ETHYLMETHANESULFONATE MUTAGENESIS IN SELECTED *VERNONIA GALAMENSIS* VARIETY *ETHIOPICA* LINES

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DECLARATION

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ABSTRACT

The overriding objective of this study was to induce genetic variation in Vernonia (*Vernonia galamensis* variety *ethiopica*) using ethylmethanesulfonate (EMS) and select mutants for subsequent selective breeding. Vernonia is an underutilised, potential novel oilseed crop with multiple applications in industry mostly due to the production of naturally epoxidised vernolic acid oil. Commercial cultivation of vernonia is significantly hampered by non-uniform seed maturity, tall plant height, seed shattering and lack of appropriate technologies for mechanical harvesting, seed threshing and cleaning.

Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. Chemical mutagenesis using EMS is an important, affordable and effective method to induce random useful genetic mutations in crop plants. Ethylmethanesulfonate mutagenesis has previously been reported to affect various agronomic traits, induce a wide variety of phenotypic mutations and alter both seed oil content and fatty acid profile on several crops. The objectives of this study were: (i) to determine an optimum EMS treatment combination i.e. exposure duration, temperature and dose that would enable 50-60% germination at minimum days to emergence in selected *V. galamensis* var. *ethiopica* lines (Vge-1, Vge-4, Vge-7 and Vge-10), (ii) to induce genetic variation using predetermined optimal treatment conditions and select mutants in *V. galamensis* variety *ethiopica* lines (Vge-1 and Vge-4) and (iii) to evaluate oil content and fatty acid compositions among seeds of chloroplast mutants, EMS treated seeds and untreated controls of Vge-1 and Vge-4.

Before any mutation is administered in plants, it is important that the optimal mutation dose is determined. The lethal dose 50 (LD_{50}) was the standard used in this study to find optimal treatment conditions. Significant interactions ($P<0.001$) existed between EMS, line, time and temperature with respect to days to 50% emergence, germination percentage and seedling height. Optimal days to 50% emergence (10-12 days) and germination (50- 58%) was achieved for Vge-1, Vge-7 and Vge-10 when treated with 0.372% EMS at 35^0C for 1 hour treatment. The optimal treatment combination for Vge-4 was 0.372% EMS at 32.5^0C for 2hr. The treatment combinations that yielded optimum results in the tested lines were utilized to induce large scale mutations in *V. galamensis* to select target mutants in the field.

Large scale mutation was conducted using the observed optimal treatment conditions. Ethylmethanesulfonate mutagenesis significantly delayed days to head formation, days to flowering and days to maturity on both lines. Delays in days to emergence were only significant in Vge-4. EMS treatment also significantly reduced germination percentage, number of seeds per head, number of fertile plants, plant height and plot yield for both Vge-1 and Vge-4. Thousand seed weight significantly increased in treated seeds of the two lines. Chlorophyll mutants were observed for tested lines associated with high count of sterility for both lines. Ethylmethanesulfonate successfully induced phenotypic mutation in selected vernonia lines, however at this stage the effect of mutation on vernonia seed oil content and fatty acid was unknown.

Liquid gas chromatography method was employed for oil and fatty acids analysis. In Vge-1, significant differences were observed in composition of linoleic and oleic acid due to the mutagenesis. Significant increases in linoleic and oleic acid composition were found in chloroplast mutants due to EMS mutagenesis. No significant differences were detected in fatty acid compositions in Vge-4 after the EMS treatment. Differential responses were observed when lines were compared at various EMS mutation levels showing significant effect on vernolic, linoleic and oleic acids compositions. In both lines no differences were detected on seed oil content, palmitic acid, steraic acid and arachidic acid compositions after the treatment. Oil content significantly and positively correlated with vernolic acid for Vge-1 ($P<0.001$; $r= 0.898$) and Vge-4 ($P<0.05$; $r= 0.65$). Vernolic acid significantly and negatively correlated with other fatty acids. The study found that EMS mutagenesis significantly changed the oleic acid and linoleic acid compositions in vernonia. However, the oil content and vernolic acid composition were not significantly affected by EMS treatment.

This study established that EMS was successful in inducing genetic variation (in agronomic traits, seed oil content and fatty acid composition) in the two tested lines of *V. galamensis*. Data from a single planting generation is insufficient to conclude fully on the effect of EMS on *V. galamensis*; therefore it is highly recommended that further multigenerational studies should be conducted with an increased number of testing lines from a wide range of environmental backgrounds.

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GENERAL INTRODUCTION

PROBLEM STATEMENT

Genus *Vernonia* belongs to the family Asteraceae. *Vernonia* comprises of more than a thousand species which vary from annual herbs and shrubs to perennial trees (Baye *et al*., 2001). *Vernonia galamensis* has a major potential as an industrial source of vernolic acid due to a high demand of natural epoxidised oil (Shimelis *et al.*, 2008). Crops like soybean (*Glycine max* L.) and linseed (*Linum usitatissimum* L.) are currently used for epoxy oil production, but the oil requires further processing through chemical epoxidation, which is expensive. Vernonia oil is naturally epoxidised, less viscous than the synthetically epoxidised oils and is pourable below 0^{o}C. Vernolic acid is an important resource in the manufacturing of paints and coatings and has great value in the oleochemical industry. Epoxy fatty acids are widely used in oleochemical industry as plasticizers and stabilizers of polyvinyl chloride (PVC), in reformulation of oil based paints, in cosmetics, and pharmaceutical applications (Bhardwaj *et al*., 2000). Cultivation and subsequent commercialisation of vernonia is significantly hampered by non-uniform seed maturity, tall plant height, seed shattering and lack of appropriate technologies for mechanical harvesting, seed threshing and cleaning. With a high demand for natural epoxidised oil, there is need to research and improve the productivity of natural producers of epoxic acids like *V. galamensis*.

IMPORTANCE OF THIS RESEARCH

Inducing genetic variation using mutation breeding has been proposed as a possible solution to phenotypic challenges experienced in vernonia (Mebrahtu *et al*., 2009). This study is a first attempt to breed *V. galamensis* utilising mutation breeding. Inducing mutation using ethylmethanesulfonate in *V. galamensis* has potential to be effectively applied to develop new vernonia lines with high plant and oil yield and/or enhanced agronomic traits (synchronous maturity, uniform branching, shorter plants and low shattering).

RESEARCH OBJECTIVES

The aim of the study was to induce genetic variation using EMS and select mutants for subsequent selective breeding of vernonia. The specific objectives were : (i) to determine an optimum EMS treatment combination (i.e. exposure duration, temperature and EMS dose) that would enable at least 50-60% germination at minimum days to emergence in selected *V. galamensis* var. *ethiopica* lines, (ii) to induce genetic variation using predetermined optimal treatment conditions and select mutants in *V. galamensis* variety *ethiopica* lines and (iii) to evaluate oil content and fatty acid analysis in seeds of chloroplast mutants, EMS treated seeds and untreated controls of *V. galamensis* variety *ethiopica* lines.

RESEARCH BACKGROUND

Primary to any mutagenesis of crops, the suitable chemical dose and optimal conditions for a particular line/cultivar should first be determined. Higher doses can produce very drastic effects that may lead to death of the organism. A relatively lower dose may result in altered growth characteristics. With respect to EMS doses the term lower and higher is relative and may be different for each crop species. Seedling emergence, seedling growth and chromosomal aberration are the commonly used criteria for selecting optimal treatment doses in plants (Shah *et al.*, 2008).

Chemical mutagens have been effective in the development of novel germplasm in crop plants (van Harten, 1998; Kodym and Afza, 2003; Gunstone 2006). Artificial mutation helps to induce genetic variation of gene loci controlling economically important traits and/or elimination of undesirable genes from breeding lines (Alacantara *et al.*, 1996). Mutants showing desirable traits can be developed directly as new cultivars. Chemical mutagenesis using ethylmethanesulfonate is a powerful tool in inducing random useful genetic mutations in crop plants (Kim *et al.*, 2005**;** Aliyu and Adamu, 2007**)**. Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. Alkylation of nucleotides induced by EMS in chromosomes can result in mispairing and changing of bases. This results in alkylation of guanine (G) which subsequently forms O^6-ethylguanine. O^6-ethylguanine then forms a base pair thymine (T) and not cytosine (C). Original G/C pairs end up being replaced by A/T base pairs. At a low EMS frequency, G/C to C/G or G/C to T/A transversions are generated by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors (Kim *et al*, 2005). The EMS induced

mispairing of base pairs can change the amino acid structure of the chromosome; hence change protein function during cell division. Ethylmethanesulfonate mutagenesis has previously been reported to affect various agronomic traits (Kumar and Kumar Rai, 2007; Shah *et al*., 2008; Berenschot *et al.*, 2009), induce a wide variety of phenotypic mutations (Vaida and Young, 1993; Alacantara *et al*., 1996; Singh *et al*., 2000) and alter both seed oil content and fatty acid profile (Osorio *et al*., 1995; Barro *et al*., 2001; Savant and Konthekar, 2011) in crops. Hence, inducing genetic variation in selection breeding of vernonia has been proposed as a plausible solution to combating agronomic challenges encountered in vernonia cultivation such as non-uniform seed maturity, irregular branching tall plant height, and seed shattering.

REFERENCES

Alcantara T., P. Bosland and D. Smith, 1996: Ethyl Methanesulfonate-induced Seed Mutagenesis of *Capsicum annuum*. The Journal of Heredity **87**, 239-241.

Aliyu H. and A. Adamu, 2007: The Effect of Diethylsulphate on some Quantitative Traits of Tomato (*Lycopersicon esculentum* Mill). Science World Journal **2**, 1-4.

Barro F., J. Fernandez-Escobar, M. De La Vega and A. Martian, 2001: Doubled Haploid Lines of *Brassica carinata* with Modified Erucic Acid Content through Mutagenesis by EMS Treatment of Isolated Microspores. Plant Breeding **120**, 262-264.

Baye T., H. Kebede and K. Belete, 2001: Agronomic Evaluation of *Vernonia galamensis* Gemplasm Collected from Eastern Ethiopia. Industrial Crops and Products **14**, 179-190.

Berenschot A., M. Zucchi, A. Tulmann-Neto and V. Quecini, 2009: Mutagenesis in *Petunia x hybrida*Vilm. and Isolation of a Novel Morphological Mutant. Brazilian Journal of Plant Physiology **20**, 95-103.

Bhardwaj H.L., A.A. Hamama, M. Rangappa, and D.A. Dierig. 2000: Vernonia Oilseed Production in the Mid-Atlantic Region of the United States. Industrial Crops and Products **12**, 119-124.

Gunstone F. D., 2006: Modifying lipids for use in food [M]. Woodhead Publishing Limited and CRC Press LLC, 273-305.

Kim Y.S., K.S. Schumaker and J.K. Zhu. 2005: EMS Mutagenesis of *Arabidopsis*. Methods in Molecular Biology **323**, 101-104.

Kodym A., R. Afza., 2003: Physical and Chemical Mutagenesis Methods in Molecular Biology. Plant Functional Genomics: Methods and Protocols **236**, 189-220.

Kumar G. and P. Kumar Rai, 2007: EMS Induced Karyomorphological Variations in Maize (*Zea mays* L.) Inbreds. Turkish Journal of Biology **31**, 187-195.

Mebrahtu T., T. Gebremariam, A. Kidane and W. Araia, 2009: Performance of *Vernonia galamensis* as a Potential and Viable Industrial Oil Plant in Eritrea: Yield and Oil Content. African Journal of Biotechnology **8**, 635-640.

Osorio J., J. Fernandez-Martinez, M. Mancha and R. Garces, 1995: Mutant Sunflowers with High Concentration of Saturated Fatty Acids in the Oil. Crop Science **35**, 739-742.

Savant K.D. and V.S. Kothekar, 2011: Induction of Variability in Fatty Acid Profile in Sesame (*Sesamum indicum* L.). Journal of Phytology **3**, 01-03.

Shah T., J. Mirza, M. Haq and B. Atta, 2008: Radio Sensitivity of Various Chickpea Genotypes in M1 Generation I-Laboratory Studies. Pakistan Journal of Botany **40**, 649-665.

Shimelis H., P. Mashela and A. Hugo, 2008: Performance of Vernonia as an Alternative Industrial Oil Crop in Limpopo Province of South Africa. Crop Science **48**, 236-242.

Singh U.P., B. Prithiviraj and B.K. Sarma, 2000: Development of *Erysiphepisi* (Powdery Mildew) on Normal and Albino Mutants of Pea (*Pisum sativum* L.). Journal of Phytopathology **148**, 591-595.

Vaida K. and M. Young, 1993: Ethyl Methane Sulfonate Induced Variation in Qualitative and Quantitative Characters of Roselle (*Hibiscus sabdariffa* L.) (Malvaceae). Brazilian Journal of Genetics **16**, 381-391.

van Harten A.M., 1998: Mutation Breeding: Theory and Practical Applications. Cambridge University Press.

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Vernonia (*Vernonia galamensis* variety *ethiopica*) is an underexploited oilseed crop and a potential novel oilseed crop. Vernonia is a natural producer of natural epoxidised oil consisting of vernolic acid, palmitic acid, linoleic acid, arachidic acid and steraic acid with vernolic acid as the dominant fatty acid. The use of vernonia oil is advantageous as processing costs of epoxidation are drastically reduced in industry; moreover vernonia oil is pourable below freezing temperature and is environmentally friendly. Other advantages include reduced emissions of volatile organic compounds in industrial products. Vernonia oil has multiple uses in the chemical and feed industry. Cultivation and subsequent commercialisation of vernonia is significantly hampered by non-uniform seed maturity, tall plant height, seed shattering and lack of appropriate technologies for mechanical harvesting, seed threshing and cleaning. Inducing genetic variation in selection breeding of vernonia has been proposed as a plausible solution to combating non-uniform seed maturity, irregular branching tall plant height, and seed shattering encountered in vernonia cultivation. Chemical mutagenesis using ethylmethanesulfonate (EMS) is an important, affordable and effective method to induce random useful genetic mutations in crop plants. Ethylmethanesulfonate mutagenesis has previously been reported to enhance various agronomic traits (Kumar and Kumar Rai, 2007; Shah *et al*., 2008; Berenschot *et al.*, 2009), induce a wide variety of phenotypic mutations (Vaida and Young, 1993; Alacantara *et al*., 1996; Singh *et al*., 2000) and alter both seed oil content and fatty acid profile (Osorio *et al*., 1995; Barro *et al*., 2001; Savant and Konthekar, 2011) on several crops such as *Jatropha*, sunflower, Abyssinian mustard and sesame.

1.2 Distribution of Vernonia

Vernonia galamensis belongs to a diverse group of angiosperms known as Asteraceae (Rahman *et al*., 2008). During the 1950's, the Agricultural Research Service (ARS) in the US Department of Agriculture (USDA) conducted an extensive search to identify plants not competing with existing crops as new sources of industrial raw materials (Perdue,

1988). Among the many species examined was *Vernonia galamensis* containing triacylglycerol oil consisting of vernolic acid (*cis*-12,13-epoxy-*cis*-9- octadecenoic).

Vernonia was first identified in 1964 by Perdue in eastern Ethiopia on the Harar-Jijiga road (9°14 N and 42°35 E at 1700 m above sea level). The collected *Vernonia galamensis* germplasms combined a high vernolic acid content with a promising seed yield and good seed retention. Local names of the crop include: 'Ferenkundela', 'Dunfare', 'Kefathebogie', and 'Noya' (Baye, 1996). Genus *Vernonia* comprises of more than a thousand species which vary from annual herbs and shrubs to perennial trees (Baye *et al*., 2001). Before a taxonomic revision of the complex, the species was referred to in earlier literature as *Vernonia pauciflora* (Pursh.) Poir. An earlier and less common name for this species was *Conyza pauciflora* Willd. *Vernonia galamensis* occurs naturally from Cape Verde and Senegal east to Eritrea and through East Africa south to Mozambique. The greatest diversity is found in East Africa; only a single variety occurs in West Africa. *Vernonia galamensis* is now being developed as a potential industrial oilseed crop in several parts of the world (van der Vossen and Mkamilo, 2007).

Most of *Vernonia* species occur in South America; more than 300 species have been described from Africa with about one-third occurring in Madagascar and about 50 in Ethiopia. *Vernonia galamensis* is highly variable. To account for the morphological variability, 10 sub-specific taxa (sub-species and varieties) have been described that are separated geographically or ecologically. Recent taxonomic studies have classified the genus to include six sub-species: *nairobensis*, *gibbosa*, *lushotoensis*, *afromontana*, *mutomonesis* and *galamensis*. The most widely distributed sub-species is *galamensis* which consists of four botanical varieties, namely *galamensis*, *petitiana*, *australis* and *ethiopica* (Gilbert, 1986). Due to the high oil and vernolic acid content and its relatively low shattering nature, subsp. *galamensis* var. *ethiopica* M.G.Gilbert has been the focus of research aiming at domestication and commercialization (van der Vossen and Mkamilo, 2007).

1.3 Taxonomic classification of *Vernonia galamensis* var. *ethiopica*

1.3.1 Vernonia galamensis phenotypic characterisation

Vernonia galamensis is herbaceous, usually annual but sometimes a short lived perennial. The shoot can grow up to 5 m in height, but are usually much shorter. The stem is ribbed, finely to coarsely hairy and sometimes branching near the top (Gilbert, 1986).

Leaves are arranged in an alternate format and are sessile and membranous. The length and breadth can be up to 25 x 0.6-5 cm, with each leaf being broadest at about the middle. The length/breadth ratio ranges from 20:1 to 2.3:1 depending on taxon. The apex is more or less acuminate and the base narrowly cuneate. The margins irregularly dentate to sharply serrate to serrulate and upper surfaces range from asperous to sparsely long-pilose with the lower surfaces sparsely to quite densely long-pilose, sometimes the hairs dimorphic with small appressed hairs as well as the normal long erect hairs. Capitula vary greatly, from slightly longer than broad to markedly broader than long. Involucre 8-25 mm long overall, phyllaries 4-6 seriate, very sparsely fine-pubescent to markedly woolly araneose, outermost phyllaries short, linear, often with well defined mid-rib. Middle phyllaries vary from simple, narrow-lanceolate, more or less appressed in certain very small-headed forms to, more usually, base and indurated, sometimes gibbous. The tip is usually foliaceous, often markedly reflexed. The innermost phyllaries are scarious, oblong to narrow-lanceolate with an acuminate tip, sometimes with prominent sub-apical teeth (Gilbert, 1986).

Florets are long and exserted at anthesis. The colour ranges from bright blue to pale mauve blue to almost white, and sometimes are flushed pale yellow or green. They can grow up to 14 mm in length. Florets are tubular on the lower half, expanding slightly and gradually above. Lobes are linear, up to 3 mm long and glabrous. The stamens and stigma are similar in colour to the corolla, slightly exserted, and anthers obscurely auriculate at base. Cypsella grow up to 8 mm long, and are narrowly obovoid with 10 equal narrow ribs. Cypsela colours range from very dark brown to black uniformly covered with dense, slightly appressed silky hairs. Outer pappus of barbellate setae are up to 1-5 mm high. The inner part of copious is off-white to brown and the sordid barbellate setae is up to 8 mm long (Gilbert, 1986). Chromosome counts of accessions are 2n = 18. Sub-species readily hybridize among themselves (Gilbert, 1986).

1.3.2 V. galamensis subspecies galamensis

Sub-species *galamensis* is strictly annual and can grow from 15-150 cm or higher. Stems are sparsely appressed. Leaves are 7-40 mm wide, the length to breadth ratio varies from 4:1 to 20:1. Involucres are 8-22 mm long, sparsely pilose to glabrescent. Phyllaries are without obvious foliaceous tips, often irregularly twisted or bent but only very rarely

regularly reflexed. Tips are 0.3-0.7 mm wide and inner phyllaries lack marginal teeth. Florets are pale blue to mauve-blue, bright blue or purple (Gilbert, 1986).

1.3.3 V. galamensis subsp. galamensis var. ethiopica

In this botanical variety, involucres are 17-22 mm long. Leaves are 7-15 mm wide. Phyllary tips are 0.5-0.7 mm and inner phyllaries are uniformly pale (Gilbert, 1986). A diagrammatic representation of this variety is shown below (Figure1.1).

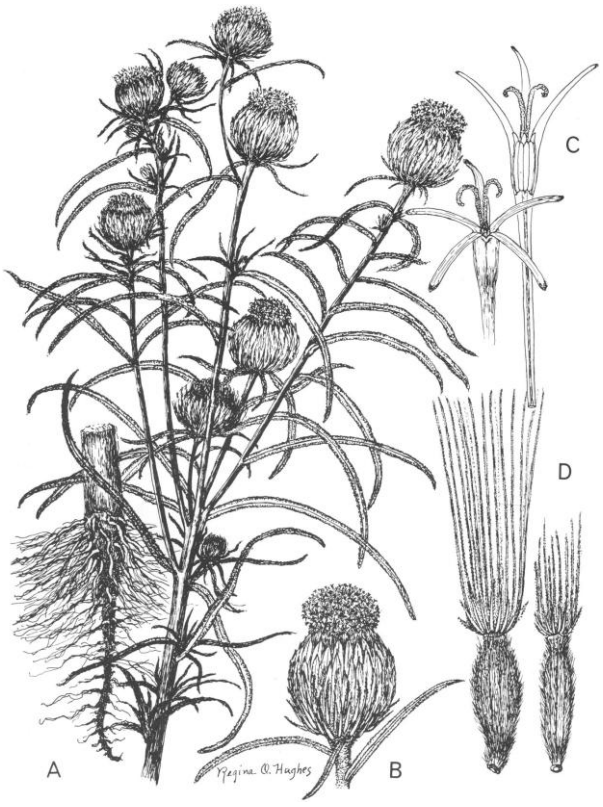

Figure 1. 1: Diagrammatic representation of *Vernonia galamensis* var. *ethiopica*. (A) root and shoot; (B) capitulum; (C) florets, one with two corolla lobes removed to reveal stamens; (D) cypsella, mature and immature (Gilbert, 1986).

1.4 Oil content and fatty acid profile of seeds and leaves

Fruit of vernonia is non-fleshy and indehiscent. Dispersal is commonly by wind by use of the hairy pappus. Vernonia seeds are dicotyledonous and the embryo does not contain chlorophyll. Seeds have a very thin endosperm. The seed contains, per 100 g: 20-27 g protein and 35-42 g of triglyceride oil. Vernolic acid is the dominant fatty acid and accounts for about 72-80% of the acids present in the seed oil triglycerides. Other fatty acids contained in *Vernonia* oil are: linoleic acid (12-14%), oleic acid (4-6%), stearic acid (2-3%), palmitic acid (2-3%) and trace amounts of arachidic acid (Ayorinde *et al.*, 1988; Thompson *et al.*, 1994). The press-cake contains per 100 g: crude protein - 44 g, crude fibre - 11 g, ash - 19 g, and carbohydrate - 7 g. The leaves contain a small amount of oil. The fatty acid composition of the leaf oil is: 12-22% palmitic acid, 41-59% linolenic acid, and 8-17% parinaric acid; vernolic acid is only found in traces in the leaves (Baye *et al*., 2005).

1.5 Economic uses of vernonia

There is a large industrial market for synthetically epoxidised vegetable oils (such as linseed and soy-bean), but the epoxidation process is expensive. Vernolic acid from vernonia is already epoxidised, and can fill in market niches. Vernolic acid is much less viscous than the synthetically epoxidised oils. The latter are semisolids at 10°C and not pourable at 0°C and below, while vernolic acid can be poured even below freezing point. The low viscosity of vernonia oil should make it a good solvent in paint manufacture, and the highly reactive epoxy group will cause it to become chemically bound in the dried paint rather than evaporating in the atmosphere (Kaplan, 1989). Other potential uses include lubricants, adhesives, protective coatings, cosmetics, detergents, a raw product for nylons and much more (Jaworski and Cahoon, 2003). Vernonia oil could also be used as a natural source of plasticizers and stabilizers for producing polyvinylchloride (PVC) plastic, which is currently manufactured from petroleum. The leaves have been smoked as a substitute for tobacco in Ethiopia. In Tanzania the leaves are cooked in porridge, or drunk as a tea to treat chest pain. In Kenya the plant is used to treat stomach pain (van der Vossen and Mkamilo, 2007). Epoxy oils have an advantage over commercially epoxidised oils in that the location, number, and configuration of epoxy groups are rigorously known. Vernolic acid is characterized by its chemically active epoxy group and due to its chemical

structure, vernolic acid and trivernolin can undergo chemical reactions characteristic of ester groups, double bonds and epoxy groups.

The potential use of vernonia as a petroleum substitute is important since the demand for petroleum is increasing each year, for instance in the U.S. it is approximately 8,500 lbs per person, of which about £500 per person is needed for production of plastics and industrial petrochemicals (Teynor *et al.*, 1992)**.** Since 1963, production of all epoxy esters has ranged from 60 to £150 million annually, a steady 7% of the 1 to £2 billion of annual plasticizer production. Growth rates in production averaged 4.3% for all plasticizers, 3.8% for all epoxy esters and 5.0% for epoxidised soybean oil (Carlson and Chang, 1985). Recently, commercial production of *Vernonia galamensis* has started in Ethiopia by Ver-Tech^{TM}. However, large-scale commercial production is still in its infancy and no data on production are available. Ver-Tech International^{TM} has identified over 70 potential uses for the *Vernonia* oil (Perdue, 2002).

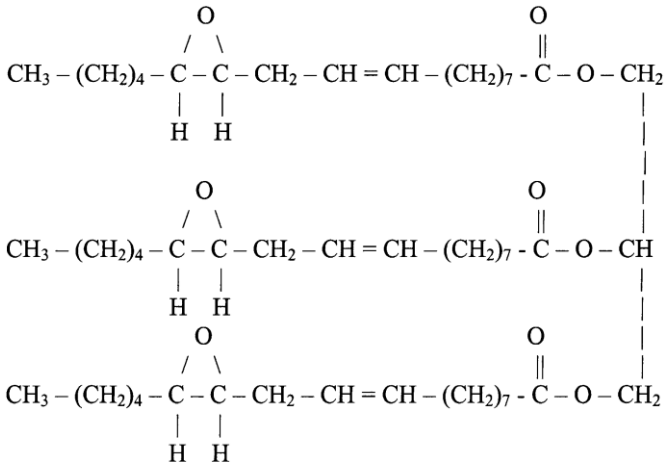

Figure 1. 2: Structure of vernonia (van der Vossen and Mkamilo, 2007).

1.5.1 Uses in paint industry

Numerous oil crops are used in coating industry due to the major different fatty acids they contain (Table 1.1). In the paint industry, vernonia oil has potential as a component of low volatile organic solvent paints. Vernonia oil provides excellent adhesion, flexibility and chipping resistance, resistance to acid, alkaline and non-polar solvents when incorporated into heat-baked films and coatings (Mebrahtu *et al*., 2009).

Table 1. 1: Plant oils used in coating industry (Derksen *et al.*, 1995).

Plant oil	Source	Major fatty acids	[a]FA content (% of total)	Iodine value	Specific gravity (g/ml, W'C)
Linseed oil	*Linum usitatissimum*	linolenic/linoleic	40/35	175-204	0.931-0.936
Tung oil	*Aleurites* spp.	elaeostearic/oleic	79/11	155-17s	0.939-0.943
Perilla oil	*Perilla frutescens*	linolenic/linoleic	43/37	192-208	0.932-0.935
Oiticica oil	*Licania rigida*	licanic/elaeostearic	76/7	179-218	0.966-0.969
Soybean oil	*Glycine max*	linoleic/oleic	55/28	125-140	0.923-0.929
Safflower oil	*Carthamus tinctorius*	linoleic/oleic	59/37	140-150	0.925-0.928
Tall oil	Pinewood pulping	fatty acids/rosin acids	50/40		
Castor oil	*Ricinus communis*	ricinoleic/linoleic	90/4	82- 88	0.958-0.969
Dehydrated castor	*Ricinus communis*	conjugated FAs/ricinoleic	80/10	135	

[a]FA= fatty acid

1.5.2 Insecticidal use

Leaf trichome extracts from vernonia have shown potential for drug (Miserez, 1999) and pesticide development (El-Sayed *et al*., 1999)**.** These trichome extracts have been shown to cause rapid mortality of both immature and adult whiteflies (Thorpe *et al*., 2006). Mass spectrometry analysis revealed that the peltate trichome is a major source of prevernocistifolide-8-O-isobutyrate. This glaucolide-type sesquiterpene lactone was previously identified as a major constituent of the aerial parts of *Vernonia galamensis* spp. *galamensis* var. *ethiopica* (Favi *et al.*, 2008).

1.5.3 Animal feed industry

The press-cake after oil extraction is suitable for animal feed. It is a valuable source of crude protein (43.75%); it also consists of crude fiber (10.90%), ash (9.50%) and the carbohydrate fraction (6.57%) with sucrose (2.36%), fructose (1.90%) and glucose (0.77%). The major mineral elements, calcium (11.08 mg/g), potassium (14.18 mg/g), magnesium (6.90 %) and high phosphorus (644 mg/g) not only meet the nutritional requirements but are also higher than in most other oilseeds (Ologunde *et al*., 1990).

1.5.4 Manufacture of plastics

Vernonia oil has potential as a plasticizer of polyvinylchloride (PVC) and as a structural component of polymers in the plastic manufacturing industry (Mebrahtu *et al., 2009)*. Polyvinylchloride is a versatile material used for various packaging materials in many countries. Polyvinylchloride suffers poor thermal stability and undergoes severe degradation at low temperatures. Degraded PVC is characterised by the development of intense discoloration resulting from the formation of conjugated polyene structures (Benaniba *et al.*, 2001). Heat stabilisers are required to stabilise the polymer during processing. Most PVC stabilisers are different metal soaps like Pb, Cd, Ca and Zn carboxylates and some di- and mono-alkylating compounds. Epoxy compounds are well known as typical non-metallic stabilisers for PVC. They are generally regarded as secondary stabilisers used to enhance the effectiveness of metal soaps (Benaniba *et al.*, 2003).

1.6 Vernonia cultivation

1.6.1 Challenges in cultivation of V. galamensis

Despite the outstanding potential of vernonia for use in the chemical, pharmaceutical and agro- industries, there remains vast areas where *V. galamensis* can be improved for profitable cultivation. Cultivation and commercialisation of vernonia is significantly hampered by lack of uniform seed-maturing varieties. Plants grow excessively tall with non-synchronised branching. These aforementioned problems accompanied with seed shattering drastically reduce yields in *V. galamensis*. There exists also a lack of relevant processing technologies such as technologies for mechanical harvesting, seed threshing and cleaning. It is expected that extensive research on mutation breeding (increasing genetic variability) is a plausible solution to combating challenges encountered in cultivation of *V. galamensis* (Mebrahtu *et al*., 2009).

1.6.2 Environmental conditions for cultivation

V. galamensis spp. *galamensis* var. *ethiopica* grows naturally in marginal areas with minimal annual rainfall as little as 200 mm and at an elevation ranging from 700 to 2400 m above sea level in the southern and south-eastern parts of Ethiopia (Gilbert, 1986).

Vernonia can tolerate and grow under substantial shade; it is therefore a potential crop in agroforestry (Baye *et al.*, 2001). *Vernonia galamensis* is adapted to the semi-arid tropics where it is found in dry bushland, but more often in disturbed places and as a weed of cultivation, up to 2000 -2500 m altitude. Rainfall may be as low as 250- 500 mm for some types, but as high as 1850 mm for other types. In cultivation, *Vernonia galamensis* requires a rainy season that provides sufficient moisture to permit the main flower heads to develop; a longer rainy season that permits secondary flower heads to develop will result in poor uniformity of maturation and a risk of seed shattering. A well-drained soil with pH 5.0-8.5 is preferred. On poorly drained soils, growth of the main stem stops before flowering; branches develop from the base of the plant, but they also wither and die. Vernonia is grown successfully in countries near the Equator because the plants with the largest seed and best seed retention only flower under day-neutral photoperiodic conditions. Therefore, more research is needed to exploit the phenotypic and genotypic variation within vernonia as an alternative crop in countries further from the equator.

Water supply for crops, livestock and a growing human population is becoming a critical commodity. It is a major concern that none of the major crop plants are adapted for cultivation under water stressed conditions (Baye *et al.*, 2000). With an annual average rainfall of 500 mm, South Africa is among the 30 driest countries on the planet (The Water Wheel, 2007). This average is way below the world's annual average of 860 mm (DWAF, 2002). According to the Water Act (RSA, 1998), South Africa's water resources are scarce and limited in extent, as a result the International Water Management Institute (IWMI) has classified South Africa as a water stressed country (IWMI, 1996). Recent climate change forecasts predicting an increase in the occurrence of droughts (Hassan, 2006) only exacerbates the situation as the frequency of crop failure is expected to increase.

Vernonia galamensis is well adapted for cultivation under arid conditions as previously it was known to be a weed colonising disturbed areas and arid agricultural lands. These characteristics make it valuable for small scale farmers on marginalized lands and a vital germplasm resource for future crop improvements. However, promotion and research of major plant crops has taken preference at the expense of crops like vernonia being underdeveloped.

1.6.3 Growth and development

Seed may show some dormancy for a few months after maturation; thereafter germination takes about 10 days. Plants form a single unbranched stem ending in an inflorescence. Growth is indeterminate. When growing conditions permit, branching starts after formation of the main inflorescence and occurs only at the higher nodes; these branches may also form flower heads. As a result, ripening of the heads of a plant may be uneven. Shattering of mature fruiting heads occurs. *Vernonia galamensis* is self-fertile, but rates of out-crossing ranging from 2.5 - 16% have been reported (Baye and Becker, 2004).

1.6.4 Seed dormancy

Seed dormancy may be defined as the inability of viable seeds to germinate within a specified period under conditions (water, air, suitable temperature, soil type etc.) considered adequate for radicle, emergence and seedling growth (Baskin and Baskin, 2004). Seed dormancy is caused by inhibition of the germination process within the imbibed seed. For example, retarded or arrested metabolism due to unmet minimum germination requirements cause dormancy in seeds, also under-developed embryos at seed dispersal require time to develop fully before germination can occur (Baskin and Baskin, 2004).

One such plant species with issues of seed dormancy is *Vernonia galamensis*, and this impedes the agricultural utilization of the crop. A study by Nyamongo *et al*. (2010) on seed dormancy and seed quality of various *Vernonia galamensis* sub-species demonstrated that germinability and desiccation tolerance was accumulated during the dry matter accumulation phase of seed development and that seeds developed dormancy 3-6 days after acquiring germinability with high levels of germination only attained under an alternating temperature regime. Different sub-species also demonstrated varying levels of seed dormancy. Seeds harvested from warm environments demonstrated decreased dormancy compared to seeds form cool environment.

1.6.5 Weed management

As to our knowledge, no herbicides are licensed for weed management in vernonia. Vernonia being previously recognised and treated as a weed colonising agricultural lands,

is vulnerable to a wide range of herbicides especially those designed for management of broad-leaved weeds.

1.6.6 Harvesting

Vernonia galamensis seed matures non-synchronously and several harvesting rounds are often necessary for thorough harvest. Harvesting of heads is done when the involucres surrounding the seeds are dry and spread out to release the fully mature seeds. Farmers therefore postpone the harvest of a heterogeneous crop until most seeds are ripe. After the harvest, first the seeds are separated from the heads, following which the pappus is removed from the seed. These are laborious and labour intensive operations if carried out manually.

1.6.7 Pests and Pathogens

Pests and pathogens can drastically reduce growth and productivity of a crop. Limited research has been undertaken to gain insight on pests and pathogen interactions with vernonia. *Vernonia* has been documented to associate with a number of diseases and pests. Some pathogens have over time co-evolved with *V. galamensis* and are specific to it. Collection and preliminary identification by Baye and Gudeta (2002) of the pest species associated with vernonia in Ethiopia indicated that various pest insects, pathogenic fungi and bacteria were associated with vernonia (Tables 1.2 and 1.3). Helmet bug was identified as a major insect pest of vernonia, with the potential of causing over 80% damage to the crop. Overall thirteen insect species belonging to seven families and six orders were identified as pests to *V. galamensis*. Damping-off caused serious losses in seedling survival (over 10%). Powdery mildew caused severe wilting and death of leaves late in the growing period, especially during flowering. *Aspergillus niger, Penicillium* sp., *Cladosporium* sp., *Pythium* sp., *Fusarium* sp., *Rhizoctonia solani, Alternaria* sp. and *Phoma* sp. have also been reported elsewhere as disease causal agents in *V. galamensis* (Tefera and Baye, 2003)

Table 1. 2: Pest insects associated with *Vernonia* in Ethiopia (Baye and Gudeta, 2002).

Common name	Scientific name	Family	Order
Aphids	*Aphis gossypii* (Glover)	Aphidiae	Homoptera
Blister beetle	*Mylabris* sp.	Meloidae	Coleoptra
Cluster bugs	*Agronoscelis pubescens* (Thunb.)	Pentatomidae	Hemiptera
Epilachna beetles	*Epilachna* sp.	Coccinelidae	Coleoptra
Green grasshopper	*Ornithacris* sp.	Acrididae	Orthoptera
Green stick bug	*Nezara viridula* (L.)	Pentatomidae	Hemiptera
Harlequin bug	*Bagrada* sp.	Pentatomidae	Hemiptera
Helmet bug	*Captosoma* sp.	Pentatomidae	Hemiptera
Leaf miner	*Liriomyza* sp.	Agromizidae	Diptra
Lygaeus bug	*Lygus* sp.	Lygaeidae	Hemiptera
Spiny boll worm	*Earias biplaga* (Wkl.)	Noctuidae	Lepidoptera
Striped blister	*Epileauta* sp	Meloidae	Coleoptra
Vernonia worm	*Indent* sp.	Noctuidae	Lepidoptera

Table 1. 3: Fungi and bacterial pathogens associated with *Vernonia* in Ethiopia (Baye and Gudeta, 2002).

Common name	Scientific name	Major damaging stage
Damping off	*Rhizoctonia solani* (Kuhn), *Fusarium* sp	Seedling
Leaf blight	Unknown bacteria	Flowering and maturity
Leaf spot	*Alternaria* sp., *Phoma* sp.	Flowering and maturity
Powdery mildew	*Erysiphe* sp.	Flowering and maturity
Rust	*Puccinia* sp.	Maturity
Wilt	*Fusarium* sp.	Seedling, flowering, and maturity

1.7 Crop breeding

Economically, yield enhancement and stability are the over-riding objective of plant breeding (Manavalan *et al*, 2009). Specific areas need to be addressed and improved which contribute to lower production costs or which help in reaching the genetic yield potential of a genotype. There is an urgent need for crop diversification to add new uses to existing crops and to introduce new crops to meet new demands for food, fibres, fuel, pharmaceuticals and chemical raw materials (Hatti-Kaul *et al*, 2007). It is driven by efforts to reach a more sustainable and environmentally friendly agriculture. These efforts also call for renewable resources, new and more specialized raw materials for the chemical

industry, vegetable oils for fuel, disease and pest resistance sources which will reduce the use of pesticides, products with modified qualities and adaptation to stress conditions.

Plant breeding efforts combining genetic resources and induced mutations using classical, in vitro and innovative molecular approaches have been responsible for much of the intensified development of industrial crops in recent decades. In addition, these efforts have changed quality characteristics, which are more exacting in industrial crops (Ruane and Sonnino, 2011). Many of the critical steps in the relevant biosynthetic pathways are controlled by one or a few major genes, which can be modified by induced mutations. These lend themselves to modification by induced mutations and breeding manipulations.

1.7.1 Mutation breeding in crops

Mutation is the ultimate source of heritable variation. As such it conditions the response to selection and adaptation through natural selection (Bataillon, 2000). Mutation breeding is the improvement of crops by inducing mutations at specific loci controlling economically important traits and/or eliminates undesirable genes from elite breeding lines (Alacantara *et al.*, 1996). Mutants of desirable traits can be developed directly as new cultivars. Mutation breeding makes extensive use of deviations from the norm in order to improve characteristics of important crops. However, an efficient genetic improvement of a cultivar depends on the knowledge of mode of gene action, genetic variability, and the interrelationship among important plant characteristics. Induced mutagenesis is a significant tool to break through the limitations of variability and to create variability in a short period of time. The degree of cytological aberrations in either mitosis or meiosis is regarded as one of the dependable criteria for estimating the effect of a mutagen. Mutagen induced anomaly of the chromosome is the primary basis of genetic change; therefore, investigations on the mechanism of chromosome breakage, type of aberrations, and their genetic consequences form an integral part of most mutation studies (Kumar and Kumar Rai, 2007).

In breeding of crop mutants using chemical mutagenesis, the most effective compounds are alkylating agents, especially ethylmethanesulfonate (EMS), N - methyl-N-nitro - guanidine (NE), Diethylsulphate (DES) and Dimethylsulphate (DMS) (Aliyu and Adamu, 2007). Ethylmethanesulfonate is a common and highly effective chemical mutagen. It is a highly recommended chemical mutagen for seeds, because it can be

applied easily and the after effects can be monitored with ease. In plants, EMS usually causes point mutations, but loss of a chromosome segment or deletion can also occur (Okagaki *et al.*, 1991). Therefore, EMS has the potential of altering loci of particular interest without inducing a great number of closely linked mutations. This allows the plant breeder to obtain useful alleles without the swarm of linked deleterious alleles present in exotic or wild germplasms or even from adapted inbred lines.

Ethylmethanesulfonate has potential to be effectively applied to develop new vernonia lines with high yield and/or enhanced agronomic traits. Primary to any mutagenesis of crops, the suitable chemical dose and optimal conditions for a particular line/cultivar should first be determined. Higher doses can produce very drastic effects that may lead to death of the organism. A relatively lower dose may result in altered growth characteristics. Under optimal mutagenesis conditions, individuals of an EMS mutant population carry a high mutation load but remain vigorous and fertile. It is important, therefore, to determine the level of mutagen treatment necessary to achieve the maximal mutation load (Stephenson *et al*., 2010). With respect to EMS doses, the terms lower and higher are relative and may be different for each crop species. Seedling emergence, seedling growth and chromosomal aberration are the commonly used criteria for selecting optimal treatment doses in plants (Shah *et al*., 2008).

1.7.2 Ethylmethanesulfonate mutagenesis

Ethyl methanesulfonate (EMS) is an alkylating agent used in chemical mutagenesis of both plants and animals. Alkylation of nucleotides induced by EMS in chromosomes can result in mis-pairing and changing of bases. This results in alkylation of guanine (G) which subsequently forms O6- thylguanine. O6-ethylguanine then forms a base pair thymine (T) and not cytosine (C). Original G/C pairs end up being replaced by A/T base pairs. At a low EMS frequency, G/C to C/G or G/C to T/A transversions are generated by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors. This can result in gain or loss of gene functions. Chemical mutagenesis can therefore be used to understand the role of specific amino acid residues in protein function. Results of many studies suggest that use of chemically induced mutants can also provide useful information for understanding the functions of essential genes by generating weak nonlethal alleles.

Ethylmethanesulfonate mutagenesis in crops can also be used to produce new breeding lines (Kim *et al.*, 2005).

REFERENCES

Alcantara T., P. Bosland and D. Smith, 1996: Ethyl Methanesulfonate-Induced Seed Mutagenesis of *Capsicum annuum*. The Journal of Heredity **87**, 239-241.

Aliyu H. and A. Adamu, 2007: The Effect of Diethylsulphate on some Quantitative Traits of Tomato (*Lycopersicon esculentum* Mill). Science World Journal **2**, 1-4.

Ayorinde F.O., J.G. Osman, R.L. Shepard and F.T. Powers, 1988: Synthesis of Azelaic Acid and Suberic Acid from *Vernonia galamensis* Oil. Journal of American Oil Chemistry Society **65**, 1774-1776.

Baskin J. M. and C. C. Baskin. 2004: A Classification System for Seed Dormancy. Seed Science Research **14**, 1-16.

Barro F., J. Fernandez-Escobar, M. De La Vega and A. Martian, 2001: Doubled Haploid Lines of *Brassica carinata* with Modified Erucic Acid Content through Mutagenesis by EMS Treatment of Isolated Microspores. Plant Breeding **120**, 262-264.

Bataillon T., 2000: Estimation of Spontaneous Genome-wide Mutation Rate Parameters: Whither Beneficial Mutations? Heredity **84**: 497- 501.

Baye T., 1996: Characterisation and Evaluation of *Vernonia galamensis* var. *ethiopica* Germplasm Collected from Eastern Ethiopia. M.Sc Thesis. Alemaya University of Agriculture, Dire Dawa, Ethiopia.

Baye T., 2000: Variation in Agronomic Characteristics of *Vernonia galamensis*, a New Industrial Oilseed Crop of Ethiopia. In: The Development of *Euphorbia lagascae* within European Community. Proceedings EC-Concerted Action Workshop II, Cambridge, UK. 49-53.

Baye T., H. Kebede and K. Belete, 2001: Agronomic Evaluation of *Vernonia galamensis* Gemplasm Collected from Eastern Ethiopia. Industrial Crops and Products **14**, 179-190.

Baye T. and S. Gudeta, 2002: Pest Survey of *Vernonia galamensis* in Ethiopia. Trends in New Crops and New Uses. ASHS Press. 1-3.

Baye T. and H.C. Becker, 2004: - Natural Outcrossing Rate in *Vernonia galamensis*. Plant Breeding **123**, 398-399.

Baye T., H.C. Becker and S. v. Witzke-Ehbrecht, 2005: *Vernonia galamensis*, a Natural Source of Epoxy Oil: Variation in Fatty Acid Composition of Seed and Leaf Lipids. Industrial Crops and Products **21**, 257-261.

Becker H.C., C. Damgaard and B. Karlsson, 1992: Environmental Variation for Outcrossing Rate in Rapeseed (*Brassica napus*). Theoretical and Applied Genetics **84**, 303-306.

Benaniba M.T., N. Belhaneche-Bensemra and G. Gelbard, 2001: Stabilizing Effect of Epoxidised Sunflower Oil on the Thermal Degradation of Poly(Vinyl Chloride). Polymer Degradation and Stability **74**, 501-505.

Benaniba M.T., N. Belhaneche-Bensemra and G. Gelbard, 2003: Stabilization of PVC by Epoxidised Sunflower Oil in the Presence of Zinc and Calcium Stearates. Polymer Degradation and Stability **82**, 245-249.

Berenschot A., M. Zucchi, A. Tulmann-Neto and V. Quecini, 2009: Mutagenesis in *Petunia x hybrida* Vilm. and Isolation of a Novel Morphological Mutant. Brazilian Journal of Plant Physiology **20**, 95-103.

Carlson K.D. and S.P. Chang, 1985: Chemical Epoxidation of a Natural Unsaturated Epoxy Seed Oil from *Vernonia galamensis* and a Look at Epoxy Oil Markets. Journal of American Oil Chemistry Society **62**, 934-939.

Clay K., and D. Levin, 1989: Quantitative Variation in Phlox: Comparison of Selfing and Outcrossing Species. American Journal of Botany **76**, 577-588.

Derksen J.T.P, F.P. Cuperas and P. Kolster, 1995: Paint and Coatings from Renewable Resources. Industrial Crops and Products **3**, 225-236.

DWAF, 2002: National Eutrophication Monitoring Programme for Surface Water: Implementation Manual. Institute for Water Quality Studies, Department of Water Affairs and Forestry, Pretoria, South Africa.

El-Sayed N.H., M.I. Mogahed, A.A. Haron and T.J. Mabry, 1999: Flavonoids and other Constituents of *Vernonia biopontani* and Insecticidal and Fungicidal Activities. Revista Latinoamericana de Química **27**, 5-8.

Favi F., C.L. Cantrelly, T. Mebrathu and M.E. Kraemer, 2008: Leaf Peltate Glandular Trichomes of *Vernonia galamensis* spp. *galamensis* var. *ethiopica* Gilbert: Development, Ultrastructure and Chemical Composition. International Journal of Plant Sciences **169**, 605-614.

Gilbert M.G. 1986: Notes on Eastern Africa *Vernonieae* (Compositae). IV. A Revision of the *Vernonia galamensis* Complex. Kew Bulletin **41**, 19-35.

Hassan R., 2006: Climate Change and African Agriculture. *Policy No.28.* Based on Durand (2006), "Assessing the Impact of Climate Change on Crop Water Use in South Africa", *CEEPA Discussion Paper No.28,* CEEPA, University of Pretoria.

Hatti-Kaul R., U. Tornvall, L Gustafssonand P. Borjesson, 2007: Industrial Biotechnology for the Production of Bio-Based Chemicals - a Cradle-to-Grave Perspective. TRENDS in Biotechnology **25**, 119-124.

Jaworski J. and E.B. Cahoon, 2003: Industrial Oils from Transgenic Plants. Current Opinions in Plant Biology **6**, 178-184.

Kaplan K.C., 1989: Vernonia New Industrial Oil Crop. Agromomic Research **374**, 10-11.

Kim Y.S., K.S. Schumaker and J.K. Zhu. 2005: EMS Mutagenesis of *Arabidopsis*. Methods in Molecular Biology **323**, 101-104.

Kumar A. and M.N. Mishra, 2004: Effect of Gamma-rays, EMS and NMU on Germination, Seedling Vigour, Pollen Viability and Plant Survival in M1and M2 Generation of Okra (*Abelmoschus esculentus* (L.) Moench). Advances in Plant Science **17**, 295-297.

Kumar G. and P. Kumar Rai, 2007: EMS Induced Karyomorphological Variations in Maize (*Zea mays* L.) Inbreds. Turkish Journal of Biology **31**, 187-195.

Manavalan L.P., S.K. Guttikonda, L. P. Tran and H.T. Nguyen, 2009: Physiological and Molecular Approaches to Improve Drought Resistance in Soybean. Plant Cell Physiology **50**, 1260-1276.

Mebrahtu T., T. Gebremariam, A. Kidane and W. Araia, 2009: Performance of *Vernonia galamensis* as a Potential and Viable Industrial Oil Plant in Eritrea: Yield and Oil content. African Journal of Biotechnology **8**, 635-640.

Miserez F., 1999: Investigation Phytochimique et Comparaison Chimiotaxonomique de Diffe´rentes espe`ces du Genre Vernonia (Asteraceae). PhD thesis. Universite´ de Lausanne.

Nyamongo D.O., M.I. Daws, J.O. Nyabundi, F.R. Hay and P.A Ayiecho, 2010: Onset of Dormancy, Dormancy Levels, and Appropriate Seed Production Environment for Two Subspecies of *Vernonia galamensis* (Cass.) Less. Journal of New Seeds **11**, 16-27.

Okagaki R. J., M. G. Neuffer and S. R. Wessler, 1991: A Deletion Common to Two Independently Derived Waxy Mutations of Maize. Genetics **128**, 425-431.

Ologunde M.O., F.O. Ayorinde and R.L. Shepard, 1990: Chemical Evaluation of Defatted *Vernonia galamensis* Meal. Journal of American Oil Chemistry Society **67**, 92-94.

Osorio J., J. Fernandez-Martinez, M. Mancha and R. Garces, 1995: Mutant Sunflowers with High Concentration of Saturated Fatty Acids in the Oil. Crop Science **35**, 739-742.

Perdue R.E., 1988. Systematic Botany in the Development of *Vernonia galamensis* as a New Industrial Oilseed Crop for the Semi-arid Tropics. Symbiotic Botany Upsal. **28**: 125-135.

Perdue R.E., 2002: Pest Survey of *Vernonia galamensis* in Ethiopia. Trends and New Uses, J. Janick and A. Whipkey (eds.). ASHS Press, Alexandria.

Rahman A.H.M.M., M.S. Alam, S.K. Khan, A. Ferdous, A.K.M. Rafiul Islam and M.M. Rahman, 2008: Taxonomic Studies on the Family Asteraceae (Compositae) of the Rajshahi Division. Research Journal of Agricultural and Biological Sciences **4**, 134-140.

Ramalema S.P., H. Shimelis, I. Ncube, K.K Kunert and P.W. Mashela, 2010: Genetic Analysis among Selected Vernonia Lines through Seed Oil Content, Fatty Acids and RAPD DNA Markers. African Journal of Biotechnology **9**, 117-122.

Republic of South Africa, National Water Act.Act 36 of 1998.

Ruane J and A. Sonnino, 2011: Agricultural Biotechnologies in Developing Countries and their Possible Contribution to Food Security. Journal of Biotechnology **156**, 356-363.

Savant K.D. and V.S. Kothekar, 2011: Induction of Variability in Fatty Acid Profile in Sesame (*Sesamum indicum* L.). Journal of Phytology **3**, 01-03.

Scotland R.W. and A.H. Wortely, 2003: How Many Species of Seed Plants are There? Taxonomy **52**, 101-104.

Shah, T., J. Mirza, M. Haq and B. Atta, 2008: Radio Sensitivity of Various Chickpea Genotypes in M1 Generation I-Laboratory Studies. Pakistan Journal of Botany **40**, 649-665.

Shimelis, H., P. Mashela and A. Hugo, 2008: Performance of Vernonia as an Alternative Industrial Oil Crop in Limpopo Province of South Africa. Crop Science **48**, 236-242.

Singh U.P., B. Prithiviraj and B.K. Sarma, 2000: Development of *Erysiphepisi* (Powdery Mildew) on Normal and Albino Mutants of Pea (*Pisum sativum* L.). Journal of Phytopathology **148**, 591-595.

Stephenson P., D. Baker, T. Girin, A. Perez, S. Amoah, G.J. King and L. Østergaard, 2010: A Rich TILLING Resource for Studying Gene Function in *Brassica rapa*. BMC Plant Biology **10**, 1-10.

Tefera T. and T. Baye, 2003: Mycoflora Associated with New Industrial Oilseed Crop (*Vernonia galamensis* var. *ethiopica*) in Ethiopia. Tropical Science **43**, 6-9.

Teynor T.M., D.H. Putman, E.S. Oplinger, E.A Oelke, K.A. Kelling, J.D. Doll, 1992: Vernonia. Alternative Field Crops Manual. Center for Alternative Plant and Animal Products, University of Wisconsin- Extension and Cooperative Extension, University of Minnesota.

The Water Wheel, 2007: Water Scarcity- Making Every Drop Count. The Water Wheel, 28-29.

Thompson A.E., D.A Dierig, E.R.Johnson, G.H. Dahlquist and R. Kleiman, 1994: Germplasm Development of *Vernonia galamensis* as a New Industrial Oilseed Crop. Industrial Crops and Products **3**, 185-200.

Thorne R.F. 2002. How Many Species of Seed Plants are There? Taxonomy **51**, 511-522.

Thorpe J., F.D. Favi and M.E. Kraemer, 2006: Toxicity of Peltate Glandular Trichome Extract of *Vernonia galamensis* against Whiteflies. Page 5 in Program and Abstracts of 14th Biennial Research Symposium of the Association of Research Directors, Atlanta.

Vaida K. and M. Young, 1993: Ethyl Methanesulfonate Induced Variation in Qualitative and Quantitative Characters of Roselle (*Hibiscus sabdariffa* L.) (Malvaceae). Brazilian Journal of Genetics **16**, 381-391.

van der Vossen H.A.M. and Mkamilo G.S. (Eds), 2007: Plant Resources of Tropical Africa 14. Vegetable Oils. PROTA Foundation, Netherlands. 178-181.

CHAPTER 2

DETERMINATION OF OPTIMUM ETHYLMETHANESULFONATE CONCENTRATION, TREATMENT TEMPERATURE AND DURATION ON MUTAGENESIS OF SELECTED VERNONIA LINES

ABSTRACT

Chemical mutagenesis using ethylmethanesulfonate (EMS) is an important method to induce random useful genetic mutations in crop plants. The objective of this study was to determine an optimum treatment combination i.e. exposure duration, temperature and EMS dose that would enable 50-60% germination at minimum days to emergence in selected *Vernonia galamensis* var. *ethiopica* lines. Seeds of four selected vernonia lines (Vge-1, Vge-4, Vge-7 and Vge-10) were treated in two replicates using three EMS concentrations (0.372, 0.744 and 1.1%), three temperature regimes (30, 32.5 and 35^0C) and four time durations (0.5, 1, 1.5 and 2hr). The treated seeds were planted in seedling trays in a tunnel using the completely randomized design at the Controlled Environment Facility (CEF) of the University of KwaZulu-Natal. Days to 50% emergence, germination percentage and seedling height were recorded for each line and treatment combination. Significant interactions ($P<0.001$) were observed between EMS, line, time and temperature with respect to days to 50% emergence, germination percentage and seedling height. Optimal days to 50% emergence (10-12 days) and germination (50- 58%) was achieved for Vge-1, Vge-7 and Vge-10 when treated with 0.372% EMS at 35^0C and an hour treatment. The optimal treatment combination for Vge-4 was 0.372% EMS at 32.5^0C for 2hr. The treatment combinations that yielded optimum results in the tested lines will be utilized to induce large scale mutation in *V. galamensis* to select target mutants.

KEYWORDS: ethylmethanesulfonate, mutation, vernonia, *Vernonia galamensis*

2.1 INTRODUCTION

Vernonia comprises of more than a thousand species which vary from annual herbs and shrubs to perennial trees (Baye *et al*., 2001). Recent taxonomic studies have classified the genus to include six subspecies. Subspecies *nairobensis*, *gibbosa*, *lushotoensis*, *afromontana*, *mutomonesis* and *galamensis*. The most widely distributed of these is subspecies *galamensis* which consists of four botanical varieties, namely var. *galamensis*, var. *petitiana*, var. *australis* and var. *ethiopica* (Gilbert, 1986). Visual images of variety *ethiopica* are shown in figure 2.1. *Vernonia galamensis* is very variable with respect to morphological traits; and cultivars are described according to geographic and ecological origin.

The oilseed crop *Vernonia galamensis* and other Vernonia species are natural producers of vernolic acid, this provides the potential to extract epoxidised oil without the use of expensive industrial epoxidation processes. Production of these seed oils at a commercial scale however is hampered by high production costs due to low seed yield. Attempts to breed for better yielding cultivars have had limited success thus far. Attempts to introduce the fatty acid biosynthetic machinery from the wild plants into a high-yielding oil crop by genetical engineering have also been attempted, with low expression of the epoxidised product (Hatti-Kaul *et al*., 2007). Research on *V. galamensis* by Mebrahtu *et al.*, (2009) recommended further investigations on development of the crop. Among the recommendations was that research on mutation breeding be conducted to breed and select for mutants with reduced shattering and synchronized maturity. The future challenges identified were lack of uniform seed maturity, problem of shattering, and lack of appropriate technologies for mechanical harvesting, seed cleaning (threshing) and processing and oil extraction.

Vernonia galamensis var. *ethiopica*, Asteracea is an underexploited potential crop colonising agricultural and arid lands which is endemic to tropical regions of East Africa including Kenya, Malawi and Ethiopia (Baye *et al*., 2001; Mebrahtu *et al*., 2009). South and south-eastern Ethiopia has been described as a natural habitat of this botanical variety (Gilbert, 1986). *V. galamensis* has a major potential as an industrial source of vernolic acid due to a high demand of natural epoxidised oil (Shimelis *et al.*, 2008). Crops like soybean (*Glycine max* L.) and linseed (*Linum usitatissimum* L.) are currently used for epoxy oil production, but the oil still requires undergoing chemical epoxidation which is expensive.

Vernolic acid is an important resource in the manufacturing of paints and coatings and has great value in the oleochemical industry. Epoxy fatty acids are widely used in oleochemical (chemicals derived from plants or animal fats which are similar to petrochemicals) industry as plasticizers and stabilizers of polyvinyl chloride (PVC), in reformulation of oil based paints, in cosmetics, and pharmaceutical applications (Bhardwaj*et al*., 2000). With a high demand for natural epoxidised oil, there is need to research and improve the productivity of natural producers of epoxic acids like *V. galamensis*.

Figure 2. 1: Ripening capitula, primary and secondary flowering, and *in vitro* germination of *V. galamensis* var. *ethiopica*.

Chemical mutagens have been effective in the development of novel germplasm in crop plants (van Harten, 1998; Kodym and Afza, 2003; Gunstone 2006). Artificial mutation helps to induce genetic variation of gene loci controlling economically important traits and/or elimination of undesirable genes from breeding lines (Alacantara*et al*., 1996). Mutants showing desirable traits can be developed directly as new cultivars. Crop mutants can be developed using chemical mutagenesis and the most effective compounds are alkylating agents, especially ethylmethanesulfonate (EMS), N-methyl-N-nitro-guanidine (NE), Diethylsulphate (DES) and Dimethylsulphate (DMS) (Aliyu and Adamu, 2007). Chemical mutagenesis using ethylmethanesulfonate is a powerful tool in inducing random useful genetic mutations in crop plants (Kim, Schumaker and Zhu, 2005). Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. Increasing the dose of EMS application alone or in combination with increasing duration of seed exposure to EMS in treatments has been shown to decrease germination and survival percentage in certain crops, and to induce variation in both qualitative and quantitative traits (Miller *et al.*, 1980; Vaida and Young, 1993; Kumar and Kumar Rai, 2007; Berenschot*et al.*, 2008; Shah *et al.*, 2008 and Ashoka Kumar *et al.*, 2009).

Ethyl methanesulfonate is an alkylating agent used in chemical mutagenesis of both plants and animals. Alkylation of nucleotides induced by EMS in chromosomes can result in mispairing and changing of bases. This results in alkylation of guanine (G) which subsequently forms O^6- ethylguanine. O^6-ethylguanine then forms a base pair thymine (T) and not cytosine (C). Original G/C pairs end up being replaced by A/T base pairs. At a low EMS frequency, G/C to C/G or G/C to T/A transversions are generated by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors (Kim *et al*, 2005). The EMS induced mispairing of base pairs can change the amino acid structure of the DNA; hence change protein function during cell division.

Ethylmethanesulfonate has potential to be effectively applied to develop new vernonia lines with high yield and/or enhanced agronomic traits. Primary to any mutagenesis of crops, the suitable chemical dose and optimal conditions for a particular line/cultivar should first be determined. Higher doses can produce very drastic effects that may lead to death of the organism. A relatively lower dose may result in altered growth characteristics. With respect to EMS doses the term lower and higher is relative and may be different for each crop species. The lethal dose 50 (LD_{50}) was the standard used in this study to find optimal treatment conditions. Lethal dose 50 refers to the mutation dose that will kill 50% of the tested group/population, therefore EMS treatment conditions that resulted in germination of 50- 60% of the treated seeds formed part of the selection criteria for determining optimal treatment conditions.

Seedling emergence, seedling growth and chromosomal aberration are the commonly used criteria for selecting optimal treatment doses in plants (Shah *et al*., 2008). Ethylmethanesulfonate has also been reported to delay seedling emergence in crops (Greer and Reinhart, 2009). This study was conducted to determine the optimum EMS concentration, treatment temperature and duration that would enable early days to emergence at 50-60% germination in inducing mutation in vernonia (*Vernonia galamensis* var. *ethiopica*). The optimum treatment combinations will be used for large scale mutagenesis in vernonia to select mutants with favorable morphological characteristics including uniform branching, early and uniform maturity and enhanced seed yield and oil content and vernolic acid composition.

2.3 MATERIAL AND METHODS

2.3.1 Pre-treatment handling of seeds

Normal shaped disease-free, dry and quiescent vernonia seeds were selected from each of the four *V. galamensis* lines (Vge-1, Vge-4, Vge-7 and Vge-10). For each line, the seeds were divided into 36 batches of about 80 seeds. The seeds were placed inside specially designed and labelled polyethylene mesh bags measuring 8 cm long and 6 cm wide. The bags were heat-sealed. Extra mesh bags were cut to double thicken the bags. Labelling was achieved by using paper tags with cotton strings, the openings of the mesh bags was closed by tying the cotton strings around them.

2.3.2 Presoaking of seeds

An EMS mutagenesis protocol proposed by Mba *et al*. (2009) was modified and applied in treating vernonia seeds. The seeds (in the mesh bags) were soaked in 70% ethanol for 1 minute, and then washed in running water under room temperature for 1 minute. Seeds were afterwards soaked in 30% JIK® for 5 minutes, and then washed in running water for 1 minute. Seeds were then soaked by placing in a dish filled with distilled water and left to stand for 16-20 h at 20-22 °C.

2.3.3 EMS preparation and treatment of seeds

Towards the end of the pre-soaking stage, fresh ethylmethanesulfonate solutions were prepared according to desired concentrations (0.372%, 0.744% and 1.1%). The following formula was used in preparing the required rate of EMS. To prepare a 1.1% EMS the required amount is:

Required EMS rate = EMS dose x [distilled water + 2% dimethyl sulfoxide (DMSO)] / 100

= 1.1 x (980ml + 20ml) / 100

= 11ml

Thus 11ml of EMS was added per 1000ml water and DMSO solution to prepare 1.1% EMS solution. Accordingly, other EMS rates were prepared for the study.

Required volumes of distilled water were mixed with 2% (v/v) dimethyl sulfoxide (DMSO) into a bottle and autoclaved at 120 °C for 15 min at 103.5 kPa (15 psi) and the

mixture left to cool at room temperature. A sterile syringe was used to add the required volume of EMS solution to the sterile water and DMSO mixture. The resulting solution was shaken vigorously to give a homogeneous emulsion. At the end of the pre-soaking period, the bags were removed from distilled water and shaken to remove excess water.

Barring only the control bags, the seeds were soaked in the EMS solutions according to the desired combinations of the three EMS concentration , three temperature (30°C, 32.5°C and 35°C) and treatment duration (0.5, 1, 1.5 and 2 hours). The temperatures were maintained in a water bath during the duration of treatment. After treatment, the seeds were washed (to remove excess EMS) under running cold tap water for 2-3 hours. The mesh bags were shaken off to remove excess water and the seeds placed on blotting paper for 2 hours to dry.

2.3.4 Planting of seeds, data collection and analysis

Seedling soil mix (National Plant Foods, South Africa) was pasteurized in a steam chamber for 4 hours and left to cool overnight. Speedling 200 trays were filled with pasteurized soil and 1cm holes were drilled in the soil. The experiment was laid out using a completely randomized design with the following factors: vernonia lines (4 levels; Vge-1, Vge-4, Vge-7 and Vge -10); EMS concentration (3 levels; 0.372, 0.744 and 1.1%); time (4 levels; 0.5, 1, 1.5 and 2h) and temperature (3 levels; 30^oC, 32.5^oC and 35^oC) with two replications. One seed was put into each hole according to the treatment combination. Extra soil was applied after planting to cover seeds. Each tray was watered thoroughly before placing in a controlled environmental facility (CEF). Days to emergence were recorded daily for each treatment combination from first emergence until no further seedling emergence was observed. Germination percentage for each treatment was also recorded until 30 days after planting (Figure 2.2). Seedling height was recorded as an average shoot length of all seedlings per treatment after 30 days.

Data was subjected to general analysis of variance (without blocking) and Pearson correlation analysis using Genstat® (12^{th} edition, VSN International, UK).

2.4 RESULTS

The interaction between time of exposure, EMS dose, temperature and line was highly significant (P< 0.001) (Table 2.1), suggesting that days to 50% emergence, germination percentage and seedling height were highly influenced by temperature, time of exposure, EMS concentration and the type of line.

Table 2. 1: Analysis of variance on days to 50% emergence, germination percentage and seedling height among four *Vernonia galamensis* var. *ethiopica* lines when tested using three EMS doses, three treatment temperature and four exposure time.

		DTE		GP		SH	
Source of variation	**d.f..**	**m.s.**	**F pr.**	**m.s**	**F pr.**	**m.s**	**F pr.**
EMS	2	1625.7	<.001	16225.6	<.001	81100.3	<.001
Line	3	9.9	0.27	328.6	0.006	493.7	0.01
Temp	2	42.1	<.001	233.2	0.051	1806.1	<.001
Time	3	289.2	<.001	6423.3	<.001	13699.4	<.001
EMS x Line	6	17.1	<.001	148.2	0.08	342.3	0.016
EMS x Temp	4	24	<.001	163.6	0.08	1099	<.001
Line x Temp	6	48.8	<.001	284.7	0.002	468.7	0.002
EMS x Time	6	42.6	<.001	2028.3	<.001	2808.8	<.001
Line x Time	9	40.9	<.001	224.4	0.003	465.1	<.001
Temp x time	6	25.3	<.001	284.1	0.002	1024.4	<.001
EMS x Line x Temp	12	33.4	<.001	285.1	<.001	666.6	<.001
EMS x Line x Time	18	23.3	<.001	253.6	0.113	682.1	<.001
EMS x Temp x Time	12	18.9	<.001	119.1	<.001	599.9	<.001
Line x Temp x Time	18	21.6	<.001	73	0.678	126.6	<.001
EMS x Line x Temp x Time	36	8.2	<.001	62	<.001	781.5	<.001
Residual	143	39.2		1.111		769	

DTE= days to 50% emergence; GP= germination percentage; SH= seedling height
d.f = degrees of freedom; m.s = mean square; F pr. = F probability

Optimum conditions for Vge-1, Vge-7 and Vge-10 seeds were achieved when treated with 0.372% EMS at 35^0C for an hour. About 50-58% of the planted seeds of these lines emerged within 10-12 days after planting. Optimum conditions for Vge-4 were achieved when treated with 0.372% EMS at 32.5^0C for 2 hrs, 53% of the seeds emerged 9 days after planting (Tables 2.2 and 2.3).

The earliest emergence was observed 9 days after planting, whilst the latest was recorded at 24days after planting. Earliest emergence was recorded from seeds treated at 0.372% EMS. Seeds exposed to 0.744 and 1.1% EMS with exposure time of 1.5 - 2 hours delayed emergence drastically (Table 2.2). Seeds treated with 0.744 and 1.1% EMS at 0.5 and 1 hour exposure time provided intermediate time to emergence. Earliest seed emergence and relatively high germination percentage was related with low EMS dose (0.372%). Prolonged exposure (2 hours) of seeds to a high EMS dose (1.1%) delayed emergence drastically. The untreated controls for all the lines emerged early (9-10 days), therefore the mutagenesis treatment of vernonia seeds significantly delayed days to emergence (Table 2.2).

Table 2. 2: Days to 50% emergence among four selected *V. galamensis* lines treated at three different EMS doses (%), three temperature regimes (°C), and four exposure times (hrs).

			Days to 50% emergence			
			Time (hrs)			
Line	**EMS (%)**	**Temp (°C)**	**0.5**	**1**	**1.5**	**2**
Vge-1	0.372	30	9	9	19.5	10
		32.5	16	15.5	10	9
		35	15	11	13	18.5
	0.744	30	14.5	19	22.5	23.5
		32.5	15.5	20	23.5	23
		35	9.5	22	23	17.5
	1.1	30	22	15	23.5	23.5
		32.5	17.5	24	18.5	23.5
		35	12	21	23	24
	untreated control			9		
Vge-4	0.372	30	9	14	14	12.5
		32.5	11	14.5	11	9
		35	15.5	10	13	18
	0.744	30	13	19.5	21.5	23.5
		32.5	13	19.5	22	23
		35	10	22	22.5	24
	1.1	30	12.5	16.5	24	23
		32.5	15.5	23	21.5	24
		35	15.5	19	22.5	24
	untreated control			9		
Vge-7	0.372	30	14.5	11.5	9.5	14
		32.5	15	11.5	11	23
		35	10.5	10	9	19
	0.744	30	13.5	18.5	24	18.5
		32.5	15	21	19	23
		35	22	18.5	22.5	24
	1.1	30	11	14	24	21
		32.5	13	22	22	23.5
		35	16	18.5	24	24
	untreated control			9.5		
Vge-10	0.372	30	10	11	9.5	13
		32.5	12	16.5	17.5	10
		35	13	11.5	12.5	9
	0.744	30	12	21.5	23	23.5
		32.5	16.5	20	23.5	23.5
		35	10	20	23	24
	1.1	30	14	15	22.5	20.5
		32.5	20.5	23	21	24
		35	9	22.5	23.5	24
	untreated control			9		
L.S.D			5.673			
C.V (%)			14.5			

L.S.D = least significant difference at 0.05; C.V = co-efficient of variation

The lowest germination percentage (5%) was observed when Vge-10 seeds were treated using 0.744% EMS for 2hr at 30^{o}C. Highest number of germinated seedlings (83%) was recorded when Vge-4 seeds were exposed to 0.372% EMS for 1.5 hr at 32.5^{o}C. All seeds treated at 0.372% EMS had the highest percentage germination. Seeds exposed to 0.744 and 1.1% EMS at 1.5-2hrs exposure time exhibited the lowest germination percentage. Seeds treated with 0.744 and 1.1% EMS at 0.5 and 1 hr exposure time had intermediate germination percentage (Table 2.3). The untreated controls for all the lines had high germination percentage (80-89%). Treatment of vernonia seeds therefore significantly reduced germination as compared to the untreated controls (Table 2.3).

Table 2. 3: Germination percentage among four selected *V. galamensis* lines treated at three different EMS doses (%), three temperature regimes (°C), and four exposure times (hrs).

			Germination %			
			Time (hrs)			
Line	**EMS (%)**	**Temp (°C)**	**0.5**	**1**	**1.5**	**2**
Vge-1	0.372	30	71.67	76.67	46.67	63.33
		32.5	26.67	46.67	68.33	76.67
		35	53.33	51.67	55	51.67
	0.744	30	35	31.67	30	16.67
		32.5	51.67	56.67	11.67	11.67
		35	15	20	15	40
	1.1	30	50	40	48.33	16.67
		32.5	41.67	23.33	30	8.33
		35	51.67	50	31.67	8.33
	untreated control		89			
Vge-4	0.372	30	71.67	43.33	61.67	40
		32.5	65	51.67	83.33	53.33
		35	46.67	61.67	33.33	51.67
	0.744	30	40	50	16.67	18.33
		32.5	41.67	40	26.67	23.33
		35	63.93	38.33	31.67	16.67
	1.1	30	63.33	51.67	28.33	13.33
		32.5	46.67	41.67	38.33	11.67
		35	53.33	43.33	33.33	18.33
	untreated control		80			
Vge-7	0.372	30	51.67	45	76.67	45
		32.5	48.33	51.67	66.67	55
		35	80	50	76.67	38.33
	0.744	30	55	46.67	20	20
		32.5	60	30	41.67	18.33
		35	55	45	31.67	31.67
	1.1	30	68.33	56.67	25	28.33
		32.5	66.67	36.67	38.33	31.67
		35	36.67	51.67	15	16.67
	untreated control		83.33			
Vge-10	0.372	30	65	61.67	68.33	56.67
		32.5	50	50	58.33	43.33
		35	51.67	58.33	58.33	75
	0.744	30	45	46.67	33.33	5
		32.5	28.33	38.33	30	20
		35	40	50	23.33	10
	1.1	30	58.33	55	26.67	16.67
		32.5	45	45	13.33	10
		35	75	48.33	33.33	23.33
	untreated control		83.33			
L.S.D				17.321		
C.V (%)				20.8		

L.S.D = least significant difference at 0.05; C.V = co-efficient of variation

The shortest seedling height at 5cm was observed when Vge-10 seeds were treated using 0.744% EMS for 2hr at 35^{o}C. Tallest seedling height at 128.5cm was recorded when Vge-1 seeds were exposed to 0.372% EMS dose for half an hour at 30^{o}C. The untreated controls for all the lines had taller seedlings (111.3 - 160cm) in comparison to respective treated seedlings; however, random increases in seedling height for EMS treated seedlings were recorded which were taller than the respective controls (Table 2.4). Treatment of vernonia seeds therefore significantly reduced vernonia seedling height compared to the untreated controls (Table 2.4).

Table 2. 4: Seedling height among four selected *V. galamensis* lines treated at three different EMS doses (%), three temperature regimes (°C), and four exposure times (hrs).

			Seedling height (mm)			
			Time (hrs)			
Line	**EMS (%)**	**Temp (°C)**	**0.5**	**1**	**1.5**	**2**
Vge-1	0.372	30	128.5	110.95	34.65	84.45
		32.5	48.8	86.55	123	65.55
		35	81.9	95	75.7	62.54
	0.744	30	46.5	36.75	23.55	19
		32.5	44.5	31.5	8.5	13
		35	94.4	22.66	22.5	69.08
	1.1	30	35.83	48.8	39.71	15.25
		32.5	35.15	22.75	80.5	21.75
		35	71.75	39.02	19.67	6.25
	untreated control			162		
Vge-4	0.372	30	113.8	52.45	70.3	66.65
		32.5	118.6	55.3	84.4	34.75
		35	88.5	125.5	61.9	51.25
	0.744	30	78.3	31.85	18	8.5
		32.5	68.15	41.15	14.35	15.75
		35	67.73	22.84	32.25	3
	1.1	30	65.46	56.05	20.4	18.5
		32.5	28.05	33.4	52.2	10.75
		35	45.44	42.22	25.21	18.75
	untreated control			115.3		
Vge-7	0.372	30	78	82	119	77.75
		32.5	76.09	96.25	93.2	59.8
		35	129	105.15	126.5	54.59
	0.744	30	71.55	48.2	12.3	28.25
		32.5	68	25.9	22.5	12.35
		35	66.72	49	21.16	5
	1.1	30	45.79	44.05	28.79	39.75
		32.5	20.1	38.65	49.3	60.9
		35	42.25	30.95	20.88	11
	untreated control			120.5		
Vge-10	0.372	30	91.55	95.3	131.5	69.5
		32.5	87.42	65.1	53.65	60.5
		35	106.6	89.75	107.5	128.3
	0.744	30	79.65	41.5	22.95	5
		32.5	60.25	37.8	12.65	13
		35	75.3	30.88	17.66	5
	1.1	30	62.73	48.65	52	42
		32.5	12.15	32.55	52.85	6.75
		35	45.31	35.2	23.46	12.37
	untreated control			111.3		
L.S.D				22.2		
C.V (%)				21.7		

L.S.D = least significant difference at 0.05; C.V = co-efficient of variation

Days to 50% emergence significantly negatively correlated to germination percentage (r = -0.732, p< 0.01) and seedling height (r = -0.815, p< 0.01). Germination percentage significantly positively correlated (r = 0.676, p< 0.01) to seedling height (Table 2.5).

Table 2. 5: Correlation coefficients for pair-wise association of agronomic characters in *Vernonia galamensis.*

	DTE	SH	GP
DTE	1		
SH	-0.8146**	1	
GP	-0.7317**	0.676**	1

DTE = days to 50% emergence; SH = seedling height; GP = germination percentage
** Significant differences at 0.01 probability level.

Figure 2. 2: Seedling emergence and germination trials of vernonia in Jolley Roger controlled environmental facility, University of KwaZulu-Natal.

2.5 DISCUSSION

The present study on *V. galamensis* indicated that EMS had an effect on days to seedling emergence and germination percentage. Chemical mutagens induce physiological damage (injury), chromosomal mutations (chromosomal aberrations) and gene mutations (point mutations) in the biological material of the M1 generation. The effect of mutagens on crop seeds can be estimated based on seed germination, plant height and percentage survival among other parameters (Ashok Kumar *et al.*, 2009).

Previous research on chemical mutagenesis of crops using EMS has focused mostly on crops whose production is already commercial. In this study interaction between all the four factors (line, EMS dose, temperature and duration) was statistically significant ($P<0.001$) in affecting both days to emergence and percentage germination on vernonia seeds (Table 2.1). Thus comparing all four factors was important in determining the optimal EMS mutagenesis conditions of *V. galamensis*.

Previous studies to optimize mutational treatments considered interaction between three factors i.e. EMS, temperature and cultivars unlike the current study where exposure time was included. An example of three factor study was conducted by Greer and Reinhart (2009) using three different EMS doses and at various temperatures on different cultivars to determine optimal mutagenesis conditions for *Hydringea macrophylla* and *Hydringea paniculata*. This study demonstrated a significant interaction between all the four factors considered.

Vernonia seeds may show some dormancy for a few months after maturation; and thereafter seed germination in the field takes about 9-11 days (Baye, 2002). Seeds used in this research were about 9 months old. The untreated controls germinated in the first 9 days after planting implying that the seeds used in this research were not dormant. The earliest emergence was observed 9 days after planting, whilst the latest was recorded at 24 days after planting. Earliest emergence was recorded from seeds treated at 0.372% EMS dose. Seeds exposed to 0.744 and 1.1% EMS dose with exposure time of 1.5 - 2 hours delayed emergence drastically (Table 2.2). Seeds treated with 0.744 and 1.1% EMS at 0.5 and 1 hour exposure time provided intermediate time to emergence. Earliest seed emergence and relatively high germination percentage was related with low EMS dose (0.372%). Prolonged exposure (2 hours) of seeds to a high EMS dose (1.1%) delayed emergence

drastically. Seeds exposed to mutagenic treatments delayed emergence compared to the controls, with the greatest delay observed in treatments with high EMS dose (1.1%) and long exposure duration (1.5-2 hours). Greer and Reinhart (2009) also reported delayed sprouting in hydrangea, and the delay in emergence was accounted to the increased EMS dose. According to Gunckel and Sparrow (1961) and Zaka *et al.* (2004), EMS mutagenic treatment on seeds causes chromosomal aberrations that can adversely affect cell division, hence delayed emergence of vernonia seedlings might be attributed to a delay in cell division due to the mutagenesis.

Seed germination in vernonia reduced significantly with increasing EMS dose and exposure time compared to untreated controls (Table 2.2). The highest reduction was observed at seeds treated at high EMS dose (1.1%) and long exposure duration (1.5- 2hr) (Table 2.3). The reduction in seed germination may be attributed to the effect of EMS on the seed meristematic tissue as increasing mutagen doses may cause disturbances at physiological or cellular level. Similar findings were observed in pepper (Alacantara *et al.*, 1996), tobacco (Amernath and Prasad, 1998), okra (Kumar and Mishra, 2004), mung bean (Tah, 2006), petunia (Berenchot *et al.*, 2008) and chick pea (Sha *et al.*, 2008). Ashok Kumar *et al.,* (2009) reported a linear decline in seed germination with increasing EMS dose. Kanakamanay (2008) reported decreased germination percentage due to increased EMS dose and speculated that the decrease in germination rate due to mutagenic treatment might be attributed to an inactivation of auxin levels in the plant with increasing exposures. Studies done on oil crops such as *Cuphea* (Campbell, 1987) and *Jatropha* (Dhakshanamoorthy *et al.*, 2010), also showed a decrease in germination due to an increase in EMS dose.

Although treatment demonstrated a reducing effect on vernonia seedling height, a few treatments demonstrated increased seedling height in comparison to the controls (Table 2.4). The random increases in seedling height may be accredited to the mutation in major or minor genes. Findings of reduced plant height (shoot length) were obtained when maize (*Zea mays* L.) was treated at verifying EMS dose and exposure duration (Kumar and Kumar Rai, 2007) and when different genotypes of chickpea were treated at varying EMS doses (Shah *et al.*, 2008). Auxin proteins play a vital role in lateral and vertical development of plants, inactivation of these proteins might be primary cause of the observed decreases in seedling height.

Days to emergence significantly and negatively correlated to germination percentage (r= -0.732) and seedling height (r = -0.815), whilst seedling height significantly and positively correlated (r = 0.676, $p< 0.01$) with germination percentage. Early emerging treatments consequentially had a higher number of germinated seedlings and taller seedlings in comparison to late emerging treatments. The lowest germination percentage and shortest seedlings were observed in crops with delayed emergence suggesting that increase in mutagen dose and exposure duration increases the lethal effect of the mutagen.

Optimal days to 50% emergence (10-12 days) and germination percentage (50-58%) were achieved for Vge-1, Vge-7 and Vge-10 when treated with 0.372% EMS at 35^0C and an hour of treatment time. The optimum treatment combination for Vge-4 was 0.372% EMS at 32.5^0C for 2 hours. The treatment combinations that yielded optimum treatment conditions in the tested lines will be utilized to induce large scale mutation in *V. galamensis* to select target mutants.

2.6 CONCLUSION

Vge-1, Vge-7 and Vge-10 had similar optimum treatment conditions (0.372% EMS, 1 hour exposure time at 35^oC) whereas Vge-4 optimum treatment conditions differed in exposure time (2 hours) and temperature (32.5^oC) while the optimal EMS remained constant as for the other lines. The difference in optimal EMS dose among the four lines implies that vernonia lines differ in time and temperatures required to achieve optimal mutagenesis treatment but do not vary in the mutagen dose.

2.7 REFERENCES

Alcantara T., P. Bosland and D. Smith, 1996: Ethyl Methanesulfonate-induced Seed Mutagenesis of *Capsicum annuum*. The Journal of Heredity **87**, 239-241.

Aliyu H. and A. Adamu, 2007: The Effect of Diethylsulphate on some Quantitative Traits of Tomato (*Lycopersicone sculentum* Mill). Science World Journal **2**, 1-4.

Amernath S. and A.B. Prasad, 1998: Induced Variability in Homozygous and Heterozygous Genotypes of Tobacco. Indian Journal of Genetics**58**, 69-77.

Ashok Kumar V., R. UshaKumari, R. Amutha, T. Siva Kumar, S. Juliet Hepziba and C. Ananda Kumar, 2009: Effect of Chemical Mutagen on Expression of Characters in Arid Legume Pulse-Cowpea (*Vigna unguiculata* (L.) Walp.). Research Journal of Agriculture and Biological Sciences **5**, 1115-1120.

Baye T., H. Kebede and K. Belete, 2001: Agronomic Evaluation of *Vernonia galamensis* Gemplasm Collected from Eastern Ethiopia. Industrial Crops and Products **14**, 179-190.

Baye T., 2002: Genotypic and Phenotypic Variability in *Vernonia galamensis* Germplasm Collected from Eastern Ethiopia. Journal of Agricultural Science **139**, 161-168.

Berenschot A., M. Zucchi, A. Tulmann-Neto and V. Quecini, 2009: Mutagenesis in *Petunia x hybrid* Vilm. and Isolation of a Novel Morphological Mutant. Brazilian Journal of Plant Physiology **20**, 95-103.

Bhardwaj H.L., A.A. Hamama, M. Rangappa, and D.A. Dierig. 2000: Vernonia Oilseed Production in the Mid-Atlantic Region of the United States. Industrial Crops and Products **12**, 119-124.

Campbell, T.A., 1987: Chemical Mutagenesis in Two *Cuphea* Species. Canadian Journal of Plant Science **67**, 909-917.

Dhakshanamoorthy D., R. Selvaraj and A. Chidambaram, 2010: Physical and Chemical Mutagenesis in *Jatropha curcas* L. to Induce Variability in Seed Germination, Growth and Yield Traits. Plant Biology **17**, 113-125.

Gilbert M.G., 1986: Notes on East African Vernonieae (Compositae): A Revision of the *Vernonia galamensis* Complex. Kew Bulletin **4**, 19-35.

Greer S. and T. Rinehart, 2009: In Vitro Germination and Dormancy Responses of *Hydrangea macrophylla* and *Hydrangea paniculata* Seeds to Ethyl Methane Sulfonate and Cold Treatment. Horticultural Science **44**, 764-769.

Gunckel J.E. and A.H. Sparrow. 1961: Ionizing Radiation: Biochemical, Physiological and Morphological Aspects of their Effects on Plants. Encyclopedia of Plant Physiology(Ed.) Ruhland, W.XVI: pp. 555-611, Springer-verlag, Berlin.

Gunstone F. D., 2006: Modifying Lipids for Use in Food [M]. Woodhead Publishing Limited and CRC Press LLC, 273-305.

Hatti-Kaul R., U. Tornvall, L Gustafssonand P. Borjesson, 2007: Industrial Biotechnology for the Production of Bio-Based Chemicals - a Cradle-to-Grave Perspective. TRENDS in Biotechnology **25**, 119-124.

Kanakamanay M., 2008: Induction of Genetic Variability in Kacholam, *Kaempferia galanga* L. Plant Mutation Reports **2**, 4-6.

Kim Y.S., K.S. Schumaker and J.K. Zhu. 2005: EMS Mutagenesis of *Arabidopsis*. Methods in Molecular Biology **323**, 101-104.

Kodym A., R. Afza., 2003: Physical and Chemical Mutagenesis Methods in Molecular Biology. Plant Functional Genomics: Methods and Protocols **236**, 189-220.

Kumar A. and M.N. Mishra, 2004: Effect of Gamma-rays, EMS and NMU on Germination, Seedling Vigour, Pollen Viability and Plant Survival in M1and M2 Generation of Okra (*Abelmoschuses culentus* (L.) Moench). Advances in Plant Science**17**, 295-297.

Kumar G. and P. Kumar Rai, 2007: EMS Induced Karyomorphological Variations in Maize (*Zea mays* L.) Inbreds. Turkish Journal of Biology **31**, 187-195.

Mba C., R. Afza, S. Bado and S.M. Jain, 2009: Induced Mutagenesis in Plants Using Physical and Chemical Agents. Plant Cell Culture **1,** 111-130.

Mebrahtu T., T. Gebremariam, A. Kidane and W. Araia, 2009: Performance of *Vernonia galamensis* as a Potential and Viable Industrial Oil Plant in Eritrea: Yield and Oil Content. African Journal of Biotechnology **8**, 635-640.

Miller P., K. Vaughn and K. Wilson, 1980: Induction, Ultrastructure, Isolation, and Tissue Culture of Chlorophyll Mutants in Carrot. In Vitro**16**, 823-828.

Shah T., J. Mirza, M. Haq and B. Atta, 2008: Radio Sensitivity of Various Chickpea Genotypes in M1 Generation I-Laboratory Studies. Pakistan Journal of Botany **40**, 649-665.

Shimelis H., P. Mashela and A. Hugo, 2008: Performance of Vernonia as an Alternative Industrial Oil Crop in Limpopo Province of South Africa. Crop Science **48**, 236-242.

Sparrow A., 1961: Types of Ionizing Radiation and their Cytogenetic Effects. Mutation Plant Breeding **891**, 55-119.

Tah P., 2006: Induced Macromutation in Mungbean [*Vigna radiata* (L.) Wilczek]. International Journal of Botany **2**, 219-228.

van Harten A.M., 1998: Mutation Breeding: Theory and Practical Applications. Cambridge University Press.

Vaida K. and M. Young, 1993: Ethyl Methane Sulfonate Induced Variation in Qualitative and Quantitative Characters of Roselle (*Hibiscus sabdariffa* L.) (Malvaceae). Brazilian Journal of Genetics **16**, 381-391.

Zaka R., C. Chenal and M.T. Misset, 2004: Effect of Low Doses of Short-term Gamma Radiation on Growth and Development through Two Generations of *Pisum sativum*. Science of the Total Environment**320**, 121-129.

CHAPTER 3

INDUCING GENETIC VARIATION ON SELECTED VERNONIA LINES USING PREDETERMINED ETHYLMETHANESULFONATE DOSAGE, TEMPERATURE REGIME AND EXPOSURE DURATION

ABSTRACT

Vernonia (*Vernonia galamensis*) is an underutilised oilseed crop. Cultivation and subsequent commercialisation of vernonia is significantly hampered by non-uniform seed maturity, tall plant height, seed shattering and lack of appropriate technologies for mechanical harvesting, seed threshing and cleaning. Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. Chemical mutagenesis using ethylmethanesulfonate (EMS) is the most effective to select mutants with desirable plant attributes and agronomic values. This study was conducted to induce genetic variation using predetermined EMS treatment conditions and select mutants in *V. galamensis* variety *ethiopica* lines. Seeds of two selected lines of vernonia (Vge-1and Vge-4) were treated using previously determined optimum mutagenesis conditions. Vge-1 was treated at 0.372% EMS dose for one hour at 35^{o}C while Vge-4 was treated at 0.372% EMS dose for two hours at 32.5^{o}C. 50,000 seeds of each line were treated and field planted at the Ukulinga Research Farm of the University of KwaZulu-Natal along with the untreated controls in a randomised complete block design. Data on days to emergence, germination percentage, days to heading, number of chloroplast mutants, percentage of sterile plants, days to flowering, days to maturity, number of seeds per head, number of sterile plants, plant height, thousand seed weight and plot yield was recorded and subjected to analysis of variance. EMS treatment significantly affected all the agronomic traits compared to checks. Treatment significantly delayed days to head formation, days to flowering and days to maturity on both lines. Delays in days to emergence were only significant in Vge-4. EMS treatment also significantly reduced germination percentage, number of seeds per head, number of fertile plants, plant height and plot yield for both Vge-1 and Vge-4. Thousand seed weight significantly increased in the treated seeds of the two lines. Chl orophyll mutants were observed for tested lines associated with high count of sterility for both lines. Ethylmethanesulfonate treatment successfully induced mutation in the tested

lines of *V. galamensis*. Each fertile chloroplast mutants were harvested at full maturity and seeds kept for further selection and breeding in subsequent generations.

Keywords: ethylmethanesulfonate, EMS, mutation, vernonia

3.2 INTRODUCTION

The *Vernonia galamensis* complex has six sub-species (*nairobensis, gibbosa, lushotoensis, afromontana, mutomonesis,* and *galamensis*). Sub-species *galamensis* is the most widely distributed and shows high diversity with four botanical varieties (*galamensis*, *petitiana*, *australis*, and *ethiopica*) (Gilbert 1986). *V. Galamensis* spp. g*alamensis* var. *ethiopica* grows naturally in marginal areas with minimal rainfall as little as 200 mm and at elevations ranging from 700 to 2400 m above sea level in the southern and south-eastern parts of Ethiopia (Gilbert, 1986).

Vernonia can tolerate and grow under substantial shade, therefore it could be a potential crop in agro-forestry (Baye *et al.,* 2001). *Vernonia galamensis* is adapted to the semi-arid tropics where it is found in dry bush lands, but more often in fertile lands as a weed, growing from 2000 - 2500 m altitude. Some accessions were found at environments receiving as low as 250 - 500 mm rainfall but other accessions were found in high rainfall areas of 1850 mm. *V. galamensis* requires a rainy season that provides sufficient moisture to permit the main flower heads to develop. A longer rainy season that permits secondary flower heads to develop results in non-uniform maturity and risks seed shattering. Vernonia prefers a well-drained soil with pH 5.0-8.5. On poorly drained soils, growth of the main stem stops before flowering; branches develop from the base of the plant, but they also wither and die (Baye *et al*., 2001).

Environmental degradation caused by soil erosion, desertification, deforestation and inappropriate agricultural practices is a major threat to agricultural sustainability. It is estimated that 80 percent of rangelands and rainfed croplands in southern Africa are degraded (Abalu and Hassan, 1998). Below-normal rainfall years are also occurring more and more frequently, resulting in poor harvests especially with the lack of early-maturing and drought-tolerant varieties in major crops (de Waaland Whiteside, 2003). *Vernonia galamensis* is well adapted for cultivation under arid conditions; hence *Vernonia* remains a

promising oil plant. The success of *Vernonia* as a commercial oil crop however depends on the economic yields that can be obtained with improved selections and further development of qualities associated with vernonia oil (van der Vossen and Mkamilo, 2007).

Research on *V. galamensis* by Mebrahtu *et al.* (2009) recommended further research on development of the crop. Among the recommendations were that research on mutation breeding be conducted to breed and select for mutants with reduced shattering and synchronised maturity. The future challenges identified were lack of uniform seed maturity, problem of shattering, and lack of appropriate technologies for mechanical harvesting, seed cleaning (threshing) and processing and oil extraction. Shimelis *et al.* (2008) conducted a study on 36 *V. galamensis* var. *ethiopica* accessions in the Limpopo province of South Africa and identified accession Vge-4 with relatively high oil yield. The study concluded this accession could be used in the further strategic improvement of vernonia to maximize seed yield and oil content as an alternative crop in the province and similar environments. Cultivation of vernonia could help as a source of raw material for agro-processing industries and as a new cash crop, enlarging the existing crop husbandry practice (van der Vossen and Mkomali, 2007; Ramalema *et al*., 2010).

In mutagenesis alkylating agents, such as ethylmethanesulfonate (EMS), are effective because they form adducts with nucleotides, causing them to mispair with their complementary bases, thus introducing base changes after replication (Greene *et al*., 2003). Mutations of a single or few genes possessing target traits are invaluable in crop improvement programs. This may lead to phenotypic changes in plant agronomic characteristics including plant height, maturity, sterility, yield, chemical composition, pathogen and pest resistance and adaptability to adverse environmental conditions. The most important parameters for inducing mutation with EMS are concentration, duration of treatment, and solution temperature (Alacantara *et al*., 1996). It has been established that chemical mutagens such as EMS provide opportunities to increase genetic variability of quantitatively inherited characters (Sharma *et al*., 1991).

The objective of this study was to induce genetic variation using optimal ethylmethanesulfonate treatment conditions and select mutants for further development in *V. galamensis* lines Vge-1 and Vge-4. As outlined in the previous experiment, optimal EMS concentration, treatment duration and temperature were established for several *V.*

galamensis lines.Vge-1 and Vge-4 vernonia lines were selected for large scale mutagenesis in this study.

3.3 MATERIALS AND METHODS

3.3.1 Pre-treatment handling of seeds

Genetically similar and normal shaped, disease-free, dry and quiescent vernonia seeds were selected from each of the two *V. galamensis* lines (Vge-1 and Vge-4). For each line, 50,000 seeds were used for treatment. Untreated seeds of both lines were included as comparative controls. Seeds for the treatment were handled inside specially designed and labelled polyethylene mesh bags measuring 12 cm long and 8 cm wide. The bags were heat-sealed. Extra mesh bags were cut to thicken the bags.

3.3.2 EMS preparation and treatment of seeds

An EMS mutagenesis protocol initially proposed by Mba *et al.* (2009) was modified and applied in treating vernonia seeds. The seed batches for each line was soaked in 70% ethanol for 1 minute, and then briefly washed in running water. Thereafter, seeds were soaked in 30% JIK® for 5 minutes, and then washed in running water for 1 minute. Seeds were then soaked by placing in a dish filled with distilled water and left to stand for 16-20 h at 20-22°C.

Towards the end of the pre-soaking stage, fresh EMS solutions were prepared according to the desired concentration of 0.372% v/v determined from the previous experiment (Chapter 2 Section 6). The following formula was used in preparing the required rate of EMS. To prepare a 0.372% EMS solution, the required amount is:

Required EMS rate = EMS dose x [distilled water + 2% dimethyl sulfoxide (DMSO)] / 100

= 0.372 x (980 ml + 20 ml) / 100

= 3.72 ml

Thus, 3.72 ml of EMS was added per 1000 ml water and dimethyl sulfoxide (DMSO) solution to prepare 0.372% EMS solution.

980 ml of distilled water were mixed with 2% (v/v) DMSO into a bottle and autoclaved at 120°C for 15 min at 103.5 kPa (15 psi) and the mixture left to cool at room temperature. Then 3.72 ml EMS solution was added into the DMSO/water solution and the solution was shaken vigorously to give a homogeneous emulsion. At the end of the pre-

soaking period, the bags were removed from distilled water and shaken to remove excess water.

Except the control bags, the seeds were soaked in the EMS solutions. Vge-1 treatment batch was soaked in 0.372% v/v EMS solution at 35^{o}C for 1 hour; Vge-4 treatment batch was soaked in 0.372% v/v EMS solution at 32.5^{o}C for 2 hours. The temperatures were maintained in a water bath during the duration of treatment. After treatment, the seeds were washed (to remove excess EMS) under running cold tap water for 2 hours. The mesh bags were shaken off to remove excess water and the seeds placed on blotting paper for 2 hours to dry.

3.3.3 Field planting

Field planting was conducted at the Ukulinga Farm, the research and training farm of the University of KwaZulu-Natal outside Pietermaritzburg in the subtropical hinterland of KwaZulu-Natal province. It lies at 30^{o}24'S, 29^{o}24'E, 775 m above sea level. Rain falls mostly in summer, between October and April. The mean annual rainfall during the study season 2010/2011 was 970 mm. The maximum and minimum mean annual temperatures were 23.6 and 12.7°C, respectively. While the maximum and minimum mean relative humidity for the year were 75.7 and 52.2%. Occasionally light to moderate frost occurs in winter (May - July). Roundup® (systemic, broad spectrum herbicide with glycophosphate active ingredient) was applied to the soil 4 weeks prior seed planting. Hand weeding was conducted between rows and plants after seed planting and germination.

3.3.4 Experimental design and data collection

Four treatment populations were planted on the 4th of February 2011. Vge-1 had two levels, i.e., seeds treated at 0.372v/v EMS at 35^{o}C for 1hr and untreated control. Similarly Vge-4 consisted of two treatment levels, i.e., seeds treated at 0.372v/v EMS at 32.5^{o}C for 2 hr and a control. Treated seeds were planted on a plot size of 10 m x 17 m and replicated six times, while untreated checks on a block size of 10 m x 17 m replicated three times. 8,600 treated seeds were planted per plot per replication and later thinned at inter-row spacing of 0.3 m and intra-row spacing of 0.3 m providing 1889 plant populations per plot per replication for treated and untreated batches, respectively.

Days to emergence (DTE) were recorded as number of days from planting to the date when 50% of the seedlings emerged. Germination percentage (GP) was recorded from emergence until 21 days post planting, as the percentage of germinated seeds from the total number of seeds planted. Days to heading (DTH) (flower head formation) were recorded as number of days from planting to the date when 50% of the plants formed heads. Days to flowering (DTF) were recorded as number of days from planting to the date when 50% of the plants showed flowers. Days to maturity (DTM) were recorded as number of days from planting to the date when 50% of the plants formed mature black seeds and brown involucres. Plant height (PHT) was measured (in centimetres) from the base to the tip of the plant, including the terminal head, during 50% maturity. Percentage sterile plants (PSP) were recorded at harvest. Seeds per head (SPH) were recorded from five randomly selected heads from primary branches at full plant maturity. Chlorophyll mutants were tagged (Figure 3.1a) when showing albinism and chlorosis. Each surviving mutant was harvested separately at full plant maturity. Number of chlorophyll mutants (NCM) and percent of mutant sterile (PMS) were recorded at maturity. Thousand seed weight (TSW) (in grams) was measured from a random sample of 1000 seeds of each line. Plot yield (PY) was measured per plot and converted to kilogram per hectare. Individual plant measurements in the lines were taken from 20 plants that were randomly tagged per replicate. Studies were conducted from February through October in 2011.

Data on agronomic traits was subjected to analysis of variance (ANOVA) using Genstat® (2011, 14th edition, VSN International, UK). The least significant difference (LSD) test procedure was used to compare means at 5% level of significance. Pearson's correlation procedure was conducted to estimate the degree of relatedness between agronomic traits.

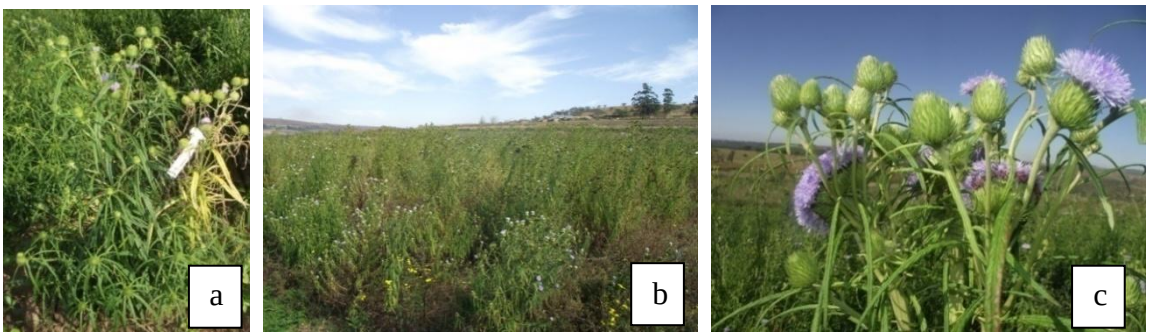

Figure 3. 1: A tagged chloroplast mutant (a), *V. galamensis* plants at secondary flowering (b) and a V. galamensis plant at primary flowering and secondary heading (c).

3.4 RESULTS

After the ANOVA test, mean square values and significance levels ($P \leq 0.05$) are presented in Tables 3.1, 3.3 and 3.5. Corresponding means and ranges, standard error values, least significant differences and co-efficient of variations for various traits are presented in Tables 3.2, 3.4 and 3.6.

In Vge-1, all agronomic traits showed significant differences ($P < 0.05$) due to the EMS treatment except for days to emergence and percentage of sterile chloroplast mutants (Table 3.1). In Vge-1, EMS treatment significantly delayed flowering (2 days), heading (5 days) and plant maturity (15 days). Germination percentage was significantly reduced; treated seeds germinated at 71% whereas the untreated control germinated at 93%. Plant height (33 cm) and plot yield (862.8 kg/ha) was significantly reduced due to EMS treatment. Treatment increased thousand seed weight (0.07 g) but the increase was not significant. Seeds per head were significantly reduced (13 seeds per head difference). Eight chloroplast mutants were observed, 22% of which were sterile. The fertility in Vge-1 was significantly reduced to about 89% in treated seeds when compared to the control (Table 3.2).

Table 3. 1: Analysis of variance indicating mean squares and levels of significance on 12 traits of line Vge-1 of *V. galamensis* for untreated controls and seeds treated with EMS.

		Agronomic traits											
Source of variation	**d.f.**	**DTE**	**GP**	**DTH**	**DTF**	**DTM**	**PSP**	**PHT**	**SPH**	**TSW**	**NCM**	**PSM**	**PY**
Replication	2	0.00	10.50	0.50	0.17	1.17	1.17	57.77	20.67	0.047	0.17	185.10	3197.6
Treatment	1	1.5n.s	770.67*	37.5*	6*	352.67*	170.67*	1631.52n.s	253.5*	0.007n.s	10.67*	740.6n.s	1116687.5*
Residual	2	0.00	77.17	0.50	0.50	1.17	1.17	13.45	6.00	0.018	0.17	185.10	876.8

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield
n.s= not significant at 0.05 probability level; *= significant at the 0.05 probability level

Table 3. 2: Means of various agronomic characteristics of line Vge-1 of *V. galamensis* for untreated controls and seeds treated with EMS.

	Agronomic traits											
Treatment	**DE**	**GP**	**DTH**	**DTF**	**DTM**	**PSP**	**PHT (cm)**	**SPH**	**TSW (g)**	**NCM**	**PSM**	**PY (kg/ha)**
Vge-1 + EMS	10	70.67	96	107	207	10.67	121.9	111	4.73	2.67	22.20	700
Vge-1 Control	9	93.33	91	105	192	0	154.9	124	4.66	0.00	0.00	1562.8
S.E (±)	0.00	2.29	0.50	0.29	0.76	0.76	5.37	3.21	0.15	0.29	9.62	40.00
L.S.D	0	30.86	2.484	2.484	3.795	3.795	12.88	8.61	0.472	1.434	13.54	104
CV%	0	2.8	0.5	0.3	0.4	14.3	3.9	2.7	3.3	21.7	86.6	3.5

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield
L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

For Vge-4 all the agronomic traits were significantly affected by EMS treatment. In Vge-4, EMS significantly delayed emergence (4 days), heading (8 days), flowering (10 days) and maturity (35 days). Germination percentage was reduced to 40% in EMS treated plants whereas 87% germination was observed in controls. Plot yield was highly and significantly reduced (1645.8 kg/ha). Fertility was reduced by 63% as a result of EMS treatment. Thousand seed weight was significantly increased (0.35g), whilst plant height was significantly reduced (52.2cm). Seeds per head were significantly reduced (124 seeds to 95 seeds).

Table 3. 3: Analysis of variance indicating mean squares and levels of significance on various agronomic traits of Vge-4 of *V. galamensis* for untreated controls and seeds treated with EMS.

		Agronomic traits											
Source of variation	**d.f.**	**DTE**	**DTF**	**DTH**	**DTM**	**GP**	**PSP**	**PHT**	**SPH**	**TSW**	**NCM**	**PSM**	**PY**
Replicate	2	0.17	1.17	1.17	7.17	18	10.17	23.97	28.5	0.013	23.97	50	590.3
Treatment	1	20.17*	160.17*	88.17*	1872.67*	3313.5*	5890.67*	4081*	1320.17*	0.19*	4081*	3750*	4062660.6**
Error	2	0.17	1.17	3.17	13.17	14	10.17	30.19	46.17	0.006	30.19	50	121.4

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield
*= significant at the 0.05 probability level;

Table 3. 4: Means of various agronomic characteristics of line Vge-4 of *V. galamensis* for untreated controls and seeds treated with EMS.

	Agronomic traits											
Treatment	**DE**	**GP**	**DTH**	**DTF**	**DTM**	**PSP**	**PHT (cm)**	**SPH**	**TSW (g)**	**NCM**	**PSM**	**PY (kg/ha)**
Vge-4 + EMS	14	40.00	100	115	225	62.67	98.6	95	5.38	6.00	50.00	32.9
Vge-4 Control	10	87.00	92	105	190	0	150.8	124	5.03	0.00	0.00	1678.7
S.E (±)	0.29	3.00	0.76	0.76	1.89	2.25	3.46	3.77	0.08	0.87	0.50	17.80
L.S.D	1.434	13.74	6.252	3.795	12.75	11.2	19.3	23.87	0.2633	4.303	24.84	38.71
CV %	2.4	4.7	0.8	0.7	0.9	7.2	2.8	3.4	1.5	28.9	33.3	2

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield
L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

The overall analysis of variance indicated that all tested agronomic characters differed significantly in respect to *V. galamensis* lines. In addition, the interaction between line and EMS treatment was significant in affecting all tested traits (Table 3.5). The effect of EMS treatment was much more pronounced on Vge-4 treated seeds compared to Vge-1 (Table 3.6).

Table 3. 5: Combined analysis of variance with degrees of freedom, mean squares and significance levels on 12 traits of two *V. galamensis* lines (Vge-1 and Vge-4) tested with and without EMS treatments using three replications.

Source of variation	d.f.	Agronomic traits											
		DTE	**GP**	**DTH**	**DTF**	**DTM**	**PSP**	**PHT**	**SPH**	**TSW**	**NCM**	**PSM**	**PY**
Replicate	2	0.08	18.75	0.08	1.00	4.00	2.33	53.56	31.08	0.05	1.08	117.60	1344
Line (L)	1	16.33**	1026.75*	16.33*	44.0833**	176.33*	2028**	558.97*	176.33*	0.8**	8.33*	578.8n.s	227861**
Treatment (T)	1	16.33**	3640.08**	120.33**	114.0833**	1925.33**	4033.33**	5436.61**	1365.33**	0.13*	56.33**	3911.8*	4719632**
LxT	1	5.33**	444.08*	5.33*	52.0833**	300**	2028**	275.9*	208.33*	0.06n.s	8.33*	578.8n.s	459716**
Error	6	0.08	33.64	1.75	0.67	6.22	6.78	23.93	23.42	0.01	0.75	117.60	1147

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield
n.s= not significant at 0.05 level; *= significant at the 0.05 probability level; **= significant at the 0.01 probability level.

Table 3. 6: Means and multiple comparisons of various agronomic characteristics among two *V. galamensis* lines (Vge-1 and Vge-4) tested with and without EMS.

	Agronomic traits											
Treatment	**DTE**	**GP**	**DTH**	**DTF**	**DTM**	**PSP**	**PHT (cm)**	**SPH**	**TSW (g)**	**NCM**	**PSM**	**PY (kg/ha)**
Vge-1 + EMS	10b	70.67bc	96bc	107a	207b	10.67b	121.9b	111a	4.73b	2.67a	22.2ab	700b
Vge-1 Control	9a	93.33a	91a	105a	192a	0a	154.9a	124a	4.66c	0a	0a	1562.8a
Vge-4 + EMS	14c	40c	100c	115b	225c	62.67c	98.6c	95b	5.38a	6b	50b	32.9c
Vge-4 Control	10b	87b	92ab	105b	190a	0a	150.8a	124a	5.03ab	0a	0a	1678.7a
S.E (±)	0.14	2.17	0.14	0.50	0.76	3.66	1.00	2.79	0.11	0.52	2.93	18.33
L.S.D	0.5767	11.59	2.643	1.631	4.984	5.201	9.77	9.67	0.2062	1.73	24.09	67.86
CV %	1.4	3	0.2	0.5	0.5	4.2	2.8	2.5	2.3	24	16.2	1.8

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield

[a] Means in a column followed by the same letter are not significantly different from each other at $P \leq 0.05$.

L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

Table 3.7 presents correlations among agronomic traits and the levels of significance thereof. In Vge-1, plot yield was positively and significantly associated with germination percentage, plant height and seeds per head. However correlations with plant development (days to emergence, heading, flowering and maturity), percentage of sterile plants and number of chlorophyll mutants were significantly negative. Correlations of plot yield with thousand seed weight and percentage of mutants sterile were not significant. In Vge-4, plot yield was positively and significantly associated with germination percentage, plant height and seeds per head. However correlations with plant development (days to emergence, heading, flowering and maturity), percentage of sterile plants, thousand seed weight, number of chlorophyll mutants and percentage of sterile mutants were significantly negative.

Table 3. 7: Pair-wise correlations of *V. galamensis* traits after testing with and without EMS. The top diagonal represents correlations of traits in Vge-4 and bottom diagonal in Vge-1.

Traits	DTE	GP	DTH	DTF	PSP	PH	DTM	SPH	TSW	NCM	PSM	PY
Days to Emergence	1	-0.967**	0.954**	0.976**	0.968**	-0.962**	0.988**	-0.883*	.901*	.962**	.924**	-.983**
Germination Percentage	-0.903*	1	-0.959**	-0.995**	-0.996**	0.961**	-0.989**	0.934**	-.916*	-.966**	-.987**	.989**
Days to Heading	0.974**	-0.807	1	0.967**	0.952**	-0.908*	0.963**	-0.871*	0.802	.958**	.930**	-.953**
Days to Flowering	0.905*	-0.804	0.940**	1	0.988**	-0.947**	0.997**	-0.901*	.924**	.985**	.973**	-.985**
Percentage of Sterile Plants	0.987**	-0.940**	0.925**	0.846*	1	-0.980**	0.985**	-0.957**	.917*	.947**	.990**	-.996**
Plant Height	-0.959**	0.866*	-0.892*	-0.77	-0.977**	1	-0.956**	0.970**	-.896*	-.888*	-.955**	.988**
Days to Maturity	0.993**	-0.862*	0.993**	0.931**	0.961**	-0.931**	1	-0.896*	.931**	.983**	.960**	-.989**
Seeds per Head	-0.909*	0.859*	-0.840*	-0.808	-0.920**	0.938**	-0.887*	1	-.825*	-.815*	-.961**	.949**
Thousand Seed Weight	0.232	-0.131	0.316	0.166	0.173	-0.094	0.269	0.1	1	.906*	.904*	-.915*
Number of Chloroplast Mutants	.0970**	-0.889*	0.945**	0.914*	0.950**	-0.927**	0.969**	-0.955**	0.158	1	.924**	-.946**
Percentage of Mutants Sterile	0.707	-0.676	0.689	0.746	0.676	-0.667	0.718	-0.857*	-0.031	.857*	1	-.974**
Plot Yield	-0.996**	0.910*	-0.978**	-0.927**	-0.978**	0.933**	-0.993**	0.886*	-0.258	-.964**	-0.698	1

DTE= days to emergence; GP= germination percentage; DTH= days to heading; DTF=days to flowering; DTM= days to maturity; PSP= percent sterile plants; PHT= plant height; SPH= seeds per head; TSW= thousand seed weight; NCM= number of chlorophyll mutants; PSM= percent of sterile mutants; and PY= plot yield

*= correlation is significant at the 0.05 level; **= correlation is significant at the 0.01 level.

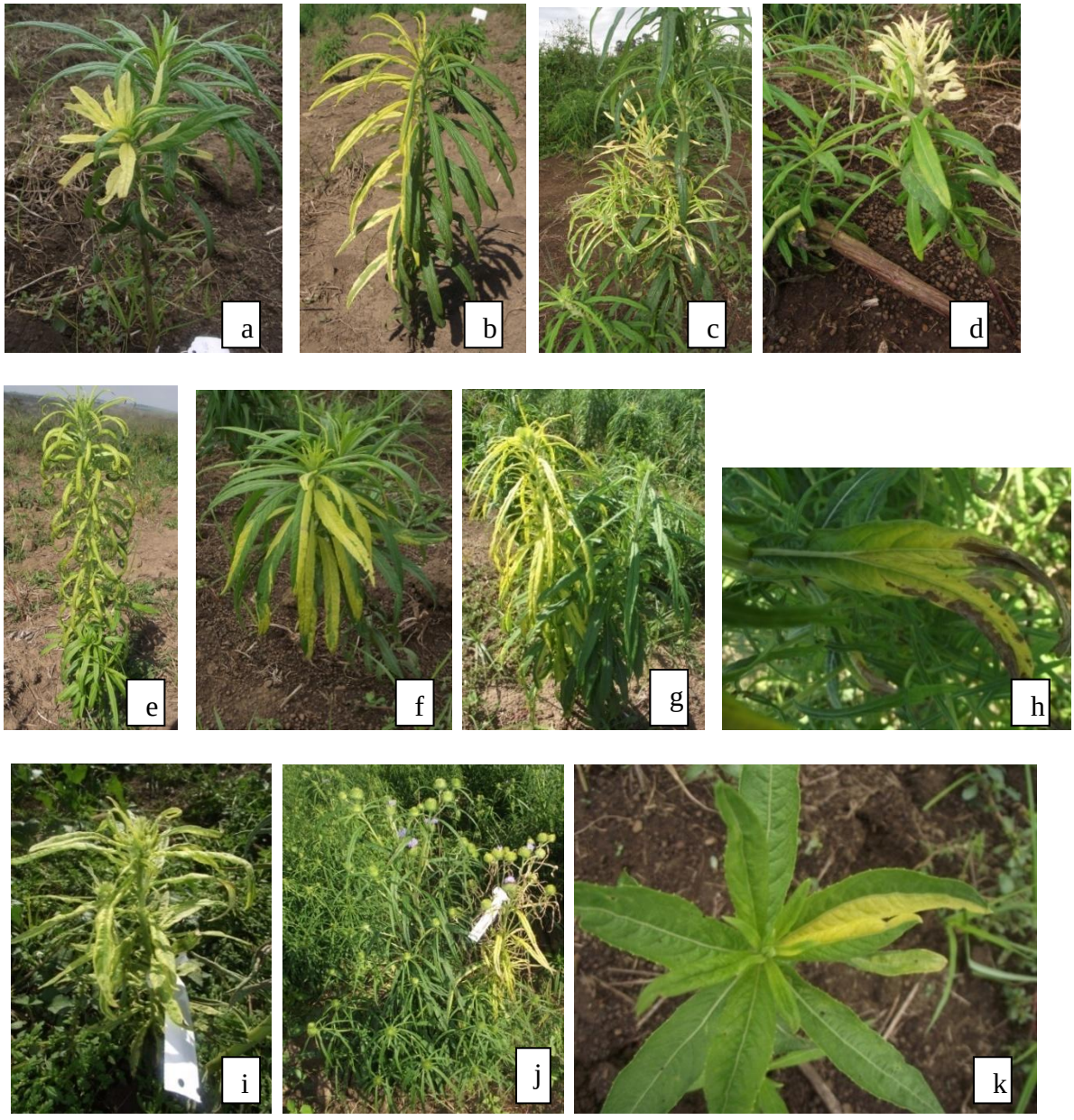

Figure 3.2: Various chlorophyll mutants in *Vernonia galamesis* after EMS mutagenesis: Branch chlorotic mutants (a, g and j); curled leaf chlorotic mutant (e); spindle leaf branch mutation (c); half plant chlorotic mutant (b); albino branch mutant (c and d); split leaf mutants (h) and; leaf chorotic mutants (f and k) of *V. galamensis* lines Vge-1 (c, d, g, h and j) and Vge-4 (a, b, e, f, i and k) after EMS treatment.

3.5 DISCUSSION

Days to emergence were delayed for both lines, but delays were only significant in Vge-4. Earliest emergence was observed in Vge-1 untreated controls at 9 days post planting, which is similar to previously conducted greenhouse trials under similar treatment conditions. Greer and Reinhart (2009) also reported delayed sprouting in hydrangea, and the delay in emergence was accounted to seed treatment with EMS. Ethylmethanesulfonate mutagenic treatment on seeds causes chromosomal aberrations that can adversely affect cell division (Gunckel and Sparrow, 1961; and Zaka *et al*., 2004), hence delayed emergence of vernonia seedlings might be attributed to a delay in cell division due to the mutagenesis.

Post-washing of the seeds, as recommended by Narayanan and Konzak (1969), lessens the deleterious effects of mutagenesis on germination percentage. Post-washing removes the surface non-reacted EMS and the hydrolysis products from the seeds. In addition, it also reduces seed mortality should seeds require redrying. Germination percentage was reduced significantly for both lines, reductions were more pronounced in Vge-4 treated seeds where only 40% of the planted seeds germinated. Due to environmental fluctuations in the field germination percentage was less compared to the greenhouse. However in Vge-1 germination percentage was 17% higher in the field as compared to similar treatment conditions in the greenhouse. Findings of significantly reduced germination percentage due to EMS treatment have also been reported in pepper (Alacantara *et al.,* 1996), tobacco (Amernath and Prasad, 1998), okra (Kumar and Mishra, 2004), mung bean (Tah, 2006), maize (Kumar and Kumar Rai, 2007), petunia (Berenchot *et al.,* 2008), and chick pea (Sha *et al*., 2008).

Days to heading, flowering and maturity were delayed significantly due to EMS treatment with more severe delays in Vge-4. Comparative studies carried out in Limpopo, South Africa on similar lines revealed that flowering occurred a week earlier in Vge-4 and two weeks earlier in Vge-1 conforming to the current study (Shimelis *et al*., 2008).

Reduction in plant height due to EMS treatment were observed in both lines, however differences were only significant in Vge-4. In respect to plant height, no significant differences were observed between untreated controls of the two lines. Reduced shoot length due to EMS treatment have also been reported in maize (Kumar and Kumar Rai, 2007), and chickpea (Shah *et al.,* 2008).

All untreated seeds produced fertile seeds. Treatment of Vge-1 seeds with EMS reduced the frequency of fertile plants by about 11%. About 63% of Vge-4 treated plants were sterile. Seeds per head were reduced for all tested lines, but differences were only significant in Vge-4. Thousand seed weight increased in all treated lines compared to untreated controls. Increases in thousand seed weight were only significant in Vge-4. Thousand seed weight (4.66 g for Vge-1 and 5.03 g for Vge-4) increased by almost twice than previously reported (2.27 g in Vge-1, and 2.58 g in Vge-4) by Shimelis *et al* (2008). Sterility (Penmetsa and Cook, 2000; Jabeen and Mirza, 2004 and Berenschot, 2008), reduced seeds per head/pod/capsule (Vaida and Young, 1993; Berenschot *et al.*, 2009) and increased seed weight (Penmetsa and Cook, 2000) have been similarly reported to accompany EMS mutagenesis.

Onset of chloroplast mutations varied with mutants, some mutants showed chloroplast mutation four weeks after emergence while others showed chloroplast mutation at primary or secondary branching. A wide variety of chloroplast mutations were observed (Figure 3.2). Various mutants formed heads however failed to flower and set seed. Certain mutants wilted and died due to lack of photosynthesis in the plant. Leaf discolorations (chlorophyll mutants) are well documented as an indication of EMS mutations in plants. Chloroplast mutagenesis with EMS has been reported in peas, carrots, soybeans, lentils, and radishes (Miller *et al*., 1980; Miller *et al*., 1984), *Capsicum annum* L. (Alacantara *et al*., 1996), Pea (Singh *et al*., 2000) and roselle (Vaida and Young, 1993).

Sterile chlorophyll mutants are a common feature of EMS mutagenesis (Alacantara *et al*., 1996). Numerous completely sterile mutants were observed in this study from both lines when seeds were treated with EMS, whilst the rest of the chlorophyll mutants were semi-sterile with a certain fertile portion of seed in a head. Leaf variegation is a common mutation which can be either a nuclear or cytoplasmic mutation. EMS may have a high specificity for mitochondrial and plastid genomes (Miller *et al*. 1984). It is known that many plastid mutations interfere with the development of the photosynthetic apparatus (Redel *et al*., 1984) and can cause male and female sterility.

Yield per plot was drastically and significantly reduced for both lines due to chemical treatment of seeds. Severity of the reduction in yields was more pronounced in Vge-4 where EMS treatments yielded 32.9 kg/ha in comparison to 1678.7kg/ha obtained in the untreated controls. Delayed growth and development, decreased germination percentage, high counts of

sterility and decreased plant height were highly associated with decreased yields in treated plants (Table 3.7). Germplasm evaluation studies also attributed increased yields to such traits as days to heading, days to flowering, plant height, number of seeds per head and thousand seed weight (Thompson *et al*., 1994; Bhardwaj *et al*., 2000; Baye and Becker, 2005; Shimelis *et al.*, 2008). Comparative EMS treatment studies under field conditions reported decreased germination percentage, seedling survival, plant height, pollen fertility, seed fertility, pods per plant, pod length, seeds per pod, 100 seed weight and single plant yield due to treatment in cowpeas (Ashok Kumar *et al*., 2009).

Chlorophyll mutants were observed for all vernonia lines with high count of sterility for both lines (Figure 3.2). Ethylmethanesulfonate treatments successfully induced mutation in vernonia. Selected chlorotic mutants will be used in further development and breeding research of vernonia.

3.6 CONCLUSION

Ethylmethanesulfonate treatments successfully induced mutation in the two tested lines of *V. galamensis*. Each fertile chloroplast mutant was harvested at full maturity and seeds kept for further breeding in subsequent mutant generations.

3.7 REFERENCES

Abalu G. and R. Hassan, 1998: Agricultural Productivity and Natural Resource Use in Southern Africa. Food Policy **23**, 477-490.

Alcantara T., P. Bosland and D. Smith, 1996: Ethyl Methanesulfonate-induced Seed Mutagenesis of *Capsicum annuum*. The Journal of Heredity **87**, 239-241.

Amernath S. and A.B. Prasad, 1998: Induced Variability in Homozygous and Heterozygous Genotypes of Tobacco. Indian Journal of Genetics **58**, 69-77.

Ashok Kumar V., R. Usha Kumari, R. Amutha, T. Siva Kumar, S. Juliet Hepziba and C. Ananda Kumar, 2009: Effect of Chemical Mutagen on Expression of Characters in Arid Legume Pulse-Cowpea (*Vigna unguiculata* (L.) Walp.). Research Journal of Agriculture and Biological Sciences **5**, 1115-1120.

Baye T., H. Kebede and K. Belete, 2001: Agronomic Evaluation of *Vernonia galamensis* Gemplasm Collected from Eastern Ethiopia. Industrial Crops and Products **14**, 179-190.

Baye T. and H. Becker, 2005: Genetic Variability and Interrelationship of Traits in the Industrial Oil Crop *Vernonia galamensis*. Euphytica **142**, 119-129.

Berenschot A., M. Zucchi, A. Tulmann-Neto and V. Quecini, 2009: Mutagenesis in *Petunia x hybrida* Vilm. and Isolation of a Novel Morphological Mutant. Brazilian Journal of Plant Physiology **20**, 95-103.

Bhardwaj H.L., A.A. Hamama, M. Rangappa, D.A. Dierig, 2000: Vernonia Oilseed Production in the Mid-Atlantic Region of the United States. Industrial Crops and Products **12**, 119-124.

de Waal A. and A. Whiteside, 2003: New Variant Famine: AIDS and Food Crisis in Southern Africa. The Lancet **362**, 1234-37.

Gilbert M.G., 1986: Notes on East African Vernonieae (Compositae) A Revision of the *Vernonia galamensis* Complex. Kew Bulletin **4**, 19-35.

Greene E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai and S. Henikoff, 2003: Spectrum of Chemically Induced Mutations From a Large-Scale Reverse-Genetic Screen in *Arabidopsis*. Genetics **164**, 731-740.

Greer S. and T. Rinehart, 2009: In Vitro Germination and Dormancy Responses of *Hydrangea macrophylla* and *Hydrangea paniculata* Seeds to Ethyl Methane Sulfonate and Cold Treatment. Horticultural Science **44**, 764-769.

Gunckel J.E. and A.H. Sparrow, 1961: Ionizing Radiation: Biochemical, Physiological and Morphological Aspects of their Effects on Plants. Encyclopedia of Plant Physiology (Ed.) Ruhland, W.XVI: 555-611, Springer-verlag, Berlin.

Hohmann U., G. Jacobs and C. Jung, 2005: An EMS Mutagenesis Protocol for Sugar Beet and Isolation of Non-bolting Mutants. Plant Breeding **124**, 317-321.

Jabeen N. and B. Mirza, 2004: Ethyl Methane Sulfonate Induces Morphological Mutations in *Capsicum annuum*. International Journal of Agriculture and Biology **6**, 340-345.

Kumar A. and M.N. Mishra, 2004: Effect of Gamma-rays, EMS and NMU on Germination, Seedling Vigour, Pollen Viability and Plant Survival in M1and M2 Generation of Okra (*Abelmoschus esculentus* (L.) Moench). Advances in Plant Science **17**, 295-297.

Kumar G. and P. Kumar Rai, 2007: EMS Induced Karyomorphological Variations in Maize (*Zea mays* L.) Inbreds. Turkish Journal of Biology **31**, 187-195.

Mba C., R. Afza, S. Bado and S.M. Jain, 2009: Induced Mutagenesis in Plants Using Physical and Chemical Agents. Plant Cell Culture **1,** 111-130.

Mebrahtu T., T. Gebremariam, A. Kidane and W. Araia, 2009: Performance of *Vernonia galamensis* as a Potential and Viable Industrial Oil Plant in Eritrea: Yield and Oil Content. African Journal of Biotechnology **8**, 635-640.

Miller P., K. Vaughn and K. Wilson, 1980: Induction, Ultrastructure, Isolation, and Tissue Culture of Chlorophyll Mutants in Carrot. In Vitro**16**, 823-828.

Miller P.D., K.C. Vaughn and K.G Wilson, 1984: Ethyl Methanesulfonate-induced Chloroplast Mutagenesis in Crops. Journal of Heredity**75**, 86-92.

Narayanan K.R. and C.F. Konzak, 1969: Influence of Chemical Post-treatments on the Mutagenic Efficiency of Alkylating Agents. In: Induced Mutation In Plants. Vienna: IAEA; 281-301.

Penmetsa V.R. and D.R. Cook, 2000: Production and Characterization of Diverse Developmental Mutants of *Medicago truncatula*. Plant Physiology **123**, 1387-1397.

Ramalema S.P., H. Shimelis, I. Ncube, K.K. Kunert and P.W. Mashela, 2010: Genetic Analysis among Selected Vernonia Lines through Seed Oil Content, Fatty Acids and RAPD DNA Markers. African Journal of Biotechnology **9**, 117-122.

Redel G.P., G.N. Acedo and S.S. Sandhu, 1984: Mutation Induction and Detection in *Arabidopsis*. In: Mutation, Cancer, and Malformation (Chu E.H.Y. and Generoso W.M., eds). New York Plenum; 285-313.

Shah T., J. Mirza, M. Haq and B. Atta, 2008: Radio Sensitivity of Various Chickpea Genotypes in M1 Generation I-Laboratory Studies. Pakistan Journal of Botany **40**, 649-665.

Sharma D., A. Sharma and S. Talukdar, 1991: Comparison of Gamma Ray and EMS Induced Variation in Green Gram [*Vignaradiata* (L.) Wilczek]. Journal of Nuclear Agricultural Biology **20**, 87-93.

Shimelis H., P.W. Mashela and A. Hugo, 2008: Performance of *Vernonia* as an Alternative Industrial Oil crop in Limpopo Province of South Africa. Crop Science.**48**, 236-242.

Singh U.P., B. Prithiviraj and B.K. Sarma, 2000: Development of *Erysiphepisi* (Powdery Mildew) on Normal and Albino Mutants of Pea (*Pisum sativum* L.). Journal of Phytopathology **148**, 591-595.

Tah P., 2006: Induced Macromutation in Mungbean [*Vigna radiata* (L.) Wilczek]. International Journal of Botany **2**, 219-228.

Thompson A.E., D.A. Dierig, E.R. Johnson, G.H. Dahlquist, R. Kleiman, 1994: Germplasm Development of *Vernonia galamensis* as a New Industrial Oilseed Crop. Industrial Crops and Products **3**, 185-200.

Vaida K. and M. Young, 1993: Ethyl Methanesulfonate Induced Variation in Qualitative and Quantitative Characters of Roselle (*Hibiscus sabdariffa* L.) (Malvaceae). Brazilian Journal of Genetics **16**, 381-391.

van der Vossen H.A.M. and Mkamilo G.S. (Eds), 2007: Plant Resources of Tropical Africa 14. Vegetable Oils. PROTA Foundation, Netherlands. 178-181.

Zaka R., C. Chenal and M.T. Misset, 2004: Effect of Low Doses of Short-term Gamma Radiation on Growth and Development through Two Generations of *Pisum sativum*. Science of the Total Environment **320**, 121-129.

CHAPTER 4

THE EFFECT OF ETHYLMETHANESULFONATE MUTAGENESIS ON SEED OIL CONTENT AND FATTY ACID COMPOSITION IN VERNONIA (*Vernonia galamensis* var. *ethiopica*)

ABSTRACT

In oil seed crops chemical mutagenesis using ethylmethanesulfonate (EMS) has been reported to induce genetic variation and alter seed oil content and fatty acid profiles. *Vernonia galamensis* produces natural epoxidised oil which has multiple industrial uses. The objective of this study was to determine the seed oil content and fatty acid compositions of vernonia selections derived from EMS mutagenesis. Seeds from two lines (Vge-1 and Vge-4) with and without chlorophyll mutations and untreated controls were subjected to oil and fatty acid analyses. In Vge-1, significant differences were observed in composition of linoleic and oleic acid due to the mutagenesis. Significant increases in linoleic and oleic acid composition were found in chloroplast mutants due to EMS mutagenesis. No significant differences were detected in fatty acid compositions in Vge-4 after the EMS treatment. Differential responses were observed when lines were compared at various EMS mutation levels showing significant effect on vernolic, linoleic and oleic acids compositions. In both lines no differences were detected on seed oil content, palmitic acid, steraic acid and arachidic acid compositions after the treatment. Oil content significantly and positively correlated with vernolic acid for Vge-1 ($P<0.001$; $r= 0.898$) and Vge-4 ($P<0.05$; $r= 0.65$). Vernolic acid significantly and negatively correlated with other fatty acids. The study found that EMS mutagenesis significantly changed the oleic acid and linoleic acid compositions in vernonia. However, the oil content and vernolic acid composition were not significantly affected by EMS treatment. Further selections should be conducted in the subsequent mutation generations to isolate useful mutants.

KEYWORDS: ethylmethanesulfonate, mutation, fatty acids, oil content, *Vernonia galamensis*

4.1 INTRODUCTION

New oilseed crop species have been investigated as potential sources of plant based oils in the oleochemical industry. Several of them contain a high proportion of industrially desirable fatty acids, such as linoleic acid (*Madia sativa* Molina), linolenic acid (*Lepidium sativum* L. and *Camelina sativa* (L.) Crtz.), calendic acid (*Calendula officinalis* L.), epoxy- oils (*Euphorbia lagascae* Sprengel and *Vernonia galamensis* (Cass.) Less.), hydroxy- (*Lesquerella fendleri* (Gray) Wats.) and petroselinic acid (*Coriandrum sativum* L.) (Angelini *et al.*, 1997). Plant oil in the form of triacylglycerol (TAG) is an attractive renewable resource to supplement or replace petroleum. Plant species have been found to contain high levels of unusual fatty acids (UFA) such as hydroxy, epoxy and acetylenic fatty acids (van de Loo *et al*., 1993)**.** For example, an epoxy fatty acid known as vernolic acid (cis-12-epoxyoctadeca-cis-9-enoic acid) constitutes up to 50-90% of total fatty acids in the seeds of *Vernonia galamensis, Euphorbia lagascae,Stokesia laevis, Crepis palaestina* and *Bernardia pulchella* (Perdue, 1989; Pascual and Correal, 1992; Bafor *et al*., 1993; Thompson *et al*., 1994; Spitzer *et al*., 1996).

There is potentially large industrial market for synthetically epoxidised plant based oils such as from linseed and soybean, but the epoxidation process is expensive. Vernolic acid derived from vernonia [*Vernonia galamensis* (Cass.) Less.] is a naturally epoxidised fatty acid, and can fill in the market niches. Vernolic acid is much less viscous than the synthetically epoxidised oils. The latter are semisolids at 10°C and not pourable at ≤ 0°C, while vernolic acid can be poured even below freezing point. The low viscosity of vernonia oil should make it a good solvent in paint manufacture, and the highly reactive epoxy group will cause it to become chemically bound in the dried paint rather than evaporating in the atmosphere (Kaplan, 1989). Other potential uses of vernonia oil include lubricants, adhesives, protective coatings, cosmetics, detergents and a raw product for nylons (Jaworski and Cahoon, 2003). Vernonia oil could also be used as a natural source of plasticizers and stabilizers for producing polyvinylchloride (PVC plastic), which currently is manufactured from petroleum. The potential use of vernonia as a petroleum substitute is important since the global price for petroleum has been volatile in the past years.

Vernonia research has focused on collection and development of germplasm, selection of accessions with increased oil content and maximization of the proportion of specific fatty

acids, specifically vernolic acid. The fatty acid composition of the seed triacylglycerols (TAGs) determines the physical and chemical properties and, hence, their use in edible oil or industrial applications. TAG composition depends on the interaction of several different groups of enzymes in the lipid biosynthesis pathway. The enzymes of the fatty acid synthase complex in the plastids of developing seeds are responsible for the biosynthesis of fatty acids including vernolic acid (Katavic, 1995). In vernonia, it has been proposed that diacylglycerol acyltransferase (DGAT; EC 3.2.1.20) is one of the rate-limiting steps in plant storage lipid accumulation and plays an essential role in controlling both the quantitative and qualitative flux of fatty acids into storage triacylglycerol (Vogel and Browse, 1996; Jako *et al.*, 2001; He *et al*., 2004; Lung and Weselake, 2006; Yu *et al*., 2006).

Single nucleotide changes that result in missense mutations are valuable in understanding gene function. In cases of quantitative and qualitative traits, it is mostly the accumulation of several point mutations that often yields the required result (Weil and Monde, 2007). Ethylmethanesulfonate (EMS) is an alkylating agent used in chemical mutagenesis of both plants and animals. Alkylation of nucleotides induced by EMS in chromosomes can result in mispairing and changing of bases. This results in alkylation of guanine (G) which subsequently forms O6- thylguanine. O6-ethylguanine then forms a base pair thymine (T) and not cytosine (C). Original G/C pairs end up being replaced by A/T base pairs. At a low EMS frequency, G/C to C/G or G/C to T/A transversions are generated by 7-ethylguanine hydrolysis or A/T to G/C transition by 3-ethyladenine pairing errors. The resulting changes are referred as point mutations and can modify gene function therefore have the potential to modify traits that such genes code for (Kim *et al*., 2005). EMS mutagenesis results in high point mutational densities with only low levels of chromosome breaks that would cause aneuploidy, reduced fertility, and dominant lethality (Greene *et al*., 2003). In nature, the heritable changes are random and can occur in any gene and are recurrent. Hence it is vital to use large scale crop populations of over 10 000 for selection of mutants (Tah, 2006). Compounds such as EMS induce single nucleotide changes by alkylation of specific nucleotides resulting in mutations that are high in density and essentially randomly distributed. Therefore, a relatively small population of individuals can provide an allelic series that includes a variety of missense changes with differing effects on protein function, and non-sense changes that cause truncation of the gene product (Till *et al*., 2007).

Cultivation and subsequent commercialization of vernonia is considerably hampered by non-uniform seed maturity, tall plant height, seed shattering and lack of appropriate technologies for mechanical harvesting, seed threshing and cleaning. Consequently, an attempt was made to apply EMS mutagenesis to induce genetic variation and select suitable plants with desirable agro-morphological attributes. Thus, mutants were selected with visual chloroplast mutations from two lines, i.e., Vge-1 and Vge-4 of *V. galamensis* var. *ethiopica* after large scale mutagenesis. The objective of this study was to determine the seed oil content and fatty acid compositions of selections derived from EMS mutagenesis.

4.3 MATERIAL AND METHODS

4.3.1 Selection of plant material

Seed was collected from selected mutant plants of lines Vge-1 and Vge-4 lines. The mutants were selected after mutation induction of 60000 seeds per line using EMS (Chapter 3 section 3.4). For each line, seeds from selected mutants and controls were separated for analysis into three batches according to three mutation occurrences namely: (i) ethylmethanesulfonate mutants with chloroplast mutation, (ii) ethylmethanesulfonate mutants without chloroplast mutation and (iii) untreated control. Seeds from six selected mutant individuals (with chloroplast mutation), six mutants (without chloroplast mutation) and 2 untreated plants were used from each vernonia line. About 1.5 g from each of the selected individual plants was manually grinded using pestle and mortar. Thereafter the seeds were subjected to oil content and fatty acid analysis.

4.3.2 Determination of seed oil content

Oil content was determined on the basis of dry seed weight and six replications per mutant were analysed, with controls only having two replications. Oil was isolated according to an established method (Folch *et al.,* 1957) with chloroformmethanol (2:1, v/v) containing butylated hydroxyl toluene (0.001%) as an antioxidant. Subsequently the weight of the fat (g), oil content (OC), fat free dry matter (FFDM) (%), and moisture content were calculated. The weight of the oil was determined as a difference of the weight of the polytops containing the extracted fat less than the original weight. The OC was calculated as a ratio of weight of the oil to its respective sample mass expressed in %.

4.3.2 Determination of fatty acids

The fatty acid composition was determined after transesterification by the addition of tri-methyl sulphonium hydroxide (TMSOH) (Butte, 1983). Fatty acids were quantified using a Varian GX 3400 flame ionisation gas chromatograph, with a fused silica capillary column, chrompack CPSIL 88 (100 m length, 0.25 µm ID, 0.2 µm film thicknesses). Column temperature ranged from 40 - 230°C (hold 2 min; 4°C min-1; hold 10 min). Fatty acid methyl

esters in hexane (1 µl) were injected into the column using a varian 8200 CX autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250℃. Hydrogen was used as the carrier gas at 45 psi and nitrogen as the makeup gas. Identification of sample fatty acids was made by comparing the relative retention times of fatty acid methyl ester peaks from samples with those of standards obtained from SIGMA (cat. No.189-19). Chromatographs were recorded with Varian star chromatography software version 4 and relative % composition of fatty acids quantitated as ratios of peak areas. Data on seed oil content and fatty acid composition was subjected to analysis of variance (ANOVA) using Genstat® (14^{th}edition, VSN International, UK). The least significant difference (LSD) test procedure was used to compare means at 5% level of significance. Pearson's correlation analysis was conducted to measure relatedness between seed oil content and fatty acids.

4.3 RESULTS

The study compared oil content and fatty acid compositions in vernonia (*V. galamensis* variety *ethiopica*) after EMS mutagenesis of lines Vge-1 and Vge-4. Mean square values and significance levels ($P \leq 0.05$) of the three EMS mutation levels (untreated control, EMS treated with chlorophyll mutation and EMS treated without chlorophyll mutation) are presented in Tables 4.1, 4.3 and 4.5. Corresponding mean and range, standard error values, least significant differences and co-efficient of variation are presented in Tables 4.2, 4.4 and 4.6.

The analysis of variance showed significant differences among the three mutation levels in linoleic acid and oleic acid compositions in Vge-1 (Table 4.1). Linoleic acid composition was significantly higher ($P< 0.05$) in mutants showing chloroplast mutation at 28.81%; linoleic acid composition e was not significantly different between mutants without chloroplast mutation at 23.75% and untreated plants at 23.91% (L.S.D= 4.461%). Oleic acid composition was also observed to be highest among chloroplast mutants at 7.38%, followed by untreated plants at 6.49% and lastly mutants without chloroplast mutation at 6.39%. Highest oil content (30.16%) and vernolic acid composition (59.98%) was observed from EMS untreated controls (Table 4.2).

Table 4. 1: Analysis of variance on seed oil content and fatty acid compositions among three groups (untreated control, with and without chloroplast mutations) after EMS mutagenesis in line Vge-1 of *Vernonia galamensis* when analysed using six replications.

		Seed oil characteristics													
		Oil Content		Vernolic acid		Linoleic acid		Oleic acid		Palmitic acid		Stearic acid		Arachidic acid	
Source of variation	d.f.	m.s.	sign.	m.s.	sign.	m.s.	sign.	m.s.	sign.	m.s.	sign.	m.s.	sign.	m.s.	sign.
Replicate	5	8.046	n.s	29.91	n.s	0.009985	n.s	0.3078	n.s	1.1791	n.s	0.01802	n.s	0.004139	n.s
Mutation level	2	23.353	n.s	118.19	n.s	0.065399	*	1.8038	*	3.8212	n.s	0.08207	n.s	0.008855	n.s
Error	6	8.473		13.69		0.005277		0.2689		0.8183		0.02337		0.002586	

[a] mutation levels= untreated control, EMS treated with and without chloroplast mutants
d.f.= degrees of freedom; m.s.= mean square; sign.= significance ($P \leq 0.05$)
ns = not significant at 0.05 level;* = significant at the 0.05 level; ** = significant at the 0.01 level

Table 4. 2: Mean seed oil content and fatty acid compositions among three groups (untreated control, with and without chloroplast mutations) after EMS mutagenesis in line Vge-1 of *Vernonia galamensis* when analysed using six replications.

	Seed oil characteristics (%)						
Mutation level	**Oil content**	**Vernolic acid**	**Linoleic acid**	**Oleic acid**	**Palmitic acid**	**Stearic acid**	**Arachidic acid**
With chloroplast mutation	25.78	52.08	28.81	7.38	7.35	3.11	0.48
Without chloroplast mutation	28.48	59.97	23.75	6.39	5.92	2.93	0.43
Untreated control	30.16	59.98	23.91	6.49	6.04	2.85	0.41
S.E (±)	2.911	3.7	2.276	0.5185	0.9046	0.1529	0.05085
L.S.D	5.705	7.251	4.461	1.0164	1.773	0.2997	0.09967
C.V %	10.56	5.5	8.77	7.59	13.81	5.11	11.35

L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

No significant differences (P> 0.05) were detected in oil content and fatty acids compositions among different mutation levels in Vge-4 (Table.4.3). In Vge-4 the highest oil content at 31.93% was observed in mutants with chloroplast mutations. In this line the highest vernolic acid composition of 67.23% was recorded in the untreated controls (Table 4.4).

Table 4. 3: Analysis of variance on seed oil content and fatty acid compositions among three groups (untreated control, with and without chloroplast mutations) after EMS mutagenesis in line Vge-4 of *Vernonia galamensis* when analysed using six replications.

		Seed oil characteristics													
		Oil Content		Vernolic acid		Linoleic acid		Oleic acid		Palmitic acid		Stearic acid		Arachidic acid	
Source of variation	d.f.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.
Replicate	5	1.997	n.s	1.871	n.s	2.045	n.s	0.1393	n.s	0.29641	*	0.0055	n.s	0.0001617	n.s
Mutation level	2	0.484	n.s	7.538	n.s	6.393	n.s	0.0733	n.s	0.09777	n.s	0.09311	n.s	0.0001388	n.s
Error	6	3.628		3.706		4.243		0.1287		0.05195		0.04346		0.0003958	

[a] mutation levels= untreated control, EMS treated with and without chloroplast mutants
d.f.= degrees of freedom; m.s.= mean square; sign.= significance ($P \leq 0.05$)
n.s = not significant at 0.05 level;* = significant at the 0.05 level; ** = significant at the 0.01 level

Table 4. 4: Mean seed oil content and fatty acid compositions among three groups (untreated control, with and without chloroplast mutations) after EMS mutagenesis in line Vge-4 of *Vernonia galamensis* when analysed using six replications.

	Seed oil characteristics (%)						
Mutation level	**Oil content**	**Vernolic acid**	**Linoleic acid**	**Oleic acid**	**Palmitic acid**	**Stearic acid**	**Arachidic acid**
With chloroplast mutation	31.93	67.23	18.83	5.29	5.19	2.77	0.35
Without chloroplast mutation	31.37	66.16	19.55	5.5	5.45	2.54	0.34
Untreated control	31.81	65.18	21.19	5.42	4.95	2.56	0.35
S.E (±)	1.905	1.925	2.06	0.3588	0.2279	0.2085	0.0199
L.S.D	3.733	3.773	4.037	0.7032	0.4468	0.4086	0.039
C.V %	6.01	2.9	10.58	6.64	4.33	7.89	5.76

L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

There were significant interactions between lines and mutation levels on the magnitude of vernolic acid, linoleic acid and oleic acid composition. No significant interactions found on seed oil content, palmitic acid, steraic acid and arachidic acid compositions (Table 4.5). Vernolic acid was the dominant fatty acid present in all accessions followed by linoleic acid, oleic acid, palmitic acid, steraic acid and arachidic acid. Higher seed oil content and vernolic acids were observed in Vge-4 as compared to Vge-1 in all mutation levels (Table 4.6). In Vge-1, untreated plants indicated highest oil content (27.30- 34.38%), whilst mutants without chloroplast mutations showed highest vernolic acid composition (56.64- 62.88%). In Vge-4, mutants without chloroplast mutation had the highest oil content (27.30- 34.38%), whilst chloroplast mutants had the highest vernolic acid content (65.37- 69.29%) (Table 4.6).

Table 4. 5: Combined analysis of variance on seed oil content and fatty acid compositions among three groups (untreated control, with and without chloroplast mutations) after EMS mutagenesis in line Vge-1 and Vge-4 of *Vernonia galamensis* when analysed using six replications.

		Seed oil characteristics													
		Oil Content		Vernolic acid		Linoleic acid		Oleic acid		Palmitic acid		Stearic acid		Arachidic acid	
Source of variation	d.f.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.	m.s.	Sign.
Replication within levels	12	2.856	n.s	0.35	n.s	0.255	n.s	0.1072	n.s	0.0587	n.s	0.00153	n.s	0.000333	n.s
Line (L)	1	118.334	**	685.01	**	292.411	**	14.2847	**	11.6056	**	0.8742	**	0.073659	**
Mutation level (M)	2	7.84	n.s	37.45	n.s	14.311	n.s	0.6188	n.s	1.3117	n.s	0.16136	*	0.004922	n.s
L x M	2	11.468	n.s	73.06	*	33.286	*	1.1656	*	2.1518	n.s	0.00465	n.s	0.002843	n.s
Error	18	6.075		11.4		5.507		0.2345		0.6149		0.0278		0.001636	

[a] mutation levels= untreated control, EMS treated with and without chloroplast mutants
d.f.= degrees of freedom; m.s.= mean square; sign.= significance ($P \leq 0.05$)
n.s = not significant at 0.05 level;* = significant at the 0.05 level; ** = significant at the 0.01 level

Table 4. 6: Mean and range of seed oil content and fatty acid composition among EMS treated plants with and without chloroplast mutations and untreated controls in lines Vge-1 and Vge-4 of *Vernonia galamensis*

		Seed oil characteristics (%)													
		Oil content		Vernolic acid		Linoleic acid		Oleic acid		Palmitic acid		Stearic acid		Arachidic acid	
Line	Mutation level	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range	Mean	Range
Vge-1	With CM[a]	25.78	20.58-30.44	52.08	43.30-57.64	28.81	24.68-34.34	7.38	6.56-8.42	7.35	6.26-9.37	3.11	2.80-3.36	0.48	0.40-0.59
Vge-1	Without CM	28.48	26.57-29.92	59.97	56.64-62.88	23.75	21.53-25.67	6.39	6.10-6.92	5.92	5.47-6.62	2.93	2.82-3.06	0.43	0.41-0.45
Vge-1	Untreated	30.16	29.97-31.40	59.98	58.79-60.80	23.91	23.20-24.61	6.49	6.36-6.62	6.04	5.96-6.12	2.85	2.78-2.92	0.41	0.40-0.42
Vge-4	With CM	31.93	30.31-33.37	67.23	65.37-69.29	18.83	17.77-20.24	5.29	4.98-5.50	5.19	4.88-5.60	2.77	2.63-2.97	0.35	0.34-0.37
Vge-4	Without CM	31.37	27.30-34.38	66.16	61.42-67.73	19.55	17.58-24.64	5.5	4.57-5.87	5.45	4.76-5.60	2.54	2.42-2.79	0.34	0.31-0.36
Vge-4	Untreated	31.81	32.15-32.51	65.18	64.87-65.13	21.19	21.08-21.31	5.42	5.40-5.44	4.95	4.94-4.95	2.56	2.56-2.58	0.35	0.34-0.35
SE (±)		0.167		3.376		2.347		0.484		0.784		0.163		0.04	
L.S.D [Line (L)]		1.965		2.692		1.871		0.386		0.625		0.133		0.015	
L.S.D [Mutation level (M)]		3.002		4.113		2.859		0.59		0.955		0.203		0.023	
L.S.D (L x M)		4.246		5.816		4.043		0.834		1.351		0.287		0.033	
C.V %		5.9		5.5		10.3		7.9		13.3		5.8		10.2	

[a]CM= chloroplast mutation

L.S.D = least significant difference at 0.05%; CV = co-efficient of variation; S.E= standard error

Vernolic acid in the Vge-1 significantly and negatively correlated with all other fatty acids (Table 4.7). A significant and positive correlation ($P<0.001$; $r= 0.898$) was observed between vernolic acid and oil content (Table 4.7). In Vge-4, vernolic acid was significantly and negatively correlated with linoleic acid. Furthermore the correlation of vernolic acid with oil content was significantly positive in Vge-4 ($P<0.05$; $r= 0.65$).

Table 4. 7: Pair-wise correlations of *V. galamensis* seed oil content and fatty acids after analysis of chloroplast mutants, seeds treated with and without EMS. The top diagonal represents correlations of traits in Vge-1 and bottom diagonal in Vge-4.

Trait	OC	VA	LA	SA	OA	PA	AA
Oil Content (OC)	1	0.898**	-0.905**	-0.741**	-0.752**	-0.876**	-0.956**
Vernolic acid (VA)	0.650*	1	-0.992**	-0.820**	-0.932**	-0.982**	-0.945**
Linoleic acid (LA)	-0.633*	-0.959**	1	0.753**	0.884**	0.955**	0.958**
Steraic acid (SA)	0.368ns	0.361ns	-0.399ns	1	0.910**	0.867**	0.722**
Oleic acid (OA)	0.537*	0.506ns	-0.657*	0.233ns	1	0.952**	0.795**
Palmitic acid (PA)	-0.618*	-0.409ns	0.252ns	-0.513ns	-0.179ns	1	0.918**
Arachidic acid (AA)	-0.088ns	-0.298ns	0.24ns	0.560*	-0.2ns	-0.147ns	1

OC= oil content; VA= vernolic acid; LA= linoleic acid; SA= steraic acid; OA= oleic acid; AA= arachidic acid

n.s= correlation not significant at the 0.05 level; *= correlation is significant at the 0.05 level; **= correlation is significant at the 0.01 level.

4.5 DISCUSSION

The study compared seed oil content and fatty acid compositions in two selected lines of vernonia after EMS mutagenesis. Comparatively the same seed oil content was found in both lines before EMS treatment. Seed oil content was 30.16% in Vge-1 and 31.81% in Vge-4 untreated seeds (Table 4.6). Shimelis et al. (2008) studying the same accessions reported oil content of 28.12% in Vge-1 and 35% in Vge-4. Oil content did not differ signicantly between different mutation levels. Findings of non-significant alterations in oil content are contrary to the reports by Savant and Konthekar (2011) whose study on EMS mutation in sesame showed significant increases in oil content. Other studies in seed oil crops such as soybean (Koo, 1972), safflower (Sahu *et* al., 1980), soybean (Rahman *et al*., 1994), groundnut (Venkatchalam *et al*., 1999) have also demonstrated improved quality and quantity of seed oil due to mutation breeding. Differences in seed oil content were significant between Vge-1 and Vge-4 and this is probably due to genotypic differences between the lines.

In Vge-1, vernolic acid significantly and negatively correlated with all other fatty acids, however a significant and positive correlation ($P<0.001$; $r= 0.898$) was observed between vernolic acid and oil content (Table 4.7). In Vge-4, correlations between fatty acids were relatively poor and non-significant. In the line vernolic acid positively and significantly correlated ($P<0.05$; $r= 0.65$) with oil content (Table 4.7), therefore breeding for lines with increased vernolic acid should focus on selecting for plants with increased oil content. Selection for increased thousand seed weight and increased seed yield is the recommended morphological selection criteria for increased oil content in *V. galamensis*. Studies on vernonia by Mebrathu *et al.* (2009) associated genotypes with low thousand seed weight with increased oil content. In comparison to Vge-4 which had 31.37 to 31.93% seed oil, low oil content was obtained in Vge-1 ranging from 25.78 to 30.16% for all mutation levels (Table 4.6). In regards to vernolic acid no significant differences were observed among tested mutation levels, however there were positive responses in Vge-4 (Table 4.4) and negative responses in Vge-1 (Table 4.2) due to treatment. The highest vernolic acid composition (69.29%) was achieved in Vge-4 chloroplast mutants suggesting that mutagenesis altered this important fatty acid in *V. galamensis* (Table 4.5).

For oleochemical applications, an increased amount of a single fatty acid content would be of considerable value as processing costs are reduced (Baye *et al*., 2005). In both

treatment groups vernolic acid that ranged from 43.30 to 62.29% was the dominant fatty acid found in the accessions followed by linoleic acid (17.77 to 34.34%), oleic (4.57 to 8.42%), palmitic acid (4.76 to 9.37%), stearic acid (2.42 to 3.36%) and arachidic acids (0.31 to 0.59%).

Ethylmethanesulfonate treatment resulted in mutations of vernonia with and without chloroplast polymorphism. In Vge-4 comparison of these mutants with the control did not provide significant differences on the composition of the fatty acids. However, in Vge-1 significant alterations were observed in linoleic and oleic acid composition following mutagenesis (Table 4.2) with significant increases in linoleic and oleic acid composition only found in chloroplast mutants. Other studies on sunflower (Osorio *et al*., 1995), *Brassica carinata* (Barro *et al*., 2001) and sesame (Savant and Konthekar, 2011) indicated similar results of significantly altered oleic and linoleic composition after EMS treatment. In Vge-1 chloroplast mutation indicated significantly (L.S.D= 4.461%) altered linoleic acid composition (28.81%), however linoleic acid composition between untreated control plants at 23.91% and mutants without chloroplast mutation at 23.75% did not differ significantly (Table 4.2). Oleic acid composition in Vge-1 significantly increased (L.S.D= 1.0164) in chloroplast mutants at 7.38%, whereas composition between mutants without chloroplast mutation and untreated plants was not significantly different.

The current study did not find significant effect of EMS on palmitic acid composition in vernonia. In another studies on sunflower Osorio *et* al. (1995) found an increased palmitic acid composition in five-folds due to EMS treatment. Interaction between lines and mutation level significantly affected vernolic acid, linoleic acid and oleic acid composition. No interaction occurred among lines and mutation levels on seed oil content, palmitic acid, steraic acid and arachidic acid composition (Table 4.5). Further selections at M_2 and M_3 generations would provide clear trend of the oil content and fatty acid profiles of selected mutants of *V. galamesis*. The inclusion of more lines may present better opportunity to isolate mutants with a wider range of variations in agronomic traits, oil content and fatty acid composition.

4.6 CONCLUSION

The study revealed that oil content and vernolic acid composition were not significantly affected by EMS treatment, however significant alterations in composition of oleic acid and linoleic acid were observed as a result of EMS treatment. Significant increases in linoleic and oleic acid composition were found in chloroplast mutants due to EMS mutagenesis. Further multigenerational studies need to be conducted with an increased number of testing lines from a wide range of environmental conditions to gain thorough insight on the potential of EMS mutagenesis on *V. galamensis*.

4.7 REFERENCES

Angelini L.G., E. Moscheni, G. Colonna, P. Belloni and E. Bonari, 1997: Variation in Agronomic Characteristics and Seed Oil Composition of New Oilseed Crops in Central Italy. Industrial Crops and Products **6**, 313-323.

Bafor M., M.A. Smith, L. Jonsson, K. Stobart and S. Stymme, 1993: Biosynthesis of Vernoleate (cis-12-epoxyoctadeca-cis-9- enoate) in Microsomal Preparations from Developing Endosperm of *Euphorbia lagascae*. Architectural Biochemistry and Biophysics **303**, 145-151.

Barro F., J. Fernandez-Escobar, M. De La Vega and A. Martian, 2001: Doubled Haploid Lines of *Brassica carinata* with Modified Erucic Acid Content through Mutagenesis by EMS Treatment of Isolated Microspores. Plant Breeding **120**, 262-264.

Baye T., H. Kebede and K. Belete, 2001: Agronomic Evaluation of *Vernonia galamensis* Gemplasm Collected from Eastern Ethiopia. Industrial Crops and Products **14**, 179-190.

Baye T., H.C. Becker and S.V. Witzke-Ehbrecht, 2005: *Vernonia galamensis*, a Natural Source of Epoxy Oil: Variation in Fatty Acid Composition of Seed and Leaf lipids. Industrial Crops and Products **21**, 257-261.

Butte W. 1983: Rapid Method for the Determination of Fatty Acid Profiles from Fats and Oils using Trimethyl Sulphonium Hydroxide for Transesterification. Journal of Chromatography **261**, 142-145.

Folch J., M. Lees and G.H. Sloane-Stanley, 1957: A Simple Method for the Isolation and Purification of Total Lipids from Animal Tissue. Journal of Biological Chemistry **226**, 497-509.

Greene E.A., C.A. Codomo, N.E. Taylor, J.G. Henikoff, B.J. Till, S.H. Reynolds, L.C. Enns, C. Burtner, J.E. Johnson, A.R. Odden, L. Comai and S. Henikoff, 2003: Spectrum of Chemically Induced Mutations From a Large-Scale Reverse-Genetic Screen in *Arabidopsis*. Genetics **164**, 731-740.

He X., C. Turner, G. Chen, J.-T. Lin,and T. Mckeon, 2004: Cloning and Characterization of a cDNA Encoding Diacylglycerol Acyltransferase from Castor Bean. Lipids **39**, 311-318.

Jako C., A. Kumar, Y. Wei, J. Zou, D.L. Barton, E.M. Giblin, P.S. Covello and D.C. Taylor, 2001: Seed-specific Over-expression of an Arabidopsis cDNA Encoding a Diacylglycerol Acyltransferase Enhances Seed Oil Content and Seed Weight. Plant Physiology **126**, 861-874.

Jaworski J. and E.B. Cahoon, 2003: Industrial Oils from Transgenic Plants. Current Opinions in Plant Biology **6**, 178-184.

Kaplan K.C., 1989: Vernonia New Industrial Oil Crop. Agromomic Research **374**, 10-11.

Katavic V., D.W. Reed, D.C. Taylor, E.M. Ciblin, D. Barton, J. Zou, S. MacKenzie, P. S. Covello and L. Kunst, 1995: Alteration of Seed Fatty Acid Composition by an Ethyl Methanesulfonate-lnduced Mutation in *Arabidomis fhaliana* Affecting Diacylglycerol Acyltransferase Activity. Plant Physiology **108**, 399-409.

Kim Y.S., K.S. Schumaker and J.K. Zhu. 2005: EMS Mutagenesis of *Arabidopsis*. Methods in Molecular Biology **323**, 101-104.

Koo F.K.S., 1972. Induced Mutation and Plant Improvement. IAEA., Vienna, 95-118.

Lung S.C. and R. Weselake, 2006: Diacylglycerol Acyltransferase: A Key Mediator of Plant Triacylglycerol Synthesis. Lipids **41**, 1073-1088.

Osorio J., J. Fernandez-Martinez, M. Mancha and R. Garces, 1995: Mutant Sunflowers with High Concentration of Saturated Fatty Acids in the Oil. Crop Science **35**, 739-742.

Pascual M.J. and E. Correal, 1992: Mutation Studies of an Oilseed Spurge Rich in Vernolic Acid. Crop Science **32**, 95-98.

Perdue R.E., 1989. Vernonia - Bursting with Potential. Agricultural Engeneering **70**, 11-13.

Rahman S.M., Y. Takagi, K. Kubota, K. Miyamoto, T. Kawakita, 1994: High Oleic Mutant in Soybean Induced by X-ray Irradiation. Biosciences, Biotechnology and Biochemistry **58**, 1070-1072.

Sahu G.R., P. Mukerji, B.B. Singh, and R.B. Singh, 1980: Induced Polygenetic Variability in Safflower (*Carthamus tinctorius* L.). Journal of Cytology and Genetics **15**, 81-85.

Savant K.D. and V.S. Kothekar, 2011: Induction of Variability in Fatty Acid Profile in Sesame (*Sesamum indicum* L.). Journal of Phytology **3**, 01-03.

Shimelis H., P.W. Mashela and A. Hugo, 2008: Performance of *Vernonia* as an Alternative Industrial Oil crop in Limpopo Province of South Africa. Crop Science.**48**, 236-242.

Shimelis H. and A. Hugo, 2011: Determination of Selection Criteria for Seed Yield and Seed Oil Content in Vernonia (*Vernonia galamensis* variety *ethiopica*). Industrial Crops and Products **33**, 436-439.

Spitzer V., W. Tomberg and M. Zucolotto, 1996: Identification of A-parinaric Acid in the Seed Oil of *Sebastiana brasiliensis* Sprengel (Euphorbiaceae). Journal of American Oil Chemistry Society **73**, 569-573.

Tah R.P., 2006: Induced Macromutation in Mungbean [*Vigna radiata* (L.) Wilczeck]. International Journal of Botany **2**, 219-228.

Teynor T.M., D.H. Putman, E.S. Oplinger, E.A. Oelke, K.A. Kelling and J.D. Doll, 1992: Vernonia. Alternative Field Crops Manual. Center for Alternative Plant and Animal Products, University of Wisconsin-Extension and Cooperative Extension, University of Minnesota.

Till B.J., J. Cooper, T.H. Tai, P. Colowit, E.A. Greene, S. Henikoff and L. Comai, 2007: Discovery of Chemically Induced Mutations in Rice by TILLING. BMC Plant Biology **7**, 1-12.

Thompson A.E., D.A. Dierig and R. Kleiman, 1994: Variation in *Vernonia galamensis* Flowering Characteristics, Seed Oil and Vernolic Acid Contents. Industrial Crops and Products **3**, 175- 183.

van de Loo F.J., B.G. Fox and C. Somerville,1993: Unusual fatty acids. *In* TSJ Moore, ed, Lipid Metabolism in Plants, CRC Press, Boca Raton, Florida, 91-126.

Venkatchalam P., N. Geetha, and N. Jayabalan, 1999: Twelve New Groundnut (*Arachis hypogea* L.) Mutated Germplasm Registered in ICRISAT Gene Bank. Mutation Breeding **44**, 8-13.

Vogel G. and J. Browse, 1996: Choline Phosphotransferase and Diacylglycerol Acyltransferase: Substrate Specifities at a Key Branch Point in Seed Lipid Metabolism. Plant Physiology **110**, 923-931.

Weil C.F. and R. Monde, 2007: Getting the Point - Mutations in Maize. Crop Science **47**, 60-67.

Yu K., C.J. McCracken, R. Li and D.F. Hildebrand, 2006: Diacylglycerol Acyltransferase from *Vernonia* and *Stokesia* Prefer Substrates with Vernolic Acid. Lipids **41**, 557-566.

GENERAL DISCUSSION

Profitable cultivation of *Vernonia galamensis* is significantly hampered by non-uniform seed maturity, tall plant height and seed shattering. In addition, commercialisation is also hindered by lack of appropriate processing technologies such as mechanical harvesting, seed threshing and cleaning. Mutation breeding makes extensive use of deviations from the norm in order to improve characteristics of important crops. Inducing genetic variation in selection breeding of vernonia has been proposed as a plausible solution to combating challenges encountered in vernonia cultivation (Mebrahtu *et al*., 2009). Therefore the aim of the study was to induce genetic variation using EMS and select mutants for subsequent selective breeding of vernonia. In order to achieve this, several objectives were set out: (i) to determine an optimum treatment combination (i.e. exposure duration, temperature and EMS dose) that would enable at least 50-60% germination at minimum days to emergence in selected *V. galamensis* var. *ethiopica* lines (Vge-1, Vge-4, Vge-7 and Vge-10), (ii) to induce genetic variation using predetermined optimal treatment conditions and select mutants in *V. galamensis* variety *ethiopica* lines (Vge-1 and Vge-4) and (iii) to evaluate oil content and fatty acid analysis among seeds of chloroplast mutants, EMS treated seeds and untreated controls in lines Vge-1 and Vge-4 of *V. galamensis* variety *ethiopica*.

Prior to EMS mutagenesis of any crop, it is important that the optimal treatment dose is established. Under optimal mutagenesis conditions, individuals of an EMS mutant population carry a high mutation load but remain vigorous and fertile (Shah *et al*., 2008; Stephenson *et al*., 2010). The first experiments (Chapter 2) investigated optimal treatment conditions (EMS dose, duration and solution temperature) for mutagenising four *V. galamensis* lines (Vge-1, Vge-4, Vge-7 and Vge-10). Results showed that highly significant interactions ($P<0.001$) existed between EMS, line, time and temperature with respect to days to 50% emergence, germination percentage and seedling height. Therefore, all four factors were used in selection for optimal EMS treatement conditions. Optimal days to 50% emergence (10-12 days) and germination (50- 58%) was achieved for Vge-1, Vge-7 and Vge-10 when treated with 0.372% EMS at 35ºC for an hour. The optimal treatment combination for Vge-4 was 0.372% EMS at 32.5ºC for 2 hours. The treatment combinations that yielded optimum results in the tested lines were utilized to induce large scale mutation in *V. galamensis* to select target mutants.

Following this, two lines (Vge-1 and Vge-4) were selected, treated at predetermined optimal treatment conditions and field planted to select for mutants (Chapter 3). Results indicated that EMS treatment significantly delayed days to head formation, days to flowering and days to maturity on both lines. Delays in days to emergence were only significant in Vge-4. Comparative studies have also reported delayed development in EMS treated plants (Greer and Reinhart, 2009). Ethylmethanesulfonate treatment also significantly reduced germination percentage, number of seeds per head, number of fertile plants, plant height and plot yield for both Vge-1 and Vge-4. Thousand seed weight significantly increased in the treated seeds of the two lines. Results of significantly altered phenotypic characteristics in EMS treated plants have also been reported elsewhere (Alacantara *et al.,* 1996; Amernath and Prasad, 1998; Penmetsa and Cook, 2000; Jabeen and Mirza, 2004; Kumar and Kumar Rai, 2007; Berenschot *et al*, 2008; Sha *et al*., 2008). Chlorophlast mutation and seed sterility are common features indicative of EMS mutation in crops (Miller *et al*., 1980; Miller *et al*. 1984; Vaida and Young, 1993; Alacantara *et al*., 1996; Singh *et al*., 2000). Chlorophyll mutants were observed from both lines associated with high count of sterility. However, the exact chromosomal changes resulting in the observed mutations are not known prompting the need for further genotypic experiments. Fertile chloroplast mutants were harvested at full maturity with the rest of the cultivated plants, and used to evaluate the effect of mutation on oil content and fatty acid composition.

Thereafter, oil content and fatty acid composition were analyzed for three mutation levels (EMS mutants with and without chloroplast mutation, and the respective untreated plants) for Vge-1 and Vge-4 lines. Differential responses were observed when lines were compared at various EMS mutation levels showing significant effect on vernolic, linoleic and oleic acids compositions. In both lines no differences were detected on seed oil content, palmitic acid, steraic acid and arachidic acid compositions after the treatment. However, significant increases in the magnitude of oil content magnitude as a result of EMS mutation have been reported on sesame (Savant and Konthekar, 2011). The highest vernolic acid composition (69.29%) was achieved in Vge-4 chloroplast mutants suggesting that mutagenesis altered this important fatty acid in *V. galamensis*. Oil content significantly and positively correlated with vernolic acid for Vge-1 ($P<0.001$; $r= 0.898$) and Vge-4 ($P<0.05$; $r= 0.65$). Vernolic acid significantly and negatively correlated with other fatty acids. The study found that EMS mutagenesis significantly changed the oleic acid and

linoleic acid compositions in vernonia. Other studies on sunflower (Osorio *et al*., 1995), *Brassica carinata* (Barro *et al*., 2001) and sesame (Savant and Konthekar, 2011) indicated similar results of significantly altered oleic and linoleic composition after EMS treatment. However, the oil content and vernolic acid composition were not significantly affected by EMS treatment. Further selections would be conducted in the subsequent mutation generations to isolate useful and stable mutants.

CONCLUSIONS

The aim of the study was to induce genetic variation using EMS and select mutants for subsequent selective breeding of *V. galamensis*. In conclusion, EMS treatment successfully induced mutation in selected lines of *V. galamensis*; this is affirmed by the significant alterations in various agronomic traits, linoleic and oleic acid content and the observed chloroplast mutants.

RECOMMENDATIONS

- Certain geographical locations are more favourable than others for production of *Vernonia* as an industrial oilseed crop (Thompson *et al*., 1994); however, no single location has been identified with any degree of certainty as most favourable for vernonia cultivation. Different *V. galamensis* accessions posses variability in genetic makeup therefore are expected to vary in response to induced chemical mutagenesis. Data from a single planting generation in a single location is insufficient to conclude fully on the effect of EMS on *V. galamensis*. To gain further insight on EMS mutagenesis of *V. galamensis*, it is therefore necessary that further multigenerational chemical mutagenesis studies conducted with an increased number of testing accessions over locations that largely differ in environmental conditions.
- Genotypic studies are necessary to understand EMS mutagenesis of vernonia at loci level. This also presents opportunity to study gene function of *V. galamensis* seeds through the mutated base pairs.

REFERENCES

Alcantara T., P. Bosland and D. Smith, 1996: Ethyl Methanesulfonate-induced Seed Mutagenesis of *Capsicum annuum*. The Journal of Heredity **87**, 239-241.

Amernath S. and A.B. Prasad, 1998: Induced Variability in Homozygous and Heterozygous Genotypes of Tobacco. Indian Journal of Genetics **58**, 69-77.

Barro F., J. Fernandez-Escobar, M. De La Vega and A. Martian, 2001: Doubled Haploid Lines of *Brassica carinata* with Modified Erucic Acid Content through Mutagenesis by EMS Treatment of Isolated Microspores. Plant Breeding **120**, 262-264.

Berenschot A., M. Zucchi, A. Tulmann-Neto and V. Quecini, 2009: Mutagenesis in *Petunia x hybrida* Vilm. and Isolation of a Novel Morphological Mutant. Brazilian Journal of Plant Physiology **20**, 95-103.

Jabeen N. and B. Mirza, 2004: Ethyl Methane Sulfonate Induces Morphological Mutations in *Capsicum annuum*. International Journal of Agriculture and Biology **6**, 340-345.

Kumar G. and P. Kumar Rai, 2007: EMS Induced Karyomorphological Variations in Maize (*Zea mays* L.) Inbreds. Turkish Journal of Biology **31**, 187-195.

Mebrahtu T., T. Gebremariam, A. Kidane and W. Araia, 2009: Performance of *Vernonia galamensis* as a Potential and Viable Industrial Oil Plant in Eritrea: Yield and Oil Content. African Journal of Biotechnology **8**, 635-640.

Miller P., K. Vaughn and K. Wilson, 1980: Induction, Ultrastructure, Isolation, and Tissue Culture of Chlorophyll Mutants in Carrot. In Vitro **16**, 823-828.

Miller P.D., K.C. Vaughn and K.G Wilson, 1984: Ethyl Methanesulfonate-induced Chloroplast Mutagenesis in Crops. Journal of Heredity**75**, 86-92.

Osorio J., J. Fernandez-Martinez, M. Mancha and R. Garces, 1995: Mutant Sunflowers with High Concentration of Saturated Fatty Acids in the Oil. Crop Science **35**, 739-742.

Penmetsa V.R. and D.R. Cook, 2000: Production and Characterization of Diverse Developmental Mutants of *Medicago truncatula*. Plant Physiology **123**, 1387-1397.

Rahman S.M., Y. Takagi, K. Kubota, K. Miyamoto, T. Kawakita, 1994: High Oleic Mutant in Soybean Induced by X-ray Irradiation. Biosciences, Biotechnology and Biochemistry **58**, 1070-1072.

Savant K.D. and V.S. Kothekar, 2011: Induction of Variability in Fatty Acid Profile in Sesame (*Sesamum indicum* L.). Journal of Phytology **3**, 01-03.

Shah T., J. Mirza, M. Haq and B. Atta, 2008: Radio Sensitivity of Various Chickpea Genotypes in M1 Generation I-Laboratory Studies. Pakistan Journal of Botany **40**, 649-665.

Singh U.P., B. Prithiviraj and B.K. Sarma, 2000: Development of *Erysiphepisi* (Powdery Mildew) on Normal and Albino Mutants of Pea (*Pisum sativum* L.). Journal of Phytopathology **148**, 591-595.

Stephenson P., D. Baker, T. Girin, A. Perez, S. Amoah, G.J. King and L. Østergaard, 2010: A rich TILLING resource for studying gene function in *Brassica rapa*. BMC Plant Biology **10**, 1-10.

Thompson A.E., D.A Dierig, E.R.Johnson, G.H. Dahlquist and R. Kleiman, 1994. Germplasm Development of *Vernonia galamensis* as a New Industrial Oilseed Crop. Industrial Crops and Products **3**, 185-200.

Vaida K. and M. Young, 1993: Ethyl Methanesulfonate Induced Variation in Qualitative and Quantitative Characters of Roselle (*Hibiscus sabdariffa* L.) (Malvaceae). Brazilian Journal of Genetics **16**, 381-391.