, 1973).

### **1.1.3 Fat**

Sorghum consists of almost the same amount of fat as maize, with sorghum containing one per cent less. The grain is made up of 2-4 % of free lipids and 0.1-0.5% bound lipids (FAO, 1995). The properties of the fat are also similar to those of maize, and are highly unsaturated with oleic and linoleic acid accounting for 76% of the total fat (ICRISAT, 2009). Oil is found primarily in the embryo. Oil content has been found to be positively correlated with protein content, so both traits can be selected for simultaneously (Dendy, 1995).

## **1.2. IMPROVEMENT OF THE NUTRITIONAL CONTENT OF SORGHUM**

Developing sorghum with enhanced nutrition and agronomic traits is one of the greatest challenges in sustainable sorghum production. Plant breeding efforts have contributed tremendously to the genetic improvement of sorghum in terms of yield and quality. However, conventional breeding alone will not be able to support this demand due to natural barriers such as sexual incompatibility and the narrow genetic variability that is available (Vinocur and Altman, 2005). Genetic modification methods have also not been successful in achieving this goal.

### **1.2.1 Conventional breeding**

The improvement of crops by crossing plants with desired traits and selecting the best offspring over multiple generations is known as conventional or classical breeding. In other cases, chemical mutagens are used in order to produce mutants with useful new traits (House, 1985). The major focus of the sorghum breeding programs in the semi-arid regions is increased yield potential through the development of elite parental lines with drought resistance (pre-flowering and post-flowering), herbicide tolerance, cold/heat tolerance, non-lodging and disease resistance (Fusarium stalk rot, charcoal rot, anthracnose, and ergot) (Morandini, 2003). Insect resistance (green bug) and forage quality are secondary priority focus areas. Adaptation of sorghum to the range of environmental conditions in semi-arid

regions has resulted in the evolution of extensive genetic variation for drought tolerance in sorghum (Blum, 1979; Doggett, 1988).

### 1.2.2 Biolistic transformation

Biolistic transformation involves the introduction of foreign DNA or RNA into target cells on micron-sized particles, usually of tungsten or gold. These microparticles are accelerated at supersonic speed. The acceleration can be provided by either an electric discharge, gun powder, gases such as helium or CO<sub>2</sub> (Hansen and Wright, 1999). Klein *et al.* (1987) observed that tungsten particles could be used to deliver macromolecules such as RNA and DNA into epidermal cells of onion resulting in transient expression of enzymes encoded by these molecules. Christou *et al.* (1996) went on to demonstrate that the process could be used to deliver biologically active DNA into living cells and result in the recovery of stable transformants.

Hagio *et al.* (1991) reported the first stable transformation of sorghum using the biolistic method. Transcripts from the introduced foreign genes were detected in two transformants, both of which had a high copy number of genes integrated into their genome. Casas *et al.* (1993) also obtained two independent events after transforming sorghum (P898012) with the *bar* gene. These plants were resistant to local application of the herbicide Ignite/Basta, and the resistance was inherited in T1 plants as a single dominant locus. To date, several groups have successfully transformed sorghum using biolistics (Zhu *et al.*, 1998; Tadesse *et al.*, 2003; Girijashankar *et al.*, 2005; Grootboom *et al.*, 2008).

Due to the physical nature of the technique employed in micro-projectile bombardment there is no biological limitation to the actual DNA delivery process, thus making genotype a non-limiting factor (Casas *et al.*, 1993). Combining the relative ease of DNA introduction into plant cells with an efficient regeneration protocol, microprojectile bombardment appears to be an effective system for transformation (Christou, 1992).

However, molecular analysis of plants obtained by biolistic transformation generally reveals a complex pattern of transgene integration. In addition, delivery of long fragment DNA is challenging because breaks can occur in the delivered DNA (Christou, 1996). Furthermore, the fate of introduced DNA (where it integrates in the genome, rearrangements and expression levels) is not predictable.

### **1.2.3 *Agrobacterium*-mediated transformation**

*Agrobacterium*-mediated transformation utilises various strains of *Agrobacterium tumefaciens* for introduction of the transgene via the natural infection pathway. In this case, part of the vector DNA contained between two unique sequences called Left and Right borders is transferred as single stranded DNA (coated with DNA binding proteins encoded by *Agrobacterium* and double stranded DNA in the nucleus) into host DNA at semi-random locations (Gelvin, 2000). This process is facilitated by wounding or by the addition of phenolic compounds, such as acetoseringone (Howe *et al.*, 2006).

The first definitive report of stable *Agrobacterium*-mediated transformation of sorghum came from the work of Zhao and co-workers (2000), who described in detail the transformation of immature embryos of two lines of sorghum under a variety of conditions using the *Agrobacterium* strain LBA4404 carrying a super-binary vector with a *bar* gene under the control of maize ubiquitin promoter. Stable transformants were obtained and Southern blot analysis revealed that a majority of the transgenic plants had a single insertion site with no apparent rearrangements. Analysis of progeny from five independent transformants indicated typical Mendelian segregation for a single locus of the transgene. However, sorghum has been categorised as one of the most difficult plant species for tissue culture and transformation (Gelvin, 2000). The main constraints of this procedure for transformation are those associated with tissue culture, which include unsuitability of explants, genotype dependence, phenolic pigment production and poor regeneration. The sorghum embryo,

which is the only explant, capable of reliable regeneration has low tolerance for *Agrobacterium*. It is genotype-dependent and produces phenolic compounds which cause necrosis.

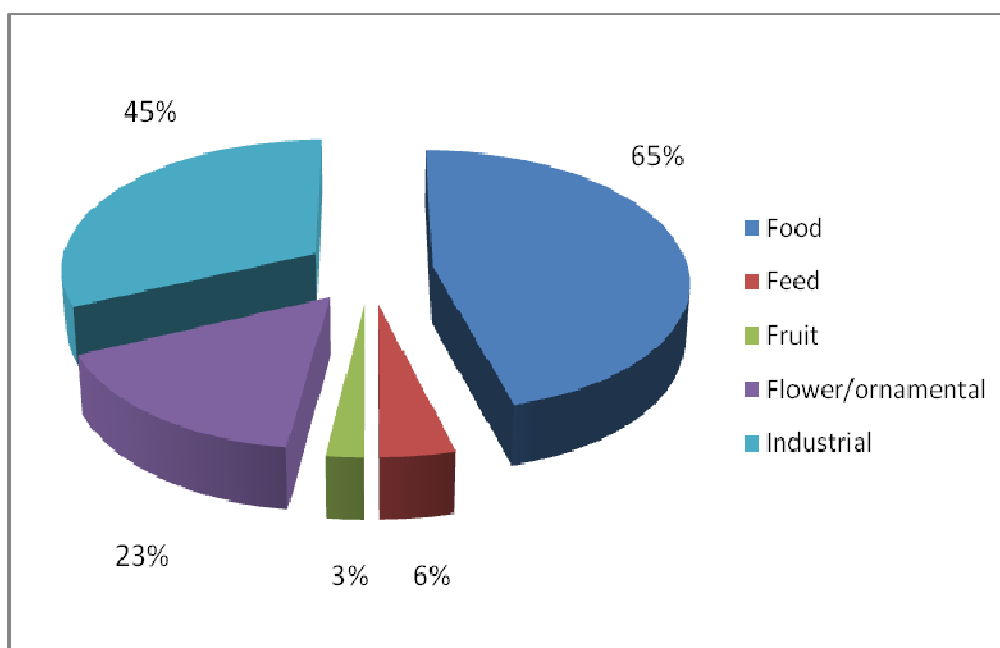
Furthermore, selection agents, such as bialaphos and imidazilone tolerance, are known to hinder the regeneration process. To improve the efficiency of sorghum, the ideal selectable marker gene would be one that is visually detectable at various stages of plant growth, is non-detrimental to regeneration, and requires no addition of substrates and cofactors (Zhao *et al.*, 2000). Gao *et al.* (2005) successfully used *gfp* as a visual selectable marker in sorghum and reported a 100% correlation between the GFP expression and the presence of the target gene.

### **1.3. PLANT MUTAGENESIS AND CROP IMPROVEMENT**

The use of physical and chemical agents that induce genetically heritable mutations is one of the most effective ways of achieving trait variations in crops (Mba *et al.*, 2010). Since the discovery of X-rays over a hundred years ago, the use of ionising radiation, such as  $\gamma$  rays, neutrons and also chemical mutagens, to induce mutations for crop improvement has become an established technology (Ahloowalia and Maluszynski, 2004). Key factors in the irradiation of genetic materials is the mutagen dose (the amount of radiation energy absorbed by the material) and the state of DNA at the time of irradiation. Fast dividing cells, with DNA undergoing fast replication favour the introduction of genetic errors and mutations in DNA which ultimately contributes towards genetic variation. The unit of measurement of radiation dose is Gray (Gy). Each genetic material; plant or seed often requires its own set of optimised mutagenic conditions, but in general crops such as rice, wheat, maize, beans and rape seed require doses in the range 60 to 700 Gy (Ahloowalia and Maluszynski, 2004).

Many crops with improved economic value have been developed through induced mutations (Table 1.1). Mutant barley varieties that thrive at about 5,000 meters altitude in the inclement highlands of Peru with 52% yield increase between 1978 and 2002 translated to significant

increases in socio-economic impact (Gómez-Pando *et al.*, 2009). Additionally, in Vietnam, mutant rice varieties that thrive in the high salinity region of the Mekong Delta ranked amongst the top 5 export rice varieties (Kharkwal and Shu, 2009). The widespread use of induced mutants in plant breeding programmes throughout the world has led to the official release of more than 2,700 mutant plant varieties (Figure 1.3). Induced mutations for crop improvement can therefore be used as a catalyst for the further development of crops and plants geared to meet the growing demand for food, fibre and energy (Morandini *et al.*, 2003). The technique is also routinely used for the discovery of genes that control important traits, and on understanding the functions and mechanisms of actions of these genes (Ahloowalia and Maluszynski, 2004).



**Figure 1.4** IAEA mutant database from plant breeding programmes as of 2010 (adapted from Shu *et al.*, 2012). A total of 2700 plant mutants were accounted for.

**Table 1.1** Examples of the mutant crop varieties cultivated in some countries  
(Forster and Shu, 2012).

Mutant variety	Country	Extent of cultivation and importance
Durum wheat	<b>Bulgaria</b>	Occupies 90% of cultivated areas since 1980's
Rice varieties: Yuanfenzao; Zhefu, Yangdao no. 6	<b>China</b>	Each cultivated on more than 10 million hectares
Wheat variety: Yangmai 156		Cultivated on more than 10 million hectares
Cotton variety: Lumian no. 1		Cultivated on more than 10 million hectares
Rice variety: Camago	<b>Costa Rica</b>	Occupies 30% of the cultivated area
Barley	<b>Europe</b>	Many of the barley varieties are derived from the barley mutant varieties: Diamant and Golden Promise
Pulses and legumes	<b>India</b>	Mutant varieties are the dominant. <b>Tau-1</b> , a mutant of blackgram occupies 95% of the blackgram acreage in the State of Maharashtra. Ground nut varieties of the TG series (TG24; TG37) occupy 40% of groundnut acreage
Durum wheat	<b>Italy</b>	The cold tolerant Creso mutant variety expanded cultivation
Rice	<b>Japan</b>	Most rice varieties carry the <i>sd1</i> mutant allele from the variety Reimei
Pear		Japanese pear cultivation was rescued from extinction by the development of disease resistant mutant varieties: Gold Nijisseiki and its derivatives.
Wheat and cotton	<b>Pakistan</b>	Mutant wheat variety (Kiran-95) and mutant cotton variety (Niab-78) occupy 30% and 80% respectively of each crops' cultivated area
Rice and Grape fruit	<b>USA</b>	The mutant variety Calrose-78 was the donor for the <i>sd1</i> mutant allele for more than 10 successful varieties. Star Ruby and Rio Red are the two most important commercial grape fruit varieties and are trade-marked, "Rio Star".
Rice	<b>Vietnam</b>	VND rice and DT soya serial mutant varieties (VND 95-20; DT 38) have been cultivated on more than half a million hectares per year in the last decade

One obvious advantage of induced mutations over genetic engineering is that crops developed through induced mutations are not regulated under GMO (genetically modified organisms) guidelines (Longman, 1999; Kharkwal and Shu, 2009). This is because induced mutation is a process that is regarded as simply speeding up natural genetic processes that occur in nature under natural evolution, a phenomenon referred to as spontaneous mutations (Medina *et al.*, 2005). Mutagenic agents can be categorised as either physical or chemical in nature. Chemical mutagens include: diethyl sulphate; ethyl methanesulphonate (EMS); isopropyl methanesulphonate; ethylamine; and sodium azide amongst others (Medina *et al.*, 2005). Physical mutagens include: ultra violet radiation; electromagnetic radiation (e.g. x-rays or  $\gamma$  rays); corpuscular radiation (fast neutrons or beta particles); and ion or electron beams (Medina *et al.*, 2005). In addition, mutants have been generated through somaclonal variation in callus cultures, and grown out and introduced into breeding programs (Cassels and Doyle, 2003; Eggum, 1979).

### **1.3.1 Chemical mutagenesis**

Mutagenesis was defined by Rieger *et al.* (1984) as the exposure or treatment of biological material with a mutagen, either physical or chemical agent that raises the frequency of mutation above the spontaneous rate. Both physical and chemical mutagens have been successfully used to generate genetic variation and subsequent development of improved crop traits such as early flowering, reduced height, disease resistance and increased yield. Recently, however, mutation induction has become a powerful tool for investigating gene function and expression (McCallum *et al.*, 2000).

The use of chemicals as mutagens was attempted over a long period of time, however, convincing data only materialised in 1939 when Thom and Steinberger found that nitrous acid caused mutations in *Aspergillus* (Steinberg and Thom, 1940). Two years later, Auerbach (1941) was the first to report the mutagenic effect of mustard gas (1,5-dichloro-3-

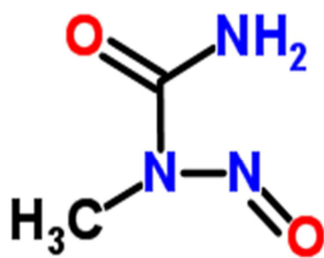
thiapentane) on fruitfly. Auerbach published the work in 1941, the most important observation being that mustard gas was highly mutagenic and capable of producing visible and lethal mutations similar in effect to X-rays. Several other chemicals such as aldehydes, diethylsulphate, diazomethane were demonstrated to have mutagenic effects between the year 1946 and 1948 (Shaw, 1970). Since then, hundreds of chemical agents such as alkylating agents, nitroso compounds, base analogues, azide etc. have been found to have mutagenic activity (Shu *et al.*, 2012).

Alkylating agents are strongly mutagenic, carcinogenic and cytotoxic (Puyo *et al.*, 2013) (Figure 1.4). They substitute hydrogen atoms on DNA with alkyl groups, resulting in the formation of cross links within the DNA chain and thereby resulting in cytotoxic, mutagenic, and carcinogenic effects. This action occurs in all cells, but alkylating agents have their primary effect on rapidly dividing cells which do not have time for DNA repair (Shu *et al.*, 2012). The alkylation process results in the misreading of the DNA code and the inhibition of DNA, RNA, and protein synthesis and therefore triggering programmed cell death (apoptosis) in rapidly proliferating tumor cells (Puyo *et al.*, 2013).

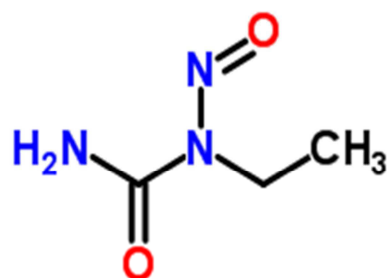
Alkylating agents can be divided into 6 classes; i) the nitrogen mustards (mechlorethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil, ii) Ethylenamine and methylenamine derivatives (altretamine, thiotepa), iii) Alkyl sulfonates (busulfan), iv) Nitrosoureas (carmustine, lomustine), v) Triazines (dacarbazine, procarbazine, temozolomide) and vi) the platinum-containing antineoplastic agents (cisplatin, carboplatin, oxaliplatin), which are usually classified as alkylating agents although they do not alkylate DNA but cause covalent DNA adducts by a different means (Bautz and Freese, 1960).

Ethyl methanesulphonate (EMS -  $\text{CH}_3\text{SO}_2\text{OC}_2\text{H}_5$ ) is amongst the most popular alkylating agents. It is a colourless liquid compound with a molecular weight of 124 and is 8% soluble in water. Alkylating agents have one of more reactive alkyl groups which are capable of

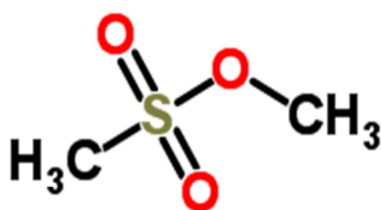
being transferred to other molecules at position of higher electron density (Shu *et al.*, 2012). According to their number of functional groups, alkylating agents are mono-, bi-, or polyfunctional alkylating agents. Bi- and poly- alkylating agents are usually more toxic than mono-. Ethyl methanesulphonate is a mono-functional alkylating agent (Mikaelsen *et al.*, 1967).



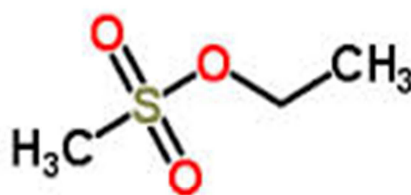
1 – Methyl-1-nitrosourea



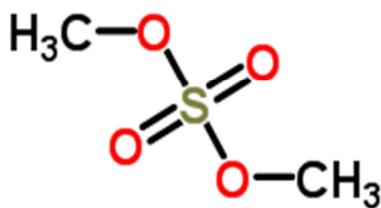
1-Ethyl-1-nitrosourea



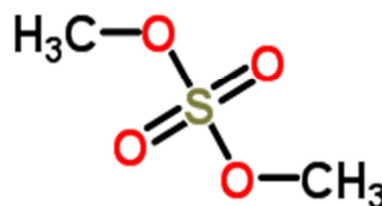
Dimethyl sulphate



Diethyl sulphate



Methyl methanesulphonate

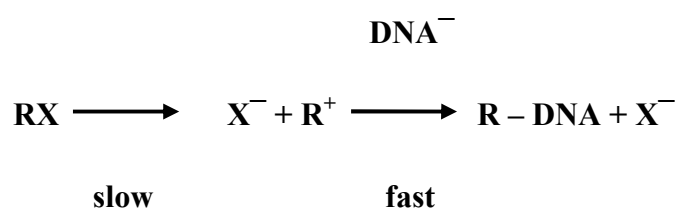


Ethyl methanesulphonate

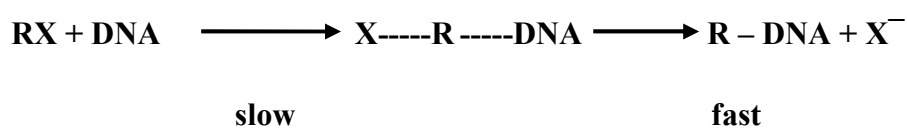
**Figure 1.5** Molecular structure of commonly used alkylating agents in plant mutagenesis. (Adapted from Shu *et al.*, 2012).

Compounds for which the rate determining step in the alkylation reaction (slow) is the first order kinetics formation of reactive intermediates independent of the substrates (for example, DNA) are designated  $S_N1$ -type alkylating agents (Puyo *et al.*, 2012). Those where the rate-determining step is a second order nucleophilic substitution reaction involving both compound and the substrate (DNA) are designated  $S_N2$ -type alkylating agents (Figure 1.5).

## S<sub>N</sub>1



## S<sub>N</sub>2



**Figure 1.6** The S<sub>N</sub>1 and S<sub>N</sub>2 mechanisms of alkylation. RX represents the alkylating agent with its alkyl group (R) and a halogen atom (X), usually chlorine (Adapted from Puyo *et al.*, 2012).

Eleven sites in the four bases and the phosphodiester groups constitute the most common targets for the alkylating agents in DNA. The most nucleophilic site in DNA, the N<sup>7</sup> (N<sup>7</sup> alkylguanine) position of the guanine is the primary alkylated site (Shu *et al.*, 2012). Although it represents the bulk of the DNA alkylation by most alkylating compounds, this altered base is non-mutagenic. On the other hand, O<sup>6</sup>alkylguanine (product of S<sub>N</sub>1-type alkylating agents) is strongly mutagenic. O<sup>6</sup>alkylguanine mispairs with thymine and gives rise to G:C – A:T transitions (Shu *et al.*, 2012).

Other chemical mutagens include nitrous acid and nitric oxide whose mutagenic effect in bacteria, fungi and yeast were documented previously (Shu *et al.*, 2012). Although they are

both routinely used for improvement of the above species, their exploitation in plant genetic improvement has been lacking (Okamoto-Hosaya *et al.*, 2003).

### 1.3.2 Physical mutagenesis

Physical mutagens include electromagnetic radiation, such as  $\gamma$  rays, X rays and UV light. Particle radiation such as fast and thermal neutrons  $\beta$  and  $\alpha$  particles also fall into this category of mutagens (Kodym and Afza, 2003). Gamma ( $\gamma$ ) rays are electromagnetic waves of very short wavelength (shorter than a few tenths of an angstrom ( $10^{-10}$  meter) but have high energy and are formed by the disintegration of radioactive atomic nuclei (Kovacs and Keresztes, 2002). They differ from fast neutrons due to their lower relative biological effectiveness which implies that in order to obtain the same biological effect, a higher dose of  $\gamma$  radiation must be given (Shu *et al.*, 2012). However, the use of  $\gamma$  rays in mutation induction is more prevalent than X-rays (Figure 1.6). This is probably on account of both wide availability and versatility of use.

When plant tissue is exposed to radiation, physical events such as ionisation and excitation of DNA, membranes, lipids etc. occurs. Chemical reactions such as formation of free radicals from  $\text{OH}^-$  and  $\text{H}^+$  and the subsequent reaction of these free radicals with oxygen molecules to form peroxy-radical are induced (Kodym and Afza, 2003). These molecules are capable of reacting with biological molecules (Sparrow *et al.*, 1974). The primary damage caused occurs randomly and is both physiological and genetic. DNA repair, in the form of undamaged DNA molecules taking over metabolic processes may occur in some cases.

The extent of the damage is dependent on the dose of mutagen used. It is therefore important to establish radio-sensitivity of the samples prior to carrying out of experiments, unless data on radio-sensitivity of the plant of interest is known from experience or published work.

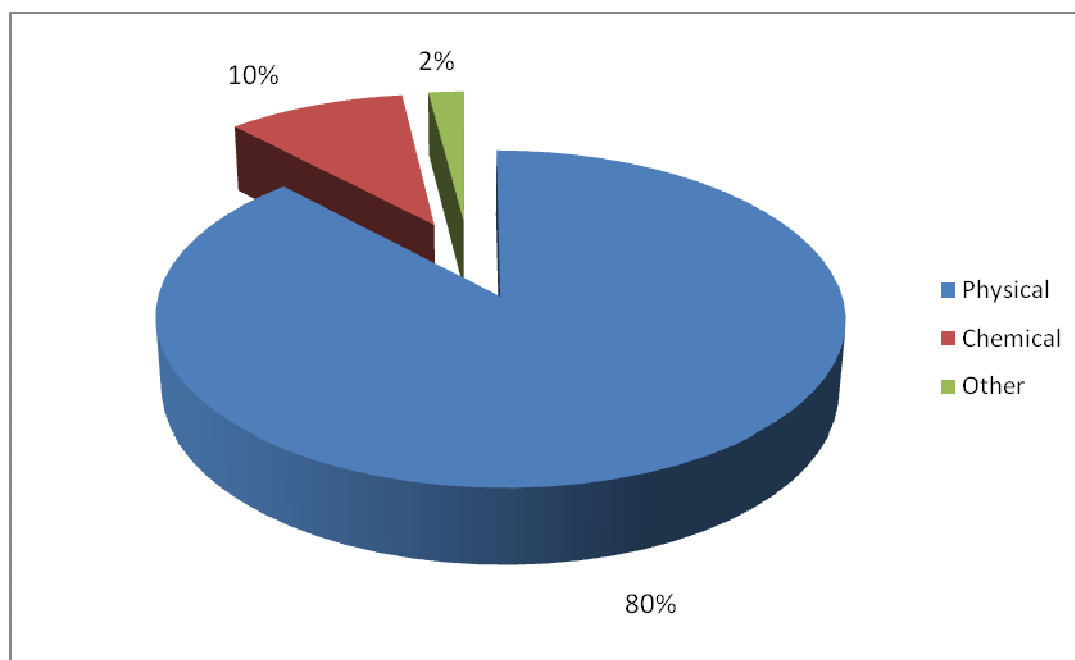
Radio-sensitivity is the measure which gives an indication of the quantity of recognisable effects of the radiation exposure on the irradiated sample. Biological factors such as genetic differences and environmental modifying factors (oxygen, water content, post irradiation storage and temperature) have an influence on radio-sensitivity (Shu *et al.*, 2012).

Gamma rays are the most energetic form of electromagnetic radiation and possess energy levels ranging from 10 to several hundred kilo-electron volts (keV) , and they are considered to be the most penetrating in comparison to other radiation such as alpha and beta rays (Kovacs and Keresztes, 2002). They interact with atoms or molecules to produce free radicals in cells. These radicals can cause damage or modify important components of plant cells and have been reported to affect differentially the morphology, anatomy, biochemistry and physiology of plants depending on the irradiation level. These effects include changes in the plant cellular structure and metabolism e.g., dilation of thylakoid membranes, alteration in photosynthesis, modulation of the antioxidative system and accumulation of phenolic compounds (Kim *et al.*, 2004, Wi *et al.*, 2007).

Gamma rays generally influence plant growth and development by inducing cytological, genetical, biochemical, physiological and morphogenetic changes in cells and tissues (Gunckel & Sparrow, 1961). Some reports showed that higher exposure to gamma rays is usually inhibitory (Kumari and Singh, 1996; Radhadevi and Nayar, 1996), whereas lower exposure is sometimes stimulatory (Mathew and Gaur, 1975; Thapa, 1999).

Other mutagens, such as ion beams (produced by particle accelerators, i.e. cyclotrons) do not only elicit energy transfer (as gamma or X-rays), but also mass deposition and charge exchange; hence could result in complex DNA damage and changes that are not found when gamma or X-rays are used (high percentage of double strand breaks and subsequent chromosome aberrations). As a result, out of the 2700 mutant varieties (Figure 1.3) registered with IAEA, 80% were developed using physical mutagenesis of which 64% is gamma

irradiation (Figure 1.6). A major bottleneck in the wide application of induced mutation for crop improvement is the laborious work of producing, handling and assaying the requisite large mutant populations. This is because mutant events usually occur in low frequencies and detection therefore requires the creation of large mutant populations.



**Figure 1.7** Percentage of usage of the different types of mutagens. The majority of mutant varieties are developed through irradiation (e.g. fast neutrons, X-rays, more than 64% of the 80 % through gamma irradiation) (Shu *et al.*, 2012)

#### 1.4. PLANT MUTAGENESIS IN FUNCTIONAL GENOMICS

Much of the understanding of the genetics of higher organisms is based on studies utilising induced mutations for analysing gene function (McCallum *et al.*, 2000). Molecular biology strategies, permitting the querying of the genome, provide neutral tools that are independent of environmental or other extraneous factors for characterising living organisms (McCallum *et al.*, 2000). One of the most direct ways of establishing gene function is to identify a mutation in a specific gene and to link that mutation to a phenotype or trait change in the mutated organism. This is called the forward genetics approach. Using this strategy, large mutated populations have been developed and screened for alterations in the trait or gene of

interest (Xin *et al.*, 2008; Gruszka *et al.*, 2011). These mutants have then been used for the identification of the genes underlying the change in phenotype. The sequence of the gene responsible for the altered phenotype is then isolated using the process of map-based cloning. Although this approach is both time-consuming and labour-intensive, it has been successfully applied for cloning several genes, even in species with large genomes, such as barley and wheat (Keller *et al.*, 2005; Komatsuda *et al.*, 2007; Krattinger *et al.*, 2009; Zhang *et al.*, 2009; Gruszka *et al.*, 2011).

With the recent advances in large scale genome sequencing, locus-to-phenotype reverse genetic strategies (the use of modifications at the molecular level to predict phenotypes) have become an increasingly popular alternative to phenotypic screens for functional analysis (Kurowska *et al.*, 2011). Sequence information alone may be sufficient to consider a gene to be of interest, because sequence comparison tools that detect protein sequence similarity to previously studied genes often allow a related function to be inferred (Gruszka *et al.*, 2011; Gruszka *et al.*, 2012). Experimental determination of gene function is desirable in other situations as well, for example, when a genetic interval has been associated with a phenotype of interest. In such cases, the functions of genes in an interval can be inferred by using reverse genetic methods (Kurowska *et al.*, 2011).

Several reverse genetics technologies, such as insertional mutagenesis with TDNA, transposon/retrotransposon tagging or gene silencing using RNA interference, have been proposed for plant functional genomics (Bolle *et al.*, 2011). However, the majority of these methods are fully applicable only for model plants with small genomes, such as Arabidopsis or rice, but even in these species, there are some drawbacks that limit their utilisation. One molecular biology strategy, which holds great promise for reducing the number of putative mutants in field trials or laboratory analysis, is the Targeted Induced Local Lesions in Genomes (TILLING). TILLING is a high throughput screening technique that allows rapid

evaluation of mutant stocks for specific genomic sequence alterations and is currently being used by many research groups in the identification of mutation events (McCallum *et al.*, 2000; Till *et al.*, 2003; 2007; Elias *et al.*, 2009).

In practice, the knowledge of the frequency of induced mutations in a population allows the calculation of the optimal population size, avoiding larger populations than necessary to be screened. Therefore, a pre-test of mutation frequency is helpful before starting whole population screening (Kurowska *et al.*, 2011). Another problem is the need to have the mutated segment in a homozygous state so that the mutation, usually recessive, could manifest as a phenotype. One major difficulty is the inherent problem of chimeras, a problem that is exacerbated in vegetatively propagated plants (Zhao *et al.*, 2013). A number of *in vitro* techniques have been shown to circumvent or significantly mitigate these bottlenecks to induced mutations. These include cell suspension cultures including somatic embryogenesis; doubled haploid production and rapid *in vitro* multiplication (Szarejko, 2003).

### **1.5. SCOPE OF STUDY**

This study demonstrates the utility of induced mutations in creating genetic and trait diversity in sorghum useful for nutritional enhancement. The sorghum genome, and information regarding its numerous useful agronomic attributes (C<sub>4</sub> efficiency of photosynthesis, efficient biomass production and drought tolerance) renders this crop an ideal example of how other cereals could be improved. Current efforts, for example, to re-engineer the rice plant to facilitate C<sub>4</sub> photosynthesis in order to enhance yield in rice is considered a potential beneficiary of the recently completed genome sequencing project of sorghum (Paterson, *et al.*, 2009; Sasaki and Baltazar, 2009). The research reported in this thesis, is very critical in addressing nutrition in marginal environments in the Sub-Saharan Africa, even though no single food source can supply all the essential nutrition required for human and animal growth (Grusak and Cakmak, 2005). However, on a global scale, research has shown that

cereals generally supply close to 50% of dietary proteins for humans and that in developing countries, this statistic rises to about 70% (Gibbon and Larkins, 2005).

The target mutagenic population for this research is the M4 (mutated generation), even though several phenotypes linked to high nutritional value in sorghum have already been identified and the mutations stabilised in the M5 generation. M0 refers to the mutagen treated seeds whereas M1 refers to the generation produced directly from the mutagen-treated seeds. M2, M3 and M4 are the subsequent generations derived from M1, M2 and M3 respectively, through selfing. Mutation breeding has been successfully used to improve all major crops globally (Table 1.1). The frequency of mutation in targeted genes is usually low and of a recessive nature. However, the mutagenic population targeted for use in this study displays a high mutation frequency and numerous phenotypic mutations (visible to the naked eye) indicative of several desirable nutritional traits. Nutritional enhancement in an important crop like sorghum, is an important accompaniment to the crops' agronomic resilience in marginal conditions such as the Sub-Saharan Africa, where other staple cereals like maize, wheat and rice command negligible yields. Meeting this challenge requires the creative integration of many disciplines as proposed in this study, including molecular genetics, biochemistry, plant physiology, and the downstream plant breeding.

### **1.5.1 Hypothesis**

It is hypothesised that induced mutations in sorghum can provide sufficient genetic variability for the development of various important nutritional and agronomic traits.

### **1.5.2 Objectives**

The following objectives were set to test the above hypothesis

- a. To derive detailed phenomics of an M<sub>3</sub>-M<sub>4</sub> mutant population of sorghum harbouring high mutation frequency.

- b. To derive the chemical and physiological data associated with mutant traits of sorghum.
- c. To determine the endosperm microstructure of mutant sorghum seed: protein bodies, starch granules under SEM and TEM.
- d. To determine the protein polymorphisms in mutant sorghum grain and assess their impact on nutritional traits (amino acid composition, digestibility).
- e. To determine the changes in important phytochemical subsets of mutant sorghum grain extracts (phenolics, condensed tannins, anthocyanins) and assess their impact on antioxidant capacity.
- f. To map the elemental distribution, localisation and concentration of 9 important microelements including iron and zinc in mutant sorghum seed using a nuclear microprobe.

### **1.5.3 Experimental design**

In order to achieve the above stated objectives, the following aims were pursued in each chapter as indicated below:

In Chapter 2

- a. To irradiate sorghum seeds at an optimal dose of 500 Gy using a Cobalt-60 source.
- b. To test for the presence or absence of a pigmented testa in the sorghum grain using the bleach chlorox test.
- c. To test the texture of the kernel (caryopsis) and delineate whether the endosperm is physically hard or soft.
- d. To assess the colour of mutant seeds visually and with a colorimeter Model CR-310.
- e. To test the moisture content of the controls and the sorghum mutants.
- f. To determine the nitrogen content of the fine milled samples using the Dumas method.

- g. To analyse the starch granule ultra-structure of representative samples from each mutant and control groups using SEM.
- h. To quantify the total phenol content in sorghum extracts using the modified Folin Ciocalteu method.
- i. To determine the tannin content of sorghum extracts using the vanillin-HCl method.
- j. To test for antioxidant activity in the sorghum extracts using the DPPH method

#### In Chapter 3

- a. To extract and analyse properties of bran proteins from the sorghum seeds.
- b. To carry out fractionated protein extractions from both the mutants and the relevant controls, in order to study different classes of proteins.
- c. To study induced protein polymorphisms in the resultant sorghum mutants.
- d. To determine the free and protein-bound amino acid content of mutant sorghum grain.

#### In Chapter 4

- a. To carry out total protein extraction from digested samples of mutants and controls.
- b. To assay protein digestibility of the mutant sorghum using SDS-PAGE.
- c. To analyse the structure of protein bodies in representative samples of each of the mutant and control groups using TEM.
- d. To determine elemental distribution in the mutant sorghum seeds as compared those of the controls.

## 1.6 REFERENCES

**Ahloowalia, B., M. Maluszynski and K. Nichterlein.** 2004. Global impact of mutation-derived varieties. *Euphytica*. **135**:187-204.

**Anglani, C.** 1998. Sorghum carbohydrates—a review. *Plant Foods for Human Nutrition*. **52**:77-83.

**Audilakshmi, S. and C. Aruna.** 2005. Genetic analysis of physical grain quality characters in sorghum. *Journal of Agriculture Science-Cambridge*. **143**:267-274.

**Auerbach, C.** 1941. The effect of sex on the spontaneous mutation rate in *Drosophila melanogaster*. *Journal of Genetics*. **41**:255-265.

**Bautz, E. and E. Freese.** 1960. On the mutagenic effect of alkylating agents. *Proceedings in National Academy of Science U. S. A.* **46**:1585-1590.

**Belton, P.S. and J. Taylor.** 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science and Technology*. **15**:94-98.

**Bhatia, I., R. Singh and S. Dua.** 1972. Changes in carbohydrates during growth and development of bajra (*Pennisetum typhoides*), jowar (*Sorghum vulgare*) and kangni (*Setaria italica*). *Journal of Science Food and Agriculture*. **23**:429-440.

**Blennow, A., A. Mette Bay-Smidt and R. Bauer.** 2001. Amylopectin aggregation as a function of starch phosphate content studied by size exclusion chromatography and on-line refractive index and light scattering. *International Journal of Biological Macromolecules*. **28**:409-420.

**Blum, A.** 1979. Genetic improvement of drought resistance in crop plants: a case for sorghum. The Volcani Center, Bet Dagan, Israel.

**Borrell, A., E.V. Oosterom., G. Hammer., D. Jordan and A. Douglas.** 2003. The physiology of “stay-green” in sorghum. *In* Anonymous Proceedings of the Australian Agronomy Conference, Australian Society of Agronomy.

**Bolle, C., A. Schneider and D. Leister.** 2011. Perspectives on systematic analyses of gene function in *Arabidopsis thaliana*: new tools, topics and trends. *Current Genomics*. **12**:1-14.

**Casas, A.M., A.K. Kononowicz., U.B. Zehr., D.T. Tomes., J.D. Axtell., L.G. Butler., R.A. Bressan and P.M. Hasegawa.** 1993. Transgenic sorghum plants via microprojectile bombardment. *Proceedings of the National Academy of Sciences*. **90**:11212-11216.

**Cassells, A and B. Doyle.** 2003. Genetic engineering and mutation breeding for tolerance to abiotic and biotic stresses: science, technology and safety. *Bulgerian Journal of Plant Physiol Special*. **13**:52-82

**Christou, P.** 1992. Genetic transformation of crop plants using microprojectile bombardment. *The Plant Journal*. **2**:275-281.

**Christou, P.** 1996. Transformation technology. *Trends Plant Science*. **1**:423-431.

**Choudhary, S., R.S. Meena., R. Singh., M.K. Vishal., V. Choudhary and A. Panwar.** 2013. Assessment of genetic diversity among Indian fenugreek (*Trigoiella foenum-graecum* L.) varieties using morphological and RAPD markers. *Legume Research-An International Journal*. **36(4)**: 289-298.

**Da Silva, L.S., R. Jung., Z. Zhao., K. Glassman., J. Taylor and J. Taylor.** 2011. Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein

body structure and protein nutritional quality in improved sorghum lines. *Journal of Cereal Science*. **54**:160-167.

**Dendy, D. A.** 1995. Sorghum and millets. *American Association of Cereal Chemists*. **12**:66-84

**Dewar, J., J.R.N. Taylor, and S. M. Joustra.** 1993. Accepted methods of sorghum malting and brewing analysis. CSIR Food Science Technology, Pretoria, SA.

**Dicko, M.H., H. Gruppen., A.S. Traoré., A.G. Voragen and W.J. Van Berkel.** 2006. Review: Sorghum grain as human food in Africa: relevance of starch content and amylase activities. *African Journal of Biotechnology*. **5**:384-395.

**Doggett, H.** 1988. Sorghum. Longman Scientific and Technical. 529 pp. Tropical Agriculture Series.UK.

**Dubreuil, P and A. Charcosset.** 1998. Genetic diversity within and among maize populations: a comparison between isozyme and nuclear RFLP loci. *Theoretical and Applied Genetics*. **96**:577-587.

**Duodu, K., J. Taylor, P. Belton and B. Hamaker.** 2003. Factors affecting sorghum protein digestibility. *Journal of Cereal Science*. **38**:117-131.

**Eggum, B.O., E.M. Villegas and S.K. Vasal.** 1979. Progress in protein quality of maize. *Journal of Science Food and Agriculture*. **30**:1148-1153.

**Elias, R., B.J. Till., C. Mba and B. Al-Safadi.** 2009. Optimising TILLING and Ecotilling techniques for potato (*Solanum tuberosum* L). *BMC Research Notes*. **2**:141-150

**FAO (Food and Agricultural Organisation).** 1995. Sorghum and millet in human nutrition. . FAO Food and Nutrition Series No. 27. ISBN 92-5- 103381-1. Consulted on 10 September 2005. doi: <http://www.fao.org/DOCREP/T0818e/T0818E00.htm#Contents>.

**FAO (Food and Agricultural Organisation).** 2005. FAOSTAT. <http://faostat.fao.org/faostat>. accessed 20 August 2011.

**Gao, Z., X. Xie., Y. Ling., S. Muthukrishnan and G. H. Liang.** 2005. *Agrobacterium tumefaciens*-mediated sorghum transformation using a mannose selection system. *Plant Biotechnology Journal*. **3**:591-599.

**Gelvin, S.B.** 2000. *Agrobacterium* and plant genes involved in T-DNA transfer and integration. *Annual Review of Plant Biology*. **51**:223-256.

**Gibbon, B.C. and B. A. Larkins.** 2005. Molecular genetic approaches to developing quality protein maize. *Trends in Genetics*. **21**:227-233.

**Girijashankar, V., H. Sharma., K.K. Sharma., V. Swathisree., L.S. Prasad., B. Bhat., M. Royer., B. San Secundo., M.L. Narasu and I. Altosaar.** 2005. Development of transgenic sorghum for insect resistance against the spotted stem borer (*Chilo partellus*). *Plant Cell Reports*. **24**:513-522.

**Grootboom, A., M. OKennedy., N. Mkhonza., K. Kunert., E. Chakauya and R. Chikwamba.** 2008. *In vitro* culture and plant regeneration of sorghum genotypes using immature zygotic embryos as explant source. *International Journal of Botany*. **4**:234-256

**Grusak, M.A. and I. Cakmak.** 2005. 12 Methods to improve the crop-delivery of minerals to humans and livestock. *Plant Nutritional Genomics*. **265**:746-752

**Gruszka, D., I. Szarejko and M. Maluszynski.** 2011. New allele of *HvBR11* gene encoding brassinosteroid receptor in barley. *Journal of Applied Genetics*. **52**:257-268.

**Gruszka, D., M. Marzec and I. Szarejko.** 2012. The barley EST DNA replication and repair database (bEST-DRRD) as a tool for the identification of the genes involved in DNA replication and repair. *BMC Plant Biology*. **12**:88-94.

**Gómez-Pando, L., A. Eguiluz., J. Jimenez., J. Falconí and E. H. Aguilar.** 2009. Barley (*Hordeum vulgare*) and kiwicha (*Amaranthus caudatus*) improvement by mutation induction in Peru. *Induced Plant Mutations in the Genomics Era*, QY Shu (Ed.) Food and Agriculture Organisation of the United Nations, Rome. 330-332.

**Gouesnard, B., J. Dallard., P. Bertin., A. Boyat and A. Charcosset.** 2005. European maize landraces: genetic diversity, core collection definition and methodology of use. *Maydica*. **50**:225-240.

**Gunckel, J. and A. Sparrow.** 1967. Ionising radiation: biochemical, physiological and morphological aspects of their effects on plants. Rutgers Univ., New Brunswick, NJ. **16**:556.

**Gupta, P. K., S. Rustgi and R.R. Mir.** Array-based high-throughput DNA markers and genotyping platforms for cereal genetics and genomics. *Cereal Genomics II. Springer*. **201**: 11-55.

**Hagio, T., A. D. Blowers and E. D. Earle.** 1991. Stable transformation of sorghum cell cultures after bombardment with DNA-coated microprojectiles. *Plant Cell Reports*. **10**:260-264.

**Hansen, G. and M. S. Wright.** 1999. Recent advances in the transformation of plants. *Trends Plant Science*. **4**:226-231.

**Henley, E., J. Taylor and S. Obukosia.** 2010. The importance of dietary protein in human health: combating protein deficiency in sub-Saharan Africa through transgenic biofortified sorghum. *Advances in Food and Nutrition Research*. **60**:21-52.

**House, L.R.** 1985. A guide to sorghum breeding. International Crops Research Institute for the Semi-Arid Tropics Patancheru, India.

**Howe, A., S. Sato., I. Dweikat., M. Fromm and T. Clemente.** 2006. Rapid and reproducible *Agrobacterium*-mediated transformation of sorghum. *Plant Cell Reports*. **25**:784-791.

**Hulse, J.H., E.M. Laing and O. E. Pearson.** 1980. Sorghum and the millets: their composition and nutritive value. *Academic Press*. **15**:56-60

**ICRISAT.** 2009. Trends in conservation farming adoption and impacts. A report of the 2009 CA panel study carried out by ICRISAT and jointly supported by FAO, and GRM International.

**Index Mundi.** 2003. United States Sorghum yield by year. <http://www.indexmundi.com/agriculture/?country=us&commodity=sorghum&graph=yield>

**Jamil, M and U. Khan.** 2002. Study of genetic variation in yield components of wheat cultivar *Bukhtwar-92* as induced by gamma radiation. *Asian Journal of Plant Sciences*. **1**:579-580.

**Jarvis, C.E. and J.R. Walker.** 1993. Simultaneous, rapid, spectrophotometric determination of total starch, amylose and amylopectin. *Journal Science Food and Agriculture*. **63**:53-57.

- Jia, F., J. Chawhuaymak., M.R. Riley., W. Zimmt and K.L. Ogden.** 2013. Efficient extraction method to collect sugar from sweet sorghum. *Journal of biological engineering*. **7**: 1-9.
- Jordan, D., R. Casu., P. Besse., B. Carroll., N. Berding and C. McIntyre.** 2004. Markers associated with stalk number and suckering in sugarcane colocate with tillering and rhizomatousness QTLs in sorghum. *Genome*. **47**:988-993.
- Keller, B., C. Feuillet and N. Yahiaoui.** 2005. Map-based isolation of disease resistance genes from bread wheat: cloning in a supsize genome. *Genetic Research*. **85**:93-100.
- Kharkwal, M. and Q. Shu.** 2009. The role of induced mutations in world food security. Induced plant mutations in the genomics era, food and agriculture organisation of the United Nations, Rome, Italy. 33-38.
- Kim, J., M. Baek., B.Y. Chung, S.G. Wi and J. Kim.** 2004. Alterations in the photosynthetic pigments and antioxidant machineries of red pepper (*Capsicum annuum* L.) seedlings from gamma-irradiated seeds. *Journal of Plant Biology*. **47**:314-321.
- Klein, T.M., E. Wolf., R. Wu and J. Sanford.** 1987. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature*. **327**:70-73.
- Krattinger, S., T. Wicker and B. Keller.** 2009. Map-based cloning of genes in *Triticeae* (wheat and barley), p. 337-357. *In* Anonymous genetics and genomics of the *Triticeae*. Springer.
- Kodym, A. and R. Afza.** 2003. Physical and chemical mutagenesis. *Plant Functional Genomics*. **201**:189-203.

**Komatsuda, T., M. Pourkheirandish., C. He., P. Azhaguvel., H. Kanamori., D. Perovic., N. Stein., A. Graner., T. Wicker and A. Tagiri.** 2007. Six-rowed barley originated from a mutation in a homeodomain-leucine zipper I-class homeobox gene. *Proceedings of the National Academy of Sciences*. **104**:1424-1429.

**Kovacs, E. and A. Keresztes.** 2002. Effect of gamma and UV-B/C radiation on plant cells. *Micron*. **33**:199-210.

**Kumari, R. and Y. Singh.** 1996. Effect of gamma rays and EMS on seed germination and plant survival of *Pisum sativum* L., and *Lens culinaris*. *Medical Neo Botanica*. **4**:25-29.

**Kurowska, M., A. Daszkowska-Golec., D. Gruszka., M. Marzec., M. Szurman, I. Szarejko and M. Maluszynski.** 2011. TILLING-a shortcut in functional genomics. *Journal of Applied Genetics*. **52**:371-390.

**Lechtenberg, V., D. Holt and H. Youngberg.** 1973. Diurnal variation in nonstructural carbohydrates of *Sorghum sudanense* as influenced by environment. *Agronomy Journal*. **65**:579-583.

**Ley, A.C. and Hardy, O.J.** 2013. Improving AFLP analysis of large-scale patterns of genetic variation—a case study with the Central African lianas *Haumania spp* (*Marantaceae*) showing interspecific gene flow. *Molecular ecology*. DOI: 10.1111/mec.12214

**Lipkie, T.E., F.F. De Moura., Z.Y. Zhao., M.C. Albertsen., P. Che., K. Glassman and M.G. Ferruzzi.** 2013. Bioaccessibility of carotenoids from transgenic provitamin A biofortified sorghum. *Journal of agricultural and food chemistry*.

<http://pubs.acs.org/doi/pdf/10.1021/jf305361s>

- Longman, P.J.** 1999. The curse of Frankenfood. Genetically modified crops stir up controversy at home and abroad. *US News World Rep.* **127**:38-41.
- Magill, C.W.** 2013. Bridging classical and molecular genetics of sorghum disease resistance: Genomics of the *Saccharinae*. Springer. **20**:347-366.
- Manners, D.J.** 1974. The structure and metabolism of starch. *Essays in Biochemistry.* **10**:37-71.
- Mathew, T. and B. Gaur.** 1975. Breaking dormancy in cocklebur (*Xanthium stramonium*) seeds with gamma radiation, temperature, and light treatments. *Indian Journal of Experimental Biology.* **13**:45-48.
- Mba, C., R. Afza., S. Bado and S.M. Jain.** 2010. Induced mutagenesis in plants using physical and chemical agents. *Plant Cell Culture: Essential Methods.* 111-130.
- McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff.** 2000. Targeted screening for induced mutations. *Nature Biotechnology.* **18**:455-457.
- McKhann, H.I., C. Camilleri., A. Bérard., T. Bataillon., J.L. David., X. Reboud, V. Le Corre., C. Caloustian., I.G. Gut and D. Brunel.** 2004. Nested core collections maximizing genetic diversity in *Arabidopsis thaliana*. *The Plant Journal.* **38**:193-202.
- Medina, J., M. Rodríguez-Franco., A. Peñalosa., M.J. Carrascosa., G. Neuhaus and J. Salinas.** 2005. *Arabidopsis* mutants deregulated in RCI2A expression reveal new signaling pathways in abiotic stress responses. *Plant. Journal.* **42**: 586-597.
- Mehlo, L., Z. Mbambo., S. Bado., J. Lin., S.M. Moagi., S. Buthelezi., S. Stoychev and R. Chikwamba.** 2013. Induced protein polymorphisms and nutritional quality of gamma

irradiation mutants of sorghum. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. **749**:66-72.

**Miedaner, T., M. Hübner., V. Korzun., B. Schmiedchen., E. Bauer., G. Haseneyer., P. Wilde and J. C. Reif.** 2012. Genetic architecture of complex agronomic traits examined in two testcross populations of rye (*Secale cereale* L.). *BMC Genomics*. **13**:706-713.

**Mikaelsen, K.** 1967. Studies on mutagenic effects of ionising radiation and chemical mutagens. *IAEA Laboratory Activities-4th Report*, TRS.

**Morandini, P. and F. Salamini.** 2003. Plant biotechnology and breeding: allied for years to come. *Trends in Plant Science*. **8**:70-75.

**Mphangwe, N.I., J. Vorster., J.M. Steyn., H.E. Nyirenda., N.J. Taylor and Z. Apostolides.** 2013. Screening of tea (*Camellia sinensis*) for trait-associated molecular markers. *Applied biochemistry and biotechnology*. **171**:437-449.

**Murray, S.C., W.L. Rooney., M.T. Hamblin., S.E. Mitchell and S. Kresovich.** 2009. Sweet sorghum genetic diversity and association mapping for brix and height. *The Plant Genome*. **2**:48-62.

**Muthusamy, A., K. Vasanth and N. Jayabalan.** 2003. Responses of physiological and biochemical components in *Gossypium hirsutum* L. to mutagens. *Journal of Nuclear Agriculture and Biology*. **32**:44-51.

**Murty, D., U. Singh., S. Suryaprakash and K. Nicodemus.** 1985. Soluble sugars in five endosperm types of sorghum. *Cereal Chemistry*. **62**:150-152.

**Nestel, P., H.E. Bouis., J.V. Meenakshi and W. Pfeiffer.** 2006. Biofortification of Staple Food Crops. *The Journal of Nutrition*. **136**:1064-1067.

- Newton, R., D. Baltuskonis., J. Goeschl., D. Meckenstock and F. Miller.** 1980. Distribution and transformation of soluble carbohydrates during germination growth of sorghum. *Crop Science*. **20**:265-268.
- Okamoto-Hosaya, Y., S. Okamoto and Ochi, K.** 2003. Development of antibiotic-overproducing strains by site-directed mutagenesis of the *rps1*. gene in *Streptomyces lividans*. *Journal of Applied Environmental Microbiology*. **69**:4256-4259.
- Paterson, A.H., J.E. Bowers., R. Bruggmann., I. Dubchak., J. Grimwood., H. Gundlach., G. Haberer., U. Hellsten., T. Mitros and A. Poliakov.** 2009. The Sorghum bicolor genome and the diversification of grasses. *Nature*. **457**:551-556.
- Price, H.J., S.L. Dillon., G. Hodnett., W.L. Rooney., L. Ross and J.S. Johnston.** 2005. Genome evolution in the genus *Sorghum* (*Poaceae*). *Annals of Botany*. **95**:219-227.
- Pushpamma, P. and S.M. Vogel.** 1982. Consumer acceptance of sorghum, p. 341. *In* Anonymous proceedings of the international symposium on sorghum grain quality, ICRISAT Center, Patancheru, India, 28-31 October 1981. ICRISAT.
- Puyo, S., D. Montaudon and P. Pourquier.** 2013. From old alkylating agents to new minor groove binders. *Crit.Rev.Oncol.* doi: 10.1016/j.critrevonc.2013.07.006.
- Radha Devi, D. and N. Nayar.** 1996. Gamma ray induced fruit character variations in *Nendran*, a varie'y of Banana (*Musa paradasiaca* L.). *Geobios-Jodphur-*. **23**:88-93.
- Ramu, P., C. Billot., J.F. Rami., S. Senthilvel., H.D. Upadhyaya., L.A. Reddy and C.T. Hash.** 2013. Assessment of genetic diversity in the sorghum reference set using EST-SSR markers. *Theoretical and Applied Genetics*. **201**:1-14.

**Rieger, R., A. Michaelis and H. Nicoloff.** 1984. Clastogenic adaptation of the *Vicia faba* root-tip meristem as affected by various treatment parameters. *Mutation research letters*. **140**: 99-102

**Rooney, L.W. and R.L. Pflugfelder.** 1986. Factors affecting starch digestibility with special emphasis on sorghum and corn. *Journal of Animal Science*. **63**:1607-1623.

**Sasaki, T. and B.A. Antonio.** 2009. Plant genomics: Sorghum in sequence. *Nature*. **457**:547-548.

**Shaw, M.W.** 1970. Human chromosome damage by chemical agents. *Annu.Rev.Med.* **21**:409-432

**Shu, Q.Y., B.P. Forster and H. Nakagawa (eds.),** 2012. Plant mutation breeding and biotechnology. *International atomic energy agency*. Vienna. Austria.

**Silva, A.D.S., E.J.D. Oliveira., F. Haddad., O.N.D. Jesus., S.A.S.D. Oliveira and M.A.P.D.C Costa.** 2013. Molecular fingerprinting of *Fusarium oxysporum* f. sp. *passiflorae* isolates using AFLP markers. *Scientia Agricola*. **70(2)**:108-115.

**Singh, R. and J.D. Axtell.** 1973. High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. *Crop Science*. **13**:535-539.

**Smith, C.W. and R.A. Frederiksen.** 2000. Sorghum: origin, history, technology, and production. John Wiley and sons Inc., New York, N.Y. 824.

**Smith, J., S. Kresovich., M. Hopkins., S. Mitchell., R. Dean., W. Woodman., M. Lee and K. Porter.** 2000. Genetic diversity among elite sorghum inbred lines assessed with simple sequence repeats. *Crop Science*. **40**:226-232.

- Sparrow, A., L. Schairer and R. Villalobos-Pietrini.** 1974. Comparison of somatic mutation rates induced in *Tradescantia* by chemical and physical mutagens. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* **26:**265-276.
- Steinberg, R.A.** 1940. Chemical induction of genetic changes in *Aspergilli*. *Journal of Heredity.***31:**61-63.
- Subramanian, V., R. Jambunathan and S. Suryaprakash.** 1980. Note on the soluble sugars of sorghum. *Cereal Chemistry.* **57:**440-441.
- Subramanian, V. and R. Jambunathan.** 1982. Properties of sorghum grain and their relationship to roti quality. In: Proceedings of the International Symposium on Sorghum Grain Quality, 28-31 October 1981, ICRISAT Patancheru.
- Sweat, V., J. Faubion., L. Palacios., G. Berry., J. Akingbala and L. Rooney.** 1984. Gelatinisation energy and temperature of sorghum and corn starches. *American Society of Agricultural Engineers.* **27:**1960-1963
- Szarejko, I.** 2003. Anther culture for doubled haploid production in barley (*Hordeum vulgare* L.). *Doubled Haploid Production in Crop Plants.A Manual.*Kluwer Academic, Dordrecht. 35-42.
- Tadesse, Y., L. Sagi., R. Swennen and M. Jacobs.** 2003. Optimisation of transformation conditions and production of transgenic sorghum (*Sorghum bicolor*) via microparticle bombardment. *Plant Cell, Tissue and Organ Culture.* **75:**1-18.
- Taylor, J., J. Taylor., M. Dutton and S. De Kock.** 2005. Identification of kafirin film casting solvents. *Food Chemistry.* **90:**401-408.

- Taylor, J. and L. Schüssler.** 1986. The protein compositions of the different anatomical parts of sorghum grain. *Journal of Cereal Science.* **4**:361-369.
- Thapa, C.** 1999. Effect of acute exposure of gamma rays on seed germination of *Pinus kesiya* Gord and *P. wallichiana* AB Jacks. *Our Nature.* **2**:120-121.
- Till, B.J., S.H. Reynolds., E.A. Greene., C.A. Codomo., L.C. Enns., J.E. Johnson., C. Burtner., A.R. Odden., K. Young and N.E. Taylor.** 2003. Large-scale discovery of induced point mutations with high-throughput TILLING. *Genome Research.* **13**:524-530.
- Till, B.J., J. Cooper., T.H. Tai., P. Colowit., E.A. Greene., S. Henikoff and L. Comai.** 2007. Discovery of chemically induced mutations in rice by TILLING. *BMC Plant Biology.* **7**:19-25.
- van Hintum, T. and L. van Soest.** 1997. Conservation of plant genetic resources in the Netherlands. *Plant Varieties and Seeds.* **10**:1-8
- Vinocur, B and A. Altman.** 2005. Recent advances in engineering plant tolerance to abiotic stress: achievements and limitations. *Current Opinions in Biotechnology* **16**:123-132.
- Vos, P., R. Hogers., M. Bleeker., M. Reijans., T. van De Lee., M. Hornes., A. Friters., J. Pot., J. Paleman and M. Kuiper.** 1995. AFLP: a new technique for DNA fingerprinting. *Nucleic Acids Research.* **23**:4407-4414.
- Wani, A. and M. Anis.** 2001. Gamma rays induced bold seeded high yielding mutant in chickpea. *Mutation Breeding Newsletter.* **45**:20-21.
- Waniska, R.D., L.W. Rooney and J. Leslie.** 2002. Sorghum grain quality for increased utilisation. *Sorghum and Millets Diseases.* **54**:327-335.

- Whistler, R. and J. Daniel.** 1984. Molecular structure of starch. *Starch: Chemistry and Technology*. **14**:153-182.
- Wi, S.G., B.Y. Chung., J. Kim., M. Baek., J. Lee and Y. S. Kim.** 2007. Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron*. **38**:553-564.
- Williams, J.G., A.R. Kubelik., K.J. Livak., J.A. Rafalski and S.V. Tingey.** 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. *Nucleic Acids Research*. **18**:6531-6535.
- Wu, X., A. Maier., T. Wu., N. Li., J.R. Pedersen and D. Wang.** 2013. Evaluation of Nebraska waxy sorghum hybrids for ethanol production 1. *Cereal Chemistry*. **90**:198-203.
- Xin, Z., M.L. Wang., N.A. Barkley., G. Burow., C. Franks., G. Pederson and J. Burke.** 2008. Applying genotyping (TILLING) and phenotyping analyses to elucidate gene function in a chemically induced sorghum mutant population. *BMC Plant Biology*. **8**:103-113.
- Zhang, L., L. Lavery., U. Gill., K. Gill., B. Steffenson., G. Yan., X. Chen and A. Kleinhofs.** 2009. A cation/proton-exchanging protein is a candidate for the barley *NecSI* gene controlling necrosis and enhanced defense response to stem rust. *Theoretical and Applied Genetics*. **118**:385-397.
- Zhao, Y., A.R. Stiles., P.K. Saxena and C.Z. Liu.** 2013. Dark preincubation improves shoot organogenesis from *Rhodiola crenulata* leaf explants. *Biologia Plantarum*. 1-4. 10.1007/s10535-012-0261-5
- Zhao, Z., T. Cai., L. Tagliani., M. Miller., N. Wang., H. Pang., M. Rudert., S. Schroeder., D. Hondred and J. Seltzer.** 2000. *Agrobacterium*-mediated sorghum transformation. *Plant Molecular Biology*. **44**:789-798.

**Zhu, H., J. Jeoung, G. Liang, S. Muthukrishnan, S. Krishnaveni and G. Wilde.** 1998.  
Biolistic transformation of sorghum using a rice chitinase gene [*Sorghum bicolor* (L.)  
*Moench-Oryza sativa* L.]. *Journal of Genetics & Breeding.* **52**:243-252

## **CHAPTER 2 EFFECT OF GAMMA IRRADIATION ON ANTIOXIDANT**

### **PROPERTIES OF SORGHUM *BICOLOR* L. MOENCH**

#### **2.1 INTRODUCTION**

Over the past 50 years induced mutations has played a major role in the development of superior crop varieties translating into a tremendous economic impact on agriculture and food production that is currently valued in billions of dollars and millions of cultivated hectares (Wani and Anis, 2001). The purpose of mutation induction is to create genetic diversity which can be exploited for selection of traits. The genetic fidelity of the regenerated plants is highly desirable for developing new improved plant varieties and useful as a tool for feeding the ever-growing human population (McCallum et al., 2000). With challenges such as climate change, malnutrition and lack of adequate arable land posing a big threat and challenge to sustainable food production worldwide, mutation induction provides a flexible, workable, unregulated, non-hazardous and low-cost alternative to genetically modified organisms (Mehlo et al., 2013).

Scientific evidence suggests that the major causes of death, such as cardiovascular disease and cancer, can be prevented or delayed by nutritional interventions (Rees *et al.*, 2013). Such interventions include reduction in fat intake and increased consumption of fruits, whole grains, and vegetables (Owen *et al.*, 2000; Griendling and FitzGerald, 2003; Blomhoff, 2005). Likewise, evidence that free-radicals are involved in the development of these diseases is also increasing (Cerutti *et al.*, 1994; Demming-Adams and Adams, 2002). Free-radicals can introduce structural changes in cancer-related genes. Point mutations, for example in the *ras*-family proto-oncogenes (Bos, 1989) and in the p53 tumor suppressor gene (Hollstein *et al.*, 1991; de Fromental and Sossi, 1992) represent the most frequent genetic changes associated with human malignancies. Human and animal endogenous antioxidant defences do not possess sufficient radical scavenging capacity, and therefore dietary antioxidants are

particularly important in diminishing the cumulative effect of oxidative damage (Block, 1992; Blomhoff, 2005).

Studies conducted on both humans and animals support the beneficial effects of dietary plants rich in total antioxidants (Reese *et al.*, 2013). The antioxidants in food, including other plant compounds may improve the endogenous antioxidant defence system through the induction of phase 2 enzymes (Demming-Adams and Adams, 2002). Some sorghum varieties, e.g. black sorghum, consist of relatively higher concentrations of health-promoting phytochemicals and antioxidants. However, these are not sufficient for the inhibition of oxidative mechanisms that lead to degenerative diseases (Dykes *et al.*, 2013). The antioxidants found in sorghum can be grouped into three major categories: phenolic acids (benzoic and cinnamic acid derivatives); tannins (condensed tannins or proanthocyanidins) and flavonoids. The major flavonoids in sorghum bran are the 3-deoxyanthocyanins, including apigeninidin and luteolinidin (Burdette *et al.*, 2010). Luteolinidin and apigeninidin have a small distribution in nature, but are abundant in sorghum, where they have validated potent antioxidant activity (Clifford, 2000; Awika *et al.*, 2004).

The highest levels of antioxidants of any cereal analysed to date can be found in tannin sorghums (Gu *et al.*, 2004). These sorghums are 15-30 times more effective at quenching peroxy radicals than simple phenolics, thus they are potential biological antioxidants (Hagerman *et al.*, 1998). Regardless of their possible beneficial effects as antioxidants, tannins have been associated with reduced protein digestibility in sorghum because they complex with proteins and inhibit proteolytic enzymes (Scalbert *et al.*, 2000; Duodu *et al.*, 2002). Tannin sorghums are used in many food products and are sometimes preferred in some areas of Africa. A company in the USA, Silver Palate, has launched a number of sorghum bran-based food products under the brand name Grainberry<sup>®</sup>. These foods have more antioxidants than found in blueberries, pomegranate juice and red wine when compared

on a gram per gram basis (Hammond, 2011). The food industry in Southern Africa has also been exploring the use of sorghum in the production of ready-to-eat premixed products like *Maltabele* and *Morvite* (Taylor, 2005).

However, widespread poverty and malnutrition compounded by the unavailability of prime agricultural land, and the general preferential cultivation of other cereals like maize means that these sorghum-based products are unaffordable to the majority of Africans. Consequently, new methodologies are required to increase genetic diversity in sorghum for various nutritional traits and the lowering of anti-nutrients. To date, there are no sorghum varieties with enhanced antioxidant activity that are readily available to the poverty-stricken majorities of Africa. This study thus demonstrates the partial enhancement of total phenols, tannin content and antioxidant activity in a well characterised public sorghum line, P898012 by gamma irradiation.

## **2.2 MATERIALS AND METHODS**

### **2.2.1 Plant material irradiation**

A parental sorghum line (P898012), used for gamma irradiation in this study is a purple plant, type II low tannin sorghum with chalky white seeds. P898012 is a public sorghum line that was originally obtained from John Axtell of Purdue University, USA. Induced mutations were achieved through gamma irradiation at a dosage level of 500 Gy using a Cobalt-60 source at the Seibersdorf, Austria Laboratories of the International Atomic Energy Agency. The optimal dose for irradiation was defined as the dose of a mutagen that achieves the optimum mutation frequency with the least possible damage and was calculated according to established radiosensitivity tests (IAEA, 1977). About 1500 irradiated seeds were initially planted.

### **2.2.2 Bleach/Chlorox test**

The presence or absence of a pigmented testa in the sorghum grain was determined using the bleach or chlorox test (Waniska *et al.*, 1992). The test is based on previous observations that if the kernels contained a pigmented testa layer then condensed tannins were present in the kernels. This distinguishes type I sorghums (without tannins) from type II and III sorghums (with tannins). Briefly, 100 seeds from each of the five mutants and control were soaked in the bleach reagent (5% sodium hydroxide in 3.5% hypochlorite solution) for 20 minutes at room temperature. In this test, kernels of tannin sorghum types (those with a pigmented testa) would be expected to turn black.

### **2.2.3 Grain hardness evaluation**

Grain hardness refers to the texture of the kernel (caryopsis) and delineates whether the endosperm is physically hard or soft. Grain hardness affects various aspects of the grains' growth and processing such as resistance to fungal infection and cooking quality (Giroux and Morris, 1998). The hardness of the sorghum kernels was assessed visually using the methods and illustrations suggested by Rooney and Miller (1982). In this scheme the hardness was therefore recorded using a scale of 1-5, based on the proportion of corneous to floury endosperm. The lower end of the scale (1) denotes corneous or hard endosperm, whereas the higher end (5) represents floury or soft endosperm.

### **2.2.4 Grain colour determination**

The sorghum kernel colour was assessed visually and also by measurement with a colorimeter (Model CR-310, Minolta, Osaka, Japan) to obtain the CIE L\* values (International commission on Illumination). The L\* values provide a measure of the lightness or darkness of the grain. Whiter grains have higher L\* values, while dark coloured or grains with red pericarps have lower L\* values. This colour scale was recommended in 1976 by the CIE (CIE, 1995).

### **2.2.5 Moisture content determination**

The binding ability and mobility of moisture have great influence on rheological property, stability, quality, appearance, and sensitivity to putridness of food. Moisture also has a major influence on biochemical analysis. It was therefore essential to determine moisture content for some of the assays to be carried out in this research. An in-house method was used, where milled samples were dried for 4 hours at 90°C in accordance with previous studies (ISTA, 1996). All samples were then weighed on a Precisa Instruments Fine Balance Model 125A. Moisture values were determined in triplicate (3 different samples) and the mean of the three values calculated.

### **2.2.6 Crude protein content determination**

The nitrogen content of the samples was determined on fine milled samples using the Dumas method and according to Official Method 990.03 of the AOAC International (2000) using a LECO FP2000 nitrogen analyser (LECO Corporation 26). Because the method does not measure the protein content directly a conversion factor (6.25) was used to convert the measured nitrogen concentration to a protein concentration and give a % absolute crude protein (AOAC, 2000). The analysis was performed in triplicate. The mean of the three values was then calculated and presented.

### **2.2.7 Quantification of total polyphenols**

The modified Folin Ciocalteu method of Kaluza *et al.* (1980) was used to quantify total phenols in sorghum extracts. Milled samples were extracted for 2 hours using acidified methanol (1% HCl in methanol), with shaking carried out at low speed in an Eberbach shaker (Eberbach Corp., Ann Arbor, MI). The extracts were centrifuged and the supernatant used for total phenol quantification. An extract (0.1 mL) was diluted with 1.1 mL water, and then reacted with 0.4 mL Folin Ciocalteu reagent and 0.9 mL of 0.5 M ethanolamine. The reaction was carried out for 20 min at room temperature and absorbance was read at 600 nm. Gallic

acid was used as standard and total phenols expressed as mg gallic acid equivalents per g (mg GA/g).

### **2.2.8 Determination of condensed tannins**

Tannin content was determined using the Vanillin-HCl method as described by Price *et al.* (1978). The milled samples were extracted for 20 min at 30<sup>0</sup>C using acidified methanol (1 % HCl in methanol). Supernatants were obtained by centrifuging the extracts for 10 minutes at 3000 x g, following which 1 mL aliquots were mixed with 5 mL vanillin reagent, and absorbances read at 500 nm after 20 min. The standard used was catechin and the tannin content was then expressed as mg catechin equivalents per g (mg CE/g).

### **2.2.9 Morphology study of Sorghum endosperm using scanning electron microscope**

Scanning electron microscopy (SEM) was used to analyse representative samples from each of the mutants and controls. Seeds from two conventional endosperm types (opaque and vitreous) and the modified mutant endosperms were assessed for possible differences in the pattern of starch granules and protein body packing in the endosperm cells. In preparation for SEM, kernels were first freeze-fractured longitudinally in liquid nitrogen using a scalpel cooled to liquid nitrogen temperature. The material was then mounted to an aluminium stub using double sticky tape before being coated with gold palladium sputter coater (Technics Hummer 1). The samples were then viewed on a JEOL SEM (JSM 5800 LVSEM, Tokyo Japan) at 5kV as previously described by Parker *et al.* (1999).

### **2.2.10 Antioxidant activity assay**

Antioxidant activities of the sorghum extracts were determined using the DPPH (1,1-Diphenyl-2-picryl-hydrazyl) method described by Awika *et al.* (2003). The DPPH assay method is based on the reduction of DPPH, a stable free radical with an odd electron. DPPH gives a maximum absorption at 517 nm (purple colour). When antioxidants react with DPPH,

which is a stable free radical, it is paired off in the presence of a hydrogen donor (e.g., a free radical-scavenging antioxidant) and is reduced to the DPPHH resulting in decrease in absorbance (Sharma and Bhat, 2009). The samples were extracted in 70% aqueous acetone. The DPPH reagent was dissolved in methanol and kept at  $-20^{\circ}\text{C}$  in the dark prior to use. The DPPH radical was reacted with sorghum extracts, after which the absorbance was measured at 517 nm. The reaction tubes were prepared in triplicate, and wrapped in aluminium foil and kept at  $30^{\circ}\text{C}$  for 30 min in dark. All measurements were conducted under dim light. The standard used was ascorbic acid, and the antioxidant activity was then reported as  $\mu\text{mol}$  ascorbic acid Equivalent Antioxidant Capacity per gram sample ( $\mu\text{mol AE/g}$ ).

#### **2.2.11 Statistical analysis**

*In vitro* protein digestibility, total polyphenol analysis using the Vanillin-HCl method and the DPPH antioxidant assay were performed in triplicate. All results are presented as the mean ( $\pm$  standard deviations). Correlation analyses of antioxidant activity (Y) versus the total phenolic content (X) were carried out using the correlation and regression programme in MINITAB 13.2 (Minitab 2002 Software Inc., Northampton, MA). Analysis of variance by the least significant difference test (LSD-test) was performed to determine whether a significant difference existed ( $p < 0.05$ ) between the means of treatments.

### **2.3 RESULTS**

#### **2.3.1 Effect of induced mutation on grain quality**

Gamma irradiation at a dose of 500 Gy had a profound impact on sorghum seed germination. Out of the total 1500 seeds planted, only 70% germinated, 17 days after planting. This contrasted with a 98% germination rate for control non-irradiated seeds that germinated only after 11 days of planting. Several phenotypic mutants were observed, including dwarf,

chlorophyll and anthocyanin mutants. Five chlorophyll mutant sorghums at M<sub>4</sub> generation were chosen for nutritional analysis. These lines were denoted, Brown mutant (BR); Red mutant (RED); Biomass mutant (BIO); White mutant (T120) and Soft Yellow mutant (SY). Mutants were compared to parental material P898012 type II tannin sorghum (wild type), yellow low-tannin sorghum (YEL) with hard endosperm (SK5912) and in some cases Macia (MC), a non-tannin variety commonly cultivated and used for food preparation in Southern Africa. One of the mutants developed (T120) is a non-tannin (Figure 2.1b) variety with a corneous endosperm. The other tested mutants had pigmented testa (tannin sorghums) (Figure 2.1). The tannin sorghums had darker coloured kernels (L\* value from 46 to 95), while the Yellow non-tannin sorghum was the lightest in colour, with the highest L\* value (119), and the white non-tannin mutant had an L\* value of 103 (Table 2.1).

The kernel weights of some mutants were significantly different from that of the parental line (36 mg), with the smallest being RED (18 mg). The average weight of the mutants was 28 mg. However, T120 recorded a weight of 37 mg and had the hardest kernel as demonstrated in Figure 2.2e, while the tannin-mutants (RED, BR, YEL and BIO) were predominantly floury. The protein content range was 8.38-16.08 %, with SY having a significantly higher content and MC the lowest. The moisture and ash content ranged from 8 - 9.71 % and 1.5 – 3 respectively (Table 2.2).



**Figure 2.1** Bleach/Chlorox analysis for the presence of testa layer (tannin), a= Macia b= T120: white mutant sorghum, c= BR: brown mutant sorghum, d= DWARF mutant, e= BIO: biomass mutant sorghum, f= SY: soft yellow mutant sorghum, g= P898012 and h=RED: red mutant sorghum

**Table 2.1** Grain qualities of parental strain P898012, yellow low-tannin sorghum (YEL), Macia (MC) and 5 mutant strains at the M<sub>4</sub> generation after Gamma ray induced mutation

Sorghum type	Pericarp colour	L* value	Bleach test	Kernel hardness	Kernel hardness score	Kernel weight
<b>P898012</b>	White	46.10 <sup>d</sup>	98	Intermediate	3	36 <sup>b</sup>
<b>MC</b>	White	NR	0	Corneous	2	NR
<b>YEL</b>	Yellow	119.34 <sup>a</sup>	0	Corneous	2	50 <sup>a</sup>
<b>BR</b>	Brown	45.37 <sup>d</sup>	98	Floury	4	34 <sup>b</sup>
<b>RED</b>	Red	56.73 <sup>c</sup>	99	Floury	5	18 <sup>d</sup>
<b>BIO</b>	White	95.86 <sup>b</sup>	97	Intermediate	3	37 <sup>b</sup>
<b>T120</b>	White	103.75 <sup>a</sup>	0	Corneous	2	30 <sup>c</sup>
<b>SY</b>	lemon yellow	60.99 <sup>c</sup>	99	Floury	5	21 <sup>d</sup>

P898012: parental sorghum; YEL: yellow low-tannin sorghum; MC: Macia; BR: Brown mutant; RED: Red mutant; BIO: Biomass mutant; T120: White mutant; SY: Soft Yellow mutant. L\*values as measured by colorimeter indicate kernel lightness or darkness with higher figures indicating darkness

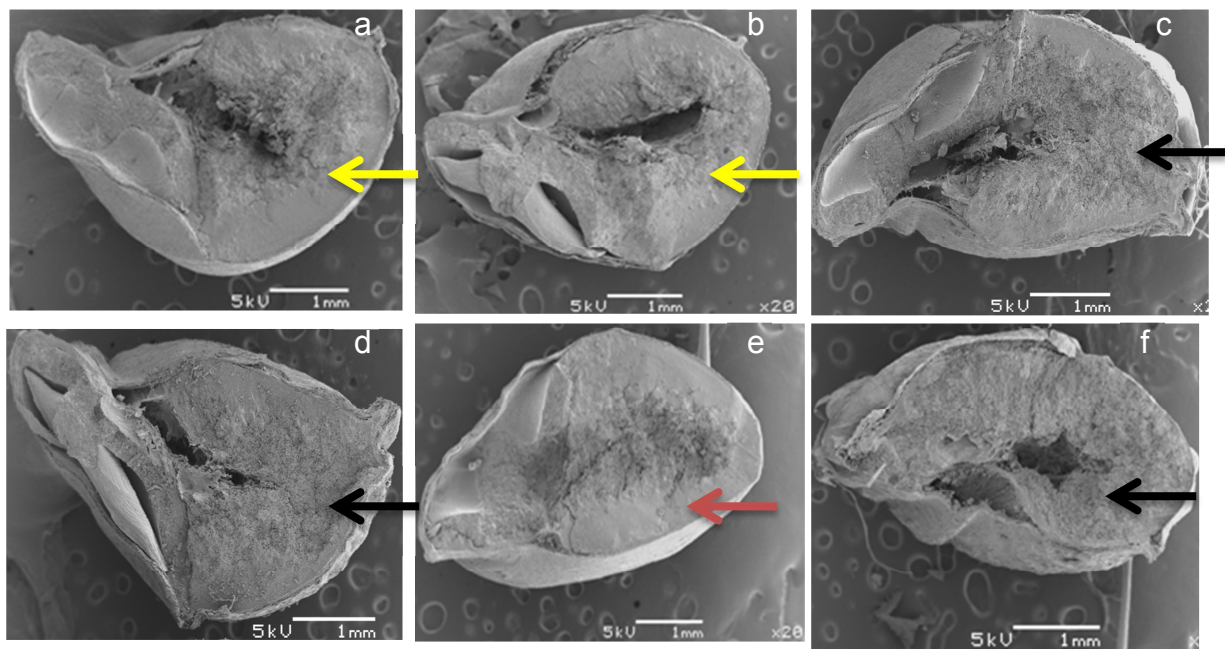
Bleach test: Number of kernels with pigmented testa layer out of the 100 evaluated (Waniska *et al.*, 1992)

Kernel hardness evaluated using a score of 1 to 5. The number 1 indicates corneous, and 5 is floury (Rooney and Miller. 1982)

Kernel weight expressed in mg

NR= data not available

Values with different letters within the same column are significantly different at P<0.05



**Figure 2.2** Scanning electron micrograph of the grain sorghum endosperm, a= parental line P898012, b= BIO: Biomass mutant, c= RED: Red mutant, d= BR: Brown mutant, e= TW: White mutant, f= SY: Soft Yellow mutant. Red arrow = hard (corneous) endosperm, black arrow = soft (floury) endosperm and yellow arrow = intermediate endosperm.

**Table 2.2** Moisture, protein and ash contents of parental, Macia, yellow low-tannin sorghum strains and 5 tested mutant sorghums.

Sorghum type	Moisture wt %	Protein content wt %	Ash wt %
<b>P898012</b>	8.73	12.33 <sup>d</sup>	3
<b>MC</b>	NR	8.38 <sup>f</sup>	1.5
<b>YEL</b>	8	10.44 <sup>e</sup>	3
<b>BR</b>	6.86	14.47 <sup>c</sup>	2
<b>RED</b>	9.75	15.09 <sup>b</sup>	3
<b>BIO</b>	9.53	12.0 <sup>d</sup>	2
<b>T120</b>	9.92	10.91 <sup>e</sup>	2
<b>SY</b>	9.71	16.08 <sup>a</sup>	3

P898012: parental sorghum; MC: Macia; YEL: yellow low-tannin sorghum; BR: Brown mutant; RED: Red mutant; BIO: Biomass mutant; T120: White mutant; SY: Soft Yellow mutant.

Moisture, protein and ash contents are expressed in percentage.

Values within the same column with different letters are significantly different at  $P < 0.05$

NR = data not available. n=3.

### **2.3.2 Effect of induced mutation on tannin content**

The levels of tannins in sorghum without pigmented testa (MC, YEL and T120) were not detectable. However, the sorghum types with pigmented testa (P898012, BR, RED, BIO and SY) had a tannin content ranging from 0.59 – 1.33 mg CE/g (Table 2.3). The tannin mutants had a significantly higher tannin content when compared to the parental line (P898012). Soft Yellow mutant (SY) in particular, had 2 fold greater tannin levels (1.33 mg CE/g) than its parental counterpart (0.59 mg CE/g).

### **2.3.3 Effect of induced mutation on antioxidant capacity**

The antioxidant contents of all sorghum types tested are shown in Table 2.3. A significant increase in antioxidant capacity of the mutants (from 142.89  $\mu\text{molAE/g}$  to 625.41  $\mu\text{molAE/g}$ ) over those of the three controls (P898012, MC and YEL) ranged from 137.09 – 158.77  $\mu\text{molAE/g}$ , was observed. Soft Yellow mutant (SY), which contained the highest tannin content as shown above, possessed a 4-fold increase (625.41  $\mu\text{molAE/g}$ ) in antioxidant capacity when compared to its wild-type counterpart (137.09  $\mu\text{molAE/g}$ ). The relationship between tannin content and antioxidant capacity is reflected in Figure 3.2 which depicts a significantly positive, strong correlation ( $r^2 = 0.8114$ ;  $p < 0.05$ ) between the two values.

### **2.3.4 Effect of induced mutation on total polyphenols**

The range of total phenol content recorded by the mutants was 3.3 to 8 mg/g. The highest phenol content was detected in SY (8 mg/g), while the parental line had the lowest 1.9 mg/g. The average total phenol content in the mutant population was 4.72 mg/g (Table 2.3). Mutants with high phenol content also had a correspondingly high antioxidant activity. SY had a 4 fold increase in phenol content when compared to its wild type counterpart, P898012, while the red mutant (RED) showed a 2-fold increase.

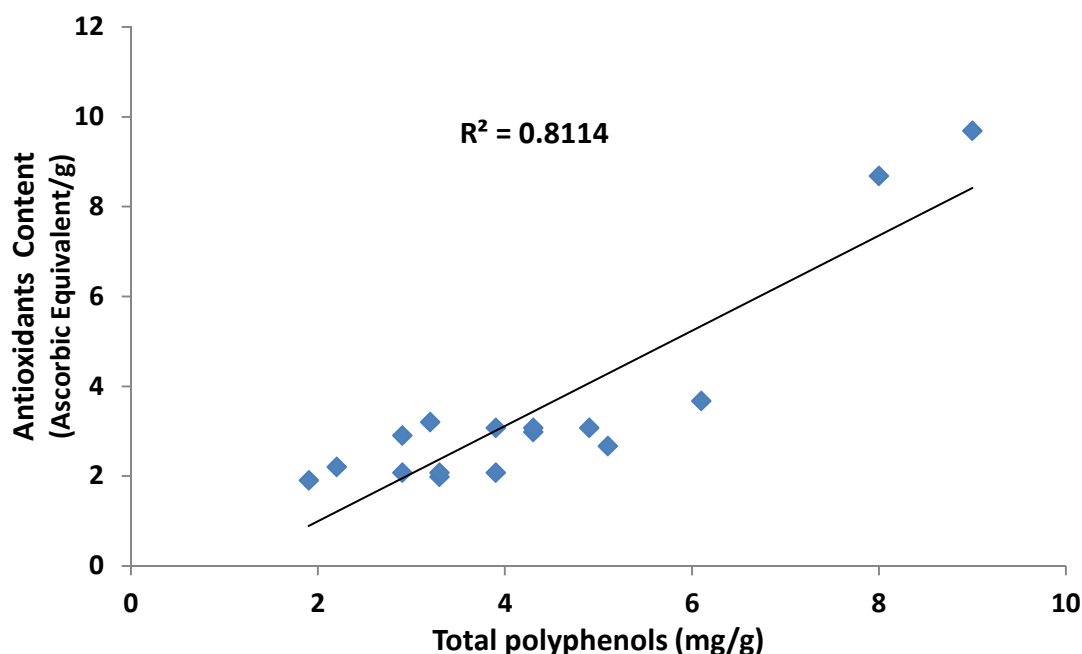
**Table 2.3** Tannin content, total polyphenol content and antioxidant capacity of tested sorghum

<b>Sorghum type</b>	<b>Tannins (mg CE/g)</b>	<b>Antioxidants (<math>\mu</math>mol/AE/g)</b>	<b>Total polyphenols(mg/g)</b>
<b>P898012</b>	0.59 <sup>f</sup>	137.09 <sup>f</sup>	1.9 <sup>g</sup>
<b>Macia</b>	ND	149.37 <sup>d</sup>	2.9 <sup>e</sup>
<b>YEL</b>	ND	158.77 <sup>c</sup>	2.2 <sup>f</sup>
<b>BR</b>	0.81 <sup>e</sup>	142.89 <sup>e</sup>	3.3 <sup>d</sup>
<b>RED</b>	0.96 <sup>d</sup>	192.35 <sup>b</sup>	5.1 <sup>b</sup>
<b>BIO</b>	1.11 <sup>c</sup>	149.34 <sup>d</sup>	3.9 <sup>c</sup>
<b>T120</b>	ND	149.35 <sup>d</sup>	3.3 <sup>d</sup>
<b>SY</b>	1.33 <sup>b</sup>	625.41 <sup>a</sup>	8 <sup>a</sup>

P898012: parental sorghum; MC: Macia; YEL: yellow low-tannin sorghum; BR: Brown mutant; RED: Red mutant; BIO: Biomass mutant; T120: White mutant; SY: Soft Yellow mutant.

Values within the same column with different letters are significantly different at  $P < 0.05$  Mg CE/g: mg Catechin equivalent per gram of sample AE: Ascorbic acid equivalent per gram of sample;

ND: not detectable. n=3.

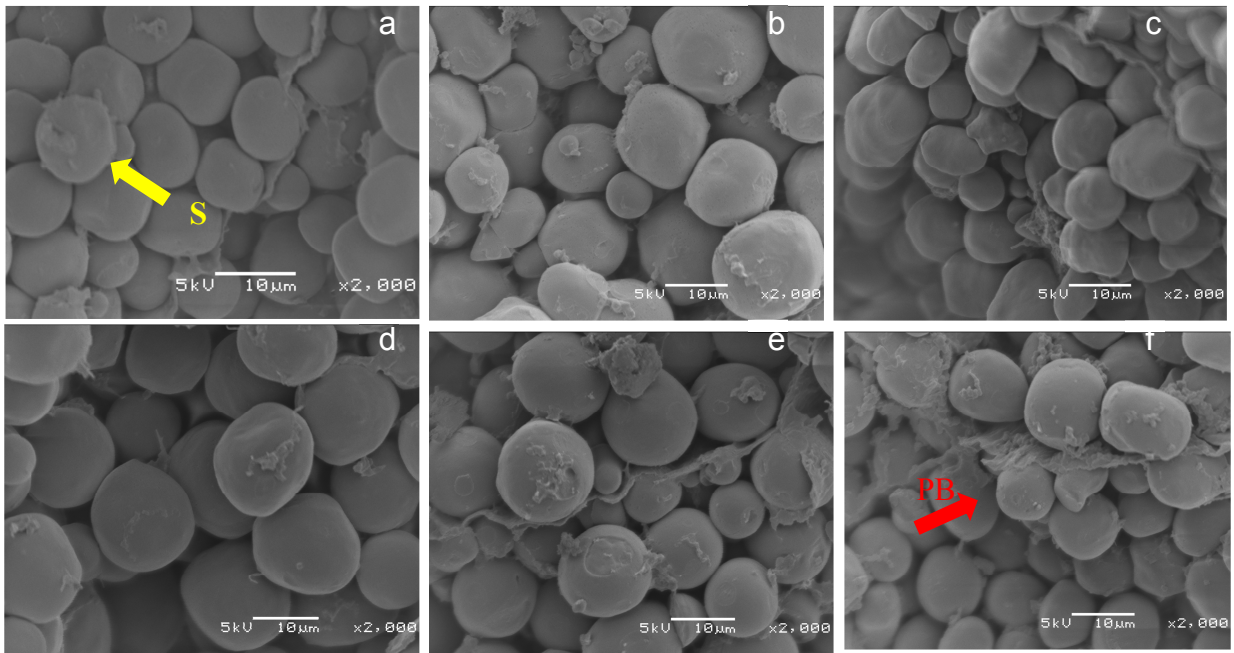


**Figure 2.3** Relationship between antioxidant activity and total phenol content of all sorghum tested. Correlation is significant at  $p < 0.05$ .

### 2.3.5 Scanning electron microscopy (SEM)

The endosperm structure of sorghum grain samples representing parental material and mutants was examined with scanning electron microscope (SEM) as shown in Figure 2.4. The soft or floury endosperm is characterised by relatively large intergranular air spaces, whereas, the hard or corneous endosperm is characterised by a tightly packed structure with no air spaces. Seeds of the P898012 parental line had oval shaped starch granules (Figure 2.4a). The oval shape of the starch granules resulted in tight packaging of the starch granules, leaving very little airspaces in between. This resulted in an intermediate endosperm structure (Figure 2.2a). On the contrary, the starch granules for the mutant SY (Figure 2.4f) were rounder in shape, bigger in size than those of P898012 parental line. As a result of their round shape, the starch granules packed in such a way that a lot of airspaces existed in between the starch granules. This resulted in a floury/soft endosperm microstructure (Figure 2.2f). The loose packing of starch granules in SY was also associated with the accumulation of more protein in this particular mutant (Figure 2.4f, Table 2.2). The other floury endosperm mutants

(RED and BR) showed a trend similar to SY with regards to packaging of starch granules. In mutant designated T120, the starch granules were irregularly shaped and varied between 5 and 10  $\mu\text{m}$  in size (Figure 2.4e). The irregular shapes allowed the starch granules to be compactly packed, thus resulting in a corneous endosperm (Figure 2.2e).



**Figure 2.4** Scanning electron micrograph of grain sorghum endosperm, a=P898012; b= BIO: Biomass mutant; c= RED: Red mutant; d= BR: Brown mutant; e= T120: White mutant; f= SY: Soft Yellow mutant; PB = Protein bodies; S = Starch granule. A lower resolution of the same image is shown in Appendix B.

## 2.4 DISCUSSION

The objective of this study was to evaluate the effect of induced mutations on antioxidant properties of five sorghum mutants generated using gamma irradiation. The parental line P898012 and two controls (MC: Macia and YEL: Yellow sorghum) were also tested. The five mutants included RED, BR, T120, SY and BIO which were chosen for their anthocyanin properties which give blue to red colour in plants. The mutants were screened for changes in tannin content, total phenols and antioxidant capacity at the M4 generation and after gamma ray induction.

A number of other grain quality parameters were determined to establish the effect of induced mutation on sorghum. These included grain colour, grain weight and protein content. Grain colour and quality are important characteristics in the baking and livestock feed industries.

Among the factors influencing the visual colour of grain sorghum are the genetics of pericarp color, pericarp thickness, the presence or absence of a testa, color and thickness of the testa, and the endosperm colour (Rooney and Miller, 1982). Grain colour in sorghum is determined by at least 10 pairs of genes. The epicarp is the outer thin layer of the sorghum pericarp and surrounds the entire seed. The genotype RR and YY determine the grain colour and its appearance, for example, combination R\_Y\_ will result in a red epicarp, R\_yy in a white epicarp, rrY\_ in lemon yellow epicarp and rryy will result in a white epicarp. These genes interact epistatically to produce the observed colours (Valencia and Rooney, 2009). It is therefore possible that gamma irradiation triggered mutations in the above mentioned genes resulting in five distinctly coloured mutants, T120 (white), SY (lemon yellow), RED (red mutant), BR (brown mutant) and BIO (brown). There are various other genes involved in colour determination such as the spreader gene S\_ and intensifier gene I\_ which determine the spread of colour throughout the epicarp and its intensity respectively (Valencia and Rooney, 2009).

Gamma irradiation had a predominantly negative effect on grain weight. The average weight of the 5 mutants was 28 mg, which is 8 mg less than the parental P898012 sorghum line. However, the white non-tannin mutant showed a slightly improved grain weight when compared to the parental line. These results are in contrast with those of Bhaskara and Reddi, 1975 who showed a 21% increase in grain weight of radiation induced sorghum mutants. The results do however concur with the hypothesis that the genes governing grain weight are disturbed by irradiation (Rahimi and Bahrani, 2011).

Out of the five tested mutants, 3 had elevated protein content (highest 16.08 %, for SY) while the other two (BIO and T120) showed a slight decrease (lowest 10.91 %) as shown in Table 2.2. In the primary irradiated material ( $M_0$ ), chemical changes in proteins that are caused by gamma-irradiation are fragmentation, cross-linking, aggregation and oxidation caused by oxygen radicals, which are generated by water radiolysis (Cho and Song, 2000). Exposure to a mutagen can cause both point mutations and small chromosomal deletions. As a result, mutagenesis can lead to development of a mutant that expresses an altered protein together with the normal protein at 50% of its normal abundance. Therefore, detection of quantitative alterations in protein expression (protein polymorphism) could, theoretically, be used to measure genetic changes that can be tested for heritability and to provide data for estimation of mutation rates (Giometti *et al.*, 1987).

Some reports have shown that higher exposure to gamma rays generally produce negative effects on plant growth, development and nutrition (Gunckel & Sparrow, 1961; Hassan *et al.*, 2009; Ikram *et al.*, 2010). In the current study, SY developed the less favourable floury endosperm phenotype resulting in lower kernel density, increased susceptibility to insects and reduced yield (Mertz *et al.*, 1964; Lending and Larkins, 1992; Tesso *et al.*, 2008; Da Silva *et al.*, 2011). These agronomic problems present major challenges for cultivation of the mutant.

However, SY had the highest lysine content (Mehlo *et al.*, 2013) which has been previously associated with a floury endosperm (Mertz *et al.*, 1964; Da Silva *et al.*, 2011).

Analysis of mutant sorghums with pigmented testa layers indicated that these lines had the highest total polyphenols. The amounts of total polyphenols were positively correlated with antioxidant activity (Figure 2.3). These results are in line with previous observations that some sorghum lines, especially the ones termed black sorghums have high content of polyphenols and antioxidants (Dykes *et al.*, 2005; Dicko *et al.*, 2005). The mutant denoted T120, had a decrease in tannin content (Table 2.3). This may be due to a mutation(s) in the gene conferring the tannin trait. Generally, non-tannin sorghums have low amount of antioxidants and phenols (Dykes *et al.*, 2006). Accordingly, lines that revealed an increase in tannin content including BR, RED and SY (Table 2.3) had higher amounts of antioxidants and phenols. These changes are attributable to the effects of gamma irradiation. However, the tannin content appeared to be correlated to the seed-coat colour. The red (RED) and yellow (SY) lines contained more tannins (0.96 and 1.33 mg catechin equivalent per gram respectively). In the White line (T120), the tannin levels were undetectable. Previous research (Abu-Tarboush, 1998) indicated that a dose of 10 and 7 kGys (kilogray) significantly reduced the tannin content of Shahlla sorghum variety from 0.35 to 0.25 mg of catechin equivalent/100 g but not that of Hamera variety, however the reasons were not stated. The variations in tannin content of Shahlla and Hamera sorghum mutants were also positively associated with variations in measurable total phenols.

Gamma irradiation significantly increased the measurable phenol content. The yellow (SY) and red (RED) mutants showed a 4-fold and 2-fold increase in phenolic compounds respectively (Table 2.3). In plant tissues many phenolic compounds are potential antioxidants. These include flavonoids, tannins and lignin precursors which may act to counteract the effects of reactive oxygen species (ROS). As a result, the observed increase in

total phenolic and tannin contents is likely to be nutritionally beneficial as far as antioxidant properties of sorghum seeds are concerned. Modulation of the antioxidative system and accumulation of phenolic compounds have also been documented in different plant species (Kim *et al.*, 2004; Wi *et al.*, 2005).

In addition to their potential for nutritional value and as functional foods, lines with improved antioxidant properties, provide an excellent resource in studies aimed at increasing understanding of the molecular mechanisms influencing antioxidant capacity of staple cereals like sorghum. Further research using techniques such as TILLING (targeted induced local lesions in genomes), would allow insight into genetic changes associated with the observed increases in antioxidant activities of these sorghum mutants.

Overall, gamma irradiation of the P898012 sorghum grain had a pronounced impact in recovered mutant progeny. Some of the mutants had elevated phenol content and associated high antioxidant activity (SY), whereas in other lines, the mutations conferred a non-tannin trait (T120) on a previously tannin line (P898012) and a change in seed colour from chalky white to brown and red. Even though the seed weight was compromised in some mutants, the overall impact on seed quality was beneficial. The results clearly demonstrate the effectiveness of gamma-irradiation induced mutations in producing substantial improvements in antioxidant properties of sorghum.

## 2.5 REFERENCES

- Abu-Tarboush, H.M.** 1998. Irradiation inactivation of some antinutritional factors in plant seeds. *Journal of Agricultural and Food Chemistry*. **46**:2698-702.
- AOAC.** 2000. Official methods of analysis. Gaithersburg: AOAC International.
- Awika, J.M., L.W. Rooney., X. Wu., R.L. Prior and L. Cisneros-Zevallos.** 2003. Screening methods to measure antioxidant activity of sorghum (*Sorghum bicolor*) and sorghum products. *Journal of Agriculture and Food Chemistry*. **51**:6657-62.
- Awika, J.M. and L.W. Rooney.** 2004. Sorghum phytochemicals and their potential impact on human health. *Phytochemistry*. **64**:1199-221.
- Bhaskara Rao, E.V.V. and V.R. Reddi.** 1975. A radiation-induced highly productive mutant in sorghum. *Radiation Botany*. **15**:29-32.
- Bos, J.** 1989. *ras* Oncogene in human cancer. A review *Cancer Research*. **49**:4682-9.
- Block, G.** 1992. The data support role for antioxidants in reducing cancer risk. *Nutrition reviews*. **50**:207-13.
- Blomhoff, R.** 2005. Dietary antioxidants and cardiovascular disease. *Current opinion in lipidology*. **16**:47-54.
- Burdette, A., P.L. Garner., E.P. Mayer., J.L. Hargrove., D.K. Hartle and J.L. Greenspan.** 2010. Anti-inflammatory activity of select sorghum (*Sorghum bicolor*) brans. *Journal of Medicinal Food*. **13**:879-87.
- Cerutti, P., R. Ghosh., Y. Oya and P. Amstad.** 1994. The role of the cellular antioxidant defense in oxidant carcinogenesis. *Environmental health perspective*. **10**:123-30

- CIE Publication No. 116.** 1995. Industrial colour-difference evaluation. *Res. Appl.* **21**: 347–352. CIE, Vienna.
- Cho, Y. and K.B. Song.** 2000. Effect of gamma-irradiation on the molecular properties of BSA and b-lactoglobulin. *Journal of Biochemical Molecular Biology.* **33**:133-137.
- Clifford, M.N.** 2000. Miscellaneous phenols in foods and beverages – nature, occurrence and dietary burden. *Journal of the Science of Food and Agriculture.* **80**:1126-37.
- Demmig-Adams, B. and W.W. Adams.** 2002. Antioxidants in photosynthesis and human nutrition. *Science.* **298**:2149-53.
- Da Silva, L.S., R. Jung., Z. Zhao., K. Glassman., J. Taylor and J. Taylor.** 2011. Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein body structure and protein nutritional quality in improved sorghum lines. *Journal of Cereal Science.* **54**:160-167.
- de Fromental, C. and T. Sossi.** 1992. The p53 tumor suppressor gene: a model for investigating human mutagenesis. *Genes Chromosomes and Cancer.* **4**:1-4.
- Dicko, M., H. Gruppen., A.S. Traore., W.J.H. Van Berkel and A.G.J. Voragen.** 2005. Evaluation of the effect of germination on phenolic compounds and antioxidant activities in sorghum varieties. *Journal of Agriculture and Food Chemistry.* **53**:2581-8.
- Duodu, K.G., A. Nunes., I. Delgadillo., M.L. Parker., E.N. Mills., P.S. Belton and J.R.N. Taylor.** 2002. Effect of grain structure and cooking on sorghum and maize *in vitro* protein digestibility. *Journal of Cereal Science.* **35**:161-74.
- Dykes, L., W.L. Rooney and Rooney, L.W.** 2013. Evaluation of phenolics and antioxidant activity of black sorghum hybrids. *Journal of Cereal Science.* **58**:278-283.

**Dykes, L., L.W. Rooney., R.D. Waniska and W.L. Rooney.** 2005. Phenolic compounds and antioxidant activity of sorghum grains of varying genotypes. *Journal of Agricultural and Food Chemistry*. **53**:6813-8.

**Giroux, M.J. and. C.F. Morris.** 1998. Wheat grain hardness results from highly conserved mutations in the friabilin components puroindoline a and b. *Proceedings of the National Academy of Sciences USA*. **95**:6262-6.

**Giometti, C.S., M.A. Gemmell., S.L. Nance., S.L. Tollaksen and J. Taylor.** 1987. Detection of heritable mutations as quantitative changes in protein expression. *J. Biol. Chem.* **262**:12764-12767.

**Griendling, K.K. and FitzGerald, G.A.** 2003. Oxidative stress and cardiovascular injury. Part II: Animal and human studies. *Circulation: Clinical Cardiology*. **108**:2034-40.

**Gu, L., M.A. Kelm., J.F. Hammerstone., G. Beecher., J. Holden., D. Haytowitz., S. Gebhardt and R.L. Prior.** 2004. Concentration of proanthocyanidins in common foods and estimations of normal consumptions. *Journal of Nutrition*. **134**:613-7.

**Gunckel, J.K. and A.H. Sparrow.** 1961. Ionising radiations: biochemical, physiological and morphological aspects of their effects on plants. *Encyclopedia of Plant Physiology*. **16**:555-611.

**Hagerman, A.E., K.M. Ried., G.A. Jones., K.N. Sovik., N.T. Ritchard., P. Hartzfield and T.L. Riechel.** 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agriculture and Food Chemistry*. **46**:1887-1892.

**Hammond, E.** 2011. Sorghum and the antioxidant craze: What benefit for Africa's farmer. *Africa Center for Biosafety*. 1-11.

[http://www.acbio.org.za/images/stories/dmdocuments/Sorghum\\_Antioxidant.pdf](http://www.acbio.org.za/images/stories/dmdocuments/Sorghum_Antioxidant.pdf)

- Hassan Amro, B., A.M. Osman Gammaa., A.H. Rushdi Mohamed., M. Etlayeb Mohaned and E.E. Diab.** 2009. Effect of gamma-irradiation on the nutritional quality of maize cultivars (*Zea Mays*) and sorghum (*Sorghum bicolor*) grains. *Pakistan Journal of Nutrition*. **8**:167-171.
- Hollistein, M.S., B. Vogelstein and C.C. Harris.** 1991. p53 mutations in human cancers. *Science*. **253**:49-53.
- IAEA.** 1977. Manual of food irradiation dosimetry. In Technical Reports Series, N. (ed.) Vienna: IAEA.
- Ikram, N., S. Dawar., Z. Abbas and Z. Javed.** 2010. Effect of (60cobalt) gamma rays on growth and root rot diseases in mungbean (*Vigna radiata* L.). *Pakistan Journal of Botany*. **42**:2165-2170.
- ISTA.** 1996. International Rules for Seed Testing, Rules 1996. International Seed Testing Association (ISTA). *Seed Science and Technology*. **24**:120-145
- Kaluza, W.Z., R.M. McGrath., T.C. Roberts and H.H. Schroder.** 1980. Separation of phenolics of *Sorghum bicolor* (L.) Moench grain. *Journal of Agriculture and Food Chemistry*. **28**:1191-1196.
- Lending, C.R. and B.A. Larkins.** 1992. Effect of the floury-2 locus on the protein body formation during maize endosperm development. *Protoplasma*. **171**:123-133.
- Kim, E.Y., I.H. Baik., J.H. Kim., S.R. Kim and M.R. Rhyu.** 2004. screening of the antioxidant activity of some medicinal plants. *Korean Journal of Food Science and Technology*. **36**:333-338.
- McCallum, C.M., L. Comai, E.A. Greene and S. Henikoff.** 2000. Targeted screening for induced mutations. *Nature Biotechnology*. **18**:455-457.

**Mehlo, L., Z. Mbambo., S. Bado., J. Lin., S.M. Moagi., S. Buthelezi., S. Stoychev and R. Chikwamba.** 2013. Induced protein polymorphisms and nutritional quality of gamma irradiation mutants of sorghum. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis.* **749**:66-72.

**Mertz, E.T., L.S. Bates and O.E. Nelson.** 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science.* **145**:279-280.

**Owen, R.W., A. Giacosa., W.E. Hull., R. Haubner., G. Wurtele., B. Spiegelhalder and H. Bartsch.** 2000. Olive-oil consumption and health: the possible role of antioxidants. *The Lancet Oncology.* **1**:107-12.

**Parker, M.L., A. Grant., N.M. Rigby, P.S. Belton and J.R.N. Taylor.** 1999. Effects of popping on the endosperm cell walls of sorghum and maize. *Journal of Cereal Science.* **30**: 209-216.

**Price, M.L., S. Van Scoyoc and L.G. Butler.** 1978. A critical evaluation of the vanillin reaction as an assay for tannin in sorghum grain. *Journal of Agriculture and Food Chemistry.* **26**:1214-1218.

**Rahimi, M.M. and A. Bahrani.** 2011. Influence of Gamma radiation on some physiological characteristics and grain protein in wheat. *World Applied Sciences Journal.* **15**: 654-659.

**Rees, K., M. Dyakova., K. Ward., M. Thorogood and E. Brunner.** 2013. Dietary advice for reducing cardiovascular risk. *Cochrane Database of Systematic Reviews.* Issue 3. Art. No.: CD002128. DOI: 10.1002/14651858.CD002128.pub4.

**Rooney, L.W. and F.R. Miller.** 1982. Variation in the structure and kernel characteristics of sorghum. In Mertin, J. V. (ed.) *Proceedings of the International Symposium on Sorghum Grain Quality.* Patancheru, India. 143-62.

- Scalbert, A., S. Deprez., I. Mila., A. Albrecht., J. Huneau and S. Rabot.** 2000. Proanthocyanidins and human health: Systemic effects and local effects in the gut. *BioFactor*. **13**:115-20.
- Taylor, J., J. Taylor., M. Dutton and S. De Kock.** 2005. Identification of kafirin film casting solvents. *Food Chemistry*. **90**:401-408.
- Tesso, T., B.R. Hamaker and G. Ejeta.** 2008. Sorghum protein digestibility is affected by dosage of mutant alleles in endosperm cells. *Plant Breeding*. **127**: 579-586.
- Valencia, R.C. and W.B.L. Rooney.** 2009. Genetic control of sorghum grain colour. IntSormil. <http://intsormil.org/smscientificpresents/CONTROLE.pdf>.
- Wani, A. and M. Anis.** 2001. Gamma rays induced bold seeded high yielding mutant in chickpea. *Mutation Breeding Newsletter*. **45**:20-21.
- Waniska, R.D., L.F. Hugo and L.W. Rooney.** 1992. Practical methods to determine the presence of tannins in sorghum. *Journal of Applied Poultry Research*. **1**:122-8.
- Wi, S.G., B.Y. Chung., J.S. Kim., J.H. Kim., M.H. Baek., J.W. Lee and Y.S. Kim.** 2007. Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron*. **38**:553-64.

**CHAPTER 3 INDUCED PROTEIN POLYMORPHISMS AND ITS NUTRITIONAL  
IMPLICATIONS IN GAMMA IRRADIATED MUTANTS OF SORGHUM**

This chapter has been published in part by Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis

**Mehlo, L., Mbambo, Z., Bado, S., Lin. J., Moagi, S., Buthelezi, S., Stoychev, S and Chikwamba, R.** 2013. Induced protein polymorphisms and nutritional quality of gamma irradiation mutants of sorghum. *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*.749:66-72

### 3.1 INTRODUCTION

On a global scale, cereals generally supply close to 50% of dietary proteins for humans and in developing countries, this statistic rises to about 70% (Gibbon and Larkins, 2005). For approximately one third of the world's population in resource-poor countries of the Sub Saharan Africa and South East Asia, one cereal staple is usually the only main source of nutrition (Sofi *et al.*, 2009). Changes in climate and its impact on global cereal crop production, particularly the increased incidences of drought and crop failure, therefore refocus attention to the cultivation of adaptable cereal crops like sorghum (Sasaki *et al.*, 2009; Elkonin *et al.*, 2010). The number of people consuming sorghum is expected to increase, especially in the semi-arid regions of the world where other cereals like rice, wheat and barley are less likely to thrive (Belton *et al.*, 2004). Worst still, the global population is expected to grow by 15% from the current 7 billion to 9.3 billion people in 2050 and to 10.1 billion people in 2100, thus suggesting a need to increase agricultural production and the nutritional quality of food (Lee, 2011). Unfortunately, a staple diet consisting of sorghum is likely to lead to malnutrition. The grains' storage proteins do not provide a balanced source of protein and calories. The major storage proteins in sorghum seeds (prolamins) are deficient in many essential amino acids like lysine, methionine and tryptophan. Sorghum storage proteins are also less digestible by proteases and are even more so upon cooking (Da Silva *et al.*, 2011). There are several reasons for the low digestibility of sorghum. Firstly, the spatial organisation of different storage proteins (kafirins) within the grains' protein bodies ensures that the less digestible  $\gamma$ - and  $\beta$ -kafirins shield the more digestible and most abundant  $\alpha$ -kafirins (Belton *et al.*, 2004; Da Silva *et al.*, 2011). Secondly, the chemical structure of kafirin molecules has abundant sulphur-containing amino acids capable of forming strong protease digestion-resistant S-S bonds. Finally, the interaction of kafirins with non-protein molecules like polyphenols and polysaccharides interferes with digestion. The sorghum grain is also deficient in vitamin A, and its content of iron and zinc has limited bioavailability due to anti-

nutrients (Kumar *et al.*, 2012). However, studies have shown that the digestibility of sorghum can be improved (Da Silva *et al.*, 2011). In this research we report gamma irradiation induced protein polymorphisms related to kaf rin protein accumulation. The data discussed also shows improved accumulation of essential amino acids.

## **3.2 MATERIALS AND METHODS**

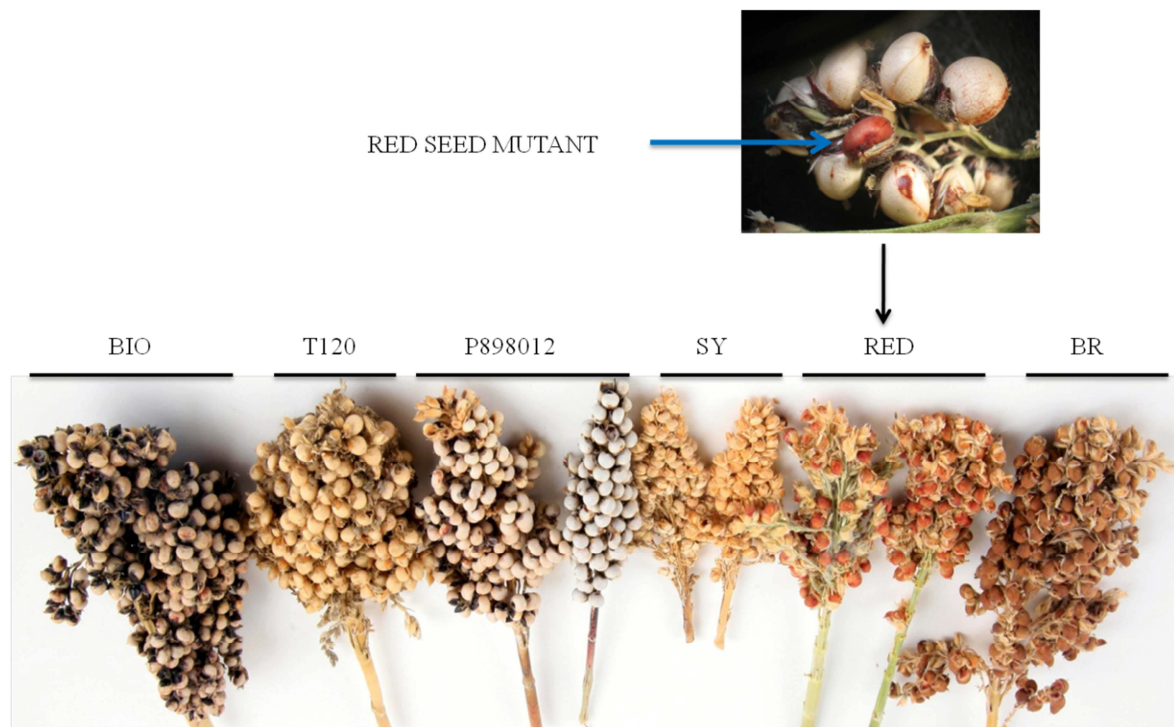
### **3.2.1 Plant material irradiation**

Parental sorghum line (P898012), a purple plant, type II low tannin sorghum with chalky white seeds was used for gamma-irradiation as described in Chapter 2 (Section 2.2.1). The generated mutants are M<sub>4</sub> generation including non-tannin white sorghum (designated T120), a low yielding, soft endosperm, lemon yellow tannin sorghum of low seed density (designated SY), a mutant with seeds whose pericarp is red (designated RED), a mutant sorghum whose seeds have a brown pericarp (designated BR) and a white tannin sorghum mutant producing many tillers (designated BIO), all shown in Figure 3.1. In M1 the RED mutant consisted of isolated red seeds (Figure 3.1, blue arrow) within each panicle, however, the number of red seeds increased with subsequent generations.

### **3.2.2 Bran protein extraction**

For the extraction of bran proteins, mutant seeds were scrapped off physically with a scalpel blade, carefully avoiding the endosperm which is just beyond the testa layer and then ground to fine powder in liquid nitrogen. The fine powder (10 mg) was used for extraction where 100 µl of Tris-HCl buffer (2% SDS, 50 mM Tris-HCl pH 6.8, 100 mM DTT) was mixed with the powder, allowed to stand for 3 hours with constant vortexing. Embryos were also physically excised and ground to fine powder in liquid nitrogen. Protein extraction was then carried out in 60% *t*-butanol for 3 hours at room temperature with constant agitation as described above

for endosperm proteins. Protein concentrations of all samples were determined as described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.



**Figure 3.1** Stabilised  $M_4$  mutant sorghum seeds used for analysis. (P898012) is chalky-white type II tannin sorghum used as a parental line for gamma irradiation. (BIO) is a mutant sorghum with enhanced biomass of multiple fertile tillers, and yielding at least five times more seed than the parental P898012. (T120) is a lemon yellow hard endosperm non-tannin mutant sorghum of P898012. (SY) is a lemon yellow mutant sorghum of P898012 with a foury-soft endosperm, low seed density and low yielding. (RED) is a mutant sorghum of P898012 with a red pericarp. (BR) is a mutant sorghum of P898012 with a brown pericarp. The top photograph shows a mutant red seed in a panicle containing chalky white seeds of the parental P898012 sorghum line

### 3.2.3 Fractionated protein extraction

For fractional extraction of proteins, half a gram of ground sorghum was defatted with excess hexanes and dried as described by Yang and Browning (2009). The following extractions were then made: water extraction (targeting sorghum albumins), sodium chloride extraction (targeting sorghum globulins) to which, 1 ml of water was added followed by continuous shaking for 2 hours at 4<sup>0</sup>C. Samples were then centrifuged at 15294 g for 15 min and supernatant collected in fresh tubes (this contained albumins). The pellet was not discarded but 1 ml water was added to it and extraction repeated for collection of supernatant two more times. The three supernatants were then pooled together (they contained albumins). Pellets were kept for the extraction of globulins. Where, 1 ml of 0.5 M NaCl was added followed by continuous shaking for 2 hours at 4<sup>0</sup>C. Samples were centrifuged at 15294 g for 15 min and supernatant collected in fresh tubes (this contains globulins). The extraction was repeated for collection of supernatant two more times and a set of three NaCl supernatants pooled together. To extract total kafirins, 1 ml of 12.5 mM sodium borate buffer (pH10.0), as previously described by Youssef (1998); 1% SDS; 2% β-Mercaptoethanol (βME) was added to the remaining pellet. The sample was then incubated with agitation at room temp for 1 hour before being centrifuged at 15294 g for 15 min at room temp. The supernatant was carefully removed to new tubes and procedure repeated twice before the 3 supernatants were pooled together. To the pooled sample, *t*-butanol was added to a final concentration of 60%. The sample was then incubated with occasional mixing at room temperature for 30 min before being centrifuged at 15294 g for 15 min also at room temperature. The supernatant containing total kafirin fraction was then collected, followed by protein concentration measurement for all three samples as described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as a standard.

### **3.2.4 Protein profiles of total kafirin fraction of sorghum using sodium dodecyl sulphate-polyacrylamide gel electrophoresis**

Protein profiles of total kafirin fraction of sorghum obtained were analysed using Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE), 15% separating gel and 4% stacking gel under reducing and non-reducing conditions according to the method of Laemmli (1970). For reducing conditions 10% 2-mercaptoethanol was added to the sample buffer. Samples were prepared by dissolving ten micrograms of protein sample in 0.5 Tris-HCl pH 6.8, 10% glycerol, 5%  $\beta$ ME and 1% bromophenol blue, following which they were heated in water bath at 100<sup>0</sup>C for 3 min before being loaded in sample wells. The electrophoresis was conducted at 150 mV for 1 hour. A broad range molecular weight standard (MW 10-170 kDa prestained protein ladder from Thermo Scientific) was used. The gel was then stained with 0.1% Coomassie brilliant Blue R250 in 40% methanol and 10% acetic acid and de-stained with 10% methanol in 7.5% acetic acid. The protein profile images were captured using a G:BOX gene documentation system (Syngene). Protein bands of interest were excised from the gels with a scalpel blade for further identification using peptide sequencing.

### **3.2.5 Peptide sequencing for the identification of isolated protein bands**

Protein bands of interest were trypsin digested using the method of Shevchenko *et al.*, 2007. The excised gel bands were de-stained using 50 mM NH<sub>4</sub>HCO<sub>3</sub>/50% MeOH, then in-gel protein reduction (50 mM DTT in 25 mM NH<sub>4</sub>HCO<sub>3</sub>) and alkylation (55 mM iodoacetamide in 25 mM NH<sub>4</sub>HCO<sub>3</sub>) was then carried out. The proteins were then digested overnight at 37<sup>0</sup>C using 5 – 50  $\mu$ L (10 ng/ $\mu$ L trypsin) depending on the gel piece size. Peptides were extracted using 50% acetonitrile (ACN)/5% formic acid (FA) and vacuum dried. Samples were re-suspended in 10  $\mu$ L 5% FA and desalted via C18 Stage Tips, followed by elution

from the Stage Tips using 70% ACN/0.1% FA containing 5 mg/mL  $\alpha$ -Cyano-4-hydroxycinnamic acid and directly spotted on to MALDI plate.

Data acquisition was performed on an Applied Biosystems QSTAR-ELITE mass spectrometer with an oMALDI source installed. Laser pulses were generated using a nitrogen laser with intensities between 11.3 – 11.7  $\mu$ J depending on sample concentration and whether single MS or MS/MS experiments were performed. Single MS spectra were acquired for 20-30 seconds and the 50 highest peaks were automatically selected for MS/MS acquisition. Tandem spectra acquisition lasted for 4 - 8 min depending on sample concentration. Argon was used as cooling gas in  $Q_0$  and as a collision gas in  $Q_2$ . The collision energy was first optimised using a 9-peptide mixture covering the scan range of 500 – 3500 Da and then automatically set during MS/MS experiments using the Information Dependent Acquisition (IDA) function of the Analyst QS 2.0 software. The instrument was calibrated externally, in TOF-MS mode, via a two point calibration using the peptides Bradykinin 1 - 7 and Somatostatin 28 ( $[M+H]^+ = 757.3992$  Da and 3147.4710 Da, respectively). Protein identification was performed using NCBI's msdb database and the Paragon<sup>TM</sup> algorithm thorough searches in Protein Pilot. An identification confidence of 95 % was selected during searches. This work was conducted at CSIR Biosciences, Pretoria.

### **3.2.6 Amino acid concentration**

Both free and bound amino acid content analysis was performed on fine milled samples according to the Official Method 994.12 of the AOAC International (2000) using a Waters Breeze HPLC with Empower software (Waters, Millipore Corp., Milford, MA) and a Pico-Tag Column (3.9 mm x 150 mm) C18. Samples (400 mg) were hydrolysed with 6 N HCL for 24 hours and then derivatised with phenylisothiocyanate (PITC) to produce phenylthiocarbamyl (PTC) amino acids. The resulting amino acids were then analysed by reverse phase HPLC using the following standards: Amino acid standard H from Pierce (Prod

no: 20088) and internal standard: L- $\alpha$ - AMINO-n-BUTYRIC ACID Sigma (A-1879). This work was conducted at the South African Grains Laboratory (SAGL) in Lynnwood, Pretoria.

### 3.3 RESULTS

#### 3.3.1. Induced protein polymorphisms in P898012 sorghum lines

Protein polymorphism as reported in Figure 3.2 refers to the variation in protein profiles which are reflected by the presence or absence of a protein band. Under reducing (Figure 3.2 A) and non-reducing conditions (Figure 3.2B) at least six monomeric kafirin bands with approximate molecular weight ranging from 18-27 kDa were observed. El Nour *et al.* (1998) described the same weight range for sorghum monomeric proteins. A number of changes in protein expression patterns of some major sorghum kafirin proteins in mutants were observed. Expression of the 27 kDa  $\gamma$ -, 24 kDa  $\alpha$ -A1 and the 22 kDa  $\alpha$ -A2 kafirins was reduced significantly in the endosperm of mutants denoted RED, BR, and SY as compared to those in others (Figure 3.2B). In T120, the 27 kDa  $\gamma$ -kafirin was over-expressed (Figure 3.2B). The mutant SY also consisted of some over-expressed  $\beta$ -kafirin proteins (Figure 3.2A and B, black arrow). The control line YEL naturally has suppressed synthesis of the  $\beta$ -kafirin proteins (Figure 3.2A, red arrow). Essentially, only kafirin monomers with similar quantity amongst all tested sorghums were observed under reduced conditions (Figure 3.2A). Data obtained from peptide sequencing of protein bands extracted from an SDS-PAGE suggested a high accumulation of kafirins in BR as observed (Figure 3.3, arrow labelled, 6). This unusually high amassing of proteins in the germ area is especially true for SY (Figure 3.3). The common and abundant sorghum oleosin protein was also detected across all embryo protein extracts of the lines analysed using peptide sequencing (Figure 3.3, arrow labelled, 5).

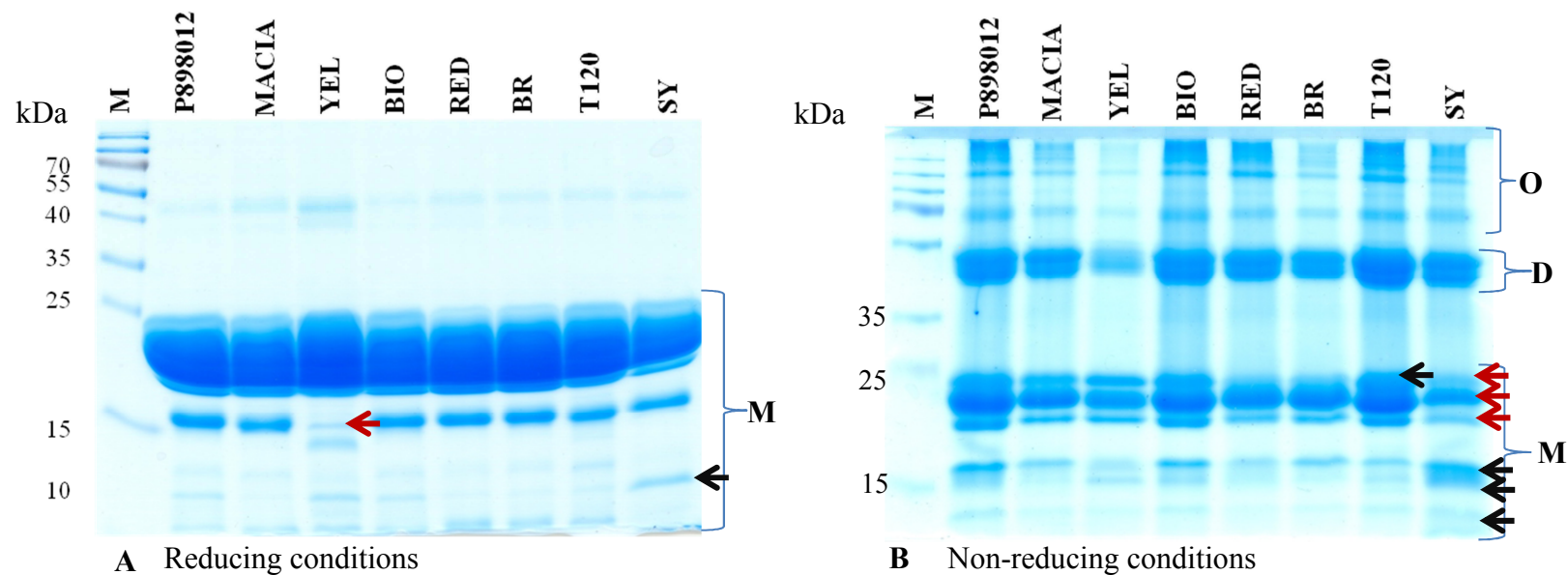
### **3.3.2. Protein variation and amino acid accumulation**

A sample as referred to in this study was constituted as an average of about 1000 crushed seeds from a single panicle of each mutant sorghum line at the M<sub>4</sub> generation. Figure 3.4 shows protein bound amino acid compositions for each of the five mutants, the parental line P898012 and two additional controls (Macia and YEL). The mutant SY was superior to all other mutants (RED, BR, BIO, T120) in the composition of lysine, arginine and aspartic acid (Figure 3.4; red arrow). Table 3.2 depicts free amino acid data showing high production of all free amino acids analysed with the exception of alpha-aminoadipic acid (Table 3.2). SY mutant had 11 times higher content of lysine (21.6 g/100g) than its parental line (1.9 g/100g) and 4-fold higher than the recommended requirements (5.2 g/100g) for a 1-2 year old child by WHO (2007) (Table 4.2). Other major changes of SY in amino acid contents were increased lysine, histidine, isoleucine, leucine and valine in essential amino acids and arginine, glutamic acid, proline, alanine, asparagine, and glutamine in non-essential amino acids. The composition of protein bound amino acids is shown in Table 4.3.

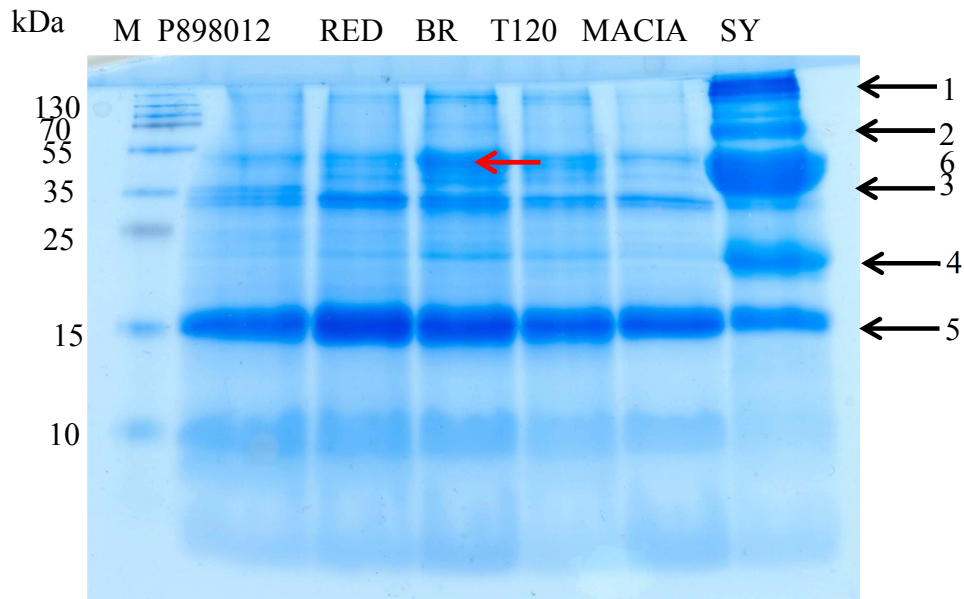
### **3.3.3 Compensatory synthesis of proteins in mutant sorghum lines with suppressed accumulation of key kafirins**

In an effort to explain the variations in kafirin protein, the study was extended to non-kafirin proteins including albumins and globulins. Variable expressions and accumulation patterns of albumin and globulin proteins across the mutant and control sorghum lines were observed. At least 7 albumin proteins and 9 globulin proteins were observed to have variable expression and accumulation patterns across the mutant and control sorghum lines analysed as shown in Figure 3.5. A further analysis of total proteins extracted from the seed pericarp of mutant and the P898012 parental sorghum also indicated that, the mutants designated RED, BR, SY and

T120, all had significantly elevated synthesis and accumulation of some undetermined proteins, which are labelled P4 in Figure 3.6.



**Figure 3.2** Representative protein polymorphisms of parental sorghum P898012 and its mutant lines. **A:** Kafirin proteins extracted from sorghum endosperms using 60% t-butanol with a reducing agent. **B:** Kafirin proteins extracted from sorghum endosperms using 60% t-butanol without a reducing agent. **M** = monomers; **D** = dimers; **O** = oligomers. Black arrow shows high band intensity. Red arrow shows decreased band intensity. Lane M is a PageRuler™ Prestained Protein Ladder (170kDa) purchased from Thermo Scientific, cat. No. SM0671.



**Figure 3.3** Representative protein polymorphisms of germ extracts of parental sorghum and mutant lines. Numbers and arrows indicate the relative position and size of variant protein profiles in seeds of mutant sorghums. Numbers (1–4) in the mutant SY and red arrow in the mutant BR represent unusual accumulation of kafirins in the germ of mutant seeds. These proteins included kafirin monomers (arrow 4), dimmers (arrow 3), trimers (arrow 2) and polymers (arrow 1) of  $\beta$ -, and  $\alpha$ -A1 kafirins. The protein band represented by the arrow (5) across all sorghum lines analysed is a sorghum oleosin protein. Lane M is a PageRuler™ Plus–Prestained Protein Ladder (10–170kDa) purchased from Thermo Scientific, cat. No. 26619.”

**Table 3.1** Proximate free amino acid composition (g/100 g) of sorghum P898012,

Amino acid	Controls			Mutants					WHO <sup>b</sup> STD
	P89 <sup>a</sup>	Marcia	YEL	BIO	BR	RED	T120	SY	
<i>Essential amino acids</i>									
lys	1.9	2.0	2.1	1.9	8.9	7.5	1.4	21.6	5.2
his	1.2	1.2	3.8	1.2	1.0	2.2	2.3	13.5	1.8
ile	0.5	0.5	0.7	0.4	2.3	1.1	0.3	24.1	3.1
leu	0.5	0.5	1.3	0.8	0.8	2.3	0.6	12.1	6.3
phe	0.0	0.0	0.0	0.6	0.5	0.7	0.0	2.8	4.6
thr	1.7	1.2	1.8	2.0	1.1	1.2	0.0	7.6	2.7
val	0.8	0.8	1.0	0.4	2.0	2.3	0.8	12.3	4.2
<i>Non-essential amino acids</i>									
arg	9.2	5.3	13.5	8.2	32.8	31.6	3.6	94.8	
asp	9.4	7.0	10.8	8.3	10.9	9.7	10.1	24.8	
ser	1.9	2.1	5.4	1.9	2.1	3.3	0.0	10.5	
glu	8.3	3.3	10.0	8.9	7.2	9.5	10.0	64.6	
pro	6.4	4.4	20.3	8.4	2.8	45.5	5.1	31.7	
gly	2.1	1.4	1.8	1.4	2.5	3.3	2.2	12.4	
ala	1.2	1.2	2.0	1.8	3.9	7.2	2.3	31.9	
met	0.5	0.0	0.4	0.4	0.6	1.0	0.0	3.7	
tyr	2.9	2.5	2.9	3.0	2.1	2.7	3.5	18.9	
asn	4.2	17.7	38.4	33.1	56.8	51.7	19.7	241.2	
β-ala	0.0	0.0	0.2	0.0	0.0	0.0	0.0	31.3	
trp	1.0	1.7	1.9	1.2	1.1	1.8	3.6	7.7	
Orn	0.4	0.3	0.4	0.2	2.0	1.4	0.4	5.3	
gln	1.9	0.0	0.0	0.0	0.0	3.0	0.0	110.7	

Marcia, YEL and sorghum mutants

<sup>a</sup>P89 = P898012 the parental line used to induce the mutations

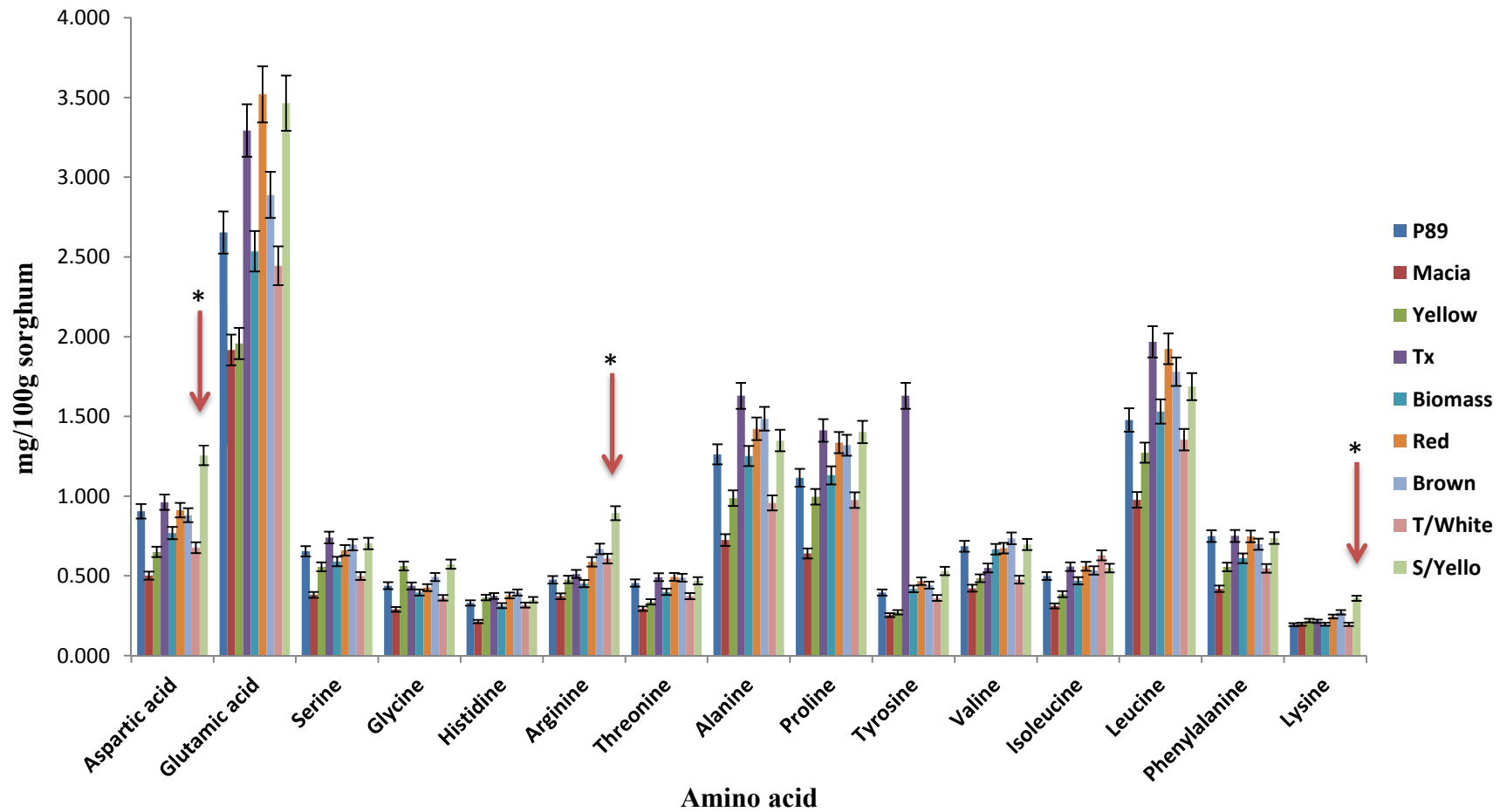
<sup>b</sup>Essential amino acid requirements of a 1-2 year old child, World health Organisation standards (WHO), 2007.

**Table 3.2**

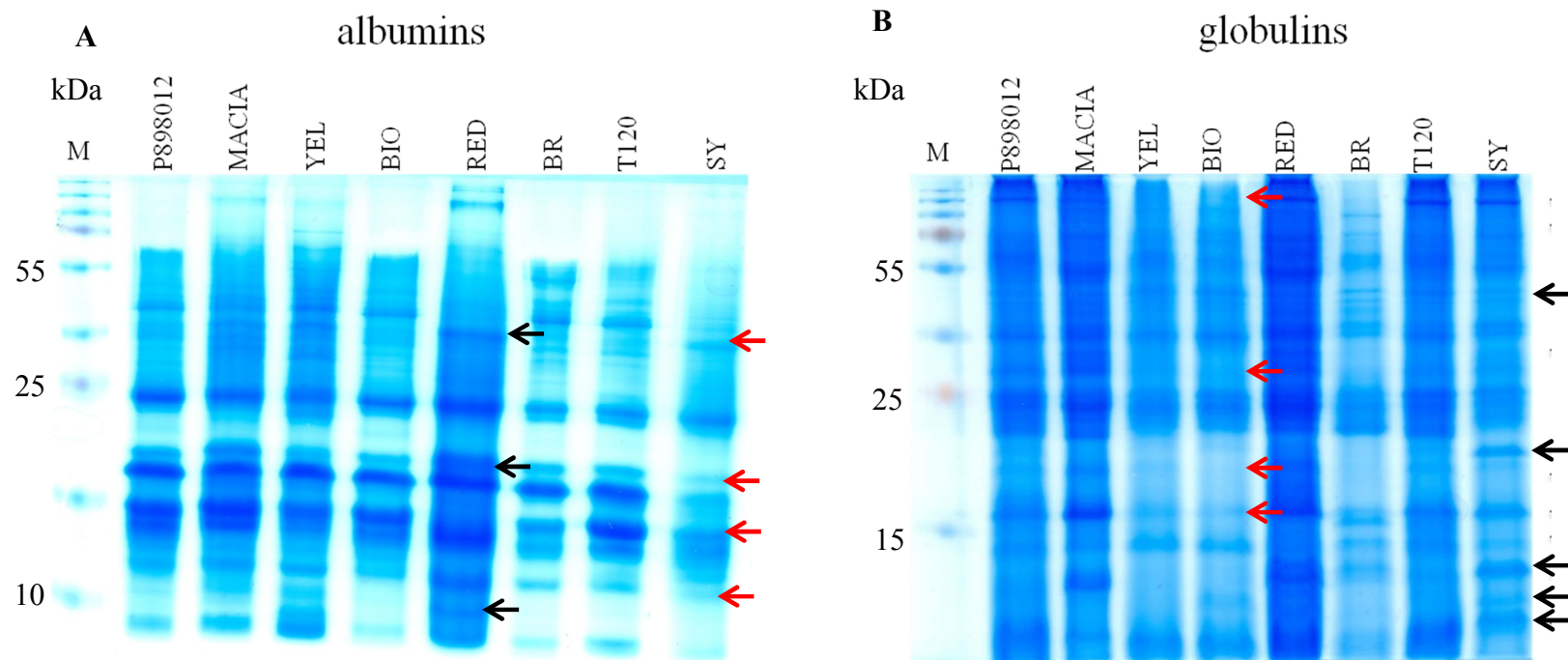
Proximate protein-bound amino acid composition (g/100 g) of sorghum P898012, Marcia, YEL and sorghum mutants

Amino Acid	Controls			Mutants				
	P89	Macia	YEL	BR	BIO	RED	T120	SY
<i>Essential amino acids</i>								
Lysine	1.3	0.7	1.0	1.5	1.3	1.4	1.0	1.3
Histidine	0.9	0.5	0.7	0.9	0.8	0.9	0.7	1.3
Isoleucine	0.3	0.2	0.4	0.4	0.3	0.4	0.3	0.4
Leucine	0.5	0.4	0.5	0.7	0.5	0.6	0.6	0.9
Phenylalanine	0.5	0.3	0.3	0.5	0.4	0.5	0.4	0.5
Threonine	0.7	0.4	0.5	0.7	0.6	0.7	0.5	0.7
Valine	0.4	0.3	0.4	0.5	0.4	0.4	0.4	0.6
<i>Non-essential amino acids</i>								
Arginine	2.7	1.9	2.0	2.9	2.5	3.5	2.4	3.5
Aspartic acid	0.5	0.3	0.4	0.5	0.5	0.6	0.6	0.5
Serine	0.7	0.4	0.6	0.7	0.6	0.7	0.5	0.7
Glutamic acid	1.5	1.0	1.3	1.8	1.5	1.9	1.4	1.7
Proline	0.4	0.3	0.3	0.4	0.4	0.5	0.4	0.5
Glycine	0.2	0.2	0.2	0.3	0.2	0.2	0.2	0.4
Alanine	1.1	0.6	1.0	1.3	1.1	1.3	1.0	1.4
Tyrosine	0.7	0.4	0.5	0.7	0.7	0.7	0.5	0.7

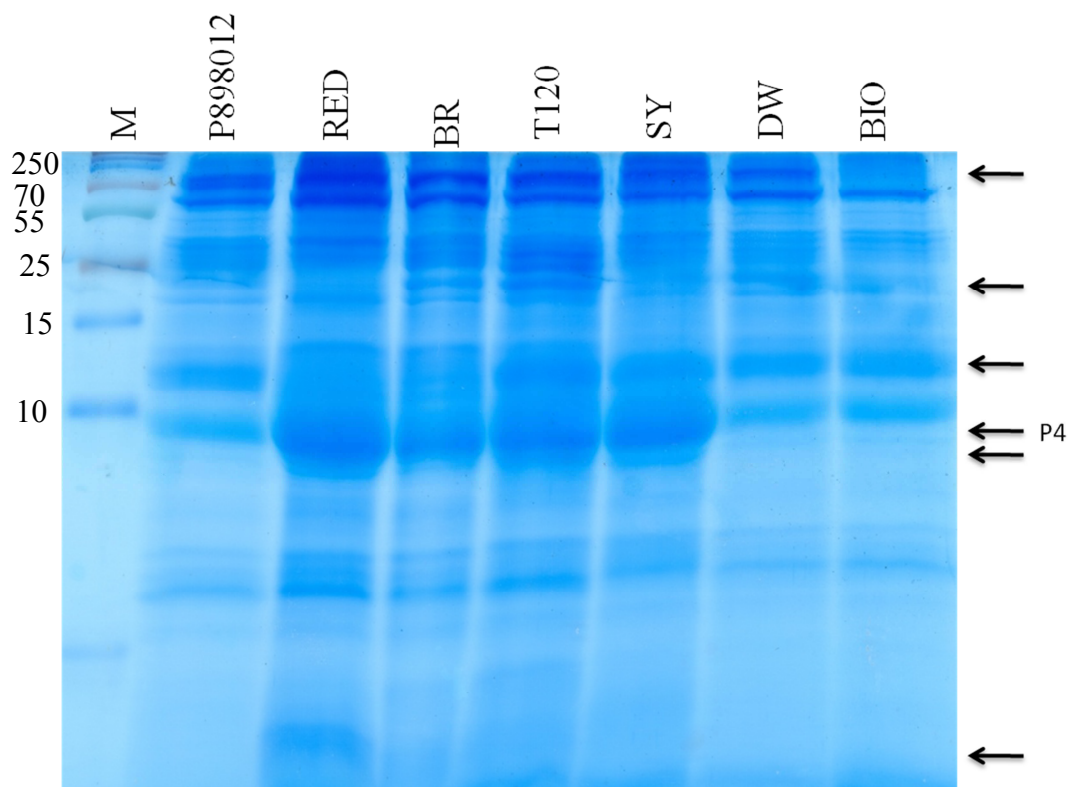
<sup>a</sup>P89 = P898012 the parental line used to induce the mutations



**Figure 3.4** Protein bound amino acids of parental sorghum P898012 and mutant lines, measured in mg/100g of sorghum flour. Each value is an average of three replicates of the samples. \*: significantly different ( $p < 0.05$ ).



**Figure 3.5** Variations in the expression pattern of albumins **A** and globulins **B** in mutant P898012 sorghum seeds. Red arrows indicate relative positions of unique down-regulation and black arrows show up-regulated proteins. Lane M is a Page Ruler™ Prestained Protein Ladder (10–170kDa) purchased from Thermo Scientific, cat. No.SM0671.



**Figure 3.6** Variations in expression patterns of proteins extracted from the pericarp of mutant P898012 sorghum seeds. Arrows indicate the relative positions where variant proteins were observed. The mutants, RED, BR, T120 and SY had particularly elevated synthesis and accumulation of proteins in the region indicated by arrow (4). (M) is a PageRuler™ Plus – Prestained Protein Ladder (10–170kDa) purchased from Thermo Scientific, cat. No. 26619.

### 3.4 DISCUSSION

In Africa, maize or sorghum is consumed at levels of up to 400-500 g/day (Van der Westhuizen *et al.*, 2010). It is therefore imperative that research is geared towards addressing nutritional shortcomings of cereals because their high rate of consumption as the main food source can lead to malnutrition in developing countries.

Analysis of mutants derived from gamma irradiation of a P898012 sorghum parental line revealed an array of induced protein polymorphisms which were associated with a significant improvement in the protein quantity and quality (balanced amino acids). The challenge therefore lay in explaining the variations in amino acid synthesis and accumulation on the basis of variant protein expression in seed storage proteins of sorghum as presented in, (Figures 3.2 – Figure 3.4), and also on the basis of any additional mechanisms that could be at play in mutant sorghum populations. As a result non-kafirin proteins were also analysed since they contribute to sorghum nutritional value, for example, albumins and globulins. This method has been used successfully in the past and in sorghum to study genetic variability using protein expression as an indicator and as a basis of traits in a study population (Elkonin *et al.*, 2010).

Under non-reducing conditions band intensities of the monomers and the number of resolving bands for dimers were significantly higher in mutant SY (black arrows) as compared to the P898012, YEL and Macia. As sample loading was consistent throughout and this therefore suggests that a lower proportion of the highly cross-linked polymeric kafirins (> 200 kDa) was formed in this mutant during protein body synthesis. Under reducing conditions only kafirin monomers could be visualised, suggesting that polymers, oligomers and dimers were reduced through disulphide bond reduction into various kafirin monomers. In YEL, a major monomeric band was missing (red arrow) when compared to the other two controls (P898012 and Macia). Presumably as a result of suppressed synthesis of that particular kafirin protein.

The most unexpected protein polymorphism described here involves a redirected accumulation of kafirin proteins in the embryo of a specific mutant sorghum designated SY (Figure 3.3). Based on knowledge that kafirins are exclusively expressed and accumulated in the endosperm of sorghum, their occurrence in the germ was not expected. Kafirins, like zeins are not known to significantly accumulate in any other plant part (Prasanna *et al.*, 2001). The exclusive expression and accumulation of kafirins in the endosperm, their relative electrophoretic positions in one dimensional SDS-PAGE, as well as the occurrence of dimers, trimers and polymers of kafirins in the reduced and unreduced state is well documented (Elkonin *et al.*, 2010). As far as we know, no major kafirin synthesis and accumulation in the germ of sorghum or in a close relative such as maize has been reported (Ahmad *et al.*, 2011; Prasanna *et al.*, 2001). SY presumably carries a mutation(s) leading to defective signal peptides, which in turn redirect the deposition of some kafirins into the embryo. Coleman *et al.*, 1997 reported similar results involving a *floury-2* mutant (*fl-2*), which encodes a 24 kDa precursor polypeptide for a 22 kDa  $\alpha$ -zein protein that had a defective signal peptide. The defective signal peptide was shown to redirect the mutant peptide towards accumulating in the membrane of the endoplasmic reticulum instead of inside the lumen. The defect in the signal peptide also altered protein body morphology, decreased storage protein synthesis, and engendered the floury endosperm.

A widespread global increase in the synthesis and accumulation of amino acids was observed (Figure 3.4). An over-expression of some uncharacterised proteins in the pericarp of the mutants RED, BR, T120 and SY compared to the parental control line P898012 was also observed (Figure 3.6). Collectively, the data suggests that induced mutations in sorghum can be a useful tool in the improvement of sorghum nutrition, especially through an increased balance of essential and conditionally essential amino acids. Free amino acids contribute to total utilisable nitrogen in human and animal diet. Initial changes of plasma free amino acid

concentrations for example, following feeding are assumed to be contributed in part by free amino acids since free amino acids are more easily absorbed than peptides, and protein-bound amino acids which have to be digested first (Agostoni *et al.*, 2000; Carlson, 1985; Pamblanco *et al.*, 1989).

The SY mutant accumulated high amino acid content including the essential amino acids lysine, tryptophan, threonine, and methionine and also the conditionally essential amino acids, arginine, cystathionine (an intermediate in the synthesis of cysteine), glycine, glutamine, histidine, proline, serine and tyrosine. These levels of free amino acids especially the most limiting essential amino acid lysine have not been previously reported in sorghum. The levels are significantly higher than those previously reported for one commercial sorghum genotype (MASSA 03, 0.0026 mg.g<sup>-1</sup>) and nine ICRISAT high-lysine genotypes which averaged 0.0068 – 0.0052 mg.g<sup>-1</sup> (Vendemiatti *et al.*, 2008). The composition of many of the free amino acids in SY mutant sorghum was also comparable and in some cases superior to free amino acids found in many powdered and liquid infant formulas like Nutrition Premium, Aptamil, Nidina, Nativa, Vivena, Eulac, Primi Giorni and Isomil as previously reported in comparisons with human milk (Agostoni *et al.*, 2000).

The pattern of increase in protein bound amino acids, particularly in mutant SY is very consistent with a mutation(s) in a biosynthetic pathway associated with the synthesis of groups of amino acids. The essential amino acids lysine, threonine, methionine and isoleucine are derived from a strictly regulated metabolic pathway and form part of the aspartate “family” of amino acids (Ferreira *et al.*, 2005; Ferreira *et al.*, 2006; Vendemiatti *et al.*, 2008). Aspartate can act as a precursor for at least two known main synthetic pathways. The first of which results in the synthesis of asparagine via the transfer of nitrogen from the amide group of glutamine, while the second pathway is anchored in aspartate acting as a precursor to the synthesis of the aspartate “family” of amino acids which include lysine, threonine,

methionine and isoleucine (Ferreira *et al.*, 2005). This is in line with the observed increase of glutamine, asparagine, aspartic acid, lysine, threonine, methionine and isoleucine in the mutant SY, thus suggesting a mutation in the asparagine/aspartate metabolic pathway.

Efforts to find cereals with improved protein quality have been on-going since the early 1960's. Early endeavours included reports of naturally occurring high lysine maize (Mertz *et al.*, 1964). The enhanced lysine content of these mutant grains was attributed to low levels of lysine poor storage proteins (prolamins), referred to as zeins in maize. Discovery of these mutants generated major interest due to their nutritional value. However, high lysine mutants are known for their agronomic inferiority, which includes a soft or floury endosperm as shown in Figure 2.2f, Chapter 2, which might result in increased susceptibility to insects, low yields and mechanical damage (Mertz *et al.*, 1964). These agronomic problems inhibited the cultivation of high lysine maize and sorghum.

The unusual accumulation of lysine in the mutant SY was consistent with all previously reported high lysine cereal mutants in terms of the associated negative agronomic traits including a brittle soft endosperm, low yield and small seeds of low seed weight (Tesso *et al.*, 2008). These impairments inhibit the widespread utilisation of high lysine sorghum and hamper the direct utilisation of these nutritionally valuable mutants (Doll, 1984). However, there have been advances in the field of quality protein cereals, making it possible to genetically breed out negative traits with modifier genes, to end up with a normal seed possessing high lysine. This is the case with quality protein maize (Prasanna *et al.*, 2001). The utilisation of SY mutant as a source of high lysine germplasm is therefore possible.

### 3.5 REFERENCES

- Ahmad, N., R. Sant., M. Bokan., K.J. Steadman and I.D. Godwin.** 2011. Expression pattern of the alpha-kafirin promoter coupled with a signal peptide from *Sorghum bicolor* L. Moench. *Journal of Biomedicine and Biotechnology*. **2012**:1-8. doi:10.1155/2012/752391
- Agostoni, C., B. Carratu., C. Boniglia., E. Riva and E. Sanzini.** 2000. Free amino acid content in standard infant formulas: comparison with human milk. *Journal of the American College of Nutrition*. **19**:434-438.
- AOAC. Association of Official Analytical Chemists.** 2000. Official methods of analysis in chemistry, 18th ed., AOAC international.
- Belton, P.S. and J. Taylor.** 2004. Sorghum and millets: protein sources for Africa. *Trends in Food Science & Technology*. **15**:94-98.
- Carlson, S.E.** 1985. Human milk non-protein nitrogen: occurrence and possible functions. *Advances in Pediatrics*. **32**:43-70.
- Coleman, C.E., A.M. Clore., J.P. Ranch., R. Higgins., M.A. Lopes and B.A. Larkins.** 1997. Expression of a mutant-zein creates the floury-2 phenotype in transgenic maize. *Proceedings of the National Academy of Sciences*. **94**:7094-7097.
- Da Silva, L.S., R. Jung., Z. Zhao., K. Glassman., J. Taylor and J. Taylor.** 2011. Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm texture, protein body structure and protein nutritional quality in improved sorghum lines. *Journal of Cereal Science*. **54**:160-167.

**Doll, H. and R. Welch.** 1984. Nutritional aspects of cereal proteins and approaches to overcome their deficiencies. *Philosophical Transactions of the Royal Society of London.B, Biological Sciences.* **304**:373-380.

**Elkonin, L., J. Italianskaya and I. Fadeeva.** 2010. Gel electrophoresis as a tool to study polymorphism and nutritive value of the seed storage proteins in the grain sorghum. *Intech.* **23**:463-478

**Ferreira, R.R., L.W. Meinhardt and R.A. Azevedo.** 2006. Lysine and threonine biosynthesis in sorghum seeds: characterisation of aspartate kinase and homoserine dehydrogenase isoenzymes. *Annals of Applied Biology.* **149**:77-86.

**Ferreira, R.R., V.A. Varisi., L.W. Meinhardt., P.J. Lea and R.A. Azevedo.** 2005. Are high-lysine cereal crops still a challenge? *Brazilian Journal of Medical and Biological Research.* **38**:985-994.

**Gibbon, B.C. and B.A. Larkins.** 2005. Molecular genetic approaches to developing quality protein maize. *Trends in Genetics.* **21**:227-233.

**Gillikin, J.W., F. Zhang., C.E. Coleman., H.W. Bass., B.A. Larkins and R.S. Boston.** 1997. A defective signal peptide tethers the floury-2 zein to the endoplasmic reticulum membrane. *Plant Physiology.* **114**:345-352.

**Kumar, T., I. Dweikat, S. Sato, Z. Ge, N. Nersesian, H. Chen, T. Elthon, S. Bean, B. P. Ioerger, M. Tilley, and T. Clemente.** 2012. Modulation of kernel storage proteins in grain sorghum (*Sorghum bicolor* (L.) Moench). *Plant Biotechnol Journal.* **10**:533-44. Doi: 10.1111/J.1467-7652.2012.00685.X. Epub 2012 Feb 21.

**Laemmli, U.K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. **227**:680-685.

**Lee, R.** 2011. The outlook for population growth. *Science*. **333**:569-573. doi: 10.1126/science.1208859.

**Lowry, O.H., N.J. Rosebrough., A.L. Farr and R.J. Randall.** 1951. Protein measurement with the folin phenol reagent. *Journal of Biological Chemistry*. **193**:265-275.

**Mertz, E.T., L.S. Bates. and O.E. Nelson.** 1964. Mutant gene that changes protein composition and increases lysine content of maize endosperm. *Science*. **145**:279-280.

**Pamblanco, M., M. Portoles., C. Paredes., A. Ten and J. Comin.** 1989. Free amino acids in preterm and term milk from mothers delivering appropriate-or small-for-gestational-age infants. *The American Journal of Clinical Nutrition*. **50**:778-781.

**Prasanna, B.M., S.K. Vasal., B. Kassahun and N.N. Singh.** 2001. Quality protein maize. *Current Science-Bangalore*. **81**:1308-1319.

**Sasaki, T. and B.A. Antonio.** 2009. Plant genomics: Sorghum in sequence. *Nature*. **457**:547-548.

**Shevchenko, A., J.H. Henrik Tomas., J.V. Olsen and M. Mann.** 2007. In-gel digestion for mass spectrometric characterisation of proteins and proteomes. *Nature Protocols*. **1**:2856-2860.

**Sofi, P.A., S.A. Wani., A.G. Rather and S. H. Wani.** 2009. Manipulation for the nutritional fortification of maize. *Journal of Plant Breeding and Crop Science*. **1**:244-253.

- Tesso, T., B.R. Hamaker and G. Ejeta.** 2008. Sorghum protein digestibility is affected by dosage of mutant alleles in endosperm cells. *Plant Breeding*. **127**(6): 579-586.
- Van der Westhuizen, L., G.S. Shephard, J.P. Rheeder and H.M. Burger.** 2010. Individual fumonisin exposure and sphingoid base levels in rural populations consuming maize in South Africa. *Food and Chemical Toxicology*. **48**:1698-1703.
- Vendemiatti, A., R.R. Ferreira., L.H. Gomes., L.O. Medici and R.A. Azevedo.** 2008. Nutritional quality of sorghum seeds: Storage proteins and amino acids. *Food Biotechnology*. **22**:377-397.
- Yang, L., J.D. Browning and J.M. Awika.** 2009. Sorghum 3-deoxyanthocyanins possess strong phase II enzyme inducer activity and cancer cell growth inhibition properties. *Journal of Agricultural and Food Chemistry*. **57**:1797-1804.
- Youssef, A.M.** 1998. Extractability, fractionation and nutritional value of low and high tannin sorghum proteins. *Food Chemistry*. **63**:325-329.

**CHAPTER 4 EFFECT OF GAMMA IRRADIATION ON THE NUTRITIONAL  
PROFILE OF SORGHUM *BICOLOR* L. MOENCH**

This chapter has been submitted in part for publication as a book chapter by Wageningen Academic Publishers, The Netherlands.

**Mbambo, Z., Minnis-Ndimba, R., Pineda, C., Ndimba, B., Bado, S., Lin, J., Chikwamba, R and Mehlo, L.** 2013. Proton induced X-ray emission (PIXE) and electron microscopy analysis of seeds of gamma irradiation induced mutants of sorghum. Tomlekova, N. Editor. Mutagenesis: Exploring Novel Genes and Pathways. *Wageningen Academic Publishers*, The Netherlands.

## 4.1 INTRODUCTION

Many communities residing in marginal agro-ecological zones of Africa are vulnerable to food insecurity and malnutrition because the only staple cereal that thrives in such harsh conditions, sorghum, is nutritionally challenged (Belton and Taylor, 2004). Furthermore, global changes in atmospheric CO<sub>2</sub>, temperature and light intensity, drought and poor soil nutrient status is likely to negatively impact future food security in the Sub-Saharan regions (Graham *et al.*, 2001; Nestel *et al.*, 2006). More than a billion people in the semi-arid regions of the world rely on sorghum to provide dietary calories and protein because other crops like maize, wheat and rice, for example, are not as well adapted to the prevailing marginal agro-ecological conditions (Belton and Taylor, 2004).

Despite sorghums' proven resilience in marginal ecologies and its immensely untapped nutritional and commercial potential, a staple diet consisting only of sorghum is impoverished, and would lead to malnutrition. Sorghums' protein, as indeed, other cereals (rice, maize, wheat), does not provide a balanced source of protein and calories (Cakmak, 2008). This is because its predominant grain storage proteins, called prolamins are deficient in many essential amino acids like lysine, methionine and tryptophan (Mesa-Stonestreet *et al.*, 2010). In addition, sorghum's protein is less digestible upon cooking. Comparative studies have revealed the following digestibility values for important cereals: 46% (sorghum), 81% (cooked wheat), 73% (cooked maize), and 66% for cooked rice (Oria, *et al.*, 2000). The grain is also deficient in vitamin A and its iron and zinc content has limited bioavailability because these micronutrients are bound by phytate (Gibbon and Larkins, 2005; Kumar, *et al.*, 2012).

In order to realise the full genetic potential of this remarkable C<sub>4</sub> plant, several of these shortcomings of sorghum have to be addressed through various strategies of genetic engineering, induced mutations and plant breeding (Casas *et al.*, 1993). One impediment to genetic engineering and plant breeding is the limited number of traits that can be addressed simultaneously or more specifically for plant breeding, the lack of genetic diversity within crossing populations of sorghum (Fowler, 1990). An additional complication is the general difficulty of transferring traits that are governed by multiple genes using plant breeding or genetic engineering (Peterson, *et al.*, 2009).

The use of physical and chemical agents that induce genetically heritable mutations is one of the most effective ways of achieving trait variations in crops (Singh and Detta, 2010). Many crops with improved economic value have been developed through induced mutations. Mutant barley varieties that thrive in altitudes of up to 5,000 meters in the inclement highlands of Peru with 52% yield increase between 1978 and 2002 translated to significant increases in socio-economic impact (Patel *et al.*, 1980; Gómez-Pando *et al.*, 2009). In addition, crops developed through induced mutations are not regulated under GMO (genetically modified organisms) guidelines which can be seen as an advantage of induced mutations over genetic engineering (Longman, 1999; Shu, 2009). This is because induced mutation is a process of speeding up natural genetic processes that occur in nature under natural evolution, a phenomenon referred to as spontaneous mutations (Medina *et al.*, 2005).

In Chapter 2, the impact of gamma irradiation on grain physicochemical properties and its implications on nutrition was investigated and compared to parental line, P898012. While grain weight was adversely affected by induced mutation, polyphenol and antioxidant content was improved. In Chapter 3, some lines showed major improvement in lysine content compared to the parent (P8998012). The levels reported in this study were far more superior to those reported for transgenic lines in a similar study (Da Silva *et al.*, 2011). In this

Chapter, transmission electron microscopy was used to study protein body structure and its implications on digestibility. In addition, the spatial distribution of elements within the mutant sorghum grain was investigated and compared to that of the parental line. The mutagenic population targeted for analysis displayed high mutation frequency as shown by numerous phenotypic mutations (visible to the naked eye) indicative of several desirable nutritional traits.

The mutant seed phenotypes selected included non-tannin white sorghum (designated T120), a low yielding, soft endosperm, lemon yellow tannin sorghum of low seed density (designated SY), a mutant with seeds whose pericarp is red (designated RED), a mutant sorghum whose seeds have a brown pericarp (designated BR) and a white tannin sorghum mutant producing many tillers (designated BIO). The mutants used in this study were M<sub>4</sub> generation. Three additional sorghum lines were used in the analysis (specified as control lines for this study). These control lines included, Tx430, a high anthocyanin black sorghum (Awika *et al.*, 2005); Macia, a white food-type non-tannin sorghum line (Dlamini *et al.*, 2007) and SK5912, a yellow endosperm, malting sorghum line (Alhassan *et al.*, 2010) designated YEL in this research.

## 4.2 MATERIALS AND METHODS

### 4.2.1 Protein digestibility

The nutritional quality of a protein depends on its amino acid content, its digestibility and bioavailability of the amino acids. To distinguish between highly and lowly digestible lines, a digestibility assay, modified from Aboubacar *et al.* (2003) was performed. Fifty mg of seed from each sample was ground and added to 1 mL pepsin solution (20 mg pepsin/mL 0.1M KH<sub>2</sub>PO<sub>4</sub>, pH 2) in a 1.5 mL tube. The mixture was vortexed and incubated at 37°C for 2 hours, with shaking at 23,897 g. The reaction was stopped by adding 100 µL of 2N NaOH to each sample. Samples were centrifuged at 20,817 g for 10 minutes, following which, the supernatant was discarded and the pellet resuspended in 1 mL 0.1 M phosphate buffer, pH 7. The resuspension was centrifuged again at 20,817 g for 10 minutes, supernatant discarded, and the pellets washed with 1 mL double distilled H<sub>2</sub>O. Following this, the pellets were centrifuged again at 20,817 g for 10 minutes, supernatants discarded, and protein extraction carried out. To extract proteins, the pellets remaining after pepsin digestion were incubated for 1 hour at 37°C in a water bath with 0.5 mL extraction buffer (12.5 mM sodium tetraborate pH 10; 1% SDS w/v; 2% βME). Samples were centrifuged at 10,621 g for 10 minutes at 4°C. From the middle layer of supernatants, 200 µL was transferred to a clean 1.5 mL tube and used in SDS-PAGE analysis, section 3.2.4.

Images were captured using a G:BOX gel documentation system (Syngene). The intensity of the undigested kafirin bands were measured using GeneTools software (Syngene), where by, higher band intensity indicated lower protein digestibility.

### 4.2.2. Total protein extraction

Whole seed powder was obtained by grinding the sorghum seeds in liquid nitrogen. Powder (200 mg) was placed into eppendorfs and 1 mL protein extraction buffer (0.0625N Tris-HCl, [pH 6.8]; 3.3% SDS (w/v); 5% (v/v) 2-βME; 10% glycerol, 0.002% bromophenol blue) and

the proteins extracted in accordance with previous research in sorghum and maize (Hamaker *et al.*, 1987). Samples were mixed for 10 minutes by vortexing, followed by 2 hours of vigorous stirring at room temperature. The samples were then centrifuged for 15 min at 20,817 g at room temperature before the supernatant was transferred to new tubes, and heated to 95°C for 5 minutes before being stored at -20°C for further analysis.

#### **4.2.3. Fractionated protein extraction**

Fractionated protein extraction was carried out as previously described in Chapter 3 (Section 3.2.3).

#### **4.2.4 Transmission electron microscope (TEM) analysis**

For TEM analysis seeds were sliced transversely into pieces (1-2 mm long) with a razor blade. The endosperm powder was scooped out and fixed in 2.5% glutaraldehyde (0.075 M phosphate buffer, pH 7.4) for 2 h, dehydrated in a graded aqueous acetone series, and then infiltrated with Spur's resin. Sections were stained with aqueous uranyl acetate and further stained in Reynold's lead citrate and then examined using a JEOL JEM 2100F field emission electron microscope (Tokyo, Japan). This method of analysis has been used successfully in previous research of a similar nature (Da Silva *et al.*, 2011).

#### **4.2.5 Elemental distribution**

Using a nuclear microprobe, 9 elements were mapped and quantified. These include, P, K, Cu, Na, Cl, Fe, Zn, Ca and S using a nuclear microprobe at the Materials Research Group, iThemba LABS, South Africa. A detailed description of the nuclear microprobe setup at iThemba Labs was previously outlined by Prozesky *et al.* (1995). Representative samples from each sorghum line were used to examine possible differences in elemental distribution. The grains selected for Micro-PIXE analysis were first embedded in a commercial resin (EpoFix, Struers) and then longitudinally sectioned with a rotating diamond-coated blade. The samples were then photographed using a stereomicroscope to provide a pictorial

reference for sample identification and to aid subsequent interpretation of the elemental maps generated. To handle the samples, no metallic implements were used, so as to avoid any inadvertent risk of extraneous contamination. To avoid charge build-up during PIXE measurements, the cut surfaces of the samples were then coated with carbon using a Balzers sputter coater. The analysis was performed using a proton beam of 3.0 MeV energy and a current of ~100 pA, focused to a 3 x 3  $\mu\text{m}^2$  spot and raster scanned over a sample area of ~50 x 124  $\mu\text{m}^2$  using square scan patterns with a variable number of pixels. The two complementary techniques: proton-induced X-ray emission (PIXE) and proton backscattering spectrometry (BS) were simultaneously used in an event-by-event mode. The X-ray spectra were detected with a high purity Ge detector using an external 125  $\mu\text{m}$  Be absorber to shield the detector from backscattered protons and to attenuate X-rays from major light elements. Quantitative elemental maps were then obtained using the Dynamic Analysis method and as part of the data processing by GeoPIXE II software (Ryan, 2000).

#### **4.2.6 Statistical Analysis**

The data was analysed by one-way analysis (ANOVA) at confidence of ( $p < 0.05$ ). Means were compared by Fisher's least significance difference (LSD) test. The calculations were performed using the Statsgraphics Centurion XV (Stat Point Herndon, VA).

### **4.3 RESULTS**

#### **4.3.1. Protein digestibility and free amino acid content**

SDS-PAGE was conducted on sorghum samples to compare the band patterns of the undigested and digested protein samples. Figures 4.1 a, b and c are representative SDS-PAGE gels of seven sorghum lines (5 mutants plus 2 controls) before pepsin digestion and after digestion. The SDS-PAGE band patterns of the undigested samples in Figure 4.1 were similar

across the lines (controls and mutants). After proteins from the same lines were digested for 2 hours using pepsin, non-kafirin protein bands could not be visualised at the resolution of the SDS-PAGE, leaving mostly  $\alpha$ -kafirins visible (Fig. 4.1 a, b & c). The amount of  $\alpha$ -kafirin remaining after 2 hours of pepsin digestion was subsequently quantified using densitometry scans. Table 4.1 shows the percentages of protein concentrations and of standard pepsin digestibility of the control and mutant lines (the corresponding densitograms are shown in Appendix A). Protein concentrations in the samples ranged from 8.38-16.08%. *In vitro* digestibility values for the mutants were 62% for BIO, 89% for T120, 65% for the BR, 64% for SY and 72% for RED. The controls showed moderate digestibility with P898012 at 53% and YEL at 64%. Of all the mutants, T120 had digestibility 20% higher than that of the parental line. However, one other line SY had high digestibility (82%) which is significantly higher than that of parental line (53%). The lines with higher protein concentrations had higher digestibility values. For example, SY which had the highest protein content (16.08 %) had a high *in vitro* digestibility value (82%), this is also true for RED which had the second-highest protein content (15.09 %), and its digestibility was 72%.

#### **4.3.2. Transmission electron microscope**

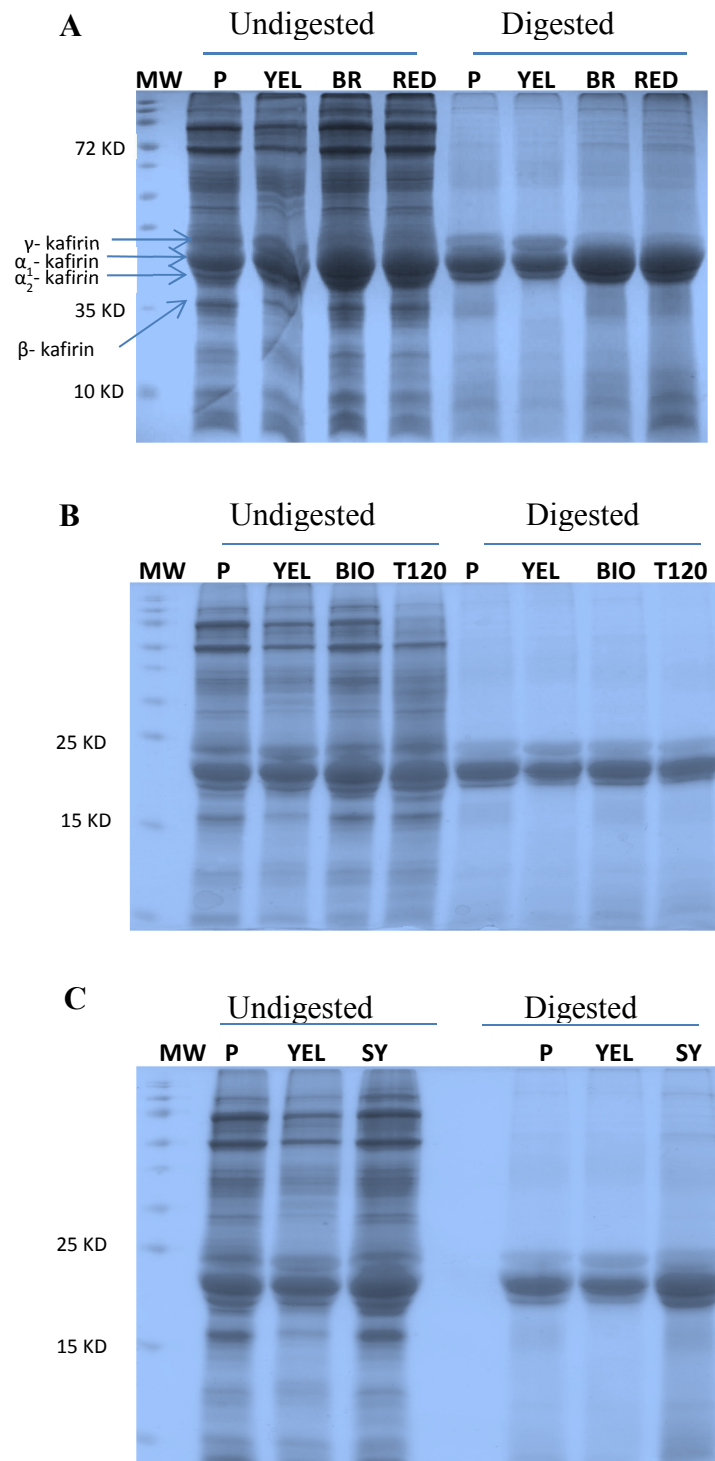
Using TEM, the ultra-structure of protein bodies of mutant sorghum grains was studied. The three notable changes to the structure of protein bodies were invaginations, shape and size (Figure 4.2). Mutants SY, RED, and BIO had invaginations on their surfaces (indicated by black arrows in SY mutant). These invaginations were extensive, deep and almost permeating to the core of the SY protein bodies. Mutant RED invaginations were also extensive; however they appeared to be confined to the surface layers of the protein bodies. Mutant BIO's protein bodies had mild invaginations confined to the surface layers of the protein body. Mutants BR, T120 and the parental P898012 lines had protein bodies with very few invaginations. As far as the shape of the protein bodies was concerned, the mutant BIO and the parental P898012

had rounded protein bodies whereas SY, RED, BR and T120 all had irregular shaped protein bodies. Mutants RED and SY had the smallest size of protein bodies whereas the biggest protein bodies were observed in mutant BR.

#### **4.3.3. Elemental distribution**

PIXE analysis allowed for the detection and quantification of Cl, K, Ca, Cu, S, Mn, P, Fe and Zn. Only the elemental maps for K, P, Fe and Zn are shown (Figure 4.3 and 4.4). The rest of the maps are shown in appendix C. The results indicate that Fe and Zn preferentially accumulated in the vacuoles of cells in the germ scutellum tissue of the mutants and the parental P898012 sorghum line (Figure 4.3). Trace amounts of zinc were also localised in the vacuoles of the pericarp and epicarp cells for all. Mutants RED, BR and BIO accumulated significantly higher concentrations (0.09%/100g) of Fe than the parental line P898012. Mutants SY, BR and BIO all accumulated significantly higher zinc in mutants, with the highest amount recorded in SY.

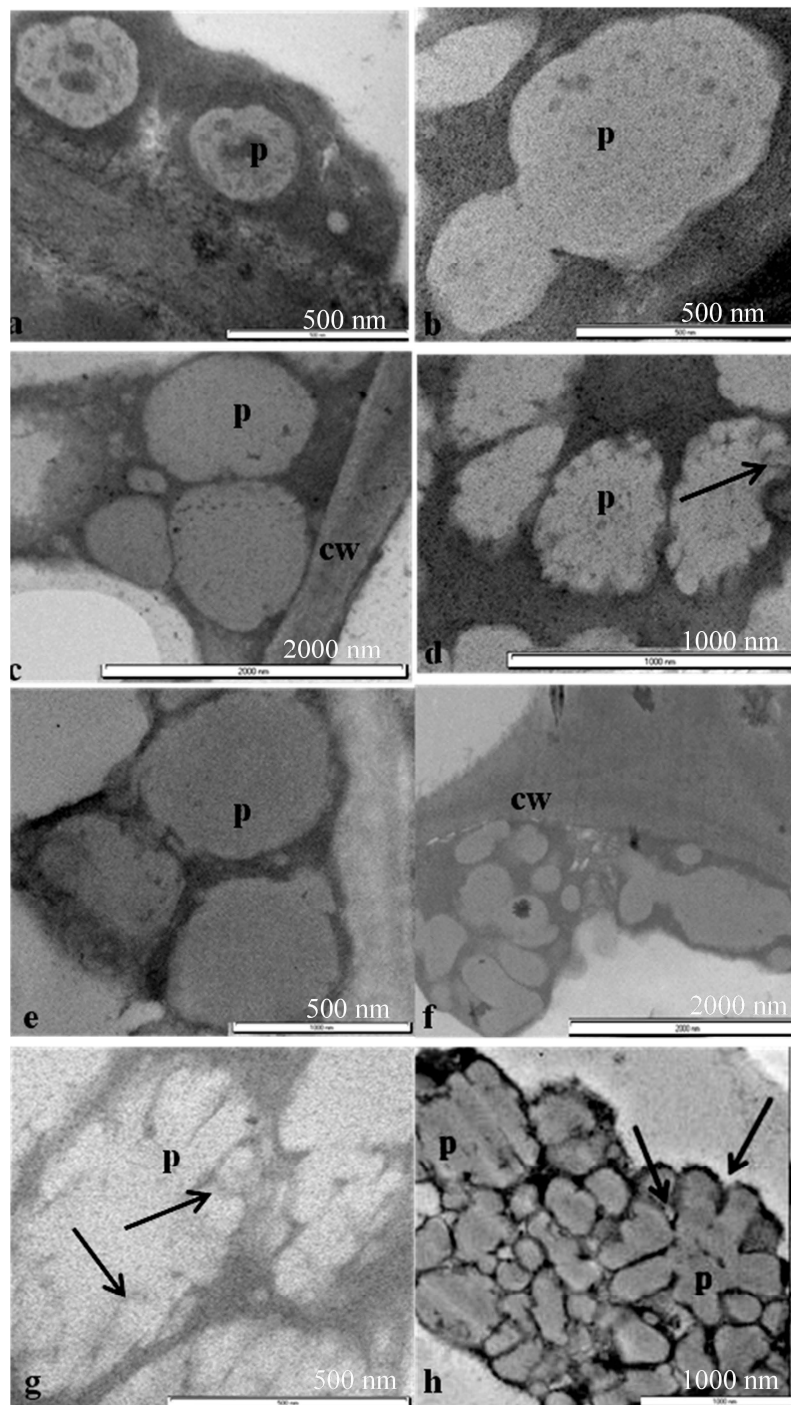
Similarly, the macroelements K and P were also predominantly localised in the vacuoles of the scutellum tissue of the sorghum germ. Traces of K were also mapped to the pericarp of RED, BR and BIO mutants (Figure 4.5). The mutants RED and BIO similarly accumulated trace amounts of K in the endosperm. In the mutant SY, two distinct layers of trace K localisation could be identified in the epicarp and pericarp of vacuoles in cells of the scutellum tissue. Mutant T120 accumulated the lowest amounts of K and P with the lowest amounts recorded for P when compared across all the mutants and the control parental line P898012 (Figure 4.5). The detected levels of (K and P) were higher than those of (Fe and Zn) across all mutants and the control parental P898012 line (Figure 4.4 and Figure 4.5).



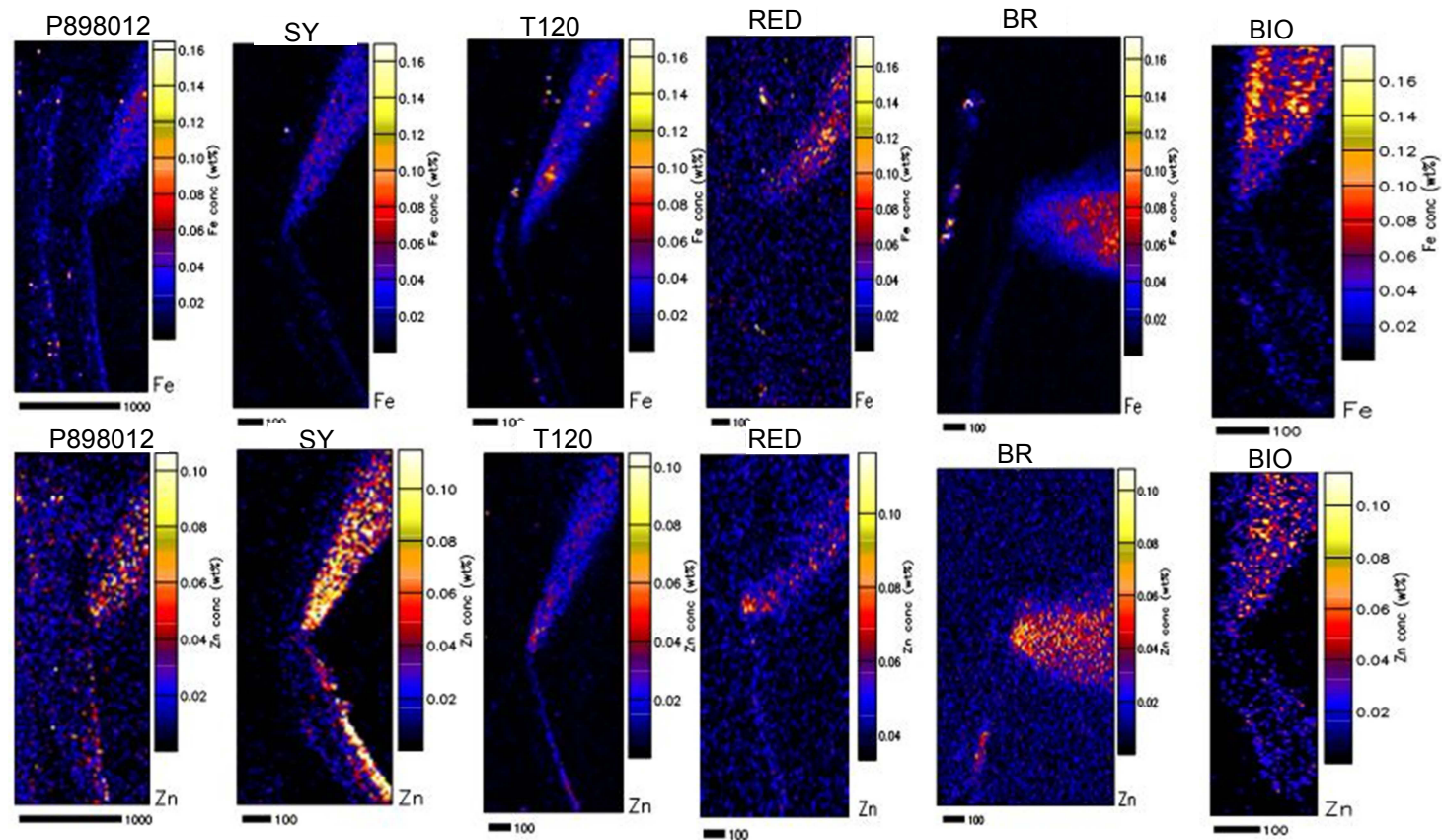
**Figure 4.1** Representative SDS-PAGE gels for digestibility assay analysis. **A:** undigested and digested proteins of P898012 (P), BR and RED mutants, **B:** undigested and digested proteins of P898012, BIO and T120, **C:** undigested and digested proteins of P898012, YEL, SY. The controls are parental line P898012 and YEL. Tracks: M: Molecular weight marker, Page Ruler™ Prestained Protein Ladder (10–170kDa) purchased from Thermo Scientific, cat. No.SM0671.

**Table 4.1** Percentages of protein concentration and digestibility by pepsin of sorghum P898012, YEL and mutants lines.

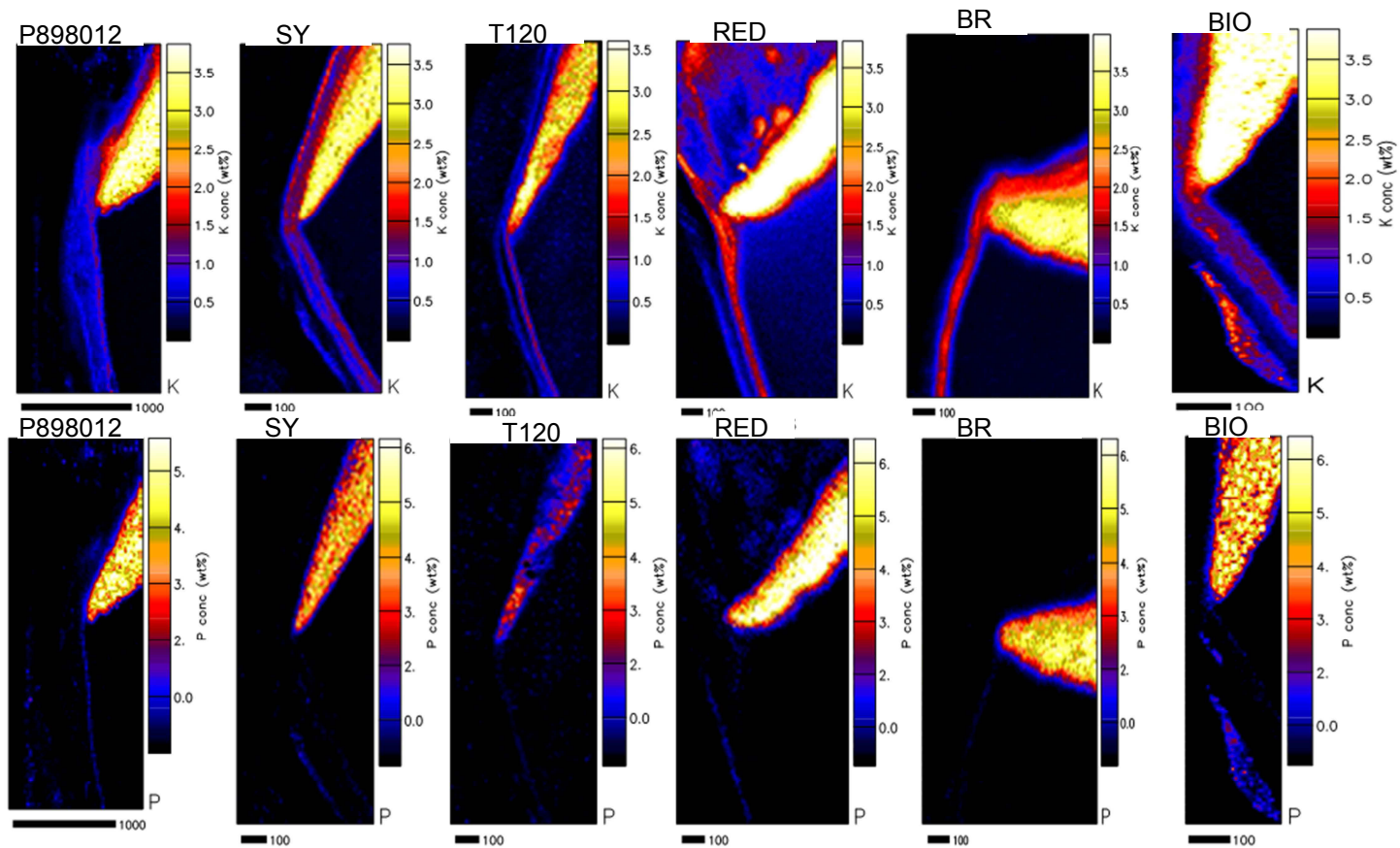
<b>Sample</b>	<b>Protein Concentration (%)</b>	<b>Standard pepsin digestibility (%)</b>
<b>P898012</b>	12.33	53.03
<b>YEL</b>	10.44	64.43
<b>BR</b>	14.47	65.08
<b>RED</b>	15.09	72.36
<b>BIO</b>	12.00	62.25
<b>T120</b>	10.91	89.05
<b>SY</b>	16.08	82.00



**Figure 4.2** Representative TEM images of sorghum protein bodies preparations a) P898012; b) YEL; c) BIO; d) RED; e) BR; f) T120; g) SY; h) HD; cw = cell wall; p = protein body, arrows show invaginations.



**Figure 4.3** Elemental distribution maps for Fe and Zn in the germ area of sorghum P898012, and sorghum mutants.



**Figure 4.4** Elemental distribution maps for K and P in the germ area of sorghum P898012, and sorghum mutants.

#### 4.4 DISCUSSION

The major objective of this study was to determine the effect that induced mutations, specifically gamma irradiation, has on nutritional and agronomic traits of sorghum. Protein digestibility is commonly used as an indicator of protein quality in sorghum as it measures the availability of a protein during digestion (Polleti *et al.*, 2004). In addition to digestibility, the nutritional quality of a protein also depends on its amino acid content and the bioavailability of the amino acids. A major factor determining bioavailability besides digestibility of a protein is its metabolism in the gut prior to absorption into the blood stream and the eventual assimilation in the cells (Aboubacar *et al.*, 2003).

The gel-based assay is routinely used for analysing protein digestion by pepsin (Aboubacar *et al.*, 2001; Nunes *et al.*, 2004). Sorghum has a relatively low digestibility when compared to other grains (Oria *et al.*, 2000). The method is reliable, requires a smaller amount of material and can accommodate more samples than another commonly used batch procedure (Mertz *et al.*, 1984). With the gel based assay, the types of undigested kafirins can be visualised, thus allowing for the estimation of the percentages digested over time. The method has also been successfully used to separate digested sorghum proteins (Aboubacar *et al.*, 2001; Bean, 2003), wheat glutenins (Kasarda *et al.*, 1998) and various other plant proteins. In this study, the differences in digestibility between the parental line and mutant sorghum lines were easily detected using this assay.

The results obtained suggests that high molecular weight components of the insoluble protein fraction, including glutelins (35–100 kDa), were readily digested, while the smaller kafirins (18–27 kDa) were more resistant (Figure 4.1a). Further gel analysis (Figure 4.1a) revealed that various kafirins  $\alpha$ ,  $\beta$ , and  $\gamma$ , were digested at different rates i.e.  $\beta > \gamma > \alpha$ . The digestion value for the T120 was approximately 40% higher than that of parental line (P898012). Of the five digested mutants, the BIO had the lowest digestibility value (62%). Amongst the

lines used as controls parental line (P898012) had lower digestion values as compared to the yellow sorghum (YEL). This is because P898012 is a type II tannin line as revealed by a bleach test for the presence of tannins (Figure 2.1, Chapter 2). Tannins are well-known for reducing sorghum protein digestibility as a result of binding and precipitating proteins including kafirins (Duodu *et al.*, 2003). In line with this observation, the non-tannin mutant (T120) had one of the highest digestibility values (89%). Relatively low tannin-protein complexes result in higher *in vitro* protein digestibility (IVPD). Emmambux and Taylor, (2003) reported on the affinity of tannins to bind kafirin proteins resulting in low protein digestibility. Likewise, Nguz and Huyghebaert (1998) using 8 different sorghum cultivars with diverse colours of pericarp and tannin contents found that a high level of tannins in sorghum grain reduces their protein digestibility for the same binding reasons stated above.

Mutant SY had a lower IVPD than the other mutants Table 4.1. This may be due to the higher tannin content in SY mutant (Table 2.3, Chapter 2). However, RED which showed the lowest IVPD did not necessarily have the highest tannin content. This is contrary to previous work which indicated that tannins bind and precipitate proteins thereby reducing their digestibility (Serna-Salvidar and Rooney, 1995; Taylor *et al.*, 2007). It is however important to note that a combination of factors influence protein digestibility, including, endosperm texture, protein body structure, amylose and amylopectin content. It is therefore possible that for IVPD to improve regardless of an enhanced tannin content.

Data on the amino acid composition makes it possible to evaluate nutritive value of protein. Lysine is the most limiting amino acid in sorghum protein. The low lysine content in sorghum is attributed to the major storage protein, kafirin being essentially free of lysine with the exceptions of two lysine-rich native Ethiopian cultivars (IS11167 & SI11758) (Singh and Axtell, 1973). Both these lines are known as ‘low prolamins’ mutants in which kafirins are

reduced to 50% resulting in compensatory increases in other lysine-rich proteins and free amino acids with up to 40-60% more lysine in the grain.

TEM revealed characteristic protein bodies for the parental line (P898012). They appeared round,  $\pm 2 \mu\text{m}$  in diameter, with internal concentric ring arrangement (Figure 4.2a) as described by Adams *et al.* (1976). In typical sorghum protein bodies, highly cross-linked kafirin proteins ( $\gamma$  and  $\beta$ -kafirins) are found at the protein body periphery and appear as dark-staining inclusions in the form of concentric circles within the interior of protein body (Oria *et al.*, 1995). In contrast, the mutants (Figure 4.2c-g) showed substantial modifications in peripheral endosperm texture and protein body structure. Protein body margins were slightly invaginated (folded) (Figure 4.2c-g, black solid arrow). The characteristic internal concentric circle structure of typical sorghum protein bodies was absent. However, these modifications were not similar to those of, high digestibility mutant (Figure 4.2h) where the protein bodies are highly invaginated with deep folds (Oria *et al.*, 2000).

It appears that gamma-irradiation of P898012 had a major effect on endosperm texture and protein body structure, which may have resulted in the improved digestibility of the mutants. The improved digestibility of mutant sorghum lines with invaginated protein bodies has been attributed to the increased body surface area and the subsequent easy accessibility of proteases to the more digestible  $\alpha$ -kafirin proteins (Oria *et al.*, 2000).

Sorghum YEL (Figure 4.2b) had protein bodies similar to those of the parental line (Figure 4.2a). This is also true for BR and BIO mutant which had generally round protein bodies which are typical of normal sorghum protein bodies. However, both these mutants still lacked the dark staining internal concentric circles visible in the control yellow sorghum and parental line. The similarity of these mutants to parental line was not surprising as these mutant lines had protein content and IVPD within normal range of most sorghum (Da Silva *et al.*, 2011)

Kafirins suppression, particularly involving a combination of gamma and alpha species is also reportedly associated with distorted protein bodies (Kumar *et al.*, 2012). This is a more likely explanation for the observed changes in protein bodies reported here (Figure 4.2). The protein polymorphisms described in Chapter 3 and published in Mehlo *et al.* (2013) also indicates suppression of kafirins. In addition, the floury endosperm, as observed in mutants RED, BR and SY, is also a trait attributed to a discontinuous protein matrix, smaller and fewer protein bodies and loosely packed starch granules with air-filled spaces that diffract light (Rooney and Miller 1982). Our results are in line with this explanation. The starch bodies of the mutants RED, BR and SY for example, as shown in Figure 2.4 of Chapter 2, are round in shape, and thus leave large spaces in between when packing. Such large spaces contribute towards refracting light as previously reported (Oria *et al.*, 2000).

Elemental distribution varied widely between tissue types. The highest amount of Fe and Zn were located in the germ area and lower concentrations in the pericarp and endosperm area (Figure 4.3). This co-localisation was also valid for the spatial distribution of P and K which approximated the repartitioning of Fe and Zn. However, small inconsistencies could be observed on the distribution of K. Potassium was detected inside the walls of aleuronic cells in addition to the above mentioned localisation. The mutants had higher levels and wider distribution of both Fe and Zn when compared to the control.

Fe is of interest because of its central role in improving crop yields and in human nutrition (Jeong and Guerinot, 2009). Still, mechanisms of subcellular compartmentalisation of iron in graminaceous plants are poorly understood. PIXE maps revealed that most of the Fe is found in the scutellum area of the germ and relatively lower amounts may be present in the pericarp (Figure 4.3). In seeds, Fe may be complexed as ferritin or phytic acid mineral salt (Nozoye *et al.*, 2007). Ferritins can store up to 4500 iron atoms in their central cavity. They are preferentially localised in plastids and were widely accepted as predominant Fe stores

(Jeong and Guerinot, 2009; Briat *et al.*, 2010). However it has been reported that Fe complexation with phytic acid may be more cost-efficient than ferritin biosynthesis (Regvar *et al.*, 2011). Both types of molecules are involved in the binding and distribution of Zn, but also other divalent cations such as Mg, Mn, and Cd (Duggleby and Dennis, 1970; Takahashi *et al.*, 2009). The understanding of Fe homeostasis in grasses may therefore also be of importance for biofortification of Zn and possibly other cations.

Research has shown that element accumulation patterns could be influenced by expression of key ion or solute transporters in certain cells. Alterations therefore of solute transporters via genetic engineering, growth under stress, and indeed induced mutations can culminate in changes to these accumulation patterns (Conn and Gilliam, 2010). In addition, the PIXE data is consistent with observations that different plants, parts of the same plant and even different cells of the same plant tissue may have different elemental profiles (Vega-Carrillo, *et al.*, 1997). Compartmentalization of certain elements, particularly those in soluble ionic form makes physiological and chemical sense for plants in order to maintain optimal function. Some elements are toxic and some react with other elements within the cells, thus the need for cell-specific distribution of some elements (Leigh, 1997). Compartmentalization of elements further provides a means to regulate cytosolic nutrient availability and accumulation (Miller and Smith 2008).

Overall, significant improvements in lysine content and protein digestibility were obtained in the mutant lines. Irregular protein body structure seemed to be associated with enhanced digestibility and spatial distribution of elements was altered and improved in mutants compared to the parental line.

#### 4.5 REFERENCES

- Aboubacar, A., J.D. Axtell., C.P. Huang and B.R. Hamaker.** 2001. A rapid protein digestibility assay for identifying highly digestible sorghum lines 1. *Cereal chemistry.* **78**:160-165.
- Aboubacar, A., J.D. Axtell., L. Nduulu and B.R. Hamaker.** 2003. Turbidity assay for rapid and efficient identification of high protein digestibility sorghum lines. *Cereal Chemistry.* **80**:40-4.
- Adams, C.A., L. Novellie and N.V.D. Liebenberg.** 1976. Biochemical properties and ultrastructure of protein bodies isolated from selected cereals. *Cereal Chemistry.* **53**:1-12.
- Alhassan, U. and A. Adedayo.** 2010. Genetic diversity in malting quality of some sorghum genotypes (*Sorghum bicolor (L.) moench*). *Production Agriculture and technology.* **6**:51-60.
- AOAC.** 2000. *Official methods of analysis.* Gaithersburg: AOAC International.
- Awika, J.M., L.W. Rooney and R.D. Waniska.** 2005. Anthocyanins from black sorghum and their antioxidant properties. *Food Chemistry.* **90**:293-301.
- Bean, S.R.** 2003. Evaluation of novel precast SDS-PAGE gels for separation of sorghum proteins. *Cereal chemistry:* **80**: 500-504.
- Belton, P.S. and J.R.N. Taylor.** 2004. Sorghum and millets: protein sources for Africa *Trends in Food Science and Technology.* **15**:94-8.
- Briat, J.F., K. Ravet., N. Arnaud., C. Duc., J. Boucherez., B. Touraine and F. Gaymard.** 2010. New insights into ferritin synthesis and function highlight a link between iron homeostasis and oxidative stress in plants. *Annals of botany.* **105**:811-822.

- Cakmak, I.** 2008. Enrichment of cereal grains with zinc: agronomic or genetic biofortification? *Plant Soil*. **302**:1-17.
- Casas, A.M., A.J.K. Kononowicz., U.B. Zehr., D.T. Tomes., J.D. Axtell., L.G. Butlers., R.A. Bressan and P.M. Hasegawa.** 1993. Transgenic sorghum plants via microprojectile bombardment. *Proceedings in National Academy of Science*. **90**:11212-11216.
- Da Silva, S.L., J. Taylor and J.R.N. Taylor.** 2011. Transgenic sorghum with altered kafirin synthesis: kafirin solubility, polymerisation, and protein digestion. *Journal of Agriculture and Food Chemistry*. **59**:9265-9270.
- Dlamini, N.R., J. Taylor and L.W. Rooney.** 2007. The effect of sorghum type and processing on the antioxidant properties of African sorghum-based foods. *Food Chemistry*. **105**:1412-1419.
- Duggleby, R.G. and D.T. Dennis.** 1970. Nicotinamide adenine dinucleotide-specific isocitrate dehydrogenase from a higher plant, the requirement for free metal-complexed isocitrate. *Journal of Biological Chemistry*. **245**:3745-3750.
- Duodu, K., J. Taylor., P. Belton and B. Hamaker.** 2003. Factors affecting sorghum protein digestibility. *Journal of Cereal Science*. **38**:117-131.
- Emmambux, N.M. and J. Taylor.** 2003. Sorghum kafirin interaction with various phenolic compounds. *Journal of the Science Food and Agriculture*. **83**:402-407.
- Fowler, C.** 1990. *Shattering: food, politics, and the loss of genetic diversity*. University of Arizona Press.
- Gibbon, B.C. and B.A. Larkins.** 2005. Molecular genetic approaches to develop quality protein maize. *Trends in Genetics*. **21**:227-33.

**Graham, R.D., R.M. Welch and H.E. Bouis.** 2001. Addressing micronutrient malnutrition through enhancing the nutritional quality of staple foods: principles, perspectives and knowledge gaps. *Advances in Agronomy*. **70**:77-142.

**Gomez-Pando, L., A. Eguilu., J. Jimenez and J. Falconi.** 2009. Barley (*Hordeum vulgare*) and kiwicha (*Amaranthus caudatus*) improvement by mutation induction in Peru. Vienna: FAO/IAEA.

**Hamaker, B., A. Kirleis., L. Butler., J. Axtell and E. Mertz.** 1987. Improving the *in vitro* protein digestibility of sorghum with reducing agents. *Proceedings of the National Academy of Sciences*. **84**:626-628.

**Jeong, J. and M. Guerinot.** 2009. Homing in on iron homeostasis in plants. *Trends in plant science*. **14**:280-285.

**Kasarda, D.D., K.M. Woodard and A.A. Elva.** 1998. Resolution of high molecular weight glutenin subunits by a new SDS-PAGE system incorporating a neutral pH buffer. *Cereal Chemistry*. **75**:70–71

**Kumar, T., I. Dweikat., S. Sato., Z. Ge., N. Nersesian., H. Chen., T. Elthon., S. Bean., B.P. Ioerger., M. Tilley and T. Clemente.** 2012. Modulation of kernel storage proteins in grain sorghum (*Sorghum bicolor* (L.) Moench). *Plant Biotechnology Journal*. **10**:533-44.

**Longman, J.P.** 1999. The curse of Frankenfood. Genetically modified crops stir up controversy at home and abroad. *US News and World Report*. 38–41.

**Medina III, F.S., S. Tano and E. Amano.** 2005. Mutation breeding manual. *Japan Atomic Industrial Forum*. Tokyo, Japan: FNCA.

**Mehlo, L., Z. Mbambo., S. Bado., J. Lin., S.M. Moagi., S. Buthelezi., S. Stoychev and R. Chikwamba.** 2013. Induced protein polymorphisms and nutritional quality of gamma

irradiation mutants of sorghum. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*. **749**:66-72.

**Mertz, E.T., M.M. Hassen., C. Cairns-Whittern., A.W. Kirleis., L. Tu and J.D. Axtell.** 1984. Pepsin digestibility of proteins in sorghum and other major cereals. *Proceedings of the National Academy of Sciences*. **81**:1-2.

**Mesa-Stonestreet, M.J., S. Alavi and S.R. Bean.** 2010. Sorghum Proteins: The Concentration, Isolation, Modification, and Food Applications of Kafirins. *Journal of Food Science*. **75**:90-104.

**Nestel, P.B., E.B. Howarth., J.V. Meenakshi and W. Pfeiffer.** 2006. Biofortification of staple food crops. *Journal of Nutrition*. **136**:1064-7.

**Nguz, K. and A. Huyghebaert.** 1998. Effect of tannins concentration on *in vitro* protein digestibility of some African sorghum (*Sorghum bicolor* L. Moench) varieties. *Sciences des Aliments* **18**:293-300.

**Nozoye, T., H. Inoue., M. Takahashi., Y. Ishimaru., H. Nakanishi., S. Mori and N.K. Nishizawa.** 2007. The expression of iron homeostasis-related genes during rice germination. *Plant molecular biology*. **64**:35-47.

**Nunes, A., I. Correia., A. Barros and I. Delgadillo.** 2004. Sequential *in vitro* pepsin digestion of uncooked and cooked sorghum and maize samples. *Journal of agricultural and food chemistry*. **52**:2052-2058.

**Oria, M., B. Hamaker and J. Schull.** 1995. *In vitro* protein digestibility of developing and mature sorghum grain in relation to  $\alpha$ -,  $\beta$ -, and  $\gamma$ -kafirin disulfide crosslinking. *Journal of Cereal Science*. **22**:85-93.

**Oria, M.P., B.R. Hamaker., J.D. Axtell and C.P. Huang.** 2000. A highly digestible sorghum mutant cultivar exhibits a unique folded structure of endosperm protein bodies. *Proceedings in National Academy of Science.* **97**:5065-70.

**Patel, M.B., M.S. Jami and J. McGinnis.** 1980. Effect of gamma irradiation, penicillin, and/or pectic enzyme on chick growth depression and fecal stickiness caused by rye, citrus pectin, and guar gum. *Poultry Science.* **59**:2105-2110.

**Paterson, A.H., J.E. Bowers., R. Bruggmann., I. Dubchak., J. Grimwood., H. Gundlach., G. Haberer., U. Hellsten., T. Mitros., A. Poliakov., J. Schmutz., M. Spannagl., H. Tang., X. Wang., T. Wicker., A.K. Bharti., J. Chapman., F.A. Feltus., U. Gowik., I.V. Grigoriev., E. Lyons., C.A. Maher., M. Martis., A. Narechania., R.P. Ojillar., B.W. Penning., A.A. Salamov., Y. Wang., L. Zhang., N.C. Carpita., M. Freeling., A.R. Gingle., C.T. Hash., B. Keller., P. Klein., S. Kresovich., M.C. McCann., R. Ming, D.G. Peterson., R. Mehboobur., D. Ware., P. Westhoff., K.F.X. Mayer., J. Messing and D.S. Rokhsar.** 2009. The *Sorghum bicolor* genome and the diversification of grasses. *Nature.* **457**:551-556.

**Prozesky, V.M., W.J. Przybylowicz., E. van Achterbergh., C.L. Churm., C.A. Pinedab., K.A. Springhorn., J.V. Pilcher., C.G. Ryan., J. Kritzinger., H. Schmitt and T. Swarta.** 1995. The NAC Nuclear microprobe facility. *Nuclear Instruments and methods in Physics Research.* **104**:36-42.

**Poletti, S., W. Gruissem and C. Sautter.** 2004. The nutritional fortification of cereals. *Current opinion in biotechnology.* **15**:162-165.

**Regvar, M., D. Eichert., B. Kaulich., A. Gianoncelli., P. Pongrac., K. Vogel-Mikuš and I. Kreft.** 2011. New insights into globoids of protein storage vacuoles in wheat aleurone using synchrotron soft X-ray microscopy. *Journal of experimental Botany.* **62**:3929-3939.

- Ryan, C.G.** 2002. Quantitative trace element imaging using PIXE and the nuclear microprobe. *International Journal of Imaging Systems and Technology*. **11**:219-30.
- Rooney, L.W. and F.R. Miller.** 1982. Variation in the structure and kernel characteristics of sorghum. In Mertin, J. V. (ed.) *Proceedings of the International Symposium on Sorghum Grain Quality*. Patancheru, India. **2**:143-162.
- Serna-salvidar, S. and L.W. Rooney.** 1995. "Structure and Chemistry of Sorghum and Millets," In: D.A.V. Dendy, Ed, *Sorghum and Millets: Chemistry and technology*, American Association of Cereal Chemists, Minnesota; USA, p. 406.
- Shu, Q.Y.** 2009. Induced plant mutations in the Genomics Era. Food and Agriculture Organisation of the United Nations. Rome. 7-8.
- Singh, B. and P.S. Datta.** 2010. Effect of low dose gamma irradiation on plant and grain nutrition of wheat. *Radiation Physics*. **03**:11-17.
- Singh, R. and J.D. Axtell.** 1973. High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. *Crop Science*. **13**:535-539.
- Takahashi, M., T. Nozoye., N. Kitajima., N. Fukuda., A. Hokura., Y. Terada and N.K. Nishizawa.** 2009. *In vivo* analysis of metal distribution and expression of metal transporters in rice seed during germination process by microarray and X-ray Fluorescence Imaging of Fe, Zn, Mn, and Cu. *Plant and Soil*. **325**:39-51.
- Taylor, J. and J.N.R. Taylor.** 2007. An *in vitro* pepsin digestibility method for cereal grains using small sample sizes. Proceedings of the first Latin America cereal science and technology conference. Rosario, Argentina. *International Association for Cereal Science and Technology*. (ICC), Vienna.

**WHO.** 2007. Protein and amino acid requirements in human nutrition. *World Health Organisation Technical Report Series.* **935**:1-265

## **CHAPTER 5 GENERAL DISCUSSION**

### **5.1 RESEARCH IN PERSPECTIVE**

Sorghum is one of the most important food crops in the world and, together with maize, rice and wheat, provides at least 30% of the food calories to more than 4.5 billion people in developing countries (Godfray *et al.*, 2010). In parts of Africa and Asia, sorghum is the primary staple for over 300 million people (ICRISAT, 2009). Sorghum is also a key ingredient in animal feed and is used extensively in industrial products, including the production of biofuels (Anandan *et al.*, 2012). Increasing demand and production shortfalls have worsened market volatility and contributed to surging prices. Climatic variability and change, and the consequent rise in abiotic and biotic stresses, further confound the problem (Godfray *et al.*, 2010).

Climate change and increased biofuel production represent major risks for long-term food security (Escobar *et al.*, 2009). Although countries in the Southern hemisphere are not the main originators of climate change, they may suffer the greatest share of damage in the form of declining yields due to the greater frequency of extreme weather events (Godfray *et al.*, 2010). Studies estimate that the aggregate negative impact of climate change on African agricultural output of up to 2080-2100 could be between 15 and 30 percent. Agriculture will have to adapt to climate change, but it can also help mitigate the effects of climate change. Many countries will continue depending on international trade to ensure their food security. It is estimated that by 2050 developing countries' net imports of cereals will be more than double from 135 million metric tonnes in 2008/09 to 300 million in 2050 (Godfray *et al.*, 2010).

Tweeten and Thompson (2008) assume linear growth in yields of major cereals and project a 79% increase in demand for all foods in 2050. They conclude that the global farm output will

need to almost double in the first half of this century to maintain historic trends in real prices for food. Unless concerted and vigorous measures are taken to address these challenges and accelerate yield growth, the outcome will be hunger and food insecurity for millions of poor consumers (Escobar *et al.*, 2009).

This study shows how induced mutations can play a key role in meeting future cereal demand. Attention is directed at the generation of highly nutritious, agronomically sound and widely-adapted varieties through a judicious combination of conventional and molecular breeding approaches. However, the use of improved varieties alone will not be enough to ensure accessibility to poor communities and will need to be complemented by a shift in focus to the biofortification of the more accessible staples and improving their agronomic practices (Qaim *et al.*, 2007). Widespread poverty and malnutrition compounded by the unavailability of agricultural land means that some of these improved products are unaffordable to the majority of Africans. Consequently, new methodologies are required to increase genetic diversity in sorghum, which is a staple for over 300 million Africans for various nutritional traits and lowering of the anti-nutrients (ICRISAT, 2009).

Thus far, studies outside of Africa have focused on evaluating sorghum for biofuel production, animal feed (Anadan *et al.*, 2012; Yahaghi *et al.*, 2013) and drought resistance (Castro-Nava *et al.*, 2012; Dalal *et al.*, 2012; Burke *et al.*, 2013). Rooney *et al.* (2007), provides a complete review on the use of Sorghum as a bioenergy source. On the other hand, studies aimed at improving sorghum lysine content only achieved lysine content of 4.10 g/100 g protein (Da Silva *et al.*, 2011). The present study therefore, focused on nutritionally and agronomically improving sorghum using a non-GMO technology. The parental line used was a purple type II low-tannin sorghum (P898012). The mutants derived from P898012 were then compared to both the parental line and a wild type yellow sorghum variety; in relevant instances, the high protein digestibility mutant from Purdue University was also

used. In order to identify the nutritionally enhanced varieties several comparative assays were also carried out.

The first part of the study involved a comparative assessment of mutant grain quality. Several physical characteristics of the kernels were studied, including grain hardness, weight and colour. No adverse changes were noted except for grain weight of RED which was 18 mg, half the weight of the parental line (36 mg). The 5 mutants also showed 5 distinct colours indicative of changes in chemical content. Batey and Reynish, 1976 hypothesised that reduced grain size was due to additional tiller survival, and therefore more kernels per plant to divide the carbohydrate produced through photosynthesis.

A remarkable increase in antioxidant activity of mutants was observed, with a 365% increase in SY and 40% increase for RED. The antioxidant activity came mainly from condensed tannins, which have a higher antioxidant activity *in vitro* than the other phenolic compounds (Hagerman *et al.*, 1998; Amarowicz *et al.*, 2003). A strong correlation between antioxidant activity and phenol content measured using colorimetric methods was observed ( $R^2 = 0.8$ ,  $P < 0.05$  Figure 2.3). This means that a high phenolic content had led to a relatively high antioxidant activity. Free radicals and other reactive oxygen species contribute to the development of many diseases (Naczki and Shahidi, 2004). Phenolic compounds scavenge free radicals by donating hydrogen atoms to the free radicals; hence they may protect cell constituents against oxidative damage and limit the risk of various degenerative diseases associated with oxidative stress (Anderson & Wolf, 1995).

In order to determine whether gamma-irradiation had an effect on microstructure, TEM and SEM were used to analyse mutant kernels. SEM did not reveal any major changes except changes in size and shape of starch granules which were shown in this study to have major implications on grain hardness. The soft or floury endosperm is characterised by relatively large intergranular air spaces. Whereas, the hard or corneous endosperm is characterised by a

tightly packed structure with no air spaces. The second part of the study involved ascertaining the changes in protein bodies using TEM. The mutants had invaginated protein bodies associated with enzyme accessibility and better digestion. Enhanced digestibility is an important trait in sorghum which has low digestibility upon cooking.

The current study was also successful in elevating protein content of the mutants. This was specifically true for SY which had 16.08 (as percentage of weight) protein content when compared to the 12.33 for parental line, P898012. The quality of a protein is primarily a function of its essential amino acid composition. Fluctuations in the protein content of the grain are generally accompanied by changes in the amino acid composition of the protein. SY mutant had 11 times as high lysine as its parental line and 4-fold higher than that recommended by WHO, (2007) for 1-2 year old children (5.2 g/100 g). Lysine is a rate limiting amino acid in sorghum, however, this study clearly showed an increase in this amino acid with the increased production of proteins. It has been reported that in high-lysine cereals such as *opaque-2*-maize, the high-lysine gene alters the amino acid pattern in the floury endosperm relative to the normal corneous endosperm tissue (Singh and Axtell, 1973). Other major changes observed were the increased content of Isoleucine, Arginine, Asparagine and Glutamine in the floury endosperm mutants.

Attempts to nutritionally improve sorghum lines started with identification of some native high-lysine sorghum genotypes from Ethiopia (Singh and Axtell, 1973) and this was then followed by chemical mutagenesis to develop a high-lysine genotype (P721 opaque) (reviewed by Mertz *et al.* (1993). Da Silva *et al.* (2011) reported protein content and amino acid content ranging from 10.6 to 13.7 % and 3.6 to 4.10 g/100 g respectively. However, these values are still below those recommended by WHO, (2007) (5.2 g/100 g).

Therefore, the substantially improved lysine content of the mutants developed in this study set precedence and far exceeds those reported by any study conducted in sorghum. However,

poor grain quality, especially soft and floury endosperm texture, moderates the value of the high-lysine line produced in this study. Breeding using SY mutant will therefore be undertaken to produce high-lysine genotypes with improved grain hardness and protein digestibility after cooking.

## **5.2 POTENTIAL FOR FUTURE WORK**

The industrial uses of sorghum have increased significantly in recent years and this has led to an increase in the global production of the crop. This highly drought resistant crop is being used increasingly in biofuel production and feed industry. However, sorghum remains a major source of protein for people in tropical and subtropical developing countries. The nutritional quality of sorghum protein is therefore a major concern in those regions. Sorghum proteins are deficient in the essential amino acid lysine, due to the kafirin storage proteins being essentially free of lysine (reviewed by Shewry, 2007). It is therefore imperative that studies be conducted for the nutritional improvement of sorghum as it remains an important food source in the continent. Of equal importance, is to ensure that any chemical changes in the composition of the grain obtained through induced mutations should not affect grain quality and agronomic performance.

The mutants developed in this study will therefore be used in further mutation breeding programs to stabilise favourable traits and improve grain quality. Further studies to ensure that the traditional and modern food processing abilities as well as consumer acceptance were not somewhat affected by the induced mutations would also be carried out.

In addition, the significance and potential of these results could only be fully exploited once the mechanisms of alteration of the micronutrient profiles and changes in protein and starch body microstructure in mutant plants, and its impact on nutrition and the environment are fully understood. Subsequent research therefore will be aimed at effectively directing the accumulation of these elements in important plants and into plant cells where they are easily

available and not bound up by anti-nutrients as is the complex case of iron and zinc being bound by phytate and phenolic acids. A further task would be to unravel the genetic basis of the mutant traits reported in this study.

### 5.3 REFERENCES

**Amarowicz, R. and A. Troszyńska.** 2003. Antioxidant activity of extract of pea and its fractions of low molecular phenolics and tannins. *Polish Journal of Food Nutrition and Science.* **12**:10-15.

**Anandan, S., H. Zoltan., A.A. Khan., D. Ravi and M. Blümmel.** 2012. Feeding value of sweet sorghum bagasse and leaf residues after juice extraction for bio-ethanol production fed to sheep as complete rations in diverse physical forms. *Animal Feed Science and Technology.* **175**:131-136.

**Anderson, R.L. and W.J. Wolf.** 1995. Compositional changes in trypsin inhibitors, phytic acid, saponins and isoflavones related to soybean processing. *The Journal of nutrition.* **125**:581-586.

**Batey, T. and D.J. Reynish.** 1976. The influence of nitrogen fertiliser on grain quality in winter wheat. *Journal of Science Food and Agriculture.* **27**:983–990.

**Burke, J.J., J. Chen., G. Burow., Y. Mechref., D. Rosenow, D., P. Payton and C.M. Hayes.** 2013. Leaf dhurrin content is a quantitative measure of the level of pre-and post-flowering drought tolerance in sorghum. *Crop Science.***53**:1056-1065.

**Castro-Nava, S., J. Ortiz-Cereceres., M.D.C. Mendoza-Castillo and A.J. Huerta.** 2012. Biomass production and grain yield of three sorghum lines differing in drought resistance. *Phyton (Buenos Aires).* **81**:149-156.

**Dalal, M., K. Mayandi and V. Chinnusamy.** 2012. Sorghum: Improvement of abiotic stress tolerance. *Improving Crop Resistance to Abiotic Stress.***1**:923-950.

**Da Silva, L.S., R. Jung., Z. Zhao., K. Glassman., J. Taylor and J.R.N. Taylor.** 2011. Effect of suppressing the synthesis of different kafirin sub-classes on grain endosperm

texture, protein body structure and protein nutritional quality in improved sorghum lines. *Journal of Cereal Science*. **54**:160-167.

**Escobar, J.C., E.S. Lora., O.J. Venturini., E.E. Yáñez., E.F. Castillo and O. Almazan.** 2009. Biofuels: environment, technology and food security. *Renewable and sustainable energy reviews*. **13**:1275-1287.

**Godfray, H.C.J., J.R. Beddington., I.R. Crute., L. Haddad., D. Lawrence., J.F. Muir and C. Toulmin.** 2010. Food security: the challenge of feeding 9 billion people. *Science*. **327**:812-818.

**Hagerman, A.E., K.M. Ried., G.A. Jones., K.N. Sovik., N.T. Ritchard., P.W. Hartzfeld and T.L. Riechel.** 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *Journal of Agricultural and Food Chemistry*. **46**:1887-1892.

**ICRISAT.** 2009. Trends in conservation farming adoption and impacts. A report of the 2009 CA panel study carried out by ICRISAT and jointly supported by FAO, and GRM International.

**Mertz, E.T., J.D. Axtell., G. Ejetu and B.R. Hamaker.** 1993. Development and recent impact of quality maize and sorghum. In: Taylor, J.N.R., P.G. Randall and J.H. Viljoen. (Eds). Cereal Science and Technology. Impact on a changing Africa. *Selected papers from ICC International symposium*. The CSIR. Pretoria. SA. pp. 115-131.

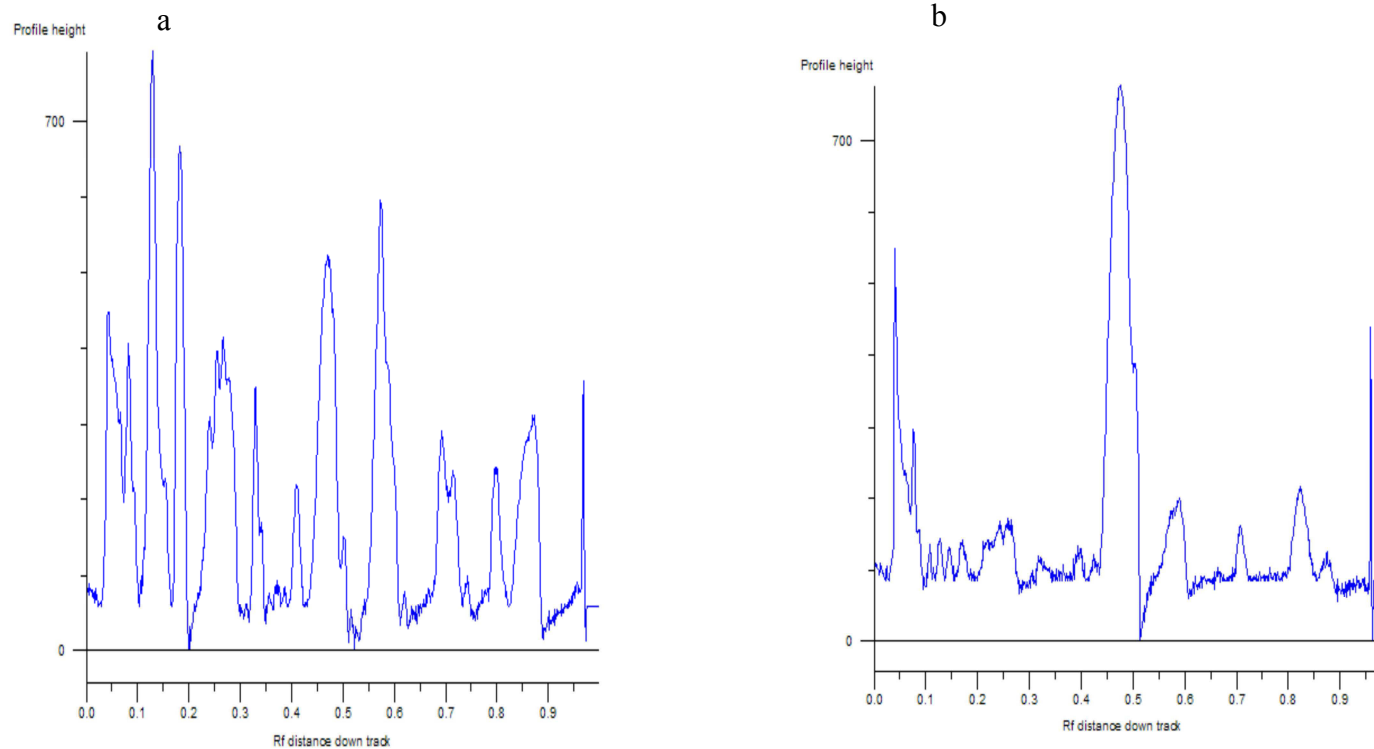
**Naczk, M. and Shahidi, F.** 2004. Extraction and analysis of phenolics in food. *Journal of Chromatography A*. **1054**:95-111.

**Qaim, M., A.J. Stein and J.V. Meenakshi.** 2007. Economics of biofortification. *Agricultural Economics*. **37**:119-133.

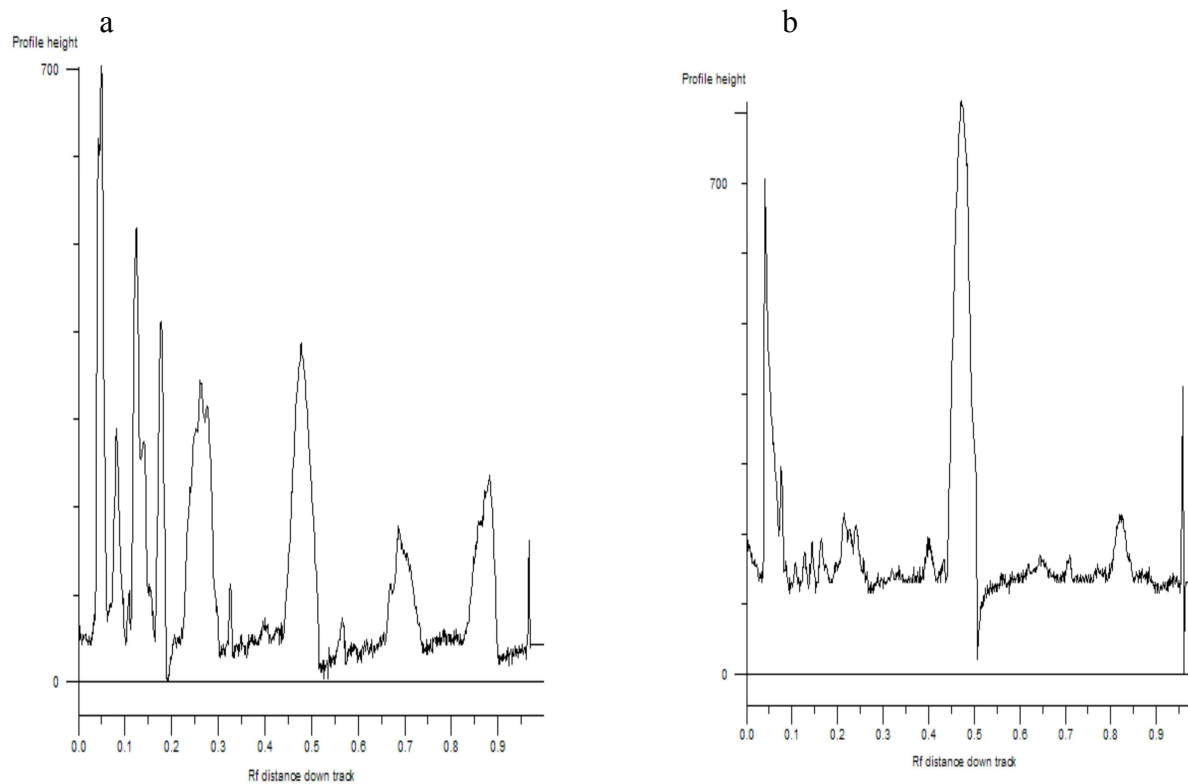
- Rooney, W.L., J. Blumenthal., B. Bean and J.E. Mullet.** 2007. Designing sorghum as a dedicated bioenergy feedstock. *Biofuels, Bioproducts and Biorefining*. **1**:147-157.
- Shewry, P.R.** 2007. Improving the protein content and composition of cereal grain. *Journal of Cereal Science*. **46**:239-250.
- Singh, R. and J. D. Axtell.** 1973. High lysine mutant gene (*hl*) that improves protein quality and biological value of grain sorghum. *Crop Science*. **13**:535-539.
- Tweeten, L.G. and S.R. Thompson.** 2008. *Agricultural Policy for the 21st century*. John Wiley and sons Inc., New York, N.Y. 824.
- WHO.** 2007. Protein and amino acid requirements in human nutrition. *World Health Organisation Technical Report Series*. **935**:1-265
- Yahaghi, M., J.B. Liang., J. Balcells., R. Valizadeh., A.R. Seradj., R. Alimon and Y.W. Ho.** 2013. Effect of substituting barley with sorghum on starch digestion, rumen microbial yield and growth in Iranian Baluchi lambs fed high concentrate diets. *Animal Feed Science and Technology*. **45**:345-370.

## APPENDIX A:

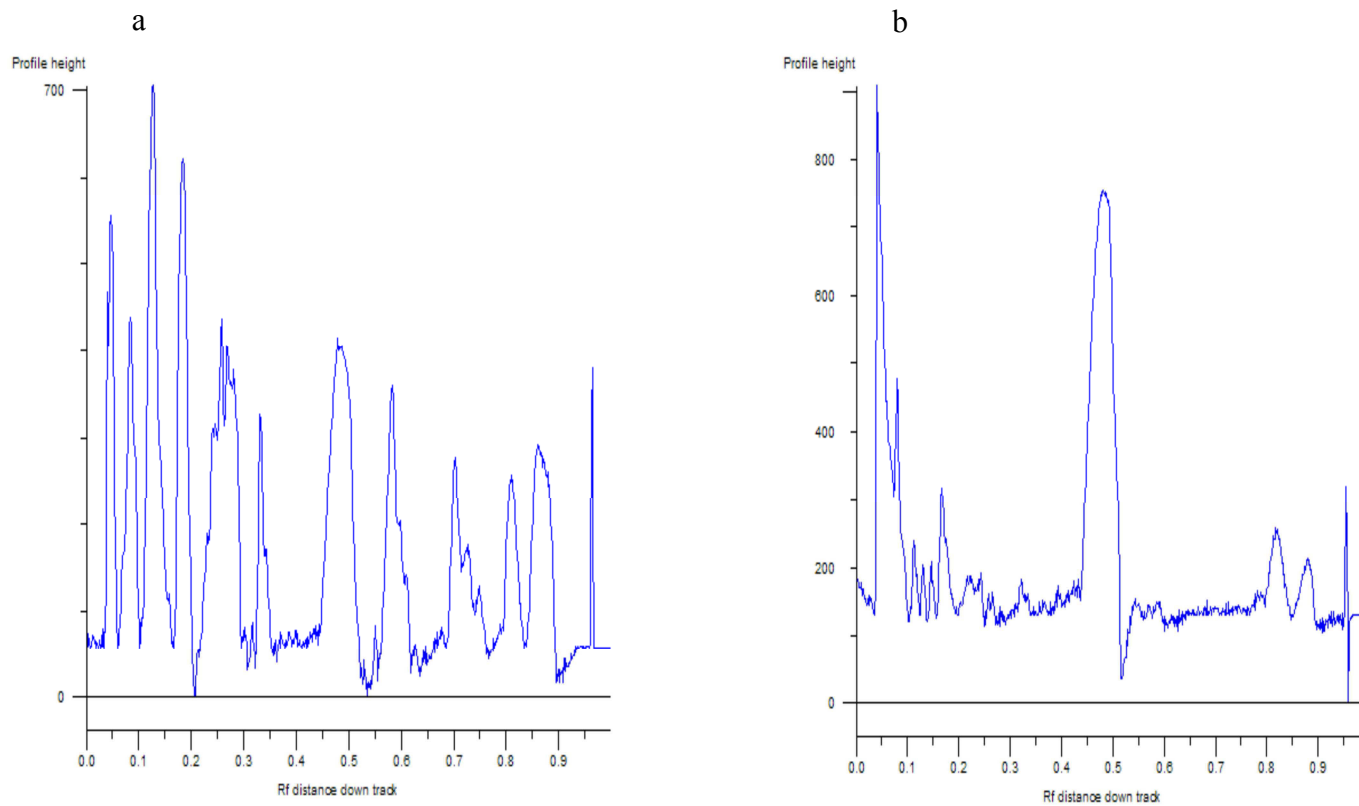
**Appendix A:** Densitographs for the 5 mutants and control (P898012). The digested sample is shown alongside its undigested counterpart. The y-axis shows Profile height and x-axis Rf distance down track.



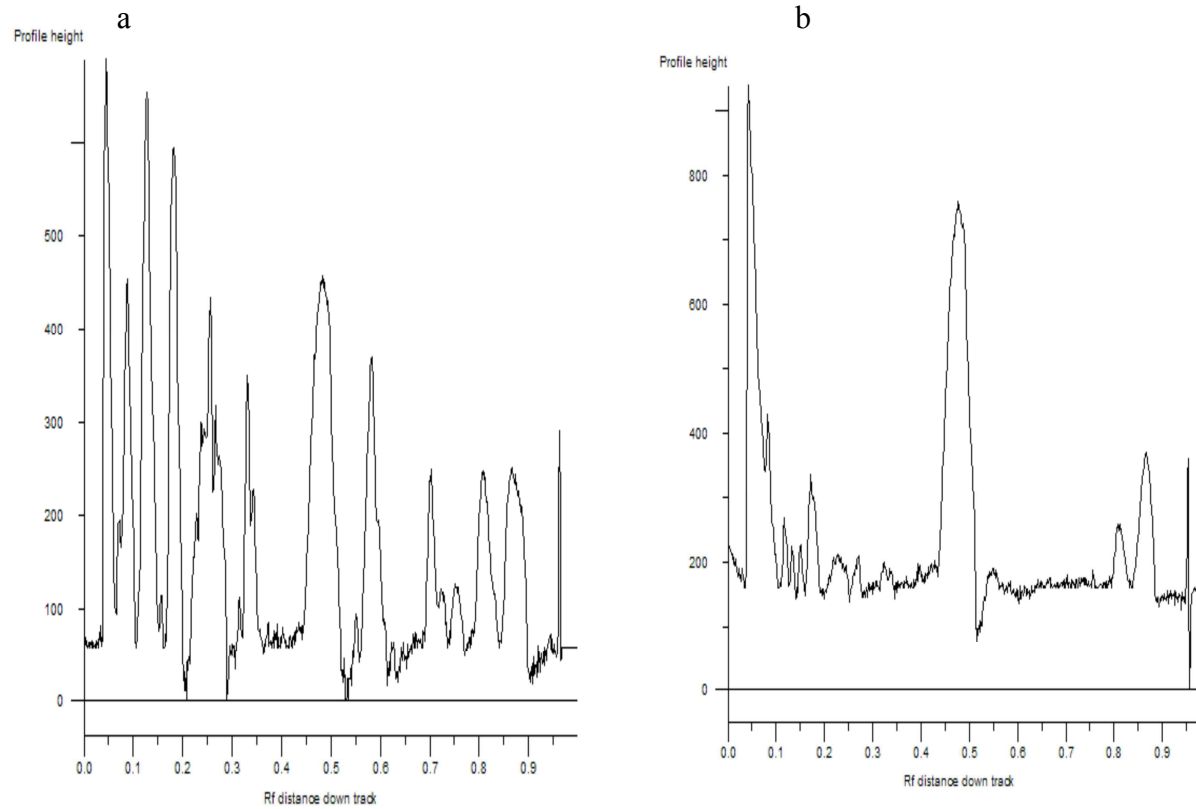
**Figure A1** Densitograms of electrophoretic spectra of sorghum proteins. a) undigested P898012; b) digested P898012



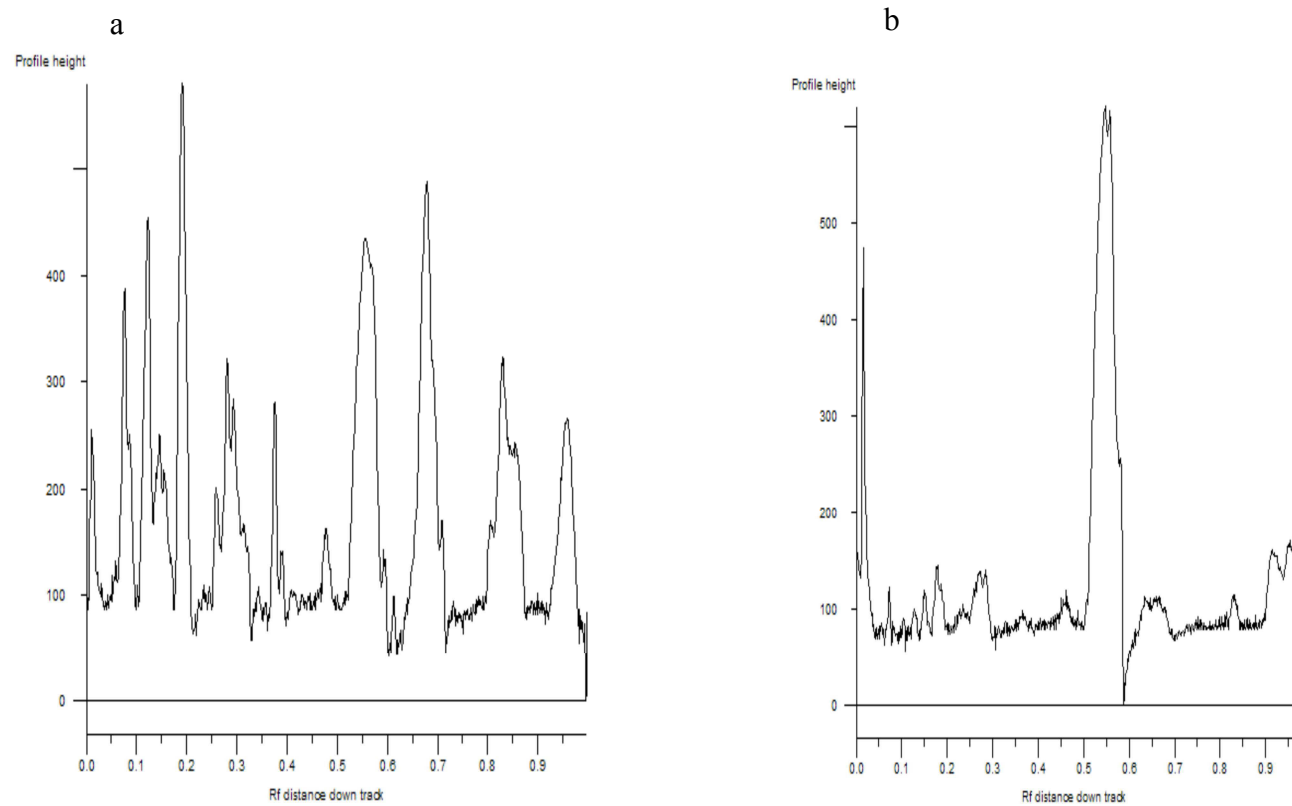
**Figure A2** Densitograms of electrophoretic spectra of sorghum proteins. a) undigested yellow sorghum (YEL); b) digested yellow sorghum (YEL).



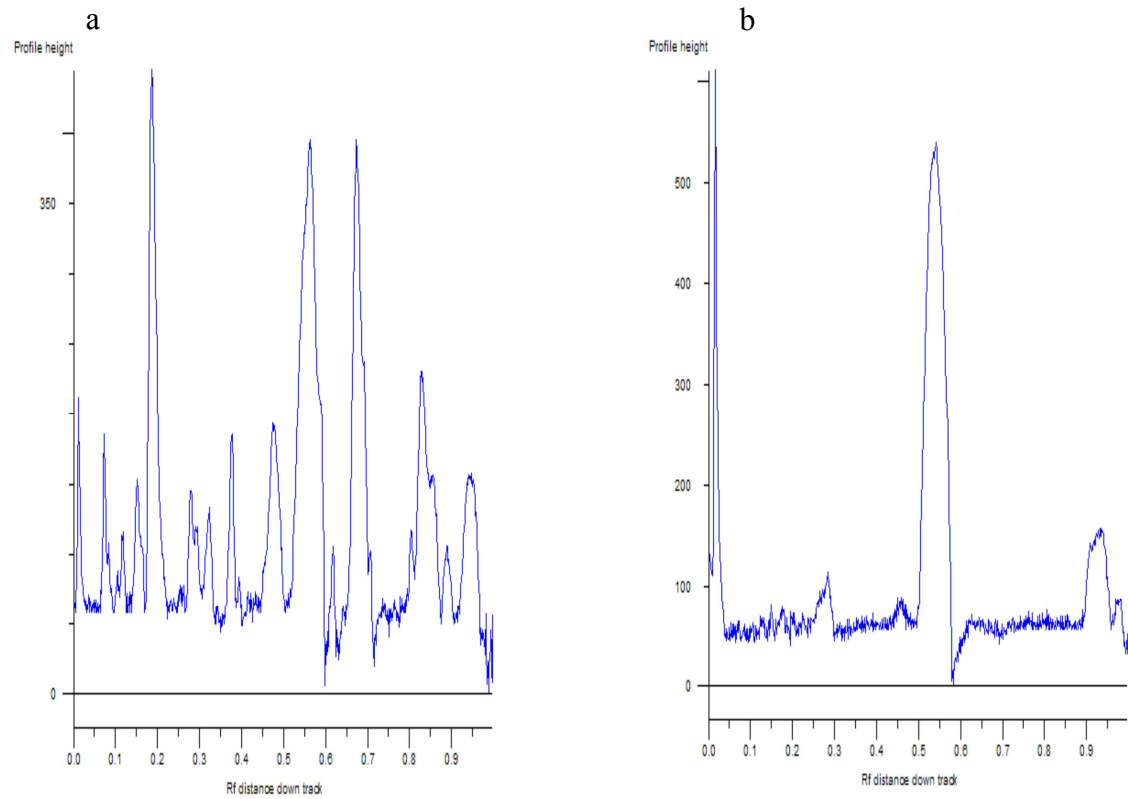
**Figure A3** Densitograms of electrophoretic spectra of sorghum proteins. a) undigested brown sorghum mutant (BR); b) digested brown sorghum mutant (BR).



**Figure A4** Densitograms of electrophoretic spectra of sorghum proteins a) undigested red sorghum mutant (RED); b) digested red sorghum mutant (RED).

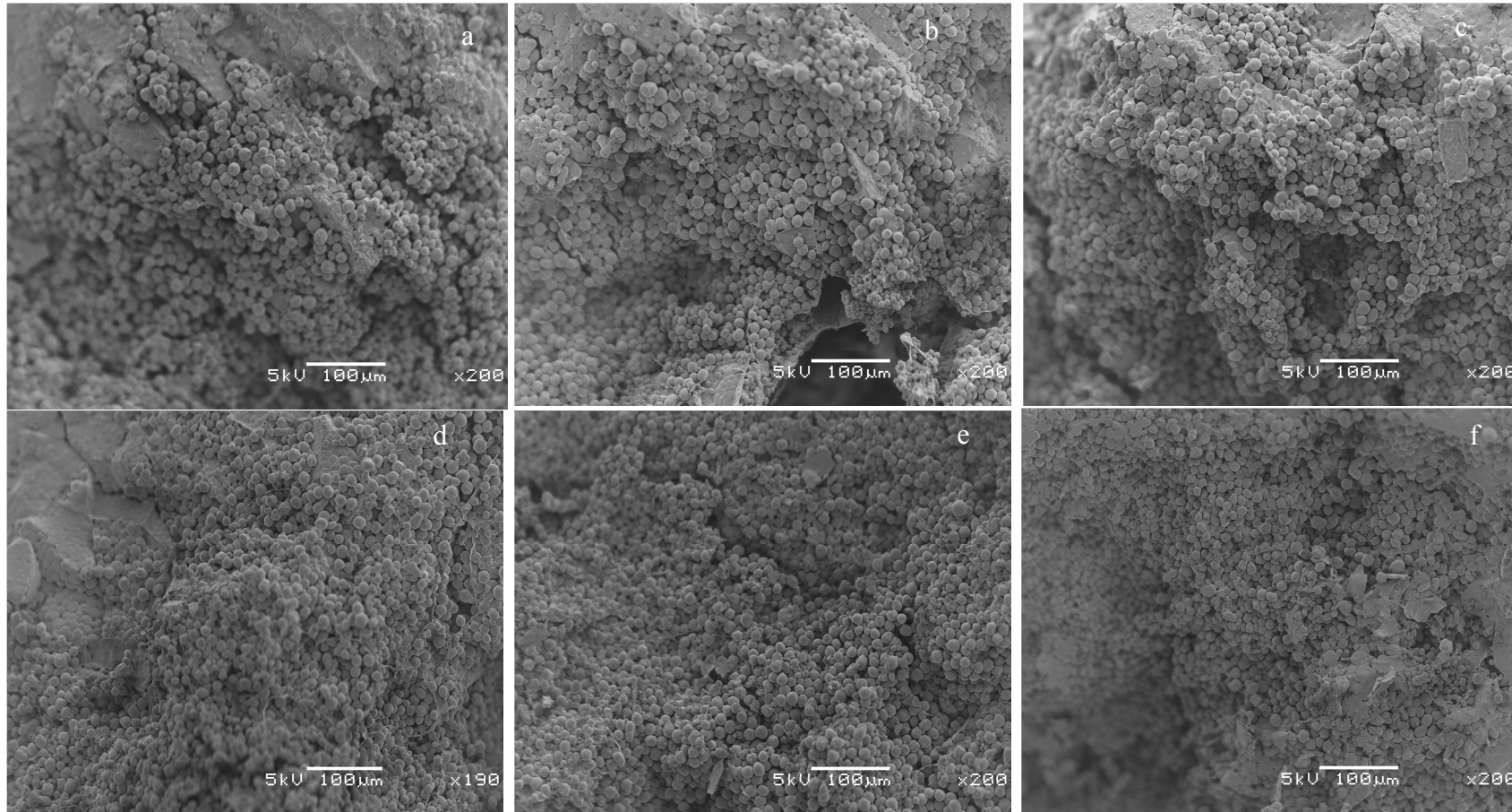


**Figure A5** Densitograms of electrophoretic spectra of sorghum proteins a) undigested Biomass sorghum mutant (BIO); b) digested Biomass sorghum mutant (BIO).



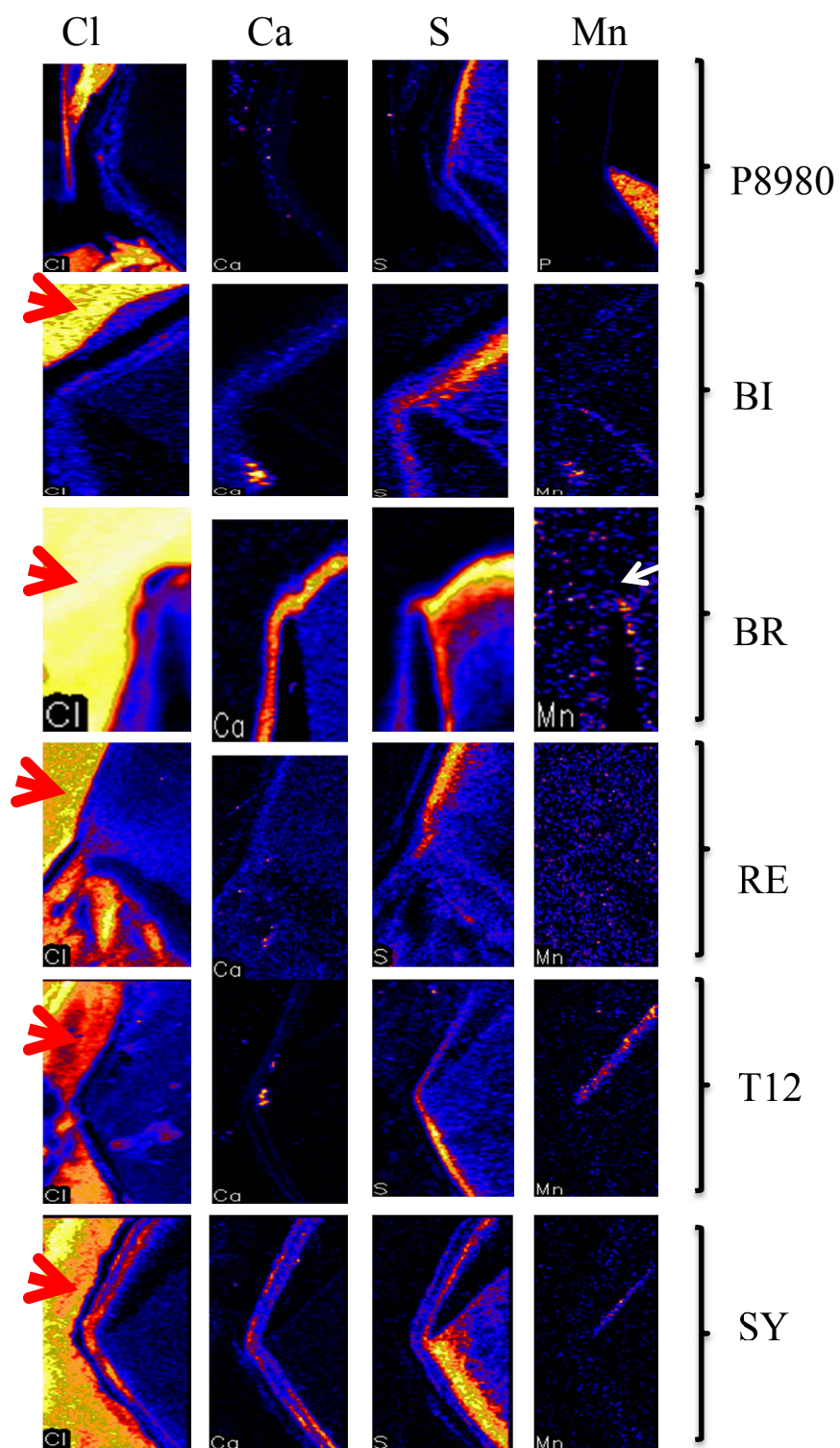
**Figure A6** Densitograms of electrophoretic spectra of sorghum proteins a) undigested White sorghum mutant (T120); b) digested T/white sorghum mutant (T120).

**APPENDIX B:**



**Figure B1** Scanning electron micrograph (SEM) of grain sorghum endosperm, a= P898012, b= (BIO), c= T120 d= BR, e= RED, f= SY.

**APPENDIX C:**



**Figure C1** PIXE analysis of the spatial distribution of Chlorine (Cl), Calcium (Ca), Sulphur (S) and Manganese (Mn) in mutant seeds of sorghum. The endosperm and germ area are pointed out with red and white arrows respectively