THE EFFECT OF CHEMOMUTAGENESIS ON ROOT NODULATION AND SEED PROTEIN IN TEPARY BEAN (*PHASEOLUS ACUTIFOLIUS*)

By

Mashifane Dipoo Charity

Student number: 14012772

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Department of Plant Production

School of Agriculture

University of Venda

South Africa

Supervisor:	Prof E.T. Gwata
Co-supervisor:	Prof P. Bessong

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Abstract

Tepary bean (*Phaseolus acutifolius*) is an important food legume originating from South America and the South-western parts of the United States. The crop is produced in many countries worldwide including South Africa. It is highly tolerant to drought and the seed contains a wide range of vitamins, minerals and protein of high nutritional quality. The genetic base of tepary bean is narrow but can be widened by chemical mutagenesis. However, there are no reports on the impact of chemical mutagenesis on the root nodulation and seed storage proteins in tepary bean. Therefore, this study was designed to examine root nodulation attributes and seed storage proteins of three tepary bean genotypes in the early mutagenic generations (M_2 to M_4) derived through treatment with varying doses (0.0, 0.5, 1.0, 1.5 and 2.0 v/v) of ethyl methanesulfonate (EMS). The experiment on root nodulation attributes was laid out as a 3 x 5 x 3 (genotypes x EMS doses x mutant generations) factorial design replicated three times. At harvest, shoot height (SHT), primary root length (PRL), dry weights (shoot, root and nodule), number of nodules per plant (NNP) and grain yield components such as the number of pods per plant (NPP) and number of seeds per pod (NSP) were measured. Highly significant ($P \leq 0.01$) dose effects were observed for SHT, PRL, shoot dry weight (SDW) and root dry weight (RDW). Highly significant ($P \leq 0.01$) interaction effects of mutant generation x genotype x dose were observed for NSP. A highly significant ($P \leq 0.01$) positive linear relationship was observed between the NNP and nodule dry weight (NDW). Increase in the PRL suggested that tepary bean mutants could be important in drought tolerance. EMS treatment led to an enhanced partitioning of dry matter (assimilates) to the shoots and roots. There was a three fold increase in most of the root nodulation traits at the 0.5% EMS dose.The Kjeldahl method was used for crude protein determination whereas the sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS PAGE) was utilized in determining the protein banding patterns of the bean. There were highly significant ($P \leq 0.01$) differences among the genotypes in crude protein accumulation. Highly significant ($P \leq 0.01$) mutant generation x genotype x dose were observed for seed protein accumulation. 'Genotype 3' attained the highest protein content (24.23%) at 1.5% EMS dose in the M_4 generation. EMS doses ≥0.5% positively stimulated protein accumulation in all genotypes but high EMS doses (2.0%) depressed protein content. There were significant variations in seed storage protein profiles among the genotypes and mutant generations. 'Genotype 6' showed a distinct 15.0kDa protein fragment which was absent in the majority of the remaining genotypes. The presence of distinct protein subunits in the three genotypes could be used in varietal identification. The results demonstrated that chemical mutagenesis using EMS could induce significant variations in both the agronomic and nutritional traits of tepary bean.

Key words: bean, dose, genotype, mutagenesis, nodulation, seed protein

Dedication

This dissertation is dedicated to the memory of my beloved Grandmother Mankepile Makweng (1936/03/22 – 2016/10/08) who to me was the epitome of strength and resilience.

“Robala ka khutšo Hunadi á Kanyane”

Declaration

I, Mashifane Dipoo Charity, hereby declare that the research submitted for the degree of Master of Science (Agriculture) at the University of Venda, is my own original work and has not been submitted for any degree or examination at any other University. I further declare that all sources cited or quoted are indicated and acknowledged by means of a comprehensive list of references.

Signature:……………………………………… Date:……………………………..

(Candidate: Mashifane D.C)

As the candidate‘s supervisor/co-supervisor, I agree to the submission of this dissertation:

Signature:………………………..………… Date:……………………….…….

(Supervisor: Prof. E.T Gwata)

Signature:……………………………………. Date:………………………………

(Co-Supervisor: Prof. P. Bessong)

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List of Abbreviations

BNF = biological nitrogen fixation

EMS = ethyl methanesulphonate

LCS = leaf color score

NDW = nodule dry weight

NEN = number of effective nodules

NNP = number of nodules per plant

NPP = number of pods per plant

NSP = number of seeds per pod

PDS = pod development score

PRL = primary root length

RDW = root dry weight

SDS PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDW = shoot dry weight

SHT = shoot height

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CHAPTER ONE: INTRODUCTION

1.1 Introduction

Tepary bean (*Phaseolus acutifolius*) is an ancient crop native to Mexico and the South-western parts of the United States (Bhardwaj, 2013). Recent studies indicate that tepary bean was most likely domesticated near the Mexico-USA border with earliest remains found in Tehuacán Valley, Mexico (Blair *et al.*, 2012). The crop has been reported as still being found growing in the wild as far south as Guatemala and as far north as central Arizona (Muñoz *et al.,* 2004).

The crop gained recognition recently as a drought tolerant and short duration legume in semi-arid parts of tropical Africa where most other grain legumes fail (Scott and Michaels, 1992; Porch *et al.,* 2013). Tepary bean is grown in many African countries including Morocco, Algeria, Malawi, Botswana, Uganda, South Africa, as well as Swaziland and Lesotho. In South Africa, it is grown mainly in the dryland area of Sekhukhune, in Limpopo Province.

The bean is primarily grown by small-holder farmers for human consumption mainly for its grain. Occasionally, it is consumed as fresh green beans or as bean sprouts. Bhardwaj and Hamama, (2004) reported that tepary bean leaves are considered edible in some parts of Africa but are tougher than those of common bean and take longer to cook. In Botswana, the grain is commonly used as supplementary feed for poultry with the stover used as animal feed (Bhardwaj and Hamama, 2005). Tepary bean grain is potentially useful in chemotherapy for treating cancer due to the presence of lectins and other compounds (De Mejia *et al.,* 2005). Moreover, legume lectins exhibit anti-tumour properties by inducing programmed cell death (apoptosis) and autophagy (Lei and Chang, 2007). Traditionally, tepary bean is ideal for people who are diabetic because of its high fibre levels which consequently makes it a slow release food, that is, sugar is released slowly. Moreover, the slow sugar release enables diabetic patients to retain protein in the body without risking an increase in body sugar levels.

Tepary bean is used in agroforestry systems for cover cropping and intercropping due to its ability to improve soil fertility through biological nitrogen fixation (Shisanya, 2003). However, the quantity of fixed N is relatively low compared to other legumes (Table 1.1). Moreover, it is unclear whether the fixation can be selected for since the process is complex and involves polygenes (Lee *et al.,* 1983). For instance, the relationship between root nodulation traits in tepary bean such as the nodule dry weight, the number of effective nodules and nodule size remains largely unclear. Therefore, it is difficult to manipulate these traits through genetic improvement approaches such as mutation breeding.

Table 1.1 Nitrogen fixation capacity of leguminous crops.

Crop species	**N_2 fixed (kg N ha^{-1})**	**Source**
Soybean (*Glycine max*)	117-237	Salvagiotti *et al.,* 2008
Groundnut (*Arachis hypogea*)	32-175	Unkovich and Pate, 2000
Common bean (*Phaseolus vulgaris*)	17-85	Dakora and Keya, 1997
Cowpea (*Vigna unguiculata*)	9-201	Hardarson *et al*., 1993
Tepary bean (*Phaseolus acutifolius*)	24-60	Shisanya, 2002

Through biological nitrogen fixation, legumes produce nitrogenous compounds in the root nodules. Crews *et al*., (2005) indicated that tepary bean transports ureides (allantoin and allantoic acid) in xylem sap as the dominant products from the nitrogen fixation process as in other legumes such as soybean (Gresshoff, 1993) and pigeonpea (Hansen and Pate, 1987). These nitrogenous compounds produced from the fixation process in the root nodules are translocated to various parts of the plant where they are catabolized and synthesized into biological molecules such as amino acids (Serraj and Sinclair, 1998). Since amino acids are the building blocks for proteins, there is a direct relationship between root nodulation and protein production in legumes.

However, the impact of induced changes in the root nodulation traits on seed protein accumulation is unclear. Mutation breeding in tepary bean could potentially broaden the gene pool of the crop. There is no information on the effect of chemomutagenesis on either seed proteins or root nodulation yet both traits (root nodulation and seed protein levels) are important traits in the development of new cultivars of tepary bean. For instance, increased levels of seed proteins would be desirable for consumers. In addition, cultivars fixing relatively large amounts of nitrogen and producing high grain yields could be desirable for farmers. Therefore, this study was undertaken to evaluate the effects of induced mutation by ethyl methanesulphonate (EMS) on both the root nodulation and seed protein traits in tepary bean.

1.2 Problem statement

Tepary bean growers in South Africa utilize traditional unimproved varieties that are low yielding. The low agronomic performance of tepary bean limits the adoption of the crop by farmers. Therefore, it is desirable to develop highly productive cultivars that can be adopted more widely by growers. However, the narrow gene pool of the species limits the genetic improvement of the crop. Mutation breeding potentially creates considerable genetic variability of agronomic traits such as root nodulation traits and seed protein accumulation thus enabling selection for these traits.

1.3 Rationale of the study

Broadening the genetic base of tepary bean through induced mutation will enable the development of new cultivars with improved agronomic and nutritional traits. Successful creation of genetic variability for several agronomic and nutritional traits in many crop species using mutation breeding approaches has been reported in previous studies. However, the use of mutation breeding approaches such as chemomutagenesis for widening the genetic base of tepary bean has not been investigated adequately in order to be applied in the genetic improvement of the crop. Successful creation of genetic variation for root nodulation and seed protein will accelerate the breeding for new cultivars that can be adopted by tepary bean growers.

1.4 Objectives of the study

The broad objective of the study was to evaluate the effect of induced mutation on root nodulation traits and seed protein in tepary bean. The specific objectives of the study were to:

(i) determine the effect of induced mutation by ethyl methanesulfonate (EMS) on the root nodulation traits of tepary bean

(ii) determine the effect of induced mutation by EMS on the quantity of seed protein of tepary bean

(iii) determine the effect of induced mutation by EMS on the profiles of seed protein in tepary bean.

1.5 Hypotheses

The study tested the following hypotheses:

(i) there were no significant differences in root nodulation traits among the mutagenized generations (M_2 to M_4) of tepary bean

(ii) there were no significant differences in seed protein accumulation among mutagenized generations of tepary bean

(iii) chemomutagenesis did not induce genetic variability in the profiles of tepary bean seed proteins.

1.6 Dissertation outline

This dissertation is comprised of five chapters. The first chapter gives an introduction of the tepary bean crop, outlining the objectives of the study and hypotheses that were tested in the study. The second chapter reviews the relevant aspects of literature on mutation breeding and key agronomic and nutritional traits of tepary bean (root nodulation and seed protein). Chapter three and four are designed as stand-alone chapters concentrating on specific components of the study. The third chapter focused on the evaluation of the effect of induced mutation by EMS on root nodulation traits of three mutagenized generations (M_2 to M_4) of the crop, while the subsequent chapter focused of the effects of induced mutation on the quantity and seed storage protein profiles of tepary bean mutants. Chapter five summarizes the findings of the study as well as outlining recommendations for future work. Due to a close relationship between the components of this study, some literature sources cited appear repeatedly in different chapters.

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CHAPTER TWO: LITERATURE REVIEW

2.1 The biology and genetics of tepary bean

Tepary bean (*Phaseolus acutifolius*) is a diploid species (2n=2x=22) native to Mexico and the South-western United States (Thomas *et al.,* 1983). It is a dicotyledonous, self-pollinating annual grain legume which is well adapted to harsh environmental conditions. The crop is distinguished from other bean species by its epigeal germination. It is a viny plant with a taproot system and can grow up to 4.0 m in length. In addition, tepary bean matures as early as 54 days after planting with flowering occurring within 27-40 days (Rao *et al*., 2013; Jiri and Mafongoya, 2016).

It is highly tolerant to drought (Beebe *et al.,* 2013), fungal diseases (Miklas and Santiago, 1996), insect pests (Pratt *et al.*, 1990), high temperatures and poor soil fertility. Partly due to these desirable traits, tepary bean was used in the introgression of useful genes into common bean (*Phaseolus vulgaris*) (Muñoz *et al.,* 2004). Wild tepary bean gene pools have disappeared gradually partly due to habitat degradation. In contrast, domesticated tepary bean suffered genetic erosion due to the shrinking area under its cultivation (Scott and Michaels, 1992).

2.2 Major uses of tepary bean

Tepary bean is mainly produced for human consumption. It is also used as a livestock feed. The grain of tepary bean is high in protein (25%), carbohydrates, dietary fibre and vitamins (Bhardwaj, 2013; Narina *et al.,* 2015). The crop can be used as a source of household income. In South Africa, it is grown in the drier southern parts of Limpopo Province by smallholder growers who trade the grain through informal markets. The crop is also used in crop rotations with cereals and vegetables. In addition, the crop is important for its ability to ameliorate soil fertility through nitrogen (N) fixation which can reach up to 260 kg N/ha (Shisanya, 2003).

Lectins found in tepary bean seed are useful in the treatment of cancer (Arteaga *et al.,* 2016; Moreno–Celis *et al.,* 2017; Valadez-Vega *et al.,* 2014). The seed also contains high levels of antioxidant enzymes, mineral and anti-cancerous properties aiding in prevention of various ailments such as colon cancer, coronary heart diseases and diabetes (Valadez-Vega *et al.,* 2011, Jiri and Mafongoya, 2016). Furthermore, its slow sugar release enables diabetic patients to retain protein in the body without risking an increase in body sugar levels (Karlstrom *et al.,* 1987). It has a lower glycaemic index (rate at which food raises blood sugar levels).

2.3 Agronomic characteristics of tepary bean crop

Tepary bean possesses a range of useful agronomic characteristics. However, the productivity of the crop is generally low under smallholder cropping systems particularly in Limpopo Province of South Africa.

2.3.1 Grain yield

The grain yield of tepary bean is generally low (<0.5 t/ha) in most production areas. Grain yield of the crop is low partly because farmers use traditional landraces in mixed cropping systems with limited production inputs (Gwata *et al.,* 2016; Molosiwa *et al.,* 2014). Grain yields under dryland (800 kg/ha) and irrigation (1700 kg/ha) conditions were reported previously (Miklas *et al.,* 1994). However, Bhardwaj *et al.,* (2002) reported >2000 kg/ha of grain yield under dryland farming.

2.3.2 Biological nitrogen fixation and soil fertility

Biological nitrogen fixation arises as a result of a symbiotic relationship between soil bacteria collectively called rhizobia and tepary bean (Mohrmann *et al.,* 2017; Vessey *et al.,* 2005). Under nitrogen limiting conditions, the rhizobial bacteria reduce atmospheric N_2 into NH_3 via nitrogenase enzyme and exchange this nitrogenous solute for photosynthates from the host plant (Peoples *et al.,* 2002; Martins *et al.,* 2014; Gresshoff, 2003). High rhizobia populations in the soil are necessary for effective symbioses without seed inoculation with commercial inoculants at planting.

Symbiosis is initiated when nod factors of the rhizobium release lipo-chitinoligosaccharide molecules and nod-genes are expressed to respond to flavonoids secreted by the plant into the soil (Colebatch *et al.,* 2004). Rhizobia penetrate differentiating root hair walls and progress across the root cortex in an infection thread (Cardoso *et al.,* 2009). Colonized plant root hairs curl and cortical tissues are invaded by an infection thread traveling into the nodule primordium. The bacterium multiplies rapidly and nod factors modify plant hormones resulting in a peri-bacteroid which subsequently develops into the root nodule. Photosynthates are transported to the root nodules in the form of sucrose through a vascular network (Puppo *et al.,* 2005). In return, nitrogen containing compounds are translocated from root nodules to the host plant where they are catabolized and assimilated into biological molecules and used for protein synthesis (Keyser and Li, 1992). The reduction of atmospheric N_2 into NH_3 by nitrogenase enzyme takes place inside the mature nodules.

In addition, tepary bean crop residues can add organic content through decomposition as well as root exudates in the rhizosphere. Beneficial effects of fixed N from leguminous crops in intercropping systems and crop rotations have been reported widely (Li *et al.,* 1999; Inal *et al.,* 2007; Mucheru-Muna *et al.,* 2010). Nitrogen is essential for optimum grain yield. Inclusion of grain legumes such as tepary bean in intercrops with maize is a strategy to improve food security for most rural communities with limited arable land in Limpopo Province. Hence, cultivation of this crop could be important for resource poor smallholder farmers as it can fix significant amounts of nitrogen (Shisanya, 2005).

2.3.3 Drought tolerance

Tepary bean is highly tolerant to drought and high temperatures (Mohamed *et al*., 2002). It can grow in areas where annual rainfall is less than 450 mm. The drought tolerance is attributed to sensitive stomata which close at relatively high-water potentials (Medina *et al.,* 2017). In addition, the crop avoids soil water deficit by completing its lifecycle quickly within approximately 90 days. It has a deep and extensive root system which enables it to penetrate deep soil levels in search of water (Butare *et al.,* 2011; Mohamed *et al.,* 2005; Gwata and Mzezewa, 2013). Moreover, tepary bean has small leaves that are associated with reduced water loss (Mohamed *et al.,* 2005; Beebe *et al.,* 2013).

2.3.4 Seed protein content and nutritional composition

Tepary bean is source of protein due to its high protein content (21-31.9%) in the seed (Porch *et al.,* 2017; Thomas *et al.,* 1983). Legumes are considered good substitution for animal protein in human diet mainly in the third world (Valizadeh, 2001). The crop contains higher levels of protein, oil, calcium, magnesium, zinc, phosphorus and potassium than common bean (pinto, navy and red kidney) (Bhardwaj and Hamama, 2004). The major nutritional advantages of the bean lie in the mature seed which contains 0.9-1.17% fat and 65.3-69.1% carbohydrates (Scheerens *et al.,* 1983). Tepary bean grain provides about 1478 kJ (353 kcal) of energy, protein and some vitamins (Table 2.1).

Table 2.1 Nutritional composition of tepary bean grain.

Nutritional component	**Quantity (per 100g of dry grain)**
Protein	23.9 %
Cabohydrates	67.3 %*
Fibre	4.8 %*
Calcium	184.2 mg
Magnesium	191.9 mg
Phosphorus	451.0 mg
Manganese	2.8 mg
Zinc	4.3 mg
Potassium	1531.0 mg
Iron	10.7 mg
Fat	1.2 %*

Adapted: Bhardwaj and Hamama, 2004; *Leung *et al.,* 1968.

2.4 Mutation breeding

Mutation breeding is the process of treating plant germplasm with mutagens such as physical (UV light, X-rays and gamma radiation) or chemical (ethyl methanesulfonate, diethyl sulfonate and ethidium bromide) to generate mutants with novel and valuable traits (Mba *et al.,* 2010). EMS mutagenesis has been noted to cause point mutations occurring when a gene's single base pair is altered. These point mutations alter the coding sequences of amino acid thus resulting in altered protein structures (Acanda *et al.,* 2014).

EMS is the most widely used mutagen in plants for development of large mutant populations because it creates large numbers of point mutations in almost all species studied and the mutation frequency seems to be independent of genome size (Greene *et al.,* 2003; Henikoff and Comai, 2003; McCallum *et al.,* 2000). Chemomutagenesis was used to induce useful mutations in a range of leguminous crops such as soybean (*Glycine max*), lentils (*Lens culinaris*), cowpea (*Vigna unguiculata*), mungbean *(Vigna radiate)* and chickpea (*Cicer arientinum*) (Table 2.2). However, the genetic variation achieved through this approach is random since any part of the gene can be mutated altering the open reading frames resulting in alterations of protein (Girija and Dhanvel, 2009; Greene *et al.,* 2003). According to Yuan and Zhang, (1993) whole chromosomes segments can be affected and the plant passes this alteration to its offspring.

Tepary bean is a source of genes for drought tolerance (Beebe *et al,* 2013), resistance to diseases and insect pests (Singh and Muñoz, 1999) that were previously introgressed into common bean (*Phaseolus vulgaris*). These novel traits could be significantly exploited through plant breeding approaches. However, plant breeding efforts aimed at developing improved cultivars are limited by the narrow gene pool of the crop. Mutation breeding is an effective tool used for generating genetic variability aimed at crop improvement.

Table 2.2 Examples of leguminous crops which utilized chemomutagens successfully.

Chemomutagen	Crop species	Reference
Ethyl methanesulfonate	Soybean (*Glycine max*)	Karthika and Lakshini, 2006
	Chickpea (*Cicer arientinum*)	Barshile, 2015
	Lentil (*Leus culinaris*)	Gaikwad and Kotheka, 2004
	Cowpea (*Vigna unguiculata*)	John, 1999
	Pigeon pea (*Cajanus cajan*)	Ariraman *et al.,* 2015
	Lablab bean (*Lablab purpureus*)	Monica and Seetharaman, 2016
Sodium azide	Groundnut (*Arachis hypogea*)	Mensah and Obadonni, 2007
	Chickpea *(Cicer arientinum)*	Mahesh *et al.,* 2011
	Mungbean *(Vigna radiate)*	Khan *et al.,* 2004
	Lentil (*Leus culinaris*)	Asad *et al.,* 2014

2.4.1 Effects of induced mutation on root nodulation

Despite the wide application of mutation breeding on both cereals and legumes, the effects of chemomutagenesis on tepary bean are largely unknown especially on both the root nodulation and seed protein traits. However, diminished seed weight (Craig *et al.,* 1999) and complete suppression of root nodulation (Gresshoff, 1993) were reported in pea (*Pisum sativum*) and soybean (*Glycine max*), respectively. Akhtar, (2014) observed that an increase in EMS doses led to lethal effects and significantly reduced morphological parameters such as shoot height. Reduction in shoot height arising from mutagenic treatments was reported in chickpea (Haq *et al.,* 1992). High (16.38g) shoot dry weight was obtained in horsegram *(Macrotyloma uniflorum)* with 0.4% and 0.5% EMS treatments in the M_2 and M_3 generations (Bolbhat and Dhumal, 2012). Moreover, root dry weight also showed a similar trend in the M_2 generation. Non-nodulating *Phaselous vulgaris* cultivars were previously induced by treatment with 0.04M EMS (Park and Buttery, 1992).

2.4.2 Effects of induced mutation on seed proteins

Alteration in seed protein contents and profiles are a result of changes induced when genes are mutated. High protein (21-35%) and amino acid content in faba bean and common bean following treatment with EMS and gamma rays were reported (Hussein and Abdalla, 1979). Increased protein content was observed in EMS, sodium azide and gamma radiation induced mutants in the M_3 generation in chickpea (Barshille and Apparao, 2009). Belele *et al.,* (2001) observed a variation of seed storage protein profiles in common bean mutants.

2.5 Evaluation of root nodulation

Examining nodule appearance, location on roots or stem, internal discoloration and structure is important in establishing root nodulation status of a legume (Sprent, 2005). The root nodule number is accomplished by carefully removing nodules by hand and counting them six to eight weeks after emergence. Root nodules are further dissected into halves with a blade to establish effectiveness. Effective root nodules are recognized generally by their pinkish–red internal colour (Gwata *et al.,* 2003; Shisanya, 2002). Nodule dry weight and shoot dry weight are also positive indicators of effective N fixation (Mubarik *et al*., 2010; Serraj and Sinclair, 1998). In practice, effective fixation in legumes is evaluated under N deficient conditions in order to visually identify genotypes that can fix N through their distinct green leaf colour (Gwata and Wofford, 2013).

2.6 Characterization of seed storage proteins

Identification and characterization of seed storage proteins of legumes can be accomplished through using various biochemical techniques. Among these methods, the sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is the most widely used in characterizing profiles of seed protein profiles due to its easiness and effectiveness (Pratt *et al.,* 1990; Siddiqui *et al.,* 2010; Ghafoor and Ahmed, 2005). Taxonomic and evolutionary relationships are effectively solved by distinguishing between genotypes based on electrophoretic mobility of proteins thus molecular weights of proteins (Campos *et al.,* 2004; Kami *et al.,* 1995; Alsohaimy *et al.,* 2007; Das & Mukarjee, 1995).

Protein polymorphisms of most grain legume species are well documented (Ghafoor *et al.,* 2003; Asghar *et al.,* 2003; Javid *et al.,* 2004). The technique was used to differentiate chickpea (Hameed *et al.,* 2009), groundnut (Valizadeh, 2001), pistachio (Ehsanpour *et al.,* 2010), mungbean (Hameed *et al.,* 2012) and tepary bean (Idouraine *et al.,* 1994) cultivars successfully. In tepary bean, it has been reported that the presence of a 33kDa protein subunit in tepary bean accession G40199 corresponding to molecular size of lectins was responsible for strong resistance to bruchids (Mbogo *et al.,* 2009). Idouraine *et al.,* (1994) indicated that albumin constitute 83% of the seed storage protein in tepary bean.

2.7 References

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CHAPTER THREE: THE EFFECT OF INDUCED MUTATION BY EMS ON ROOT NODULATION TRAITS OF TEPARY BEAN (*Phaseolus acutifolius*)

Abstract

Tepary bean (*Phaseolus acutifolius*) is an annual grain legume originating from South America and southwestern United States. The crop is cultivated in many countries worldwide including South Africa. It is highly tolerant to drought and is important in soil fertility amelioration through biological nitrogen fixation. This study examined root nodulation attributes of three tepary bean genotypes in the early mutagenic generations (M_2 to M_4) derived through treatment with ethyl methanesulfonate in a nursery at the University of Venda (22°56′ S; 30°28′ E, 724 m a.s.l.). The experiment was laid out as a 3 x 5 x 3 (genotypes x EMS doses x mutant generations) factorial design replicated three times. At harvest, shoot height (SHT), primary root length (PRL), number of nodules per plant (NNP), number of effective nodules (NEN), dry weights (root, shoot and nodule) and grain yield components such as number of pods per plant (NPP) and number of seeds per pod (NSP) were measured. Highly significant ($P \leq 0.01$) dose effects were observed for SHT, PRL, shoot dry weight (SDW) and root dry weight (RDW), while significant ($P \leq 0.05$) mutant generation x genotype effects were observed for NPP. Generation x dose interaction effects were highly significant ($P \leq 0.01$) for SHT, PRL, SDW and RDW. There was a notable increase in most of the root nodulation traits at the 0.5% EMS dose. There were no effective root nodules observed in the M_4 generation. However, there was effective symbiosis between the rhizobial strain and tepary bean as indicated by the pink coloration of the internal nodular tissue. Highly significant ($P \leq 0.01$) positive correlation (0.897) was observed between NNP and nodule dry weight (NDW) suggesting that an increase in NNP led to increased NDW. The study demonstrated that some positive attributes of root nodulation could be induced by EMS in tepary bean.

Key words: bean; dose; genotype; mutagenesis; nodulation

3.1 Introduction

Tepary bean (*Phaseolus acutifolius*) is a grain legume native to southwestern United States, Texas and Mexico (Mapp *et al.,* 2016). The tepary bean plant is more drought and heat tolerant than other *Phaseolus* species. This grain legume can flourish under extreme drought stress (Jury and Vaux, 2007). Tepary bean is a source of useful genes for disease resistance, insect pests and drought tolerance. However, the crop has a characteristic narrow genetic base limiting genetic improvement of this crop (Gwata *et al.,* 2016). Genetic variability was successfully achieved in many crop species using mutation breeding approaches aimed at crop improvement.

Mutations can occur spontaneously or be induced by using radioactive or chemical mutagens. Comai and Henikoff, (2006) indicated that ethyl methansulphonate (EMS) is considered as an effective and powerful mutagen. EMS induces chemical modification of nucleotides resulting in mispairing and base changes (Comai and Henikoff, 2006). The base pair substitution results in amino acid changes which can alter the function of proteins. Several studies have indicated that mutagenic effectiveness is an index of the response of genotypes to increasing doses of mutagens (Khan *et al*., 2009; Wani, 2009; Badere and Chaudhary, 2007).

Mutation breeding studies usually include segregating generations since only dominant mutations are visible in the M_1 generation. According to Page and Grossniklaus (2002), in the M_2 generation, the mutations segregate creating homozygotes for recessive and dominant alleles thus visual screening is an effective way in identifying phenotypic mutations. Visual screening is used as a primary indicator for selection of plants with desired characters (Østergaard and Yanofsky, 2004). Mutation breeding has been used in creating considerable genetic variability in a range of agronomic traits such as seed yield and quality, insect and disease resistance as well as seed coat color (Moh, 1971). Various studies have reported successful generation of mutants in biological nitrogen fixation (BNF) (Carroll *et al.,* 1985; Park and Buttery, 1989), increased root nodule number (Magori *et al.,* 2009), non-nod and hypernodulation mutants with enhanced nitrogen fixation (Ishikawa *et al.,* 2008). In tepary bean, these attributes have not been evaluated particularly after EMS treatment.

Legumes establish a beneficial association with compatible soil bacteria in a specialized organ called the root nodule. Bacteria obtain photosynthates from the host and differentiate into endosymbiotic bacteroid forms (Pierre *et al.,* 2014). Using the nitrogenase enzyme, the microsymbiont reduces atmospheric nitrogen to ammonia which is subsequently metabolized by the plant. Nitrogen fixation by legumes is often inconsistent, with or without inoculation (Maingi *et al.,* 2001). Biological nitrogen fixation leads to increased crop vigor, increase in crop yields and soil fertility improvement. Mohammadi *et al.,* (2012) indicated soil factors such as soil pH, drought and high temperatures (above 35^{o}C) have a negative impact on the nodulation process.

Dry matter accumulation in various plant parts (shoots, roots or nodules) was previously measured as a reliable indicator of N fixation in legumes (Ames *et al.,* 1991; Rao *et al.,* 2013). Shisanya, (2002) reported that inoculated tepary bean treatments had significantly higher dry weights than controls. However, Bala *et al.,* (2010) indicated that nodule dry weight (NDW) rarely gives significant differences as it is difficult to completely clean nodules from adhering soil. In a study conducted in soybean, it was stated that measuring NDW takes into account the presence of non-functional nodules (Gwata *et al.,* 2003).

In tepary bean, there is inadequate information regarding the effects of EMS on root nodulation. Therefore, the objective of this study was to determine the effect of induced mutation by EMS on the root nodulation traits of tepary bean. It was hypothesized that there are no significant differences in root nodulation traits among the mutagenized generations (M_2 to M_4) of tepary bean.

3.2 Materials and methods

3.2.1 Study location

The study was conducted in a nursery at the University of Venda in Thohoyandou (22°56′59″ S; 30°28′59″ E) at an altitude of 724 m. Mean daily temperatures at Thohoyandou vary from about 25^{o}C to 40^{o}C in summer and between 18^{o}C and 26^{o}C in winter. Rainfall is highly seasonal occurring between October and March, often with a mid-season dry spell during critical periods of growth. Mid-summer drought often leads to crop failure and low yields (FAO, 2009).

3.2.2 Genetic material

Germplasm (seed) of tepary bean obtained originally from growers in Sekhukhune District (Limpopo Province) was used in this study. Seed of the three genotypes (GEN 3, GEN 4 and GEN 6) was treated with EMS. The M_1 generation was selfed in order to derive the mutant generations (M_2 to M_4) that were used in the study (Appendix I).

3.2.3 Chemical treatment of the seed

Dry and healthy seed of each of the genotypes was treated with EMS following the procedure described previously by Bashir *et al.,* (2013). The mutagen treatment was performed in a laboratory at the University of KwaZulu Natal. Prior to EMS treatment, the seed was surface sterilized by 70% ethanol solution for 1 minute followed by soaking in a sodium hypochlorite solution for 10 minutes and rinsed for a couple of times using tap water. The seed was pre-soaked in distilled water at room temperature for 12 hours before being transferred into solutions containing the various doses of EMS (0.0, 0.5, 1.0, 1.5, 2.0 v/v) and incubated for 1 hour. The excess EMS was rinsed off with tap water for 2 hours and dried to enable safe handling (Jabeen and Mirza, 2004). This treated seed (M_1 generation) was planted, germinated seedlings grown and subsequently selfed in the greenhouse in order to obtain the M_2, M_3 and M_4 mutant generations (Appendix I).

3.2.4 Planting

The seed of each genotype was surface sterilized as described by Goethal *et al.,* (1989) and planted in a Leonard jar (Fig 3.1) filled with sterilized river sand (Vincent, 1970) which had been passed through a 2.0 mm sieve. Prior to planting, the Leonard jars were cleaned with a sodium hypochlorite (NaOCl) bleach solution. Hoagland (5%) medium was used as a nutrient solution and topped up every 10 – 14 days.

The seed was inoculated with X521 rhizobial strain cultured at the University of Venda. Inoculation of the seed was performed following procedure described by Gwata *et al.,* (2003) with modifications. Pieces of agar supporting rhizobial colonies were excised from subculturing dishes to prepare the inoculum. These excised pieces were then placed in a bottle containing 500 ml of distilled water and two droplets of dishwashing liquid. The rhizobial cells were then dispersed by vigorously shaking the bottle which was subsequently wrapped with aluminium foil to protect the rhizobia from UV light. A seed was placed in the centre of each Leonard jar in a hole 3 cm deep. After saturating the seed with inoculum, the seed was immediately covered with sand (Mapp *et al.,* 2016). The resultant plants were then allowed to grow for eight weeks after emergence before harvesting.

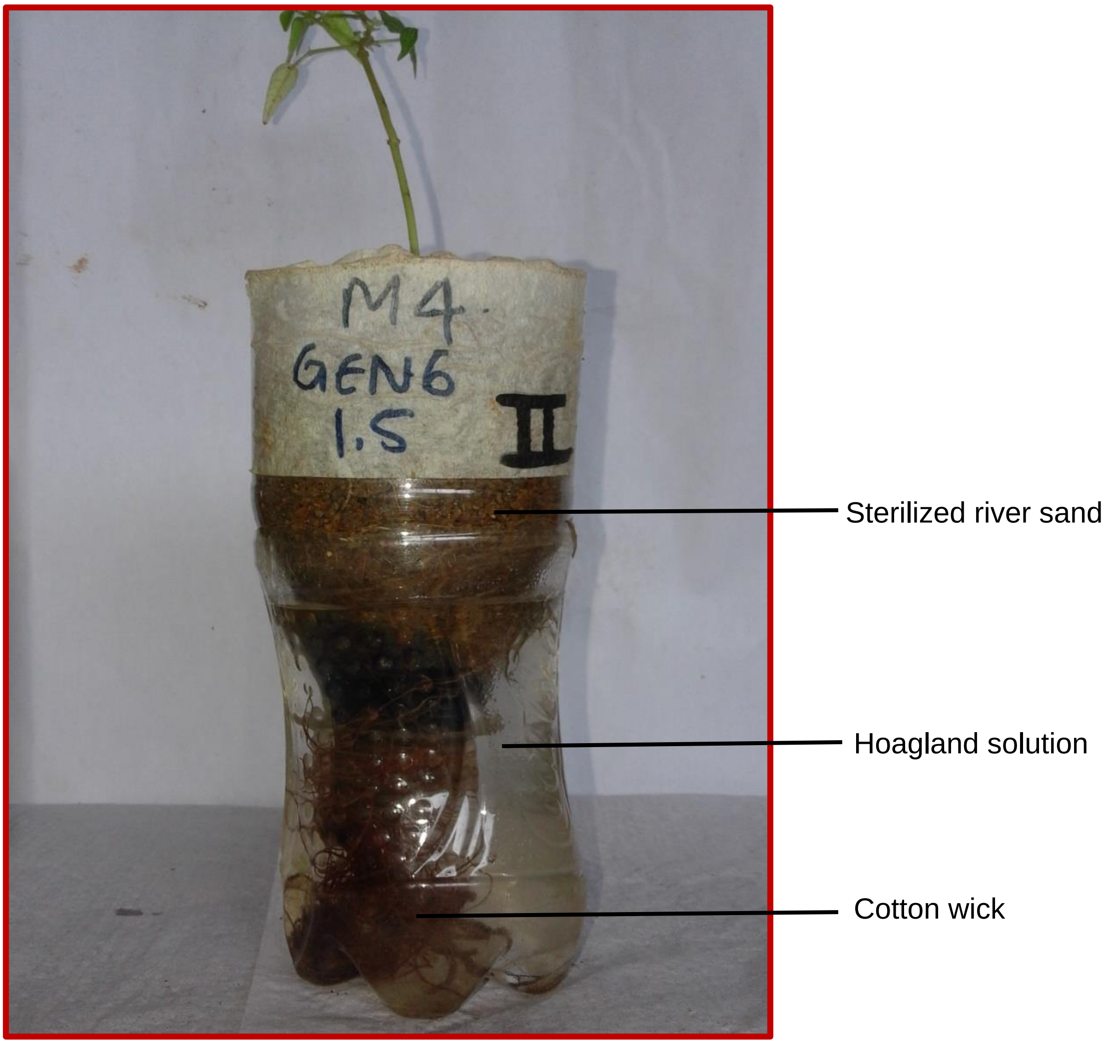

Fig 3.1 A mutant tepary bean plant growing in a modified Leonard jar with sterilized river sand and Hoagland solution.

3.2.5 Measurements

At harvest, tepary bean roots were gently washed with tap water and the nodule number was counted. Nodule internal color was determined (Appendix II). In addition, the following variables were measured or scored:

(i) shoot height (mm) (SHT)

(ii) primary root length (mm) (PRL)

(iii) number of nodules per plant (NNP)

(iv) number of effective nodules (NEN)

(v) shoot dry weight (g) (SDW)

(vii) root dry weight (g) (RDW)

(viii) nodule dry weight (g) (NDW)

(viii) number of pods per plant (NPP)

(ix) number of seeds per pod (NSP)

(x) leaf color score (LCS) (1 = chlorotic leaves; 5 = dark green leaves)

(xi) pod development score (PDS) (1 = poorly developed pods; 5 = well developed pods)

The dry weights (shoot, root and nodule) were measured after oven drying for 72 hours at 70°C (Delić *et al.,* 2010)**.** Leaf color score (LCS) and pod development score (PDS) were determined at the 8wk growth stage.

3.2.6 Experimental design and data analysis

The experiment was laid out as a 3 x 5 x 3 (genotypes x EMS doses x mutant generations) factorial design with three replications. Each replication consisted of 45 Leonard jars comprising of the three mutagenic generations, three genotypes and five levels of EMS doses. Data obtained for the variables were subjected to analysis of variance (ANOVA) using Statistix (2017) version 10.0. Tukey's test was used for mean separation at $P \leq 0.05$. Pearson's correlation was used to test the strength of linear associations between the traits studied. The following statistical mixed model was used:

$Y_{ijkl} = \mu + R_i + G_j + M_K + S_l + GM_{jk} + GS_{jl} + MS_{kl} + GMS_{jkl} + E_{ijkl}$

where:

Y_{ijkl} = observation from i^{th} rep, j^{th} genotype, k^{th} mutation treatment and l^{th} generation

μ = overall mean

R_i = random effect of the i^{th} replication

G_j = j^{th} genotype fixed effect

M_K = k^{th} mutation treatment fixed effect

S_l = l^{th} generation fixed effect

GM_{jk} = fixed interaction effect of j^{th} genotype by k^{th} mutation treatment

GS_{jl} = fixed interaction effect of j^{th} genotype by l^{th} generation

MS_{kl} = fixed interaction effect of k^{th} mutation treatment by l^{th} generation

GMS_{jkl} = fixed interaction effect of j^{th} genotype by k^{th} mutation treatment by l^{th} generation

E_{ijkl} = residual error

3.3 Results

The results demonstrated the effects of chemomutagenesis induced by varying doses of EMS on root nodulation characteristics of tepary bean genotypes over three successive generations. EMS treatment induced chlorophyll mutation (albino with pale yellow leaves) in some plants that eventually withered and died (Fig 3.2).

Fig 3.2 An albino tepary bean mutant plant observed in the nursery.

3.3.1 Effects of EMS on root nodulation attributes

There were highly significant (P≤0.01) differences due to dose effects on the SHT, PRL, SDW, RDW and LCS (Table 3.1). A similar pattern on the same attributes was observed for the generation x dose interaction. Similarly, significant (P≤0.05) mutant generation x genotype effects were observed for NPP, LCS and PDS (Table 3.1). Significant mutant generation x genotype x dose interactions were observed only for NSP.

There were no significant (P>0.05) differences among generations in terms of the shoot and root attributes. In addition, the pod development score (PDS) was similar among the three genotypes under the EMS dose treatments. However, M_3 generation showed the tallest shoots (137.40mm). ‘Genotype 3’ attained the longest primary roots (88.78mm). In comparison with ‘Genotype 6’, the shoots for ‘Genotype 3’ were >15.0% taller.

Highly significant (P≤0.01) dose effects were observed for the SHT, PRL, SDW, RDW and LCS (Table 3.2). The longest shoots (173.85mm) and longest primary roots (99.52mm) were attained with 0.5% EMS dose. Similarly, the SDW observed for 0.5% EMS dose was three times heavier than that of the control (Table 3.2).

Table. 3.1 Analysis of variance for root nodulation attributes among mutant tepary bean genotypes over three mutagenic generations.

Source	Df	Mean Squares									
		SHT (mm)	PRL (mm)	NNP	NEN	NPP	NSP	SDW (g)	RDW (g)	LCS	PDS
Replication	2	7184.5	3783.1	0.0889	0.2074	0.2074	0.4222	0.0041	0.0139	1.6222	0.0667
Generation (G)	2	2503.9	1593.6	0.1556	0.2074	0.4519	0.0889	0.0109	0.0093	0.2000	3.6222
Genotype (Gt)	2	5438.7	2793.7	0.6222	0.0963	0.1407	0.9556	0.0089	0.0062	0.6000	1.7556
Dose (D)	4	48963.1**	18801.9**	0.0259	0.0370	0.7296*	0.6852	0.0803**	0.0173**	10.49**	4.0259
G x Gt	4	3316.8	842.6	0.0444	0.0629	0.6629*	0.7111	0.0101	0.0032	1.6333*	7.7778*
Gt x D	8	2821.3	1188.8	0.0759	0.0593	0.1963	0.6407	0.0076	0.0019	0.6926	2.6259
G x D	8	34574.6**	13988.6**	0.3870	0.1148	0.4796*	0.9129*	0.0450**	0.0138**	5.8481**	7.9926**
G x Gt x D	16	1178.6	695.6	0.1370	0.0537	0.5151	0.8269**	0.0676	0.0011	0.5315	3.0926
Mean	-	129.20	79.74	0.1556	0.0741	0.4148	0.4444	0.1504	0.0858	1.756	1.267
C.V. (%)	-	37.63	37.28	28.6	91.7	16.4	21.8	6.3	20.4	45.75	3.0
R^2 (%)	-	72.84	74.53	30.48	35.63	45.59	52.31	64.00	64.99	67.80	48.57

**, * = Significant at the 1.0% and 5.0% probability level respectively. (LCS = leaf color score; NEN = number of effective nodules; NNP = number of nodules per plant; NPP = number of pods per plant; NSP = number of seeds per pod; PRL = primary root length; PDS = pod development score; RDW = root dry weight; SDW = shoot dry weight and SHT = shoot height).

Table 3.2 The main effects of EMS dose on root nodulation attributes of tepary bean.

EMS dose (%)	**Traits**										
	SHT (mm)	**PRL (mm)**	**NNP**	**NEN**	**NPP**	**NSP**	**SDW (g)**	**RDW (g)**	**(NDW (g)**	**LCS**	**PDS**
0.0	58.52 c	33.59 b	0.1481 a	0.0741 a	0.1481 b	0.2593 a	0.0668 c	0.0427 b	0.000307 a	0.6667 b	0.8184 a
0.5	173.85 a	99.52 a	0.1852 a	0.0370 a	0.5556 a	0.6296 a	0.2195 a	0.1078 a	0.000422 a	2.2593 a	1.7407 a
1.0	139.11 ab	92.07 a	0.1111 a	0.0370 a	0.3704 ab	0.3704 a	0.1572 ab	0.0971 a	0.000200 a	1.9259 a	1.1111 a
1.5	132.96 b	84.11 a	0.1481 a	0.1111 a	0.4814 ab	0.5926 a	0.1489 b	0.0863 a	0.000326 a	1.9630 a	1.5926 a
2.0	141.52 ab	89.41 a	0.1852 a	0.1111 a	0.5185 a	0.3704 a	0.1595 ab	0.0952 a	0.000652 a	1.9630 a	1.0741 a

Means followed by the same letter in a column are not significantly different ($P \leq 0.05$) (Tukey's test). (LCS = leaf color score; NDW = nodule dry weight; NEN = number of effective nodules; NNP = number of nodules per plant; NPP = number of pods per plant; NSP = number of seeds per pod; PRL = primary root length; PDS = pod development score; RDW = root dry weight; SDW = shoot dry weight and SHT = shoot height).

3.3.2 Shoot height

There was a three fold increase in the SHT for 0.5% EMS dose in comparison with the control in all the three genotypes but followed by a gradual decrease thereafter at 1.0% EMS dose (Fig 3.3). ‘Genotype 4’ had stable SHT from 1.0% EMS dose to the maximum dose (2.0%). The tallest shoots (184.33mm) were observed for ‘Genotype 4’ at 0.5% EMS dose, while the shortest roots were attained at the 1.5% EMS dose by ‘Genotype 6’ (Fig 3.3). Interestingly, the increase in the EMS dose from 1.0% to 2.0% had no detectable effect on the SHT in ‘Genotype 4’ (Fig 3.3).

3.3.3 Primary root length

A three fold increase in the primary root length (PRL) in all the three genotypes in comparison with the control was observed (Fig 3.4). However, there was a reduction in PRL in the 0.5% to 1.0% EMS dose interval for both ‘Genotype 4’ and ‘Genotype 6’. In contrast, the PRL for ‘Genotype 3’ increased when treated with 1.0% EMS dose but did not change significantly when treated with 1.5% EMS dose as was the case for the other two genotypes (Fig 3.4). The longest roots (111.33mm) were attained by ‘Genotype 3’ at 1.5% EMS dose.

3.3.4 Shoot and root dry weight

At 0.5% EMS dose, all the genotypes showed a marked increase in shoot dry weight (SDW) in comparison with the control (Fig 3.5). ‘Genotype 4’ attained the highest (0.24g) SDW at 0.5% EMS dose. At 1.5% EMS dose, both ‘Genotype 3’ and ‘Genotype 6’ showed an improvement in SDW but ‘Genotype 4’ was negatively affected by the same EMS concentration, while the root dry weight (RDW) declined steadily in both ‘Genotype 4’ and ‘Genotype 6’ at EMS dose >0.5%, ‘Genotype 3’ showed a slight increase in the RDW at the same EMS dose (>0.5%) (Fig 3.6).

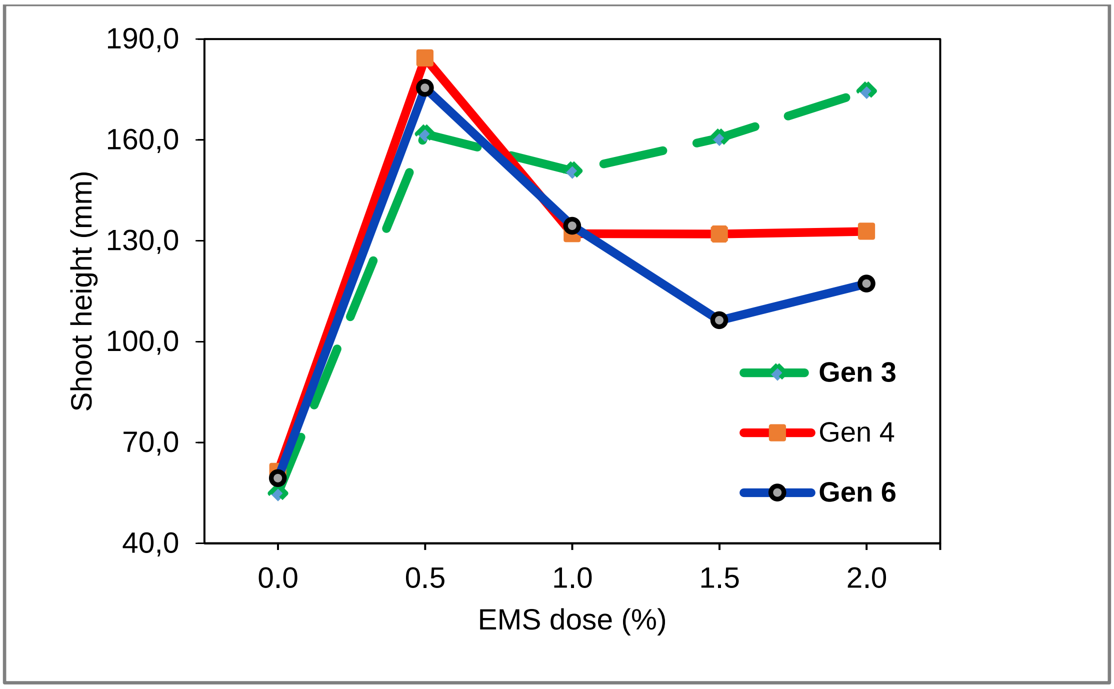

Fig 3.3 The effect of induced mutation by varying levels of EMS doses on the shoot height of three tepary bean genotypes over three mutant generations.

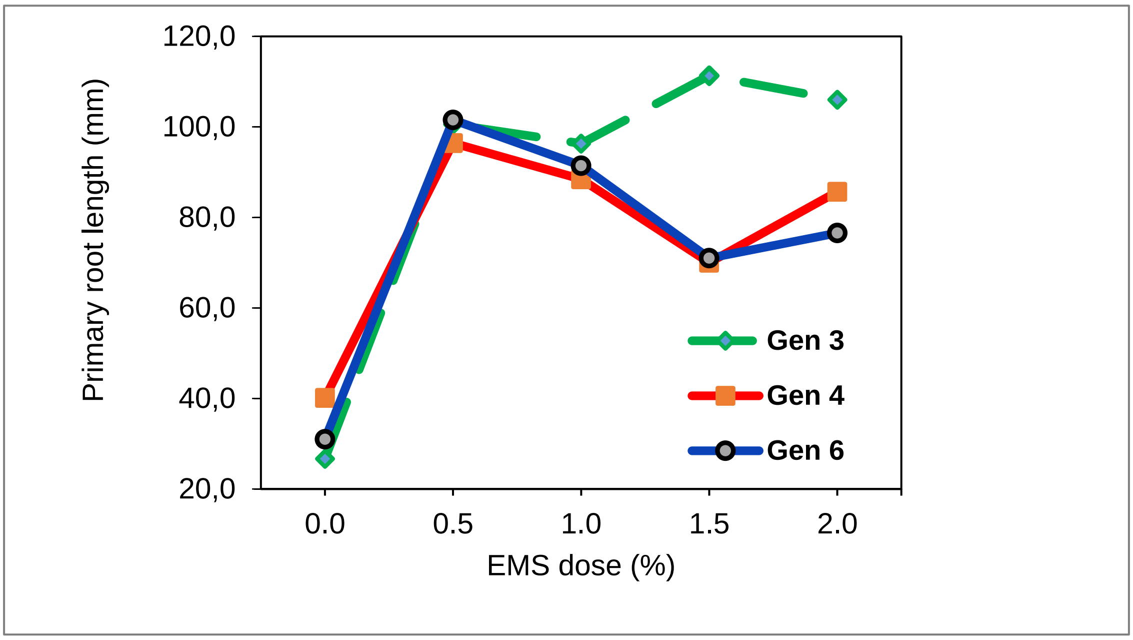

Fig 3.4 The effect of EMS on primary root length of three tepary bean genotypes over three mutant generations.

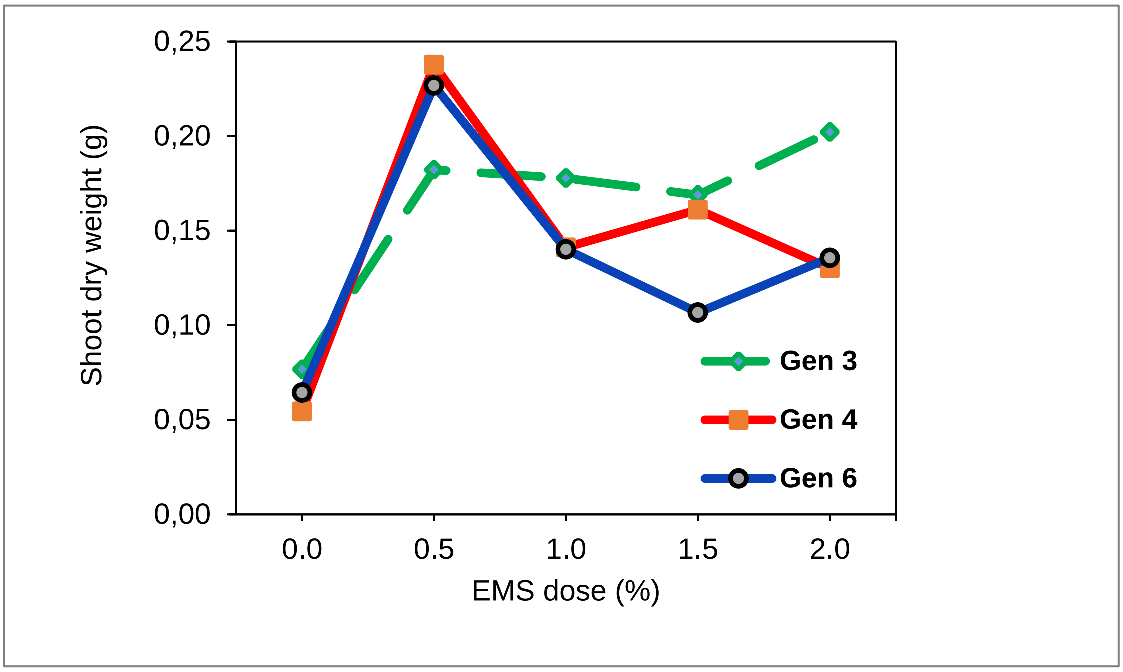

Fig 3.5 The effect of induced mutation by varying levels of EMS doses on the shoot dry weight of three tepary bean genotypes over three mutant generations.

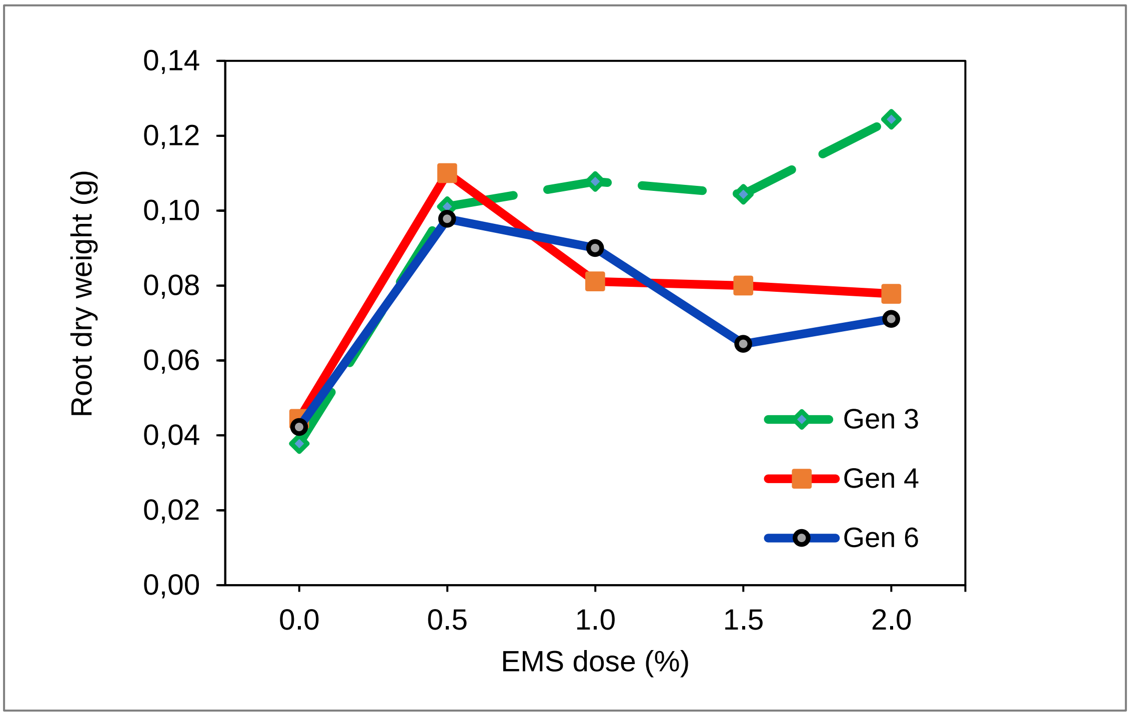

Fig 3.6 The effect of induced mutation by varying levels of EMS doses on the root dry weight of three tepary bean genotypes over three mutant generations.

3.3.5 Number of pods per plant

Prior to harvesting (terminating the experiment), some of the plants produced pods (Fig 3.7). Nonetheless, the number of pods per plant (NPP) was low on average. The highest NPP was observed for both ‘Genotype 4’ and ‘Genotype 6’ at the 0.5% EMS v/v but a clear general trend in terms of the impact of EMS on NPP was not established (Fig 3.8).

Fig 3.7 A mutant tepary bean plant with a well developed pod.

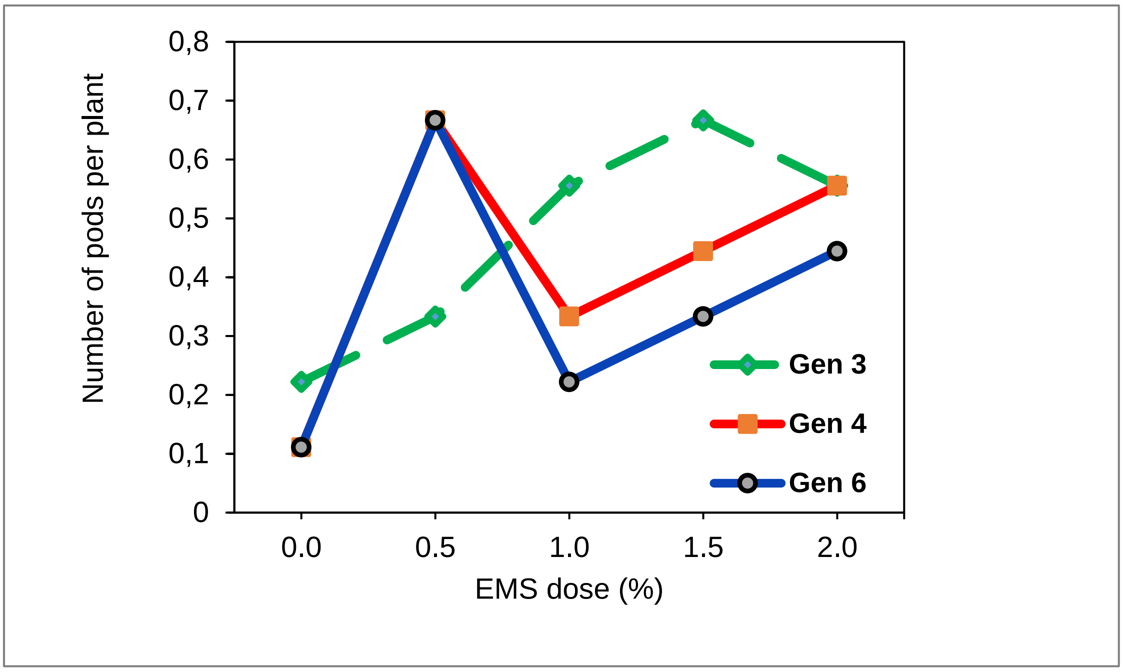

Fig 3.8 The effect of induced mutation by varying levels of EMS doses on the number of pods per plant of three tepary bean genotypes over three mutant generations.

3.3.6 Number of nodules per plant, effective nodules and nodule dry weight

The nodule load per plant was low and did not exceed two in general. Specifically, ‘Genotype 4’ developed nodules only at the 2.0% EMS dose. Overall, there was no clear impact of the EMS on nodule development among the three genotypes. Similarly, there was no clear trend of the impact of EMS on the number of effective nodules. Nonetheless, the effective nodules as characterized by the pinkish internal colour were evident in some of the plants (Fig 3.9). In addition, the nodule dry weight was also very low since it depended directly on the NNP which was low as indicated above.

Fig 3.9 A mutant tepary bean plant with effective nodules.

3.3.7 Correlation coefficients between root nodulation attributes.

There were positive correlations observed for all the root and shoot attributes observed (Table 3.3). Highly significant (P≤0.01) positive relationships were observed between SHT, PRL, RDW and SDW with almost all the other attributes. A highly significant (P≤0.01) positive relationship was observed between SHT and PRL indicating that taller plants had longer roots. Similarly, a highly significant (P≤0.01) relationship was observed between NNP and NDW suggesting increased NNP led to increased NDW (Table 3.3).

Table 3.3 Pearson's correlation coefficient (r) for EMS derived tepary bean mutants that were evaluated in N-depleted media. (LCS = leaf color score; NDW = nodule dry weight; NEN = number of effective nodules; NNP = number of nodules per plant; NPP = number of pods per plant; NSP = number of seeds per pod; PRL = primary root length; PDS = pod development score; RDW = root dry weight; SDW = shoot dry weight and SHT = shoot height).

	SHT	**PRL**	**NNP**	**NEN**	**NPP**	**NSP**	**PDS**	**LCS**	**SDW**	**RDW**	**NDW**
SHT	1.000										
PRL	0.896**	1.000									
NNP	0.202*	0.144	1.000								
NEN	0.144	0.098	0.713**	1.000							
NPP	0.508**	0.361**	0.143	0.144	1.000						
NSP	0.407**	0.266**	0.089	0.131	0.695**	1.000					
PDS	0.455**	0.288**	0.242**	0.289**	0.818**	0.858**	1.000				
LCS	0.825**	0.812**	0.183*	0.147	0.437**	0.300**	0.353**	1.000			
SDW	0.853**	0.712**	0.131	0.078	0.548**	0.531**	0.580**	0.728**	1.000		
RDW	0.853**	0.774**	0.193*	0.116	0.538**	0.392**	0.468**	0.753**	0.828**	1.000	
NDW	0.183*	0.141	0.897**	0.738**	0.054	0.050	0.129	0.129	0.083	0.127	1.000

**; * = Significant at the 1.0% and 5.0% probability levels, respectively.

3.4 Discussion

The results observed in this study were in agreement with those reported previously in similar studies. For instance, EMS induced chlorophyll mutations resulting in some albino plants that eventually withered and died. Kumari *et al.,* (2009) reported that chlorophyll mutants were due to mutation in genes responsible for chlorophyll biogenesis and functioning of photosynthetic apparatus. Ankele *et al.,* (2005) observed that RuBisCo which is the most abundant protein involved in the photosynthetic processes was either not expressed or expressed in low levels in albino plants suggesting a relationship between the absence of this protein and albinism. Similar observations were reported in mungbean (Wani *et al.,* 2011), cowpea (John, 1999) and blackgram mutants (Vannirajan *et al.,* 1993). However, Arisha *et al.,* (2015) reported on pepper mutants which survived until maturity although they exhibited diminished chlorophyll levels. Several studies have indicated that higher mutagenic efficiency can be achieved from lower doses of mutagens since lethality, injury and sterility increases with concentration of the mutagen at a faster rate than frequency of mutations (Solanki and Sharma, 1994; Wani, 2009; Dhanavel *et al.,* 2008; Bashir *et al.,* 2013; Begum and Dasguta, 2010).

The results also revealed a constant increase in the PRL with each successive mutant generation (M_2 to M_4). An increase in the PRL was observed with all the EMS doses (0.5 – 2.0%) in all the three tepary bean genotypes, whereas a notable three fold increase in this trait was attained at the 0.5% EMS dose. This suggested that these tepary bean mutants could be important for drought tolerance if grown in the field as they would be able to tap into deeper soil depths. Tepary bean has a deep and extensive root system enabling it to penetrate the soil in search of water (Butare *et al.,* 2011; Beebe *et al.,* 2013). Polania *et al.,* (2017) demonstrated the relationship between deep rooting system in water extraction from lower soil depths and superior resistance to drought in common bean. However, in other crops such as rice (Talebi *et al.,* 2012) and blackgram (Usharani *et al.,* 2017) an increase in EMS concentration and gamma radiation led to drastic reductions in root size in comparison with the control. This reduction was attributed to the effects of meristems inhibiting the synthesis of the growth stimulating auxins and consequent inhibition of cell divisions (Rupinder and Kole, 2005).

A significant three fold increase in SHT was observed at the 0.5% EMS dose level in all genotypes. Such genotypes could be important in soil amelioration if the crop residues are ploughed under. In other similar studies involving mungbean (Raza *et al.,* 2004) and groundnut (Sajid *et al.,* 2011), the increment in SHT was attributed to sufficient N-fixation. The findings in this study were also supported by those of Wi *et al.,* (2007) who reported that low doses (1 - 5Gy) of gamma radiation in *Arabidopsis* significantly increased the seedling growth. Kim *et* *al.,* (2004) proposed that mutagenesis induced growth stimulation by either changing the hormonal signaling networks in plant cells or by increasing the antioxidative capacity of cells. Moreover, the high coefficients of variance for traits such as the SHT, PRL and LCS could be due to the variation in EMS doses. Ganapati *et al.,* (2015) attributed the high genotypic coefficient of variation (GVC) and phenotypic coefficient of variation (PVC) in sugar beet (*Beta vulgaris*) to the induction of gamma rays.

The NPP was significantly influenced by EMS doses. Li *et al.,* (2007) indicated that crop yield is directly determined by yield components such as NPP and NSP. Kavera and Nadaf, (2017) reported on groundnut mutants with a higher number of NPP and NSP induced by EMS (0.5%) and gamma rays (200 and 300Gy) respectively. An increase in the yield of legumes such as the M_3 generation blackgram and pigeonpea was achieved by enhancing pod number (Sri Devi and Mullainathan, 2012; Singh and Malhotra, 1973). In the present study, highly significant ($P \leq 0.01$) interaction effects of mutant generation x genotype x dose were observed for NSP suggesting that breeding programs aimed at improving yield attributes should consider each of these factors.

Dry matter accumulation as measured by SDW and RDW were previously measured as reliable indicators of N fixation in legumes (Ames *et al.,* 1991; Rao *et al.,* 2013). Heavier shoots and roots were recorded for EMS doses ≥0.5%. 'Genotype 4' attained the highest SDW at 0.5% while 'Genotype 3' attained the highest RDW at 2.0% EMS dose suggesting that EMS treatment led to an enhanced partitioning of dry matter (assimilates) to the shoots and roots. Peanut genotypes with heavier root dry weight gave higher yields under drought conditions (Rucker *et al.,* 1995).

There were no significant differences in NNP and NDW observed for all the three factors (mutation generation, genotype and EMS dose). Nonetheless, a positive correlation between the two traits suggested that the increase in the NNP led to an increase in NDW which was in agreement with observations reported in other legumes such as cowpea and kidney bean (Ames *et al.,* 1991; Daniels-Hylton and Ahmed, 1994). Sinclair *et al*., (1991) reported a significant correlation between nodule number and weight in soybean genotypes. The positive correlation between the nodule number and nodule dry weight highlighted the effectiveness of the rhizobial isolate. However, Bala *et al.,* (2010) observed that the NDW rarely gives significant differences due to difficulties in cleaning nodules from adhering soil, whereas measuring NDW takes into account also nonfunctional nodules. The NNP observed in this study was lower than those recorded in other legumes such as soybean, adzuki bean and cowpea (Ngakou *et al.,* 2009; Delić *et al.,* 2010; Ballard *et al.,* 2004) suggesting that probably tepary bean nodules are more efficient in N-fixation in comparison with the other legumes. It would be interesting to investigate the variation in N-fixation among these legumes under uniform experimental conditions.

The three tepary bean genotypes were able to nodulate indicating that they were inoculated with viable rhizobia. Cross sections of the nodules and internal nodular tissue color indicated that the tepary bean genotypes were fixing nitrogen. However, no effective nodules were observed in the M_4 generation probably due to early senescence but this could not be concluded unequivocally since the plants were all grown in a uniform environment. In other studies, the leaf color score particularly dark green colored versus yellow (chlorotic) leaves was used to determine effective symbiosis (Gwata *et al.,* 2003; Fujihara *et al.,* 2006).

3.5 Conclusion

The current study demonstrated interesting effects of EMS treatment on root nodulation attributes in tepary bean. EMS doses ≥0.5% showed some advantages over the control. EMS induced a notable increase in the PRL suggesting that tepary bean mutants could be important in drought tolerance since they can be able to tap into deeper soil depths. The results also showed improved partitioning of dry matter in shoots and root in tepary bean mutants. A significant increase in the SHT in tepary bean suggested that such genotypes could be important in soil amelioration if crop residues are ploughed under. The results of this study showed that EMS mutagenesis could be effective in generating variability in some useful attributes of root nodulation in tepary bean.

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CHAPTER FOUR: THE EFFECTS OF INDUCED MUTATION ON THE QUANTITY AND SEED PROTEIN PROFILES OF TEPARY BEAN MUTANTS

Abstract

Tepary bean is an important food legume. The seed contains a wide range of vitamins, minerals and protein of high nutritional quality. The crop is characterized by a narrow gene pool which can be widened by chemical mutagenesis. However, the impact of chemical mutagenesis on the seed protein content and profile in this crop has not been determined adequately. Therefore, this study was designed to determine the seed protein content and profiles among the early filial generations (M_2 to M_4) of three tepary bean genotypes that were derived through treatment with ethyl methanesulfonate (EMS). The experiment was laid out as a completely randomizedd design with two replications. The data sets were subjected to analysis of variance using Statistix version 10.0 with mean separation at 5% level of significance. The Kjeldahl method was used for crude protein determination. The sodium dodecyl sulphate – polyacrylamide gel electrophoresis was utilized in determining protein banding patterns of the bean. There were highly significant ($P \leq 0.01$) differences observed for genotypes and EMS dose effects on crude protein accumulation. Highly significant ($P \leq 0.01$) interaction effects of mutant generation x genotype, doses x genotype and mutant generation x genotype x dose were observed for seed protein accumulation. 'Genotype 3' accumulated the highest protein content (24.23%) at 1.5% EMS dose in the M_4 generation. EMS doses ≥0.5% positively stimulated protein accumulation in all genotypes but high EMS dose (2.0%) depressed protein content. The results showed significant variations in the seed storage protein profiles among the genotypes and across the mutant generations. 'Genotype 3' showed a unique 20.0kDa protein subunit at the 0.5% EMS dose which was absent in the rest of the EMS doses in the M_2 generation. 'Genotype 4' showed two extra protein fragments at 40.0kDa and 100.0kDa that were absent in the control. 'Genotype 6' showed a distinct protein profile depicting the presence of a fragment (15.0kDa) which was absent in the majority of the remaining genotypes. The results demonstrated that chemical mutagenesis using EMS could induce variations in the levels and profiles of crude seed protein which could be useful in genetic studies aimed at the improvement of tepary bean.

Key words: bean; mutagenesis; profile; seed protein; variation

4.1 Introduction

Tepary bean is an annual leguminous crop which possesses protein of higher nutritional quality (Narina *et al.,* 2014). It is mainly produced for both human consumption and as a livestock feed. The bean possesses protein content of 17.19 to 26.26% (Narina *et al.,* 2014; Bhardwaj and Hamama, 2004), carbohydrates, dietary fibre (Bhardwaj, 2013), vitamins and 15 essential minerals (Grusak, 2002). Jiri and Mafongoya, (2016) indicated that the protein rich grains of tepary bean could aid in prevention of malnutrition associated with cereal based diets in the developing countries.

The seed storage proteins in tepary bean consist of four groups namely the globulins, albumins, glutelins and prolamins according to their solubility. The seed contains 83.2% albumins, 13.7% globulins, 0.5% prolamins and 2.6% glutelins (Idouraine *et al.,* 1994). Seed storage proteins are significant in determining the amount of protein in the seed, functional quality for various end uses as well as exhibiting metabolic activities.

The nutritional potential of the crop revealed high levels of antioxidant enzymes, anti-cancerous properties as well as soluble fiber significant in controlling various health ailments such as colon cancer, coronary heart diseases and diabetes (Turkan *et al.,* 2004; Valadez-Vega *et al.,* 2011, Jiri and Mafongoya, 2016). Tepary bean grain is potentially useful in treatment of cancer due to the presence of lectin proteins (De Mejia *et al.,* 2005; Valadez-Vega *et al.,* 2014). Moreover, legume lectins showed anti-tumour properties by inducing programmed cell death (apoptosis) and autophagy (Lei and Chang, 2007). Kamble *et al.,* (2015) indicated that seed proteins impart a variety of functional properties such as texture, structure, flavour as well as sinks for surplus nitrogen in mature seed.

Seed storage proteins are a mixture of components exhibiting polymorphism arising from presence of multigene families, proteolytic processing and glycosylation (Miernyk and Hajduch, 2011). The seed proteins were used successfully in cultivar identification studies in various legumes (Alege *et al.,* 2014; Berber and Yasar, 2011; Makri *et al.,* 2005; Uppal and Matta, 1996). The sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS PAGE) technique was used to effectively distinguish between genotypes based on the electrophoretic mobility of proteins (molecular weights) (Kami *et al.,* 1995; Wadood *et al.,* 2016). Previously, this technique was used to differentiate tepary bean (Idouraine *et al.,* 1994), pomegranate (*Punica granatum*) (Rouhalamin and Saei, 2016) and date palm (*Phoenix dactylifera*) (Khoshroo *et al.,* 2013).

Prasad *et al.,* (1986) reported on the alteration of seed proteins due to changes induced by mutation. Variation in seed storage protein profiles in common bean mutants was observed previously (Belele *et al.,* 2001). However, the impact of chemical mutagenesis on the seed protein content and profiles in tepary bean has not been determined. Therefore, this study was undertaken to evaluate the genetic variability induced by EMS mutagenesis on seed protein content and profiles of tepary bean. The study tested the hypotheses that (i) there are no significant differences in seed protein accumulation among mutagenized tepary bean generations and (ii) chemical mutagenesis does not induce genetic variability in seed storage protein profiles of the crop.

4.2 Materials and methods

4.2.1 Study location and genetic material

The study was conducted at the University of Venda at Thohoyandou (22°56′59″ S; 30°28′59″ E) at an altitude of 724 m. Tepary bean mutant generations (M_2, M_3 and M_4) of three genotypes were raised in the greenhouse. Each generation was obtained by successive selfing in the greenhouse and used in the study. The genetic material used was similar to that utilized in the previous chapter (Section 3.2.2). The harvested seed was refrigerated until it was required for protein quantitation and electrophoresis.

4.2.2 Crude protein determination

Total nitrogen was determined by the Kjeldahl's method (AOAC, 2000). In the procedure, 1.0g (flour) of each seed sample was mixed with 20.0ml of concentrated sulphuric acid (H_2SO_4) in heating tubes. Two Kjeldahl selenium catalyst tablets were added to each tube and mixture heated inside a fume cupboard. The mixture was boiled and allowed to digest thereafter. Subsequently, 40% NaOH solution was pumped into distillation flasks and distilled using Buchi distillation unit K-350. The liberated NH_4-N by steam distillation was collected in a beaker containing 20.0ml 4% boric acid (H_3BO_3) solution containing three droplets of methyl red indicator prior to titrating 50.0ml of the distillate with 0.1M HCl using Mettler Toledo DL15 autotitrator with pH electrode. Percentage crude protein was calculated as total nitrogen x 6.25.

4.2.3 Electrophoresis

The seed proteins were extracted from mature dry seed of tepary bean following the procedure described previously by Akbar *et al.,* (2012). Seed was ground to obtain a fine powder with a mortar and pestle. To extract seed storage proteins from flour (0.5g), 10.0ml of phosphate buffered saline was added to the flour in a 50.0ml centrifuge tube and samples sonicated for five minutes. The mixture was left to settle at room temperature for 15 minutes. For SDS PAGE protein separation, 100.0µl of the supernatant was mixed with 20.0µl of cracking buffer (20.0ml containing 0.8g SDS, 0.2mg bromophenol blue, 0.8ml mercaptoethanol, 10.0ml 0.5M Tris, pH 6.8, 8.0ml glycerol and 1.2ml dH_2O) in a 2.0ml Eppendorf microcentrifuge tube and the mixture was centrifuged at 15000rpm for four minutes. For further denaturing of proteins, the samples were placed on a heating block at 95°C for five minutes and subsequently stored in a refrigerator until electrophoresis (Mbogo *et al.,* 2009).

SDS PAGE of seed protein profiles of the tepary bean genotypes was carried out in a 12.0% separating gel in a discontinuous buffer system according to Laemmli (1970) with modifications. A vertical slab gel was organized in a glass sandwich. The 12.0% separating gel contained 4.0ml 30% acrylamide, 3.5ml distilled water (dH_2O) and 2.5 ml 1.5M Tris, pH 8.8. The gel was polymerized by adding 25.0µl 10% ammonium persulphate (APS) and 17.0µl Tetramethylenediamine (TEMED). The 5.0% stacking gel contained 0.6ml 30% acrylamide, 3.0ml dH_2O, 1.25ml 0.5M Tris, pH 6.8 and chemically polymerized with 25.0µl 10% APS and 17.0µl TEMED. Sample loading wells were made in the stacking gel with a comb. Two microliters of protein marker were loaded into the first well and 20.0µl of protein samples were loaded into the subsequent wells made in the stacking gel.

Electrophoresis was carried out in a Biorad Mini-Protean® tetra vertical electrophoresis tank. The electrode buffer for electrophoresis was prepared by dissolving 6.05g Tris, 28.8g Glycine and 2.0g SDS in 2.0L of distilled water. The electrophoresis of seed proteins was run in a Tris-Glycine electrode buffer at a voltage of 120V, 300mA for 90 minutes (Fig 4.1). A Thermo Scientific pageruler prestained protein marker in the range of 10-170kDa was used as molecular weight protein marker. After size separating the denatured polypeptides, the gel was stained with Coomasie blue solution for 120 minutes and destained in a solution containing 10% glacial acetic acid, 40% methanol and distilled water in the ratio of 10:40:50 (v/v) until the background became transparent (Sreeramulu and Singh, 1995).

4.2.4 Experimental design and data analysis

The crude protein experiment was laid out as a completely randomized design (CRD) with two replications. Data sets collected were subjected to analysis of variance (ANOVA) using Statistix (2017) version 10.0. Tukey's test was used for mean separation at $P \leq 0.05$. The following statistical model was used

$$Y_{ijkl} = \mu + R_i + G_j + M_K + S_l + GM_{jk} + GS_{jl} + MS_{kl} + GMS_{jkl} + E_{ijkl}$$

where:

Y_{ijkl} = observation from ith rep, jth genotype, kth mutation treatment and lth generation

μ = overall mean

R_i = random effect of the ith replication

G_j = jth genotype fixed effect

M_K = kth mutation treatment fixed effect

S_l = lth generation fixed effect

GM_{jk} = fixed interaction effect of jth genotype by kth mutation treatment

GS_{jl} = fixed interaction effect of jth genotype by lth generation

MS_{kl} = fixed interaction effect of kth mutation treatment by lth generation

GMS_{jkl} = fixed interaction effect of jth genotype by kth mutation treatment by lth generation

E_{ijkl} = residual error

Seed storage protein bands on each gel were scored manually across all the varieties by comparison with the reference protein ladder. Proteins fragments were classified into three groups based on protein subunit masses designated as small (MW < 35kDa), medium (35 < MW < 70kDa) and relatively large protein subunits (MW > 70kDa).

4.3 Results

4.3.1 Effects of EMS on seed crude protein accumulation

There were highly significant ($P \leq 0.01$) differences observed for genotypes and EMS dose effects on crude protein accumulation (Table 4.1). Similarly, there were highly significant ($P \leq 0.01$) interaction effects of mutant generation x genotype, doses x genotype and mutant generation x genotype x dose. The mean tepary bean crude protein content was 16.93% (Table 4.1). There were no significant differences observed for mutant generations. Nonetheless, the M_3 generation attained the highest seed protein content (17.06%).

Table 4.1 Analysis of variance for the tepary bean seed crude protein

Source	df	Mean square
Replication	1	7.915
Generation (Gn)	2	1.225
Genotype (Gen)	2	160.471**
Doses (D)	4	9.429**
Gen x Gn	4	12.303**
Gen x D	8	11.532**
D x Gn	8	2.405*
Gen x Gn x D	16	4.287**
Mean (%)	-	16.93
CV (%)	-	5.49
R^2	-	46.58

**, * = Significant at the 1.0% and 5.0% probability level, respectively.

There were highly significant ($P \leq 0.01$) genotypic differences observed in seed protein accumulation. The highest grand average crude protein (19.48%) was accumulated by ‘Genotype 3’ whereas ‘Genotype 6’ attained the lowest grand average crude protein content (14.96%) (Table 4.2). The highest average mutant generation crude protein of 20.81% was observed in the M_4 generation by ‘Genotype 3’. Moreover, this genotype accumulated the highest crude protein in all the mutant generations respectively.

The highest protein content (24.23%) in ‘Genotype 3’ was attained in the M_4 generation with 1.5% EMS dose while the lowest crude protein percent was observed with the M_3 generation control (0.0%). There was a considerable increment in crude protein accumulation from the M_3 to the M_4 generation in all EMS doses except the 1.0% treatment (Table 4.2).

The highest crude protein (18.68%) in 'Genotype 4' was accumulated by the control (0.0%) in the M_3 generation whereas the lowest protein accumulation was observed in the subsequent mutant generation at the 1.5% EMS dose (Table 4.2). There was a consistent decline in seed crude protein content observed for 1.0% EMS dose over the three successive mutant generations. However, no clear pattern could be deduced with the other EMS doses (Table 4.2).

'Genotype 6' accumulated the highest crude protein content of 18.73% in the M_3 generation with the 1.5% EMS dose treatment (Table 4.2). However, the highest crude protein accumulated (18.73%) in this genotype was observed with the 1.5% EMS dose in the M_3 generation (Table 4.2). There was an increment in seed protein content from the M_2 to M_3 generation with 0.5 – 2.0% EMS dose treatments followed by a decline thereafter in the succeeding generation (Table 4.2).

Table 4.2 Percent crude protein of tepary bean genotypes over three successive mutant generations

		% Crude protein		
Generation	**Dose**	**GEN-3**	**GEN-4**	**GEN-6**
2	0.0	18.04 cde	17.32 abc	14.56 def
	0.5	20.88 ab	14.98 de	16.39 bcd
	1.0	18.05 cde	17.69 abc	12.20 f
	1.5	20.18 bc	16.08 bcd	16.83 bc
	2.0	17.71 de	18.59 ab	12.37 ef
Mean		18.97	16.93	14.47
3	0.0	16.39 e	18.68 a	12.91 ef
	0.5	18.73 bcd	14.66 de	17.38 ab
	1.0	21.08 ab	17.19 abc	16.45 bc
	1.5	18.79 bcd	16.53 bcd	18.73 a
	2.0	18.25 cde	15.01 de	15.18 cde
Mean		18.65	16.41	16.13
4	0.0	18.61 bcd	16.42 bcd	13.51 ef
	0.5	21.96 ab	15.92 cde	16.61 bc
	1.0	19.16 bcd	17.05 abc	15.11 cde
	1.5	24.23 a	14.19 e	13.72 def
	2.0	20.10 bc	15.06 de	12.44 ef
Mean		20.81	15.73	14.28
Grand Mean		**19.48**	**16.36**	**14.96**

Means followed by the same letter in a column are not significantly different (P ≤ 0.05).

4.3.2 Seed storage protein profiles

Seed storage proteins were resolved into 17 detectable peptides using SDS PAGE. Molecular weights of the resolved peptides ranged from 10kDa to 170kDa. There were significant differences induced by EMS mutagenesis observed on the protein profiles among the genotypes and mutant generations. The proteins were classified into three groups based on protein subunit masses designated as small (< 35kDa), medium (36 - 70kDa) and relatively large protein subunits (> 70kDa).

SDS PAGE showed a homologous number of bands with similar electrophoretic mobility between the control (0.0%) and three EMS doses (1.0%, 1.5% and 2.0%) in ‘Genotype 3’ (Fig 4.1). However, 0.5% EMS dose showed a presence of a unique protein band corresponding to the 20kDa subunit that was absent in the rest of the EMS doses. A relatively high intensity of protein bands was found in the 25-40kDa region (Fig 4.1). In M_2, the protein banding pattern of the mutagenized seed in ‘Genotype 4’ showed two extra protein subunits (at 40kDa and 100kDa) that were absent in the control (Fig 4.2).

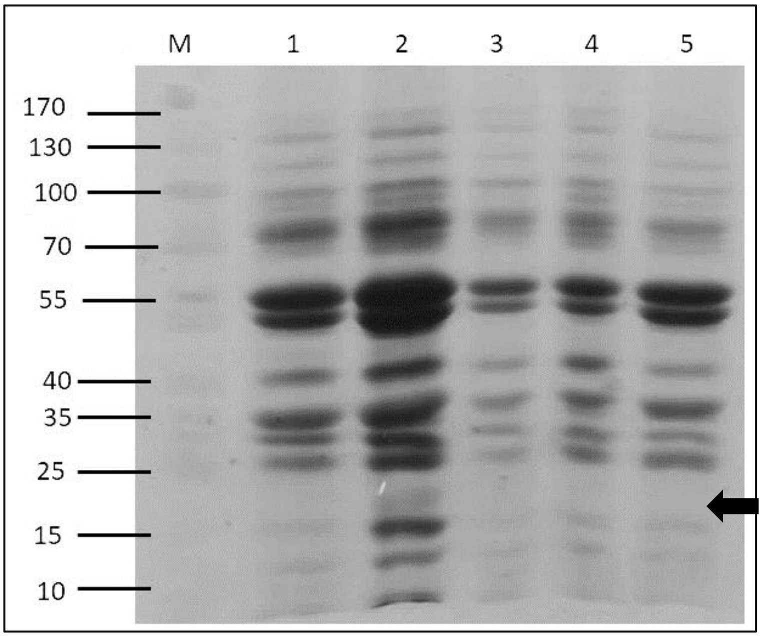

Fig 4.1 Electrophoretic banding pattern of EMS derived seed storage proteins in M_2 tepary bean ‘Genotype 3’. Lanes: M = Protein marker; 1 = control (0.0%); 2 = 0.5%; 3 = 1.0%; 4= 1.5% and 5 = 2.0% EMS dose. Arrow represents the 20kDa subunit that was present only in lane 2.

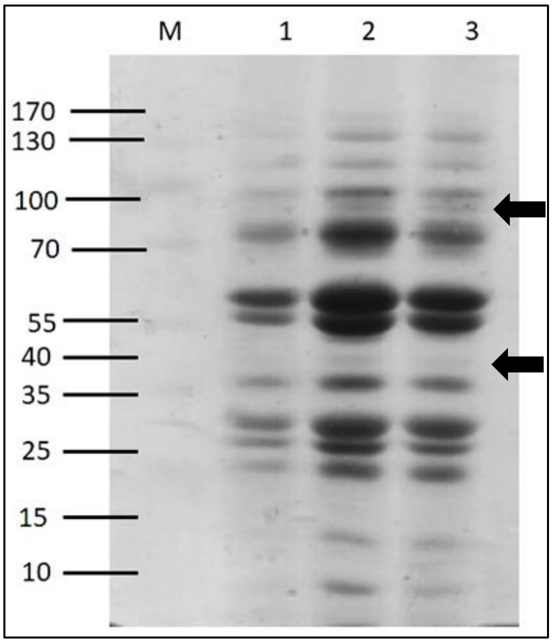

Fig 4.2 Electrophoretic banding pattern of EMS derived seed storage proteins in M_2 tepary bean ‘genotype 4’. Lanes: M = Protein marker; 1 = control (0.0%); 2 = 0.5% and 3 = 2.0% EMS dose. Top and bottom arrows represent the 40kDa and 100kDa, respectively.

4.3.2.1 Genotypic variation in protein profiles

Significant differences in seed storage proteins were observed between genotypes of tepary bean within the same mutant generation. The main genotypic differences were observed among the relatively large protein subunits (> 70kDa).

‘Genotype 3’ and ‘Genotype 4’ revealed differences between these two genotypes in protein banding pattern in the M_3 generation (Fig 4.3). A majority of the protein profiles had 17 bands with similar electrophoretic mobility whereas both 1.0% EMS dose in ‘Genotype 3’ and 1.5% EMS dose in ‘Genotype 4’ had 16 bands respectively. EMS induced an absence of the 130kDa protein subunit in these two profiles (Fig 4.3).

The genotypic variation between ‘Genotype 4’ and ‘Genotype 6’ in protein profiles were observed in the M_4 (Fig 4.4). The main differences were observed in the small (< 35kDa) and relatively large protein subunits (> 70kDa). ‘Genotype 4’ (0.0 – 2.0%) and three other profiles (0.0, 0.5 and 2.0%) in ‘Genotype 6’ had the 130kDa protein fragment that the 1.0 and 1.5% EMS dose profiles in ‘Genotype 6’ lacked. However, the 1.5% EMS dose in ‘Genotype 6’ showed a distinct 15kDa protein subunit that was only present in this profile (Fig 4.4).

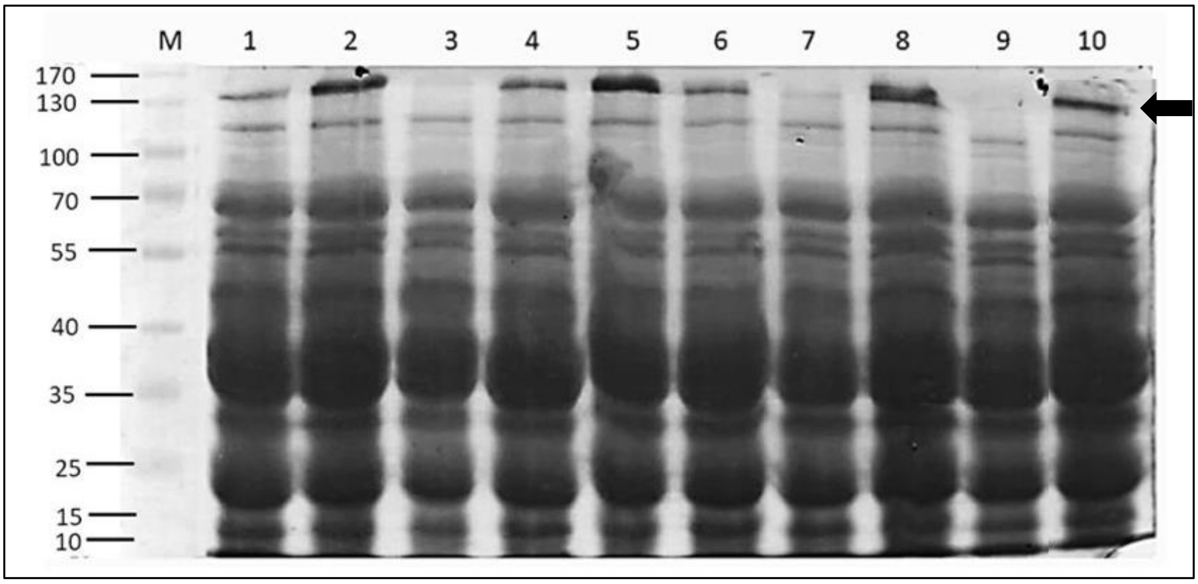

Fig 4.3 Electrophoretic banding pattern of seed storage proteins induced by EMS mutagenesis between M_3 tepary bean 'Genotype 3' and 'Genotype 4'. M = Protein Marker; 'Genotype 3': 1 = control (0.0%); 2 = 0.5%; 3 = 1.0%; 4 = 1.5% and 5 = 2.0% EMS dose. 'Genotype 4': 6 = control (0.0%); 7 = 0.5%; 8 = 1.0%; 9 = 1.5% and 10 = 2.0% EMS dose. Arrow represents absence/ presence of a 130kDa protein subunit.

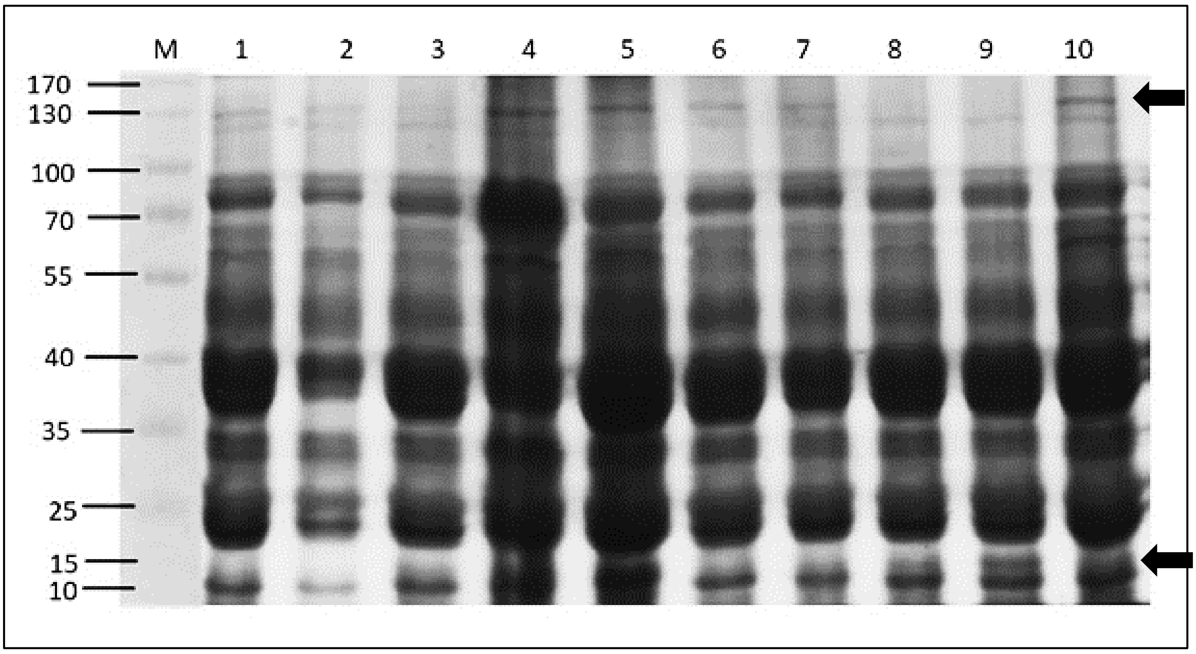

Fig 4.4 Electrophoretic banding pattern of seed storage proteins induced by EMS mutagenesis between M_4 tepary bean 'Genotype 4' and 'Genotype 6'. Protein Marker; 'Genotype 4': 1 = control (0.0%); 2 = 0.5%; 3 = 1.0%; 4 = 1.5% and 5 = 2.0% EMS dose. 'Genotype 6': 6 = control (0.0%); 7 = 0.5%; 8 = 1.0%; 9 = 1.5% and 10 = 2.0% EMS dose. Top and bottom arrows represent the absence/ presence of a 130kDa and 15kDa protein subunits, respectively.

4.3.2.2 Protein polymorphism

Significant differences between mutant generations were observed only for M_3 'Genotype 4' and M_4 'Genotype 3' (Fig 4.5). In the M_4, 'Genotype 3' 0.5% - 1.5% EMS doses had 16 protein bands while the control had 15 bands respectively thus absence of both the 80 and 130kDa protein subunits. However, there was homogeneity in the rest of the protein profiles. High intensity bands were observed in the 35-40kDa, 45-58kDa and 120kDa regions respectively (Fig 4.5). Maximum variation was observed mainly with relatively large protein subunits (> 70kDa).

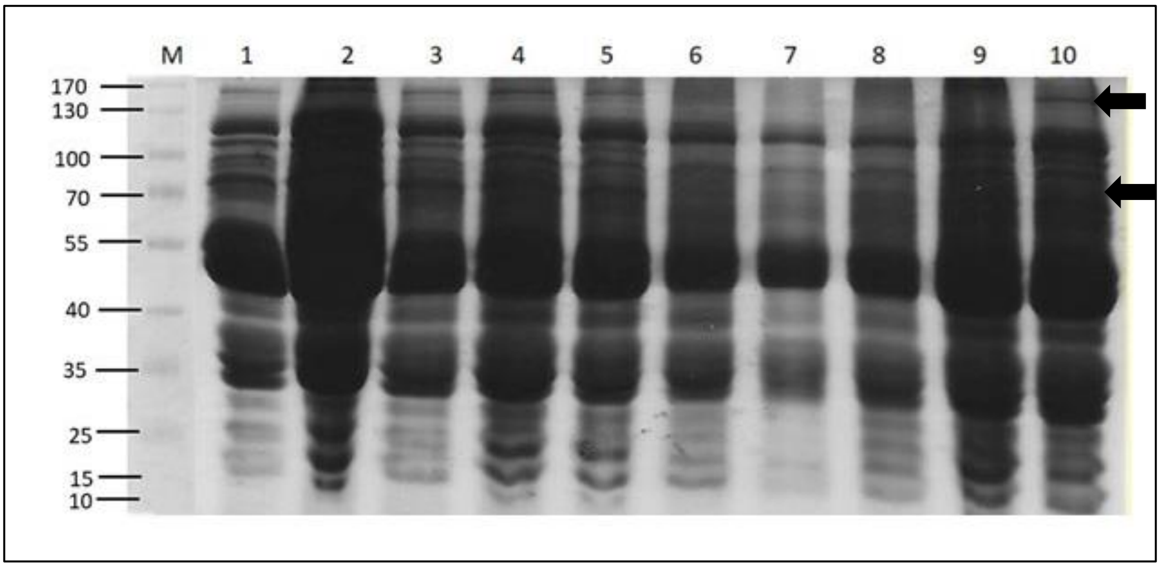

Fig 4.5 Electrophoretic banding pattern of mutant generation differences in seed storage proteins induced by EMS mutagenesis between M_3 'genotype 4 and M_4 'genotype 3'. M = Protein Marker; M_3 'Genotype 4': 1 = control (0.0%); 2 = 0.5%; 3 = 1.0%; 4 = 1.5% and 5 = 2.0% EMS dose. M_4 'Genotype 3': 6 = Control (0.0%); 7 = 0.5%; 8 = 1.0%; 9 = 1.5% and 10 = 2.0 EMS dose. Top and bottom arrows represent absence of 80kDa and 130kDa protein subunits.

4.4 Discussion

The study demonstrated the variability in seed protein content and protein profiles in tepary bean induced by EMS. There were no significant ($P \leq 0.05$) differences in crude seed protein content accumulation over the three successive mutant generations. Nonetheless, there was an increase in crude seed protein observed with each successive mutant generation. Enhanced protein content accumulation over successive mutant generations was previously documented in studies involving gamma radiated cowpea (Adekola and Oluleye, 2007) and soybean mutants (Pavadai *et al.,* 2010). Moreover, high protein (21 – 34.95%) and amino acid content *Vicia faba* mutants treated with combined treatments of EMS and gamma radiation were reported in the M_5 (Hussein and Abdalla, 1979). The induction of high protein mutants was previously attributed to micromutations with positive effects (Singh and Sastry, 1977; Kamble and Petkar, 2015).

Highly significant ($P \leq 0.01$) genotypic differences were observed in crude seed protein accumulation. The overall seed protein content ranged from 14.96% to 19.48% amongst the genotypes. The high crude protein (19.48%) which was observed in 'Genotype 3' suggested that this genotype could be desirable for people who cannot afford animal protein since increased and stabilised seed protein is a significant component of seed nutritional value. Moreover, this genotype accumulated the highest average crude seed protein in each of the three mutant generations. Drought tolerant tepary bean genotypes with higher protein content accumulation were previously documented (Narina *et al.,* 2014). Nonetheless, amino acids such as histidine, proline, serine, arginine and aspartic acid decreased with an increase in EMS treatment from 10mM to 50mM in groundnut (Muniappan *et al.,* 2016). Soybean, greengram and wheat genotypes with increased crude seed protein derived through mutation breeding were previously reported (Imsande, 2001; Samiullah and Wani, 2006; Yanagisawa *et al.,* 2004).

EMS doses ≥0.5% induced significant variability in crude seed protein content accumulation. For instance, the highest protein content (24.23%) was accumulated by 'Genotype 3' at 1.5% EMS dose in the M_4 generation. The highest average protein content amongst the doses was observed at the 1.5% EMS dose treatment. Generally, mutant tepary bean showed a significant improvement in protein content accumulation in comparison with the control. Mahamune *et al.,* (2017) attributed the increase in protein content at different doses of mutagens to an increase in auxin levels. Jain and Suprasanna, (2011) indicated that EMS treatment significantly modified biochemical pathways involved in the accumulation of proteins. Previous studies have shown that mutagens alter the physiochemical properties of nutrients and also improve nutritional qualities in various crops (Singh and Datta, 2010; Al-

Kaisey *et al*., 2003; Mokobia and Anomoharan, 2005). Lhuillier-Soundele *et al.,* (1999) and Burstin *et al.,* (2007) revealed that seed protein content depends on the availability of nitrogen during seed filling and the relative accumulation of starch and proteins.

The total seed protein extracts of tepary bean subjected to SDS PAGE analysis revealed significant variation in polypeptide banding patterns. A total of 17 protein bands with molecular weight range of 10 – 170kDa were recorded. The results depicted a medium genetic diversity between the tepary bean genotypes. Variation in seed protein banding patterns was observed in the number of protein bands in each profile. Moreover, variation was also observed in the density or sharpness of protein bands. Apart from variations in seed protein fragments, there were also common protein bands present in all the tepary bean genotypes. SDS PAGE was previously used in determining *Lens culinaris* (Sultana and Ghafoor, 2008) and chickpea (Ghafoor *et al.,* 2003) protein profiles. Differences in tepary bean protein profiles were predominantly observed with the medium to large protein subunits (> 35kDa). Similar results were reported in *Vicia faba* genotypes with variations in medium to large protein subunits (Sammour, 1992).

There were significant differences observed in protein profiles among the different EMS doses. For instance, 'Genotype 3' showed a presence of a distinct 20kDa protein subunit that was absent in the rest of the EMS doses and control in the M_2 generation at the 0.5% EMS dose. This suggested that SDS PAGE can be used for varietal identification in tepary bean. Similar observations were reported previously in other bean species (Mudzana *et al.,* 1995; Buckseth and Singh, 2016). The protein banding pattern of the mutagenized seed in 'Genotype 4' showed two extra protein fragments at 40kDa and 100kDa that were absent in the control. The intensity of protein bands indicated abundance of the protein occurrence in that region (Animasaun *et al.,* 2017). In other crops for instance in soybean, peanut and sesame, the intensity of albumin fractions was slightly decreased with an increase in gamma radiation dose whereas globulin protein fractions showed strong intensities with the 7.5kGy dose (Afify *et al.,* 2011). A similar observation of variations in the number and intensity of protein bands was reported by Azza *et al.,* (2011). Prasad *et al.,* (1986) alluded that since proteins are direct results of genes, mutation in genes responsible for specific protein synthesis may be reflected in the polypeptides. Srivalli *et al.,* (1999) indicated that seed proteins are used as genetic markers in genetic variation studies because they are the primary products of structural genes thus any change in the coding sequence of a gene generally reflects the corresponding change in the primary structure of proteins. Gamma radiation treatment modified physiochemical properties of soybean proteins (Hafez *et al.,* 1985). Hameed *et al.,* (2009) successfully differentiated Kabuli chickpea mutants from controls based on seed protein profile differences.

The main genotypic differences in protein profiles were observed with relatively large protein subunits which was consistent with previous observations (Mashifane and Gwata, 2016). Differences in seed protein profiles were also observed in *Mucuna pruriens* genotypes using SDS PAGE (Kumar, 2017). Gaafar *et al.,* (2016) reported the alteration in protein electrophoretic banding patterns of cowpea mutants by gamma radiation that resulted in presence and/or absence of some protein subunits. The results are similar to earlier reports where variations in seed protein composition in crops such as soybean, pea and mungbean were observed (Karthika and Subba Lakshmi, 2006; Mehta and Nair, 2011; Ignacimuthu and Arockiadass, 1993).

SDS PAGE revealed mutant generational differences between M_3 generation ‘Genotype 4’ and M_4 generation ‘Genotype 3’ tepary bean profiles. Most bean varieties possess legumin (23kDa), β-lactoglobulin (25kDa) and ovalbumin (50KDa). High intensity (dense) protein bands corresponding to 25-40kDa and 50-55kDa regions were present in all the genotypes. Previous studies have characterized protein bands corresponding to these regions as tepary bean lectins (Pusztai *et al,* 1987; De Mejía, 1990; Idouraine *et al.,* 1994). These plant lectins possess anticancer properties by binding cancer cell membrane proteins causing cytotoxicity, apoptosis and autophagy (Wu *et al.,* 2014; García-Gasca *et al.,* 2012). Therefore, these results from the present study suggested that the seed protein found in tepary bean could be responsible for the anticancer properties associated with this bean (Monroe *et al.,* 2003; Reynoso-Camacho *et al.,* 2007; Thompson *et al.,* 2012). Similarly, Mbogo *et al.,* (2009) identified a major 33kDa tepary bean lectin responsible for conferring strong resistance to bruchids. Most *Phaseolus* spp contain lectins and lectin like proteins associated with tolerance against seed storage pests (Li *et al.,* 2017; Kusolwa and Myers, 2012). Idouraine *et al.,* (1994) reported on major legume globulin bands at 29, 45 and 49kDa in tepary bean. Yuan *et al.,* (2009) indicated that the composition of seed storage proteins significantly influences the functionality of proteins. However, in the present study the individual protein functions were not determined. Future studies could focus on determining the function of individual proteins in tepary bean.

4.5 Conclusion

The results showed significant variations induced by EMS on the percent seed protein and profiles among the genotypes and across the generations. Genotypic variation was observed in crude seed protein accumulation. The highest crude protein was attained by ‘Genotype 3’ in all the mutant generations suggesting that this genotype could be desirable for people who cannot afford relatively expensive animal protein. Mutant tepary bean showed a significant improvement in crude seed protein accumulation in comparison to checks. Moreover, SDS PAGE revealed significant variations in seed storage protein patterns between tepary bean genotypes. Alteration in genes due to EMS mutagenesis resulted in the presence and/or absence of some protein subunits. There were also common protein subunits which were present in all genotypes indicating that the gene coding system for these proteins is conserved. The presence of distinct protein subunits in the three genotypes could be used in varietal identification. The results demonstrated that chemical mutagenesis using EMS could induce variations in crude seed proteins and seed storage protein profiles which could be utilized in genetic studies aimed at the improvement of tepary bean.

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CHAPTER FIVE: CONCLUSIONS AND RECOMMENDATIONS

The results of this study revealed new insights on the effect of induced mutation by EMS on both the agronomic (root nodulation) and nutritional (seed storage proteins) traits of the tepary bean crop. The study revealed highly significant (P≤0.01) dose effects for shoot and root attributes such as SHT, PRL, SDW and RDW. The increase in PRL suggested that tepary bean mutants could be important in drought tolerance since they can be able to tap into deeper soil depths. Similarly, the significant increase in the SHT indicated the potential of tepary bean mutants in soil amelioration if crop residues are ploughed under. The results also showed improved partitioning of dry matter in shoots and roots in tepary bean mutants as indicated by the SDW and RDW. A highly significant (P≤0.01) positive linear relationship was observed between NNP and NDW.

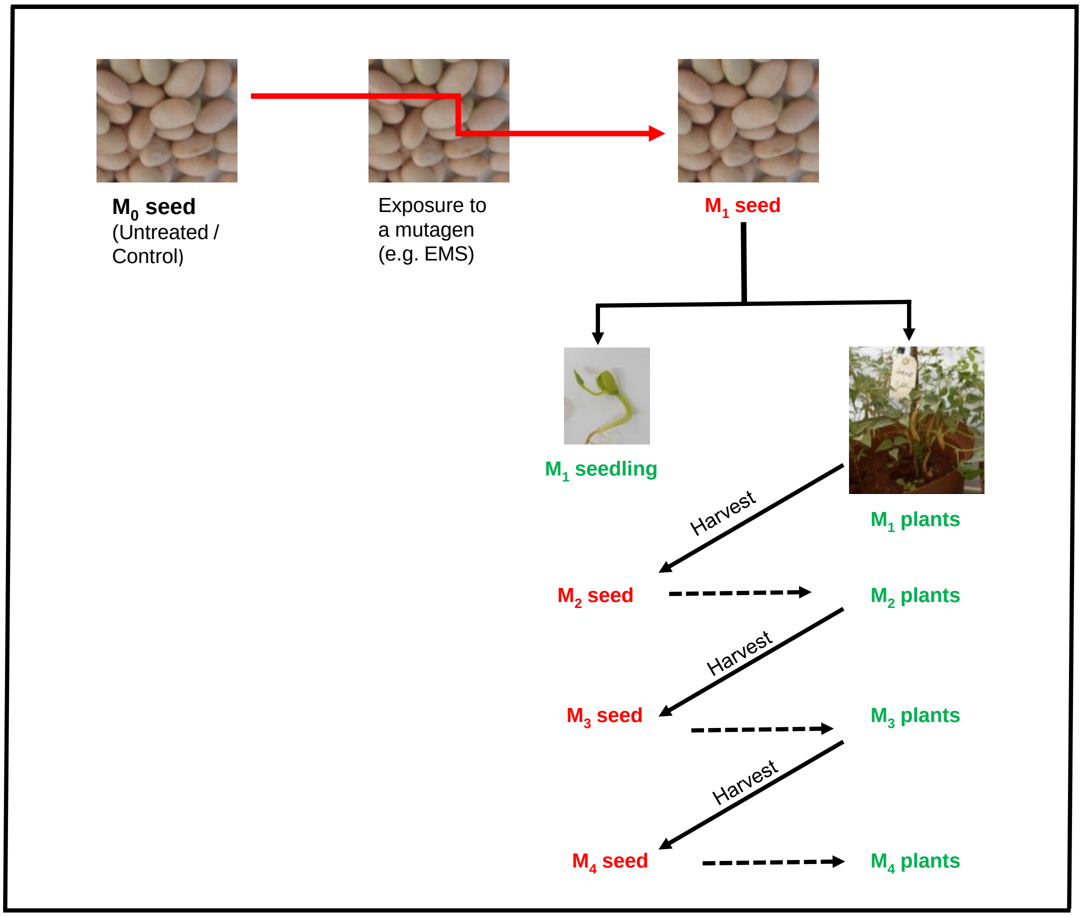
There were highly significant (P≤0.01) differences observed for genotypes and EMS dose effects on crude seed protein accumulation. EMS doses ≥0.5% positively stimulated protein accumulation in all the genotypes. There was a significant improvement in crude seed protein accumulation in comparison with the control. Across all the three mutant generations, ‘Genotype 3’ accumulated the highest crude seed protein suggesting that this genotype could be desirable for people in the rural areas who cannot afford animal protein. SDS PAGE revealed significant variations in the seed storage protein profiles among the genotypes and across the mutant generations. The presence of distinct protein subunits in the three genotypes could be used in varietal identification. The common protein subunits which were present in all tepary bean genotypes indicated that the gene coding system for these proteins was conserved.

The study suggested that tepary bean nodules could probably be more efficient in N-fixation in comparison with other legumes such as soybean and cowpea as observed with the NNP. Future work could be aimed at investigating the variation in N-fixation capabilities among these legumes under uniform experimental conditions. The presence and/or absence of some seed storage protein subunits induced by EMS demonstrated that chemical mutagenesis could be utilized in genetic studies aimed at the improvement of tepary bean. Moreover, the composition of seed storage proteins significantly influences the functionality of proteins hence future research endeavours could be designed to determine the function of individual tepary bean proteins. Breeding programs envisioned at improving yield of tepary bean should take into account the three factors since highly significant (P≤0.01) interaction effects of mutant generation x genotype x dose were observed for NSP as well as crude seed protein accumulation.

APPENDICES

Appendix I

Successive selfing of mutant generations (M_2 to M_4) derived from the M_1 generation.



Appendix II

(a) A tepary bean plant with root nodules, (b) dissected root nodules indicating effective N-fixation