MORPHOLOGICAL AND GENETIC DIVERSITY ANALYSIS IN SELECTED VERNONIA LINES

BY

SEGANKA PIET RAMALEMA

A MINI-DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN AGRICULTURE (CROP SCIENCE)

SCHOOL OF AGRICULTURAL AND ENVIRONMENTAL SCIENCES, FACULTY OF SCIENCE AND AGRICULTURE, UNIVERSITY OF LIMPOPO

DECEMBER 2007

SUPERVISOR: DR HA SHIMELIS

CO-SUPERVISOR: PROF I NCUBE

DECLARATION

I declare that the mini-dissertation hereby submitted to the University of Limpopo for the degree of Master of Science in Agriculture (Crop Science), has not previously been submitted by me for a degree at this or any other University; that is my work in design and in execution, and that all material contained has been duly acknowledged.

_____

S.P. Ramalema

ACKNOWLEDGEMENTS

First of all I would like to thank God, the Almighty for giving me a chance to live and accomplish my dream of finishing my masters degree. All my special thanks go to Dr H.A. Shimelis and Prof I. Ncube for their guidance and valuable comments throughout my study. My thanks are also extended to Professors K. Kunert and C. Cullis for inviting me to the workshop on marker assisted selection at University of Pretoria that enabled me to expose myself to the outside academic environments. I would also take this opportunity to extend sincere thanks to my parents, brothers and my sister and friends for supporting me in my academic adventures and life in general. I would not have made it without all of you.

The National Research Foundation (NRF) is acknowledged for providing financial assistance for this study.

TABLE OF CONTENTS

Title	Page
DECLARATION	ii
ACKNOWLEDGEMENTS	iii
TABLE OF CONTENTS	iv
LIST OF TABLES	viii
LIST OF FIGURES	ix
ABSTRACT	x
CHAPTER 1: INTRODUCTION	1
CHAPTER 2: LITERATURE REVIEW	7
2.1 Description and origin of vernonia species	7
2.2 Reproductive behaviour of *V. galamensis*	7
2.3 *V. galamensis* spp. *galamensis* var. e*thiopica* as a potential industrial oilseed crop	8
2.4 Composition of vernonia seeds	9
2.5 Breeding and genetics on vernonia species	10
2.6 The use of plant oils as renewable resources	10
2.7 Vernonia oil compared to other crop oils	13
2.8 Measuring genetic diversity	14
2.9 Genetic diversity in *Vernonia galamensis*	15
2.10 Genetic diversity at a morphological level	16
2.11 Genetic diversity at a molecular (DNA) level	16

TABLE OF CONTENTS (continued)

Title	Page
CHAPTER 3: MORPHOLOGICAL DIVERSITY ANALYSIS AMONG SELECTED VERNONIA LINES	20
3.1 INTRODUCTION	20
3.2 MATERIAL AND METHODS	22
3.2.1 Study site, growing conditions and experimental design	22
3.2.2 Test lines	23
3.2.3 Data collection	24
3.2.4 Data analysis	24
3.3 RESULTS	25
3.3.1 Seed yield peer plant	25
3.3.2 Days to 50 % flowering	27
3.3.3 Plant height	27
3.3.4 Number of primary heads	27
3.3.5 Number of secondary heads	28
3.3.6 Two hundred seed weight	28
3.3.7 Seed yield per hectare	29
3.4 DISCUSSION	29
3.5 CONCLUSIONS	33

TABLE OF CONTENTS (continued)

Title	Page
CHAPTER 4: SEED OIL CONTENT AND FATTY ACID ANALYSIS AMONG SELECTED VERNONIA LINES	34
4.1 INTRODUCTION	34
4.2 MATERIAL AND METHODS	36
4.2.1 Test lines, growing conditions and experimental design	36
4.2.2 Seed oil content and fatty acid profiles determination	36
4.2.2.1 Seed oil content	36
4.2.2.2 Fatty acid profiles	38
4.2.3 Data analysis	38
4.2.4 Relatedness of oil content and fatty acid profiles	39
4.3 RESULTS	39
4.3.1 Seed oil content	39
4.3.2 Vernolic acid	41
4.3.3 Linoleic acid	41
4.3.4 Oleic acid	41
4.3.5 Palmitic acid	42
4.3.6 Stearic acid	42
4.4 Correlation analysis for seed oil content and different fatty acids	42
4.5 DISCUSSION	44
4.6 CONCLUSIONS	48

TABLE OF CONTENTS (continued)

Title	Page
CHAPTER 5: USE OF RAPD ANALYSIS TO DETERMINE GENETIC DIVERSITY AMONG TEN SELECTED VERNONIA LINES ------	49
5.1 INTRODUCTION ----------	49
5.2 MATERIALS AND METHODS ----------	51
5.2.1 Test lines ----------	51
5.2.2 Reagents ----------	52
5.2.3 Genomic DNA isolation ----------	52
5.2.4 DNA quantification ----------	54
5.2.5 RAPD analysis ----------	54
5.2.6 Agarose gel electrophoresis ----------	57
5.2.7 Data analysis ----------	57
5.3 RESULTS ----------	58
5.3.1 DNA yield and quality ----------	58
5.3.2 Optimisation of RAPD conditions for amplification ----------	58
5.4 DISCUSSION ----------	60
5.5 CONCLUSIONS ----------	61
6. SUMMARY OF THE STUDY ----------	62
7. FUTURE RESEARCH ----------	63
8. REFERENCES ----------	64
9. APPENDICES ----------	83

LIST OF TABLES

Title **Page**

Table 2 Fatty acid profiles of common edible oils (% total fatty acids) adapted from Erasmus (1993) ---------- 12

Table 3.1 List of test lines, their geographic locations and coordinates ---------- 23

Table 3.2 Responses for seed yield per plant, days to 50 % flowering, plant height, number of primary heads, number of secondary heads, 200-seed weight and seed yield per hectare among ten selected vernonia lines ---------- 26

Table 4.1 Responses for seed oil content and fatty acid profiles among ten selected vernonia lines ---------- 40

Table 4.2 Correlation coefficients for pair-wise comparison for the degree of relatedness of seed oil content and fatty acid profiles among selected vernonia lines ---------- 43

Table 5.1 List of 13 RAPD primers screened for amplification and polymorphism ---------- 55

Table 5.2 Composition of the PCR reaction mixture for 25 µl reaction volume - 56

LIST OF FIGURES

Title **Page**

Figure 5.1 DNA isolated with the CTAB method of DNA isolation from ten selected *Vernonia galamensis* lines ran on a 1 % agarose gel electrophoresis ---------- 58

Figure 5.2 RAPD assays conducted with the DNA isolated from line 4 (Vge-16) by CTAB method ---------- 59

Figure 5.3 RAPD assays conducted with the DNA samples isolated from ten vernonia lines by CTAB method of genomic DNA isolation ---------- 60

ABSTRACT

Vernonia (*Vernonia galamensis*) is a new potential industrial oilseed crop. The seeds of this crop contain unusual naturally epoxidised fatty acids which are used in the production of various industrial products. The objective of this study was to evaluate the genetic diversity of selected vernonia lines in Limpopo Province through morphological, seed oil content and RAPD DNA markers. Significant differences were observed for days to 50 % flowering (93 - 140 days), plant height (141.80 - 166.33 cm), number of productive primary heads (29 - 60 head/plant), number of productive secondary heads (12 - 30 head/plant), thousand seed weight (1.85 - 3.52 g) and seed yield (454.44 - 786.85 kg/ha) between lines. Further results from oil analysis showed differences in the contents of seed oil (22.4 - 29.05%), vernolic acid (73.09 - 76.83%), linoleic acid (13.02 - 14.05%), oleic acid (3.77 - 5.28%), palmitic acid (2.48 - 2.98%) and stearic acid (2.26 - 2.75%). Among 13 RAPD DNA primers screened, primer OPA10 amplified DNA samples and resulted in four distinct groupings among tested lines. Four promising lines were selected viz. Vge-16, Vge-20, Vge-27 and Vge-32 displaying greater seed yield, increased vernolic acid content and reduced number of days to 50 % flowering.

CHAPTER 1

INTRODUCTION

Vernonia (*Vernonia galamensis*) is an annual member of the Asteraceae and is widely distributed in regions of Africa. The *V. galamensis* species complex is now recognized, according to Gilbert (1986), to include six subspecies, namely *galamensis*, *mutomoensis*, *nairobensis*, *afromomntana*, *gibbosa*, and *lushotoensis*. *Subsp. galamensis* is the most widely distributed; it is highly diverse and has four botanical varieties, namely var. *galamensis*, var. *petitiana*, var. *australis and* var. *ethiopica* (Gilbert, 1986). The subspecies *galamensis* and *mutomoensis* in general are found in areas of low rainfall, some as little as 200 mm per year, with no well defined dry season. Higher elevations and areas of high rainfall are the ideal regions for the subspecies *afromontana* and *lushotoensis* (Perdue *et al.*, 1986).

V. galamensis is new potential industrial oilseed crop, which originates from Eastern Africa. This plant was identified for the first time in Eastern Ethiopia by Perdue in 1964 at 7 km south east of Harar town, 9 ° 14' N and 42° 35' E. Due to the high oil and vernolic acid content and its relatively low shattering nature, *var. ethiopica* has been the focus of research and at present its production in some parts of the world reaches semi-commercial scale (Baye *et al.*, 2001). *V. galamensis* spp. *galamensis* var. *ethiopica* has a potential to become an industrial oilseed crop.

Vernonia seems to prefer well drained soil, and is fairly drought tolerant once established (Dierig *et al.*, 1996a). There is of variation in terms of vernonia morphology and flower

biology. The flower heads (capitula) vary considerably in size. The petals that surround the seeds on the capitula are between 8 and 25 mm long (Thompson *et al.*, 1994a). The colors of the florets are either blue to purple, or white. The capitulum is made up of 50 to 150 florets. This roughly corresponds to the number of seed produced per capitula, depending on pollination events. Hairs attached to the seed (pappus) can be up to 8 mm long. Subspecies are readily hybridised among themselves (Thompson *et al*., 1994b).

In its natural distribution vernonia in eastern and western Hararghe, which is known to farmers by the local names 'Ferenkundela', 'Dunfare', 'Kefathebogie', and 'Noya', have different connotations in different localities (Tesfaye, 1996). *Vernonia galamensis* produces high quantities of epoxy fatty acids (at least 60 %) in a trivernolin form, useful in the reformulation of oil-based (alkyd-resin) paints to reduce emission of volatile organic compounds (Perdue *et al.*, 1986). About 38% of the Vernonia seed is oil of which about 72 % is vernolic acid. Vernonia has "reactive dilutant" oil properties that produce less pollutants.

Other potential markets for the fatty acids include plasticisers, additives in polyvinyl chloride (PVC), coatings, cosmetic, and pharmaceutical applications (Carlson *et al.*, 1981). The plant could be also of interest to farmers in developed countries wishing to diversify and find an alternative and environment friendly crop (Baye, 2004). Preliminary investigations also showed that the meal after seed oil extraction 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 also are higher than in most other oilseeds (Ologunde *et al*., 1990).

Epoxidised oils, currently manufactured from animal fats or vegetable oils treated with peracetic acid or from petrochemicals are widely used in oleochemical industry as plasticisers and stabilisers for plastic, in reformulation of oil based paints, in cosmetics, and for pharmaceutical applications (Groot, 1990). Seeds from crops, such as soybeans and linseed, are currently used to extract oils. In 1950, the Agricultural Research Service (ARS) in the US Department of Agriculture (USDA) made 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 were *V. galamensis,* native to East Africa, and *Euphorbia lagascae*, native to Spain, both containing triacylglycerols consisting of vernolic acid (*cis*-12,13-epoxy–*cis*-9-octadecenoic) (Baye *et al.*, 2005).

The naturally occurring epoxidised oil (vernolic acid) of vernonia and the chemically epoxidised soybean (*Glycine max* L.) and linseed (*Linum ustitatissimum*) oils are similar. Epoxidised soybean and linseed oils are highly viscous and are non-pourable below 0 °C. Vernonia oil, however, has low viscosity and can be stored below 0 °C. The low viscosity will permit the oil to be used as a solvent alkyd-resin paint, which will become part of the dry paint surface and prevent the evaporation (emission) of volatile organic compounds that contribute to production of smog and pollute the air unlike conventional solvents such as terpentine. Another disadvantage towards the use of soybeans and linseed oils is

that it requires industrial processing or chemical epoxidation (Carlson *et al.*, 1981). Activities involved in the processing of these oils produce volatile organic compounds that contribute to the production of smog. In general, there is limited germplasm available containing naturally occurring epoxy oils, with good potential for commercialisation.

To enhance our understanding of genetic diversity and relatedness among plant species and varieties, molecular techniques like restriction fragment length polymorphism (RFLP; Fatokun *et al*., 1993), random amplified polymorphic DNA (RAPD; Mignouna *et al*., 1998; Fall *et al*., 2003), DNA amplification fingerprinting (DAF; Spencer *et al*., 2000), amplified fragment length polymorphism (AFLP; Fatokun *et al*., 1997; Coulibaly *et al*., 2002; Tosti and Negri, 2002), and microsatellites or simple sequence repeats (SSR; Li *et al*., 2001) have been used. These molecular markers are not influenced by endogenous and exogenous factors (Tanksley *et al*., 1989). Through the development of RFLP markers in the early eighties, indirect selection in plant breeding using DNA markers became technically feasible. However, the laborious nature of the RFLP technique has prevented a broad application of RFLP markers for marker assisted breeding.

In the late eighties and the early nineties, molecular diagnostic methods based on PCR technology have been developed, such as RAPD, AFLP and microsatellites. These methods allowed an efficient detection of DNA fragments starting from small amounts of DNA. The AFLP technique offers the additional advantage that it is a robust technology which, unlike microsatellites, can be applied instantaneously on any crop. In an

appropriate laboratory set-up this technology can now be used as a tool for marker-assisted breeding in a cost-effective way.

Different choices (alleles) may exist for a gene (i.e. a purple allele or a white allele). In each case, the same gene determines flower color, but the exact DNA sequences of the alleles of that gene are different. The population is said to have a low genetic diversity when all or nearly all the members of that population have the same allele at a gene level (Moran and Hopper, 1987). If many variants exist for a gene sequence, that population has high genetic diversity at that gene level. It was also elaborated by Polunin (1983), that individuals belonging to the same species share certain characteristics.

There are several DNA fingerprinting techniques that are used to evaluate genetic diversity, most of which use polymerase chain reaction (PCR) for the amplification of specific DNA fragments (Gilbert *et al.*, 2002). PCR uses two oligonucleotide primers complementary to opposite strands of a duplex DNA and consists of multiple cycles of annealing of the oligonucleotides, synthesis of a DNA chain, and denaturation of the synthesised DNA.

The PCR is used widely in molecular cloning, pathogen detection, genetic engineering, mutagenesis and in genetics producing molecular markers. These fingerprinting techniques include randomly amplified polymorphic DNA (RAPD) (Williams *et al.*, 1990), arbitrary primed PCR (AP-PCR; Welsh and McClelland, 1990), and DNA

amplification fingerprinting (DAF; Caetano-Anolles *et al.*, 1991) and amplified fragment length polymorphism (AFLP) analysis (Vos *et al.*, 1995).

Vernonia galamensis (Cass.) Less. produces an unusual fatty acid with an epoxy group attached to 18:1 molecules. *V. galamensis* is limited in distribution and is endemic primarily to East African countries as a weed colonising disturbed areas and bare agricultural lands under a wide range of agroecological conditions (Baye and Becker, 2005a). 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 variabilities within this species as an alternative crop in countries far from the equator. The phenotypic and genotypic variabilities will reveal about the genetic diversity as a measure of the possible choice of information provided by a gene or genes. The main objective of this study was to investigate the genetic diversity of ten selected Vernonia lines grown in Limpopo Province through morphological, seed oil content, fatty acid, and RAPD analysis for possible exploitation as an industrial oil crop.

CHAPTER 2

LITERATURE REVIEW

2.1 Description and origin of vernonia species

There are limited germplasm containing naturally occurring epoxy oils, with good potential for commercialisation (Gunstone, 1993). In recent years many new oilseed species have been investigated as potential sources of vegetable oils for oleochemical uses (Knapp, 1990). Many of them contain a high proportion of industrially desirable fatty acids, such as linoleic (*Mandia sativa* olina), linolenic (*Lepidium sativum* L. and *Camelina sativa* (L.) Crts.), calendic (*Calendula officinalis* L.), epoxy-(*Euphorbia lagascae* Sprengel and *Vernonia galamensis* (Cass.) Less.), hydroxyl-(*Lesquerella fendleri* (Gray) Wats.) and petroselinic (*Coriandrum sativum* L.) acids (Angelini *et al.*, 1997).

Vernonia galamensis (Cass.) Less. produces an unusual fatty acid with an epoxy group attached to 18:1 molecules. *V. galamensis* is limited in distribution and is endemic primarily to East African countries as a weed colonising disturbed areas and bare agricultural lands under a wide range of agroecological conditions (Baye and Becker, 2005a). Recent studies on herbarium materials in Addis Ababa, Ethiopia, and Kew, London, indicated the existence of about 40–50 species in Ethiopia (Naliaka, 1990).

2.2 Reproductive behaviour of *V. galamensis*

The natural outcrossing rate of vernonia ranged from 2.5 – 16.1 % (Baye and Ling, 2004). However, Dierig and Thompson (1993) found that *var. petitiana* was self-sterile,

allowing for ease of crossing with var. *ethiopica*. These crosses were made forming the intraspecies hybrids, and selections were performed for self-fertility, day-neutral photoperiodic response, good seed retention, non-dormant seed, and high yield (both for seed and oil) (Thompson *et al.*, 1994b). The intraspecies hybrids have been very successful, but only a limited number of accessions have been used in the crosses to date.

2.3 *V. galamensis* spp. *galamensis* var. *ethiopica* as a potential industrial oilseed crop

V. galamensis is a new potential industrial oilseed crop. All of the taxa, except for a few accessions of *V. galamensis spp. galamensis var. petitiana*, are short-day plants (Dierig and Thompson, 1993). Vernonia seems to prefer well drained soil, and is fairly drought tolerant once established (Dierig *et al.*, 1996b). The seeds of *V. galamensis* contain substantially higher amounts of vernolic acid (above 74%) than that of *V. anthelmintica* (67 %) and *Euphorbia lagascae* (60 %) (Higgins, 1968; Pascual-Villalobos *et al*., 1993; Thompson *et al*., 1994a). The company Ver-Tech International identified over 70 potential uses for the vernonia oil.

V. galamensis is a tropical, indeterminate annual plant which requires a well-drained soil and can grow under low rainfall and marginal conditions and is most suitable for dry land farming (Gilbert 1986; Baye 2000). It tolerates substantial shade, which makes it ideal for agroforestry; it may also prevent erosion and prevents desertification (Perdue, 1988). Altogether, the plant offers plentiful uses and substantial progress using a limited germplasm has been made on its use in different activities, including the chemistry of oil extraction and processing in some parts of the world (Carlson *et al*., 1981; Ayorinde *et*

al., 1988; Ayorinde *et al*., 1989, 1990a,b; Dirlikov *et al*., 1990; Ayorinde *et al*., 1993; Liu *et al*., 1998).

2.4 Composition of vernonia seeds

Gunstone (1954) discovered that the seed oil of *Vernonia anthelmintica* contained high amounts of epoxy fatty acids. This plant species produce unusual fatty acids with special characteristics, such as variations in chain length (shorter or longer than 18C) or functional groups within the fatty acid molecule like conjugated double bonds, hydroxy or epoxy groups (Robbelen, 1987). A collection of 41 accessions was evaluated for seed and leaf fatty acid composition. In vernonia seed, vernolic acid (C18:1>0) varied from 54 to 74 %, and linoleic acid (C18:2) from 3 to 32 % (Baye *et al.*, 2005). In the leaf linolenic acid (C18:3) varied from 41 to 59 %, palmitic (C16:0) from 12 to 22 % and C18:4 from 8 to 17 % (Thompson *et al*., 1994b). Correlation analysis between the seed fatty acids showed that vernolic acid is negatively correlated with palmitic, vernolic, linoleic and linolenic acid.

A seed of this crop also contains active lipase, which has unique acidolysis and hydrolysis selectivity properties toward vernolic acid (Ayorinde *et al*., 1993). Vernonia seeds are characterised by the rapid induction of lipolytic activity when the seeds are crushed, leading to high free fatty acid levels in the extracted oil and resultant meal. Therefore, precautions should be taken by introducing a heating step in the oil extraction processing scheme (Carlson *et al*., 1981; Ayorinde *et al*., 1990b).

2.5 Breeding and genetics on vernonia species

A program was initiated in America to conduct utilisation research on vernonia oil from *Vernonia anthelmintica*. The breeding and agronomic research led to the development of improved lines. But as a result of lack of seed retention and non-uniform maturity of seed heads, the program was discontinued (Massey, 1971). The collections of this plant and subsequent evaluation (Carlson *et al.*, 1981; Thompson *et al.*, 1994b) indicated that this species is substantially better because of the quantity and quality of the seed-oil and better seed retention. To date, research has been restricted to a narrow genetic base (fewer than 10 accessions), collected in eastern Ethiopia. Among other characters, a seed yield of up to 4000 kg/ha and an oil content of 40 % using these unimproved local materials were obtained, which seem to be higher than those found elsewhere (Baye 1996, 1997, 2000; Baye *et al*., 2001). But there are obvious gaps in the existing material, such as lack of difference in maturity group and different morphological plant types.

The genetic variations in fatty acid compositions of diverse sources of germplasm have hardly been studied. So far, research was more focused on the commercial importance of the oil and on the chemistry of oil extraction and processing (Dirlikov *et al*., 1990; Ologunde *et al*., 1990; Liu *et al*., 1998) and the study of plant lipids was restricted to seed.

2.6 The use of plant oils as renewable resources

Renewable resource-based polymers can yield a platform to substitute petroleum-based polymers through innovative ideas in designing the new biobased polymers which can

compete or even surpass the existing petroleum-based materials on a cost-performance basis with the added advantage of eco-friendliness. Many oils are currently converted through more or less extensive oleochemical processing to value added products, such as lubricants, polymer additives or surfactants. In most cases these products are based on fatty acids, their methyl esters or fatty alcohols as intermediates, because the reactive headgroup of these compounds is a ready candidate for chemical derivatisation (Dierig *et al.*, 1996b).

Unsaturated triglyceride oils, such as soybean, crambe, linseed and castor oil, constitute one major class of renewable resources. The main composition of these oils is saturated and unsaturated fatty acids (Patterson, 1989). Besides food source, they also have wide industrial application. These fatty acids are typically used directly or as their derivatives from some chemical modification in painting, coating, varnishes, cosmetics and polymer industry (Erickson *et al.*, 1980). Currently, no oilseed crop has been commercialised as a source of natural epoxidised oils (Thompson *et al*., 1994a). Hemp is also one of the oilseed crops (Rumyantseva and Lemeshev, 1994). The two polyunsaturated essential fatty acids, linoleic acid (C18:2) and linolenic acid (C18:3), usually account for approximately 50-70% and 15-25 %, respectively, of the total seed fatty acid content (Theimer and Mölleken, 1995). Different oilseed crops together with their oil content percentages are shown in Table 2 (Erasmus, 1993).

Table 2 Fatty acid profile of common edible oils (% total fatty acids) adapted from Erasmus (1993)

	Less healthy/Chemically stable <----> More nutritious/Chemically unstable				
	"Saturated"		"Monounsaturated"	"Polyunsaturated"	
Crop	Palmitic (C16:0)	Stearic (C18:0)	Oleic (C18:1Ω9)	Linoleic (C18:2Ω6)	Linolenic (C18:3Ω3)
Hemp	6-9	2-3	10-16	50-70	15-25
Soy	9	6	26	50	7
Canola	0	7	54	30	7
Wheatgerm	0	18	25	50	5
Safflower	0	12	13	75	0
Sunflower	0	12	23	65	0
Corn	0	17	24	59	0
Cottonseed	0	25	21	50	0
Sesame	0	13	42	45	0
Peanut	0	18	47	29	0
Avocado	0	20	70	10	0
Olive	0	16	76	8	0
Palm	85	0	13	2	0
Coconut	91	0	6	3	0

Most other oilseed plants, such as sunflower (Seiler and Brothers, 1999), *Brassica napus* (Mandal *et al*., 2002), soybean, peanut, sesame, have significant negative correlations among stearic, oleic and linoleic acids (Weiss, 1983). These differences perhaps indicate variations in the different species in the activities of the different steps in the fatty acid biosynthesis pathways (Fernandez-Martinez *et al*., 1993).

Epoxidised oils, currently manufactured from animal fats or vegetable oils treated with peracetic acid or from petrochemicals are widely used in oleochemical industry as plasticisers and stabilisers for plastic, in reformulation of oil based paints, in cosmetics, and for pharmaceutical applications (Groot, 1990). Seeds from crops, such as soybeans and linseed, are currently used to extract oils.

2.7 Vernonia oil compared to other crop oils

The naturally occurring epoxidised oil (vernolic acid) of vernonia and the chemically epoxidised soybean (*Glycine max* L.) and linseed (*Linum ustitatissimum*) oils are similar. Epoxidised soybean and linseed oils are highly viscous and are non-pourable below 0 °C. Vernonia oil, however, has low viscosity and can be stored below 0 °C. Vernonia oil is rich in vernolic acid, a fatty acid of high interest for oleochemical applications, mainly for manufacturing paints and coatings (Baye and Becker, 2005b). Vernolic acid (18:1>0 or 12, 13-epoxy-18:1-9) is an unusual fatty acid that occurs primarily in seeds of different plant species particularly belonging to the families of the Asteraceae and Euphorbiaceae (Earle, 1970). The structure of vernolic acid differs from that of oleic acid (18:1-9), a common plant fatty acid, by the presence of an epoxy group at carbon positions 12 and

13. This fatty acid composes up to 80 % of the total fatty acid of *V. galamensis* seeds but is virtually absent from leaves and other tissues (Baye *et al*., 2004).

The low viscosity will permit the oil to be used as a solvent alkyd-resin paint, which will become part of the dry paint surface and prevent the evaporation (emission) of volatile organic compounds that contribute to production of smog and pollute the air unlike conventional solvents such as terpentine. Vernonia oil can also be used in the animal feed industry and as a medicine to treat a variety of diseases (Harborne and Williams, 1977).

Another disadvantage towards the use of soybeans and linseed oils is that it requires industrial processing or chemical epoxidation (Carlson *et al.*, 1981). *V. galamensis* seeds are characterised by the rapid induction of lipolytic activity when the seeds are crushed, leading to high free fatty acid levels in the extracted oil and resultant meal (King *et al.*, 2001). Activities involved in the processing of these oils produce volatile organic compounds that contribute to the production of smog.

2.8 Measuring genetic diversity

To measure the genetic diversity of a crop both morphological and molecular diversity analysis are required (Frankel and Bennett, 1970). Of the identified 250 000 plant species in the global flora, the germplasm utilised in modern plant production has continuously narrowed. Selection for uniformity and closely defined breeding objectives resulting in pure lines, has led to a marked reduction in genetic variation.

Genetic diversity in some cultivated crops has been assessed on the basis of morphological and physiological markers (Ehlers and Hall, 1997). Additionally, biochemical markers, such as isosymes, have been used in order to determine genetic similarities among some varieties (Panella and Gepts, 1992; Pasquet 1993, 2000). These markers may be influenced to various degrees by plant-endogenous and environmental factors and, thus, are not reliable tools for genetic diversity assessment.

2.9 Genetic diversity in *Vernonia galamensis*

The Shannon-Weaver Diversity Index (H´) showed that most traits in *V. galamensis* are polymorphic and the highest H´ was noted for internode size (0.93) and the lowest for stem color (0.47). The overall diversity index for all traits was 0.76 (Baye, 2004). The majority of the genetic diversity, 89 % and 95 %, was observed within region of origin and altitudinal group, respectively. Baye and Becker (2005a) found that about 20 traits from 122 vernonia accessions exhibited significant variations except for days to emergence. Genotypes and locations interacted significantly ($P \leq 0.01$) for all traits. Broad-sense heritability estimates ranged from 11 % (for days to emergence) up to 79 % (for days to maturity). Expected genetic advance was between 1.3 % (for days to emergence) and 44.8 % (for seed oil yield). In another study by Baye *et al.* (2001), seventeen yield and yield component traits from eight vernonia accessions was conducted at three contrasting agroclimatic zones.

The considered traits showed significant differences at all locations except for the characters days to emergence and days to maturity. These materials could be used as

parent materials to start breeding programs for each specific location. There was a wide range of variation between the minimum and maximum values of most characters. Thus, even within the limited germplasm studied, selection for improved characteristics appears to be possible in *V. galamensis* var. *ethiopica*. These, therefore, permit the use of morphological traits as markers to measure genetic diversity of vernonia species.

2.10 Genetic diversity at a morphological level

With morphological diversity analysis, morphological markers are used to help in measuring the genetic diversity of a species. Morphological traits were among the earliest genetic markers used in germplasm management (Stanton *et al.,* 1994). However, they have a number of limitations, including low polymorphism, low heritability, late expression, and vulnerability to environmental influences (Smith and Smith, 1992).

These limitations in turn may affect the estimation of genetic relationships. Therefore, to be useful, morphological measurements should be accomplished in replicated trials. This may be expensive and time consuming. However, if the trials are highly heritable, morphological markers are one of the choices for diversity studies because the inheritance of the marker can be monitored visually without specialised biochemical or molecular techniques (Taba *et al.*, 1998).

2.11 Genetic diversity at a molecular (DNA) level

Molecular markers are becoming essential tools in plant breeding (Staub *et al.*, 1996; Mohan *et al.*, 1997; Gupta *et al.*, 1999). They have several advantages over the traditional

phenotypic markers that are difficult or time-consuming to select by plant breeders. These DNA markers are not influenced by environmental conditions and are detectable at all plant growth stages.

During the last few years, complementary molecular methods have been developed for strain typing including: (i) PCR amplification of antigen-encoding genes (gp63 and cpb) followed by analysis of restriction fragment length polymorphism (PCR-RFLP, (Harrington and Wingfield, 1995), (ii) PCR-RFLP of kDNA minicircles (Morales *et al*., 2001), (iii) random amplification of polymorphic DNA (RAPD) (Chague *et al.*, 1996) and (iv) multilocus microsatellite typing (MLMT) (Bulle *et al*., 2002; Ochsenreither *et al*., 2006).

Restriction fragment length polymorphism (RFLP) markers have routinely been used for agronomic crops linkage analysis and genome mapping (Tanksley *et al.*, 1989). However, construction of RFLP maps has been very difficult due to the low level of polymorphism in a self-pollinated crop such as wheat (Chao *et al.*, 1989). DNA markers such as RFLPs have been applied to interspecific studies of the genus *Helianthus* (Choumane and Heismann, 1988; Riesberg *et al*., 1998; Gentzbittel *et al*., 1992).

AFLP markers are highly polymorphic and reproducible and thus represent a powerful technique for DNA analysis that has revolutionised fingerprinting and diversity studies (Vos *et al*., 1995). AFLP analysis detects genetic variation throughout the genome by using a pair of specific restriction enzymes and their corresponding adapters combined

with 2 selective rounds of PCR. AFLP technique has been used to identify markers linked to disease resistance genes (Thomas *et al.*, 1995; Harlt *et al.*, 1999) and assess genetic diversity in several important agronomic crops including wheat (Breyne *et al.*, 1997; Gupta *et al.*, 1999).

SSR markers or microsatellites are tandem repeats interspersed throughout the genome and can be amplified using primers that flank these regions (Grist *et al.*, 1993). The primers for SSRs can be synthesised based on a repeat sequence of $(CA)_n$ (Godwin *et al.*, 1997). SSR has been successfully used to construct detailed genetic maps of several organisms and to study genetic variation within populations of the same species, such as grapes, honeybees and tropical trees (Brown *et al.*, 1996).

RAPD reactions are PCR reactions, but they amplify segments of DNA which are essentially unknown to the scientist (random). Randomly amplified polymorphic DNA (RAPD) analysis is widely used for studying taxonomy of various genera (Devas and Gale, 1992), species (Faroog *et al.*, 1995; Igbal *et al.*, 1995), for differentiation of intraspecies (Mackil, 1995; Sweeny and Danneberger, 1995) and to study the genetic diversity of various cultivars and lines. RAPD (Williams *et al.*, 1990) is based on the polymerase chain reaction (PCR) and is widely adapted in genetic diversity analysis. Recently, RAPDs have also been extensively used for assessment of genetic variation in inbred lines of sunflower (Isaacs *et al.*, 2003). These markers are of particular interest and DNA profiles based on arbitrary primed PCR are both time- and cost-effective (Williams *et al.*, 1990).

Principal component analysis and a dendrogram constructed from the Shannon-Weaver diversity index (H') indicated the close relationship of some of the vernonia populations both at molecular (RAPD) and morphological marker level (Baye, 2004). Though clustering matches, there was a low correlation between the RAPD based molecular diversity and phenotypic traits diversity. RAPD analyses (Welsh and McClelland, 1990) are capable of detecting differences among strains of a single species. The simplicity and fast sample processing of RAPD technique makes it useful for assessing population genetic parameters such as within-population and between-population genetic diversity. An additional advantage is that knowledge of the DNA sequences is not necessary to apply this technique (Weising *et al*., 1995).

CHAPTER 3

MORPHOLOGICAL DIVERSITY ANALYSIS ON TEN SELECTED VERNONIA LINES

3.1 INTRODUCTION

Vernonia galamensis is a new potential industrial oilseed crop originated in eastern and southeastern parts of Ethiopia. Its unique properties make it economically and environmentally interesting. *V. galamensis* spp. *galamensis* var. *ethiopica* is an annual crop, growing naturally in marginal areas with as little as 200 mm seasonal rainfall and at an elevation ranging from 700 to 2400 m above sea level (asl) in the southern and southeastern parts of Ethiopia (Gilbert, 1986). This plant in its natural distribution in eastern and western Hararghe is known to farmers by the local names 'Ferenkundela', 'Dunfare', 'Kefathebogie', and 'Noya', which have different connotations in different localities (Tesfaye, 1996).

Vernonia is grown successfully in countries near the Equator because the plants only flower under day-neutral photoperiodic conditions. Research on *Vernonia* galamensis was hindered by poor seed retention capacity of this crop in the America. Agronomic research on *V. galamensis* was initiated in Zimbabwe in 1983, where small trial plantings were made at the Botanical Garden in Harare and at four field stations of the Ministry of Agriculture's Department of Research and Specialist Services. The Harare trial and another at the Lowveld Research Station, Chiredzi, confirmed the good seed retention initially observed in Ethiopia. In another 1984 trial at Chipenge, plants were "toppled" (upper part of plants removed) to determine how they would respond to removal of apical

dominance. Plants produced lateral branches which tended to produce mature flower heads at the same time. This encouraged uniformity of seed maturity and reduced time from planting to maturity (Perdue (Jr) *et al.*, 1989).

Vernonia has limited possibilities as an oilseed crop for the northern continental United States. This crop flowers and sets seed too late in the growing season because shorter days are required for flower initiation and development. Frosts follow flowering too quickly to allow complete seed development and maturation. From trial plantings conducted at Experimental field, Georgia and Glenn Dale greenhouses, Maryland, it was concluded that poorly drained soil and/or insufficient length of growing season have contributed towards the failure of vernonia to produce a mature crop (USDA, 1986).

A breeding and agronomic research on the production of vernonia in America for its oil was hindered by the lack of seed retention and non-uniformity in the maturity of heads in America (Massey, 1971). Bhardwaj *et al.* (2000), conducted a research to specifically evaluate available vernonia germplasm for seed yield, oil content, and oil quality, and to determine suitable production practices on mid-Atlantic region of the United States and seed shattering continued to be prevalent in the available vernonia germplasm in their tests.

Limited attempts to study the naturally existing variability in different accessions of *V. galamensis* have been made since 1990 (Baye *et al*., 2001). Further collections and evaluations, however, showed that this species is substantially better because of the

quantity and quality of the seed oil and seed retention. Very recently, the plant was found to be a potential crop for inclusion into the agricultural system in Ethiopia. Among other characters, a seed yield up to 4000 kg/ha and an oil content of 40 % using unimproved local materials was obtained which is much higher than found elsewhere (Baye, 1996, 2000; Baye *et al.,* 2001). The objectives of this study were (1) to determine the phenotypic variations present among selected vernonia lines for future breeding programmes, (2) to determine seed yield and yield components and (3) to select lines that could possibly be adapted for production under irrigated dry-lands of Limpopo province (Capricorn district).

3.2 MATERIAL AND METHODS

3.2.1 Study site, growing conditions and experimental design

The study was conducted at Syferkuil, the University of Limpopo's experimental farm, which is characterised by hot dry summers and cool dry winters. The farm is situated at 23° South and 29° East and 1 261.6 m altitude. The mean average day temperature varies from 28 °C to 30 °C. The farm has sandy loam soil, of the Hutton form, Glenrosa family, with the pH ranging from 6.0 - 6.2. The experiment was laid out as a randomised complete block design with 5 replications. The plot size was 3 m X 2.4 m with 60 cm inter-row and 60 cm intrarow spacings.

3.2.2 Test lines

The ten selected vernonia lines used in the experiment together with their geographic location and coordination are all listed in Table 3.1. The lines were originally collected from southern and eastern Ethiopia, where it is believed to be the variety's centre of diversity.

Table 3.1 List of test lines, their geographic locations and coordinates

Lines	Geographic locations and coordinates
Vge-1	Gelemso (08° 49' N, 40° 31' E)
Vge-5	Melkabelo (09° 12' N, 41° 25' E)
Vge-9	Harar Zuria (09° 19' N, 42° 07' E)
Vge-14	Metta (09° 25' N, 41° 34' E)
Vge-16	Gelemso (08° 49' N, 40° 31' E)
Vge-20	Yirgalem (06° 42' N, 38° 21' E)
Vge-23	Leku (06° 52' N, 38° 27' E)
Vge-27	Awassa (06° 52' N, 38° 27' E)
Vge-32	Areka (06° 48' N, 37° 43' E)
Vge-36	Arsi-Negele (07° 00' N, 38° 35' E)

3.2.3 Data collection

From each line the following data were collected:

- Seed yield in grams per plant
- Days to 50 % flowering
- Plant height in centimeters
- Number of primary heads
- Number of secondary heads
- 200-seed weight in grams, and
- Seed yield in kilograms per hectare

3.2.4 Data analysis

Data on morphological traits were subjected to an analysis of variance (ANOVA) procedure using SAS statistical program (SAS, 1989). Traits that showed significant differences from the ANOVA were further subjected to Least Statistical Difference (LSD) mean comparison at a 5 % level of significance. Means were recorded together with coefficient of variation in percentages.

3.3 RESULTS

3.3.1 Seed yield per plant in grams

There was a significant difference between lines and replication for seed yield per plant (Appendix 9.1). The mean result allowed separation into six statistically different groupings, denoted by alphabets from a to f (Table 3.2). The line with the highest seed yield was Vge-16 with 28.33 g per plant whereas Vge-27 was the second highest with 26.13 g per plant. Vge-16 and Vge-27 were significantly different from each other (Table 3.2). Vge-14 recorded the lowest seed yield of 16.36 g per plant with Vge-20 as the second lowest in seed yield as it recorded 17.59 g. The overall mean of seed yield of lines was at 22.15 g per plant with the coefficient of variation of 5.04 %.

Table 3.2 Responses for seed yield per plant (gram), days to 50 % flowering, plant height (cm), number of primary heads, number of secondary heads, 200-seed weight (gram) and seed yield per hectare (kg) among ten selected vernonia lines

	Traits[a, b]						
Lines	SYP	DTF	PH	NPH	NSH	TSW	SYHA
Vge-1	20.22de	113.33cd	154.20cd	37.33c	17.67cd	2.27f	561.67de
Vge-5	24.00c	96.67f	151.06de	46.00b	24.00b	2.85d	666.67c
Vge-9	21.67de	120.00b	147.80e	45.33b	17.33cde	2.56e	601.85de
Vge-14	16.36f	120.00b	162.67b	30.67d	12.00f	2.67e	454.44f
Vge-16	28.33a	93.33f	151.00de	60.00a	30.67a	1.85g	786.85a
Vge-20	17.59f	114.33cd	166.33ab	30.33d	14.67def	3.52a	488.70f
Vge-23	21.92d	103.33e	153.00cd	34.33cd	20.67bc	3.66a	608.89d
Vge-27	26.13b	16.33bc	156.00c	56.00a	16.00def	2.63e	725.93b
Vge-32	25.40bc	110.00d	141.80f	48.67b	20.67bc	3.03c	705.93bc
Vge-36	19.87e	140.00a	167.85a	29.33d	12.67ef	3.22b	551.85e
Mean	22.15	112.73	155.17	41.80	18.63	2.83	615.24
Cv (%)	5.04	2.64	1.54	7.22	14.83	3.28	5.04
LSD (0.05)	1.91	5.11	4.10	5.18	4.74	0.16	53.16

[a] SYP = Seed yield per plant; DTF = Days to 50 % flowering; PH = Plant height; NPH = Number of primary heads; NSH = Number of secondary heads; TSW = 200-seed weight; SYHA = Seed yield per hectare.

[b] Means followed by the same letter in a column are not significantly different at P=0.05.

3.3.2 Days to 50 % flowering

The ten lines showed significant difference in days to 50 % flowering (Appendix 9.2). Six different mean groupings were found according to the LSD procedure of mean separation (Table 3.2). Vge-36 took more days to flower (140 days), followed by Vge-9 and Vge-14 with 120 days. Vge-16 took few days to reach 50 % flowering, which were 93 days, followed by Vge-5 which took 96 days to reach 50 % flowering. The grand mean for the ten lines to reach 50 % flowering were 112 days with low coefficient of variation estimated at 2.64 % (Table 3.2).

3.3.3 Plant height

This character showed significant differences between lines and replications (Appendix 9.3). Mean comparison among the ten lines resulted in six different groupings using the LSD procedure, which are denoted by letters from a to f, (Table 3.2). Vge-32 was the shortest with the height of 141.80 cm followed by Vge-9 with 147.80 cm and Vge-5 with 151.06 cm. Vge-32 and Vge-9 showed a statistical significant difference. The tallest line was Vge-36 with a height of 167.85 cm followed by Vge-20 with 166.33 cm. The ten lines had the grand mean height of 155.17 cm with CV of 1.54 %.

3.3.4 Number of primary heads

The lines showed a highly significant difference with respect to the number of primary heads (Appendix 9.4). The LSD mean comparison resulted in four different groupings shown as letters from a to d, (Table 3.2). Vge-16 produced the highest number of primary heads with 60 heads per plant followed by Vge-27, Vge-32 displaying 56 and 48 heads,

respectively. Vge-16 Vge-27 did not show significant difference. The line with the lowest mean primary heads per plant was Vge-20 (30 heads), followed by Vge-14 with the mean head of 31 per plant. A grand mean of 42 head per plant was displayed by the lines with the coefficient of variation of 7.22 %.

3.3.5 Number of secondary heads

This trait showed significant difference between lines (Appendix 9.5). Six different statistical groupings were found using the LSD procedure of mean comparison (Table 3.2). Vge-16 produced the highest number of secondary heads (31 per plant) while Vge-5 was the second highest with 24 heads. The lowest number of secondary heads was produced by Vge-14 with 12 heads followed by Vge-20 which had 15 heads. The grand mean among the ten lines was 19 heads per plant with the coefficient of variation of 14.83 %.

3.3.6 Two hundred seed weight

Lines had significant differences with respect to 200-seed weight (Appendix 9.6). This character resulted in seven different groupings using the LSD procure, denoted by letters from a to g, (Table 3.2). The line with the heaviest seed weight per 200 seeds was Vge-23 with 3.66 g, whereas Vge-20 was the second showing 3.52 g. Vge-1 had relatively low seed weight showing 2.27 g followed by Vge-9 with 2.56 g. A grand mean of 2.83 g was recorded among the ten lines with the coefficient of variation of 3.28 %.

3.3.7 Seed yield per hectare

Lines displayed significant differences with respect to seed yield in kilograms per hectare (Appendix 9.7). Six different groupings were found (Table 3.2). Vge-16 had the highest seed yield of 786.85 kg per hectare followed by Vge-27 showing 725.93 kg per hectare. The two lines displayed a significant statistical differences at P=0.05. Vge-14 on the other hand recorded the lowest seed yield of 454.44 kg per hectare followed by Vge-20 with 488.70 kg per hectare.

3.4 DISCUSSION

Seed yield per plant showed a highly significant difference among the selected vernonia lines. The mean yield among the ten selected lines was 22.15 g ranging from 16.36 to 28.33 g. Baye and Becker (2005a) reported mean yield of 13.85 per plant with ranges from 1.59 to 48.99 g, which showed a wider variations. Even though they reported a bigger maximum yield, their grand mean is about half of the present study. Their average yield was taken across two climatically differing locations, namely Babile and Alemaya in Ethiopia, and Babile showed better results compared to Alemaya, where vegetative growth was encouraged over reproductive growth due to cooler climates. However, Angelini *et al*. (1997) reported mean seed yield per plant of 3.8 g which are significantly lower than the present findings. This is due to the fact that their plants did not receive sufficient short-day photoinduction to produce flowering and resulted into excessively tall vegetative growth – more than 3 m.

Lines varied in terms of the time needed to reach 50 % flowering which ranged from 93 to 140 days. The reason to this variation might be the fact that the ten lines were originally collected from different geographic locations. This would mean that their responses to climate change would be different and hence triggered by different degree of coldness. Corresponding results were reported by Baye *et al*. (2001) who indicated 126 days for most *Vernonia* accessions to reach 50 % flowering. However, at other locations, such as Babile and Harar, less than 117 days and 105 days, respectively, were reported to reach flowering (Baye *et al*., 2001).

Plant height had a highly significant difference among lines and had lower coefficient of variation (2.64 %). The shortest line recorded was Vge-32 with the mean height of 141.80 cm, while Vge-36 was the tallest with the mean height of 167.85 cm with the grand mean height of 155.17 cm. Baye *et al.* (2001) found similar corresponding results when testing different vernonia accessions across three different locations. Much wider variation in terms of plant height was reported by Thompson *et al.* (1994b) when they reported plant height ranging from 23 to 88 cm sown in different locations of the USA. Baye and Becker (2005a) reported a minimum plant height of 16.95 cm and a maximum of 132.96 cm with the mean height of 97.95 cm in their trial under two different locations.

Lines also showed differences in terms of the number of primary heads per plant. The minimum and maximum values were 29 and 60 and these results are higher than the reported by Baye and Becker (2005a). The authors reported 37.82 and 36.62 mean values

of primary head number at Babile and Alemaya, respectively. Baye *et al.* (2001) reported much lower results at Babile where the minimum and maximum values were 4.38 and 10.95, respectively. The variations in the number of primary heads in this study can be attributed to the fact that these ten lines have different habits when it comes to primary branch production. Vge-16 seems to have contributed much of its metabolites towards primary branch production as compared to other lines and again it seems prominent that it is the one more adapted to the environmental conditions than others. Vge-36, which had the least number of heads, might have been interrupted and forced to enter the other developmental stages either by the environmental factors that favored reproductive growth and could not produce enough branches for high heads production.

The results from this study showed that different lines produce varying number of secondary heads. The mean responses among the ten lines ranged from 12.00 to 30.67 heads with the grand mean of 18.63 heads. Baye *et al.* (2001) reported corresponding results at Alemaya Research Station when they reported values ranging from 12.4 to 31.3 heads with the grand mean of 13.3 among eight accessions of vernonia. They further reported fewer secondary heads at Harar with values ranging from 11.2 to 16.1 and at Babile with ranges from 9.6 to 19.1 heads. The fewer primary heads number might been compromised by the relatively tall accessions at Harar, which promoted vegetative growth.

The 200 seed weight had mean value of 1.67 that ranged from 1.50 to 1.79. Angelini *et al*. (1997) reported corresponding results with a grand mean of 3.20 g with values ranging

from 2.7 to 3.8 g per 1000 seeds. Baye *et al.* (2001) also reported supporting results at three research stations with individual grand means of 4.3, 3.9 and 3.3 g per 1000 seeds among eight vernonia accessions. Contrastingly, Thompson *et al.* (1994a) reported lower values of 1.87–2.91 g/1000 seeds among 17 different *V. galamensis* accessions. Their accessions had shorter period from heading to maturity and as a results grain filling was might have been compromised.

This study found mean yield of 615.24 kg/ha with ranges from 454.44 to 786.85 kg/ha. Baye *et al.* (2001) reported supporting results with a grand mean of 638.8 kg/ha of seed yield and values ranging from 510 to 810 kg/ha from eight different vernonia accessions sown at Babile Research Station. Results from the present study are comparable to the mean yield of 861 kg/ha with ranges from 493 to 1394 kg/ha reported by Bhardwaj *et al.* (2000) when evaluating the performance of 14 different vernonia genotypes at Petersburg, Virginia. However, much higher results were found at Harar with the grand mean of 2 733.8 and values ranging from 2 470 to 3010 kg/ha of seed yield. This is about four times higher than the yield found in the present study. The contributing factor towards the low yield in the present study could be due to the spacings used in the establishment of the experiment. Baye *et al.* (2001) maintained their plant spacings at 0.5 m X 0.15 m corresponding to 133 333 plants per hectare, which is about four times larger than that of the present study which was approximately 27 700 plants per hectare from the spacings of 0.60 m and 0.60 m as inter-and intrarow spacings.

3.5 CONCLUSION

The results showed significant variation in terms of morphological traits, which confirm results of other researchers. Seed yield per plant, days to 50 % flowering, plant height, number of secondary heads, and seed yield per hectare grouped the ten selected lines into six different grouping. The best performing line was Vge-16 with best seed yield (28.33 g per plant), reduced days to flowering (93 days), higher number of primary (60) and secondary heads (30 per plant).

CHAPTER 4

SEED OIL CONTENT AND FATTY ACIDS ANALYSIS AMONG TEN SELECTED VERNONIA LINES

4.1 INTRODUCTION

In the last three decades a number of new oilseed crops have been introduced on the market providing the industry with new or unusual fatty acids, such as fatty acids with hydroxy or epoxy groups (Mikolajczak *et al*., 1961; Princen and Rothfus, 1984; Abbott *et al*., 1997). These fatty acids are used as ingredients in the production of various products such as paints and coatings, detergents, cosmetics, lubricants, flavours or biodegradable polymers (Muuse *et al*., 1992; Watkins, 1999). One important point for the introduction of a new industrial oil crop is to find new end-markets (Haumann, 1991). Oil and residue are strongly tied together, so that the success of an oil crop depends on the utilisation of both products. The residue of the oil-pressing process can be used as fuel for power stations, but from an economical point of view this residue should be used in the diet of animals to get a better commercial exploitation of the agricultural product (Steg *et al*., 1994).

Many plants produce seed oils with epoxy fatty acids (Earle, 1970). A few produce as much as 80 % of one epoxy acid, vernolic acid (12,13-epoxy-*cis*-9-octadecenoic). Those with potential as crops are *Vernonia galamensis* (Perdue *et al*., 1986), Stokes Aster (Earle, 1970), and *Euphorbia lagascae* (Kleiman *et al*., 1965). These epoxy oils have an advantage over commercially epoxidised oils in that the location, number, and configuration of epoxy and olefinic groups are rigorously known. The oil of *Vernonia*

galamensis forms excellent baked coatings on steel (Carlson *et al.*, 1981) and interpenetrating polymer networks with other polymers (Sperling *et al.*, 1983). These oils have potential in the production of plastics, paints, and lubricants.

V. galamensis (Cass.) Less. was identified for the first time in Eastern Ethiopia by Perdue in 1964 at 7 km south east of Harar town, 9º 14' N and 42º 35' E at 1700 m asl (Perdue, 1988). The seeds of *V. galamensis* contain substantially higher amounts of vernolic acid (more than 74 %) than that of *V. anthelmintica* (67 %) and *E. lagascae* (60 %) (Higgins, 1968; Pascual-Villalobos *et al*., 1993; Thompson *et al*., 1994b). Thompson *et al*. (1994b) using *V. galamensis* found a wide variation in oil content (24.4-33.8 %) and vernolic acid (60.2-75.5 %). They found a mean oil content of 28.7 and 30.4 % at Phoenix and Yuma, USA, respectively. They further stated that environmental factors undoubtedly play a quantitative and qualitative role in the biosynthesis of both oil and fatty acids.

Vernonia galamensis was also recognized as a new potential oilseed during a three year trial in Central Italy, after determining seed oil content and fatty acids from eight oilseed crops (Angelini *et al.*, 1997). *V. galamensis* was one of the crops that produced high levels (more than 50 %) of a single fatty acid in their seeds. Vernolic acid is a natural epoxy fatty acid that is present in new oil seed crops like *Euphorbia lagascae* and *Vernonia* spp (Sperling *et al*., 1983). Their seed oils consist over 60% vernolic acid allowing a relatively easy recovery of this versatile starting material for the synthesis of a wide range of chemicals (Cuperus and Derksen, 1996). The synthesis described above, using functionalized fatty acids as a renewable starting material, provide good alternatives for existing syntheses (Dasardhi *et al.*, 1991) and can reduce the amount of

hazardous chemicals required. This experiment was conducted in order to determine the seed oil content and fatty acid profiles from ten selected vernonia lines as well as to assess the relatedness of oil content and various fatty acids.

4.2 MATERIALS AND METHODS

4.2.1 Test lines, growing conditions and experimental design

For a list of the ten selected vernonia lines used in this experiment, their geographic locations and co-ordinations refer to Table 3.1 in Chapter 3. The growing conditions and experimental design for the ten selected vernonia lines were as described in section 3.2.1 in Chapter 3.

4.2.2 Seed oil content and fatty acid profiles determination

The procedures employed for seed oil content and fatty acid composition determination have been indicated in sub-sections 4.2.2.1 and 4.2.2.2.

4.2.2.1 Seed oil content

The oil content was determined on the basis of dry seed weight and three replications per entry were analysed. The oil was isolated according to an established method (Folch *et al.*, 1957) with chloroform-methanol (2:1 v/v) containing butylated hydroxyl toluene (0.001 %) as an antioxidant. Ground seed (0.5 g) was used for lipid extraction in a 250 ml round bottom flask. Chloroform:methanol (30 ml 2:1 v/v) was added into the flask containing the ground seeds and left overnight in a refrigerator at 4 °C. The sample was then transferred into a separatory funnel by filtering through a pre-weighed 18.5 cm

Whatman No. 1 filter paper. The round bottom extraction flask was washed three times with 12 ml of chloroform:methanol (2:1 v/v) and transferred to the separatory funnel through the filter paper. After allowing everything to run through, the filter paper was removed. Then 16.5 ml distilled water was added to the separatory funnel, which was recapped, shaken thoroughly, and allowed to stand for one hour for separation. After that the lower phase was drained into a 500 ml round bottom flask and 50 ml of chloroform:methanol:water (86:14:1 v/v) was added to the separatory funnel. After 15 minutes, the lower phase was drained again into the 500 ml round bottom flask and the step was repeated and the upper phase discarded.

Contents of the 500 ml round bottom flask were allowed to evaporate under vacuum in a rotary evaporator at 60°C for 20 minutes. The remaining water was removed by the addition of 30 ml methanol and then evaporated under vacuum at 60°C. Contents of the flask were washed six times with 5 ml portions of diethyl ether into a pre-weighed polytop. Diethyl ether was removed from the polytop by evaporation under a stream of nitrogen on a heating block at 60°C for 20 minutes. The filter papers together with the retentate and polytops were then dried at 50°C in a vacuum oven overnight and weighed separately the next day. 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 the original weight. The OC was calculated as a ratio of weight of the oil to its respective sample mass expressed in percentage. The FFDM was obtained as a ratio of the difference of the weight of the filter paper that contained the dry matter less its original

weight divided by initial measured sample mass and expressed in the percentage. The MC is 100 less the sum of OC and FFDM.

4.2.2.2 Fatty acid profiles

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 per minutes; hold 10 minutes). 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°C. 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 the Varian Star Chromatography Software version 4 and relative percentage composition of fatty acids quantitated as ratios of peak areas.

4.2.3 Data analysis

Data on seed oil content and fatty acids profiles were subjected to an analysis of variance (ANOVA) procedure of the SAS statistical program (SAS, 1989). Mean comparisons among lines was carried out for characters that showed significant differences from the ANOVA using the LSD procedure at 5 % level of significance.

4.2.4 Relatedness of oil content and fatty acid profiles

Correlation analysis was carried out to describe the pattern of association between agronomic variables and the various fatty acids. The correlations were calculated and significance of Pearson correlations was assessed on the basis of the available data.

4.3 RESULTS

4.3.1 Seed oil content

There was no significant difference observed for seed oil content in terms for both treatment and replication (Appendix 9.8). The mean responses together with grand mean, LSD value and the coefficient of variation are presented in Table 4.1. The best performing line in terms of seed oil content was Vge-32 with 33.11 % oil, while the worst performing line was Vge-9 with 22.40 % oil.

Table 4.1 Mean responses for seed oil content and fatty acid among ten selected vernonia lines

	Traits[a, b]					
Lines	SOC	VA	LA	OA	PA	SA
Vge-1	28.12	75.51	13.62	4.44bcde	2.92a	2.64
Vge-5	27.31	75.90	13.06	4.10cdef	2.81a	2.75
Vge-9	22.40	76.83	13.08	3.83ef	2.48b	2.26
Vge-14	23.81	74.34	13.02	4.51bcd	2.92a	2.61
Vge-16	26.44	74.66	13.20	4.86ab	2.93a	2.59
Vge-20	29.05	73.09	13.05	5.28a	2.83a	2.52
Vge-23	27.77	74.17	14.05	4.69abc	2.81a	2.74
Vge-27	26.04	74.65	13.59	4.40bcde	2.55b	2.30
Vge-32	33.11	75.92	13.37	3.99def	2.98a	2.26
Vge-36	28.38	74.67	13.13	3.77f	2.85a	2.47
Mean	27.24	74.97	13.31	4.38	2.81	2.51
LSD (0.05)	7.84	2.59	1.44	0.613	0.20	0.34
Cv (%)	12.73	1.53	4.77	6.18	3.08	6.06

[a] SOC = Seed oil content; VA = Vernolic acid; LA = Linoleic acid; OA = Oleic acid; PA = Palmitic acid; SA = Stearic acid.

[b] Means followed by the same letter in a column are not significantly different at P=0.05.

4.3.2 Vernolic acid

The analysis of variance for vernolic acid showed that there were no significant difference between both replications and lines (Appendix 9.9). The mean performances for the ten selected vernonia lines in terms of vernolic acid composition are presented in Table 4.1. The best line was Vge-9 with 76.83 %, whereas the poor line was Vge-20 with 73.09 % vernolic acid.

4.3.3 Linoleic acid

From Appendix 9.10, it was shown that both replication and lines resulted in no significant differences for linoleic acid percentage composition. Table 4.1 presents the mean performances of the ten selected vernonia lines for linoleic acid content. The best line was Vge-23 as it recorded 14.05 % of linoleic acid, while line Vge-14 recorded 13.02 % of linoleic acid which is the lowest among the ten selected lines.

4.3.4 Oleic acid

Significant differences were obtained between both the replications and lines for oleic acid composition (Appendix 9.11). The mean comparison for oleic acid composition for vernonia lines through LSD procedure is presented in (Table 4.1). Vge-20 had the highest oleic acid composition of 5.28 %, while Vge-36 had the lowest percentage composition of 3.77 %.

4.3.5 Palmitic acid

Appendix 9.12 shows that no significant difference was obtained between replications, whereas highly significant difference was obtained between lines. Vge-32 had the highest palmitic acid composition with 2.98 %, while the line with the lowest palmitic acid percentage composition was Vge-9 with 2.4 (Table 4.1).

4.3.6 Stearic acid

Appendix 9.13 shows no significant differences from both replications and lines for stearic acid composition among the ten selected vernonia lines. The mean performances among the selected lines for palmitic acid composition are shown in Table 4.1 together with the grand mean and coefficient of variation. The best line in terms of stearic acid composition was Vge-5 with 2.75 %, whereas the poorest lines were Vge-9 and Vge-32 as they both had 2.26 %.

4.4 Correlation of seed oil content with different fatty acids

The correlation coefficients for pair wise comparison of the degree of relatedness between seed oil content and the different fatty acids among ten selected vernonia lines is presented in Table 4.2.

Table 4.2 Correlation coefficients for pair-wise comparison for the degree of relatedness of seed oil content and fatty acid profiles among ten selected vernonia lines

	Traits[a, b]				
	SOC	VA	LA	OA	PA
VA	0.17^{ns}				
LA	-0.15^{ns}	-0.30^{*}			
OA	0.01^{ns}	-0.76^{*}	0.15^{ns}		
PA	0.41^{*}	-0.10^{ns}	0.05^{ns}	0.09^{ns}	
SA	-0.05^{ns}	-0.29^{ns}	0.30^{*}	0.37^{*}	0.41^{*}

[a] SOC = Seed oil content; VA = vernolic acid; LA = Linoleic acid; OA = Oleic acid; PA = Palmitic; SA = Stearic acid.

[b] ns = non significant; * = significant at $p<0.05$.

Seed oil content showed negative non-significant correlations to vernolic, linoleic, oleic and stearic acid, whereas it showed significantly positive correlations to palmitic acid. Vernolic acid on the other hand had negatively significant correlations with linoleic and oleic acids acid and stearic acid. Linoleic, oleic and palmitic acid showed positive significant correlations to stearic acid.

4.5 DISCUSSION

Seed oil content for this study ranged between 22.40 and 33.11 % and this corresponds to those reported by Angelini *et al.* (1997) in another study using *Vernonia galamensis* accessions. They reported seed oil content varying between 22.10 and 31.20 % with the mean of 26.70 % which is just lower than 27.24 % realised from the present study. However, Baye *et al.* (2001) investigated the performance of seed characters of eight *V. galamensis* var. *ethiopica* accessions sown at Alemaya, Harar and Babile and reported higher seed oil content averaging at 38.70, 39.50 and 34.70 % for each respective location. Hiruy and Getinet (1990) reported that higher altitudes and lower temperatures improve the biosynthesis of both oil and fatty acids, qualitatively and quantitatively, in most cultivated oil crops. This might be the reason for the lower yield found in the present study compared to those reported for Alemaya, Harar and Babile because the three locations were higher in terms of altitudes than Syferkuil, with the difference in altitudes being about 530 m.

Vernolic acid in the present study had a mean of 74.97 % with values ranging from 73.09 to 76.83 %. Corresponding results were reported by Angelini *et al.* (1997) when characterising different *V. galamensis* accessions, showing mean of 70.30 % which is lower than the one reported for this study. Baye and Becker (2005b) also reported supporting results, when reporting the mean vernolic acid content of seed oil of 74 % which ranged from 34 % to 87 %. Slightly lower results were reported by Baye and Becker (2005a), with the mean of 68.28 % vernolic acid from two different locations.

These results suggest that vernonia is stable in terms of vernolic acid synthesis across different agro-climatic regions.

Linoleic acid composition from this study showed a narrow variation compared to other researches. Values ranging from 13.02 to 14.05 % for linoleic acid were found in the present study whereas, much wider variations were reported by Baye *et al.* (2005) when 41 vernonia accessions, collected from different eco-geographic regions from Ethiopia, with values ranging from 2.40 % to 31.80 % linoleic acid composition. In another study conducted by Baye and Becker (2005a) on 122 vernonia populations, with 115 accessions from all regions of Ethiopia and seven accessions introduced from outside of Ethiopia, reported mean values ranging from 12.74 to 21.65 % linoleic acid content. Their results also showed a considerable variation for linoleic acid content and this was accompanied by a mean of 16.33 % linoleic acid, which is higher than the results found in the present study. The reason to these great variations reported by other researchers might be that they used many accessions 41 and 122, respectively, which possibly had great genetic variations. In the study few lines were used and these lines might have had a narrow genetic variation between them.

Oleic acid composition across the ten selected vernonia lines showed significant variations and their mean percentage had not differed from other researchers. Baye and Becker (2005b) evaluated 41 *V. galamensis* accession and reported supporting results with a mean of 4.42 %, which is not different from 4.38 realised from the present study. However, their results reported values ranging from 2.10 to 7.20 %, which is a wider than

3.77 to 5.28 % realised from the present study. The fewer number of accessions used in the present study might have been responsible for the narrow range found in this study as opposed to a total of 122 populations used by Baye and Becker (2005b).

The present study found significant differences between lines for palmitic acid. Supporting results were reported by Baye and Becker (2005b) with a mean of 3.32 % with ranges between 2.50 and 4.01 % which are higher compared to those recorded from the present study. However, Baye *et al.* (2005) reported lower percentage composition of palmitic acid, with a mean of 3.33 % and values ranging from 1.90 to 4.30 %. Their results reported slightly wider variations as well than the present study probably due to the higher number of accessions used in their study compared to 10 lines used in the present study. Their mean value might have been counteracted by the presence of the worst performing lines in their study due to large number of different accessions used as opposed to the present study.

The lack of variation among the lines used in the present study was also confirmed with stearic acid composition. Baye *et al.* (2005) reported a slightly wider range from 2.10 to 3.50 % as compared to 2.26 to 2.75 % stearic acid composition found in the present study. However, the mean percentage composition (2.88 %) across the accessions reported by these researchers were supportive of that found in the present study (2.75 %) for stearic acid. Even more corresponding results were reported with a mean composition of 2.55 % and values ranging from 1.4 – 6.5 % stearic acid. In this case, a much wider range was reported but it seems there were more poor lines than better lines and that

lowered the grand mean to 2.55 %. Better results were reported by Baye and Becker (2005a) as they reported a mean composition of 4.02 % with values ranging from 3.33 to 5.16 averaged from two different locations. It seems there was no wider range reported but still their results were better and this might suggest that they used better performing lines in terms of stearic acid synthesis as their minimum value is even higher than every other mean stearic acid composition reported, including the present study.

The results from the correlation analysis of seed oil content and various fatty acids indicate that selection for oil content would have positive and significant effects on palmitic acid and negative and non significant effects on linoleic acid and stearic acid. Nearly contrasting results have been reported by Baye and Becker (2005a) as they found that seed oil content had highly significant effects on all five fatty acids with vernolic acid showing the only positive correlation value. Their results meant that it is possible to improve vernolic acid by selecting for the high oil content in vernonia. It is also shown for the correlation analysis that selection for vernolic acid will results in negative and significant effects on linoleic acid and oleic acid. This means that palmitic and stearic acid cannot be improved by selection for vernolic acid, but they must be selected for directly. Linoleic recorded positively non-significant correlations to oleic, palmitic and stearic acid, and oleic acid had positive and non-significant correlations to palmitic and stearic acid and also palmitic acid followed on their trends. The results further suggest that linoleic, oleic, palmitic and stearic acid would have no effect on one another when selection is emphasised on either of them.

4.6 CONCLUSIONS

The results of this study confirmed less variation in seed oil content among ten selected *Vernonia galamensis* as shown by other studies conducted on this crop. All in all a good seed oil content was found in this study and five different types of fatty acids were found vernonia oil and vernolic acid comprised more than 75 percent followed by linoleic acid. The best line was Vge-9 in terms of vernolic acid composition though its seed oil content was the lowest. The correlation between seed oil and fatty acids showed that they are significantly correlated which means that selection for seed oil content will definitely affect the composition of other fatty acids and therefore conscious selection should be made in attempts to improve any of these.

CHAPTER 5

USE OF RAPD ANALYSIS TO DETERMINE GENETIC DIVERSITY AMONG TEN SELECTED VERNONIA LINES

5.1 INTRODUCTION

From the commercial point of view, DNA fingerprinting is a useful tool for varietal protection to prove ownership or derivation of plant lines. Moreover, the analysis of genetic diversity and relatedness between or within different species, populations and individuals are a prerequisite towards effective utilisation and protection of plant genetic resources (Weising *et al*., 1995). Genotypic characterisation of organisms has gained a lot of momentum over morphological characterisation since the expression of genes is not affected by the environment unlike the morphological expression of characters. There are several fingerprinting techniques that can be used to genotype or genetically characterise and determine genetic diversities.

In principle, genetic uniqueness is brought about by two factors, inheritance and new mutations, and since all genetic differences between individuals are laid down in the primary sequence of their genomic DNA, the most straightforward method is identifying an individual sequence for genomes under comparison (Krawczak and Schmidtke, 1994). Morphological and biochemical or protein markers may be affected by environmental factors and growth practices (Xiao *et al*., 1996, Ovesna *et al*., 2002, Higgins 1984). DNA markers portray genome sequence composition, thus, enabling to detect differences in the genetic information carried by the different individuals.

A wide variety of DNA-based markers have been developed in the past few years (Jatoi *et al.,* 2006). For instance, Palombi and Damiano (2002) compared RAPD and SSR-markers to detect genetic variability in kiwifruits. Similarly, Ferdinandez and Coulman (2002), Corazza-Munes *et al*. (2002), Archak *et al*. (2003) and McGregor *et al*. (2000) either compared the efficiency of RAPD, SSR and AFLP or used the different classes of markers for genotype identification. Recently, the efficacies of different classes of PCR-based markers were also used to characterise barley and rice cultivars (Saker *et al*., 2005 and Virk *et al*., 2000).

One other widely used technique is randomly amplified polymorphic DNA (RAPD) polymerase chain reaction (PCR) or arbitrarily primed PCR. This technique has been used for genetic diversity analysis in *Changium smyrnioides* (Apiaceae), which is an endangered plant (Fu *et al.,* 2003) because of the generic capabilities and relatively low cost of equipment for PCR. This method requires no previous knowledge of the template DNA sequence (Zhang *et al.,* 1997). By using one arbitrary primer and low-stringency conditions, the primer hybridises to both strands of template DNA, where it is matched or partially matched, resulting in strain-specific heterogeneous DNA products. RAPD is a PCR-based assay for the detection of genetic po1ymorphisms (Williams *et al*., 1990). In brief, 10 bp oligonucleotides of arbitrary nucleotide composition (with a minimum GC content of 60 %) are used as primers with the notion that annealing sites will be scattered throughout the genome in a strain-specific manner. There are two restrictions placed on the primer annealing sites: 1) they must be within 2kb of one another and 2) they must be in opposite orientation (Gupt *et al.,* 1999). PCR will optimally amplify stretches of DNA

between 0.2 and 2.0 kb (Haymer, 1994); primer annealing sites which are separated by a distance falling outside this range will likely not yield a reliable amplification product (Caetano-Anolles *et al.,* 1991).

A modified CTAB procedure described by Doyle and Doyle (1990) is one method that can be used for the extraction of DNA from plant materials especially from young and mature leaves. To carry out the PCR reaction, the CTAB method is relatively simple and is the method of choice for obtaining good quality total DNA from many plant species. The method is applicable to both fresh and dehydrated plant material and was tested on plants with polyphenols and polysaccharides. The procedure uses polyvinylpyrrolidone (PVP) and NaCl to remove contaminations e.g. polyphenols and polysaccharides (Sharma *et al*., 2002). Part of this study was to characterise ten selected vernonia lines through RAPDs.

5.2 MATERIALS AND METHODS

5.2.1 Test lines

Ten different vernonia lines collected from different geographical locations in Ethiopia were used for this study (see Table 3.2 in Chapter 3). Seeds of these lines were grown in the greenhouse at the University of Limpopo to obtain seedlings from which leaves were sampled. A total of thirty pots were used to grow the plants whereby five seeds per line were planted in three individual pots. Germination took place and seedlings were thinned to one seedling per pot. They were allowed to grow for three to four weeks before leaf sampling. Four to five young leaves were sampled from individual lines and immediately

placed in liquid nitrogen and then frozen in a - 80°C refrigerator until genomic DNA extraction.

5.2.2 Reagents

Acetic acid glacial, chloroform, acetic acid glacial, chloroform, ethanol, ether, glycerol were obtained from Saarchem, Johannesburg, South Africa; agarose bromophenol blue, Cetyl trimethyl ammonium bromide (CTAB), βmecarptoethanol, Ethylene diamine tetraacetic acid (EDTA), ethidium bromide, isoamylalcohol, sodium chloride (NaCl), phenol, polyvinyl pyrrolidone (PVP), Triisopropylphenylsulfonyl (TRIS) were obtained from Sigma, Johannesburg, South Africa; Liquid nitrogen was obtained from Microscopy and Micraoanlysis unit, University of Limpopo; primers were obtained from Qiagen, Germany; RNaseA, PCR components ($MgCl_2$, 5 U *Taq* DNA polymerase, 1X PCR buffer and 2 mM dNTPs) were obtained from Fermentas, Ontario, Canada.

5.2.3 Genomic DNA isolation

Leaf samples to the weight of 3 – 5 g were crushed to powder in liquid nitrogen with a mortar and pestle. A 10 ml extraction buffer pre-heated at 60°C containing 100 mM Tris-HCl (pH 8); 1.4 M NaCl; 20 mM EDTA; 0.1 % mercaptoethanol and 2 % CTAB was added to the crushed leaf material. The resulting slurry was poured into a 50 ml polypropylene centrifuge tubes, followed by rinsing of the pestle and mortar with 1 ml of extraction buffer and adding the wash off to the initial extract.

To the leaf slurry 50 µl (PVP) was added and mixed by inverting the tube several times. The mixtures were incubated at 60°C for 30 minutes and then allowed to cool to room temperature. Chloroform:isoamyl alcohol (24:1) was added to the tubes and mixed gently by inverting the tubes several times to form emulsion. The mixtures were centrifuged at 6000 rpm on a MISTRAL 1 000 centrifuge fitted with a MSE 935 rotor for 15 minutes at room temperature. The supernatant was collected, and half a volume of 5 M NaCl and two volumes of cold (-20 °C) 95 % ethanol were added to precipitate DNA. The precipitated mixture was then centrifuged at 3 000 rpm for three minutes, followed by 5 000 rpm for another three minutes to sediment the DNA pellet on the tube. Since CTAB and NaCl sometimes precipitate with DNA, the CTAB/NaCl residual was removed by a three-times wash with 70 % ethanol followed by a short centrifugation run. The supernatant was discarded, followed by washing the pellet with 76 % ethanol. The pellet was dried by leaving the tubes uncovered on a water bath at 37 °C for 30 minutes.

The dried pellet was resuspended in 100 µl sterile distilled water (sdH_2O). The DNA was collected by centrifugation for 2 minutes at 4 °C at 14, 000 rpm and the pellet was washed with 70 % ethanol, dried and re-suspended in 250 µl sdH_2O (sterile distilled water). In order to remove RNA and protein contamination, the solution was treated with 5 µl RNaseA (10mg/ml) and incubated at 37°C for 1hour. The RNaseA treatment was followed by addition of 300 µl phenol/chloroform/isoamylalcohol (25:24:1) precipitation (Cheng *et al.,* 2003) and centrifugation for 15 minutes at 10 000 rpm in a Joan micro-centrifuge. To the supernatant, 100 µl of 5 M NaCl and 400 µl water-saturated ether were added followed by centrifugation for 10 minutes at 10 000 rpm. The bottom phase was

collected and two volumes of pre-chilled 95 % ethanol was added, which was followed by incubation at 4 °C until the DNA strand is appeared. The precipitated mixture was centrifuged at 5000rpm for three minutes and the formed pellet was washed with 70 % ethanol until a colourless pellet was obtained. The pellet was dried by speed vacuum (SC110, New York) and then resuspended in 100 µl of sdH_2O. The DNA was kept at -20°C until use.

5.2.4 DNA quantification

To determine the quality and amount of isolated DNA, samples of isolated DNA 5 µl were run on a 1 % agarose gel in TAE buffer (0.04 M Tris-acetate; 1 mM EDTA, pH 8) as outlined by Sambrook *et al.* (1989). After staining, the gel with ethidium bromide for 15 minutes, a 100 base pair marker (1 kb Mass ruler DNA ladder mix) was used to determine the quantity. DNA quality was determined on a white/UV-transilluminator and photographed.

5.2.5 RAPD analysis

For RAPD amplification, 15 arbitrary nucleotide primers (see Table 5.1) were initially screened for amplification and polymorphism; those that gave reproducible and distinct banding patterns were selected. All the primers used were random sequence primers with GC contents between 60 and 70 %.

Table 5.1 List of 13 RAPD primers screened for amplification and polymorphism

Primer code	Nucleotides Sequence (5' to 3')	% G+C	Melting/ annealing temperature (T_m) $T_{m=}$ 4(G+C) + 2(A+T)
OPA 02	TGCCGAGCTG	70	34
OPA 03	AGTCAGCCAC	60	32
OPA 04	AATCGGGCTG	60	32
OPA 05	AGGGGTCTTG	60	32
OPA 06	GGTCCCTGAC	70	34
OPA 07	GAAACGGGTG	60	32
OPA 08	GTGACGTAGG	60	32
OPA 09	GGGTAACGCC	70	34
OPA 10	GTGATCGCAG	60	32
OPA 11	CAATCGCCGT	60	32
OPA 12	TCGGCGATAG	60	32
OPA 13	CAGCACCCAC	70	34
OPA 14	TCTGTGCTGG	60	32

RAPD analyses were carried out with varying concentrations of $MgCl_2$ (1.5 mM, 2 mM and 4 mM), *Taq* DNA polymerase (1 U, 1.25 U and 2 U) and DNA template (50-200 ng) to optimise PCR conditions. Two different brands of *Taq* were used, namely Ex *Taq* (Takara, Japan) and recombinant *Taq* polymerase (Fermentas, Germany). In the optimised PCR, RAPD reactions were performed in a total volume of 25 µl in sterile 0.2

ml eppendorf tubes containing 10×PCR buffer (10 mM Tris-HCl, pH 8.0), 2 mM dNTPs, 5 U *Taq* polymerase, 0.5 μM primers and 200 ng template DNA. For the initial primer screening, a reaction mixture contained 1.5 mM 1X buffer, 0.2 mM dNTPs, 0.5 μM primer, 2 U *Taq* and DNA template. To reduce the PCR artifact or possibility of cross contamination and pipetting errors for each primer, a reaction master mixture (Table 5.2) of all the reagents except the genomic DNA was used and then aliquoted. The manipulation of RAPD reaction was done on ice.

Table 5.2 Composition of the PCR reaction mixture for 25 μl reaction volume

Components	Stock concentration	Final concentration	Volume per reaction
PCR buffer	10 mM	1mM	2.5
$MgCl_2$	25 mM	4 mM	4
Taq polymerase	5 U/ μl	2 U	0.4
dNTPs	2 mM	0.2 mM	2.5
Primer	20 μM	0.5 μM	1
Water			12.6
DNA		50-200 ng	2

The best concentration of $MgCl_2$ was selected based on the number, sharpness and the intensity of bands. Different annealing temperatures (30°C, 37°C, and 40°C) and alternations of the program were made until best amplification and polymorphism were obtained. The following program was used on a Gene Ampli PCR systems 9 700 thermocycler (Applied biosystems, USA): An initial pre-denaturation step at 94°C for

120 seconds, followed by 30 cycles denaturation (94°C for 15 seconds), annealing (40°C for 30 seconds) and extension (72°C for 80 seconds) with final extension (72°C for 420 seconds). After completion of the amplification cycles, the reactions were held at 4°C until the products were used. The reaction was repeated at least once to ensure reproducible banding patterns.

5.2.6 Agarose gel electrophoresis

The first step in agarose gel electrophoresis was to combine 12.5 µl of PCR products with 3 µl of loading buffer (30 % glycerol and 0.25 % bromophenol blue). The amplification products were separated on 2 % (w/v) agarose gel submerged in 1×TAE buffer (100 mM Tris-acetic acid and 0.5 M EDTA, pH 8). Ethidium bromide (10 mg/ml) was added to the gel for visualisation of DNA bands. A 1kb mass ruler DNA ladder was used to estimate the size of RAPD bands. Electrophoresis was conducted at a voltage of 90 V for 40 minutes. A photograph of the gel was taken with a Polaroid camera under Ultraviolet (UV) light on an UV transilluminator.

5.2.7 Data Analysis

The polymorphism was analysed using RAPD bands. The reproducible, polymorphic and monomorphic bands were visually scored as present (1) or absent (0). Faint reproducible RAPD bands were also scored as present. The total number of bands per line for each primer was recorded and the percentages of polymorphic band were determined and entered.

5.3 RESULTS

5.3.1 DNA yield and quality

Enough DNA (800-1 400 ng) was extracted using CTAB method of genomic DNA isolation from ten *V. galamensis* lines (see Figure 5.1). The gel photograph shows some RNA contamination at bottom of DNA lanes with some small fragments of degraded DNA in the form of smears.

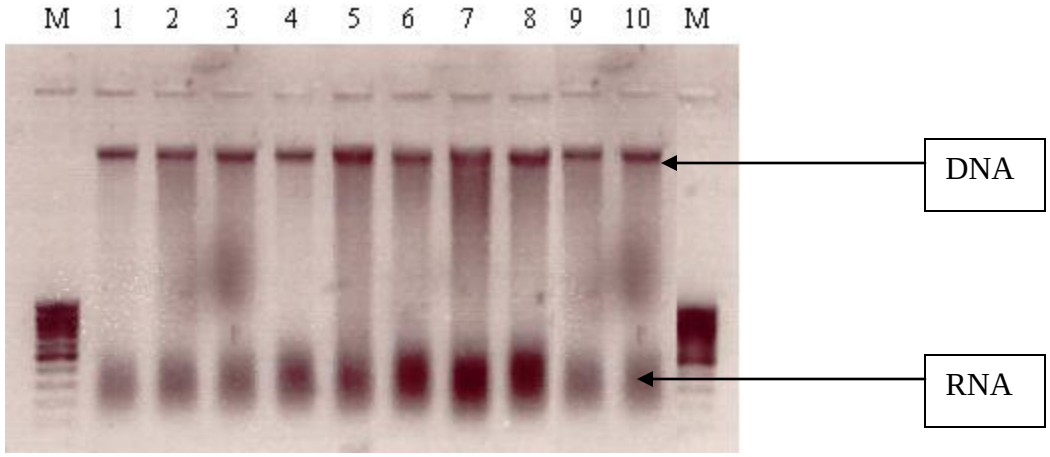

Figure 5.1: DNA isolated with the CTAB method of DNA isolation from ten selected *Vernonia galamensis* lines ran on a 1 % agarose gel electrophoresis. Lanes: 1: Vge-5; 2: Vge-9; 3: Vge-14; 4: Vge-16; 5: Vge-1; 6: Vge-20; 7: Vge-23; 8: Vge-27; 9: Vge-32; 10: Vge-36.

5.3.2 Optimisation of RAPD conditions for amplification

A total of 13 random primers were initially screened for amplification of DNA from *V. galamensis*. Individual DNA samples isolated from lines were subjected to a set of 13 primers, resulting in a total of 130 reactions, and 10 µl of the PCR products ran on a 2 % agarose gel. The effect of $MgCl_2$, type and amount of *Taq* polymerase, and annealing temperature were also examined in the process. Amplifications were obtained using 2 U of recombinant *Taq* DNA polymerase. The nucleotide primers amplified at 37 °C annealing temperature. From the 13 primers screened, only three, OPA 10, OPA 11 and

OPA 12, produced RAPD amplification products with line 4 (Vge-16) (Figure 5.2). OPA 10 produced three bright and clear bands compared to all other primers. OPA 11 produced one bright band, while OPA 12 produced faint single. OPA 10 was then selected as the best primer for amplification across all the 10 DNA samples (Figure 5.3).

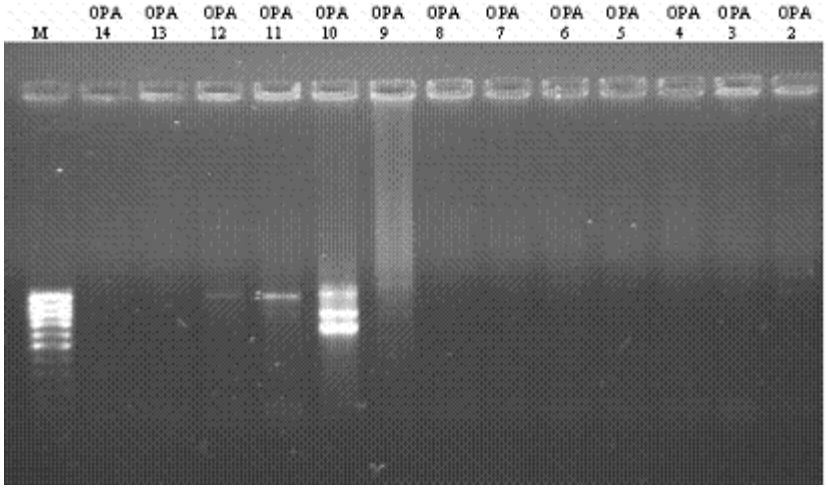

Figure 5.2: RAPD assays conducted with the DNA isolated from line 4 (Vge-16) by CTAB method representing 13 screened primers OPA 2 – 14 with lane 1 showing the mass ruler (marker).

Only three DNA samples did not amplify (Lines 7, 8 and 9) with OPA 10 whereas the remaining seven DNA samples were amplified. A total of 22 RAPD bands were produced by OPA 10 across all the ten selected vernonia lines including faint with 3.14 bands per line averaged across the ten lines. The DNA sample isolated from Vge-16 produced three bright and two faint RAPD bands totaling five, whereas Vge-5, Vge-9, Vge-1, Vge-20 and Vge-36 produced one faint upper band and two brighter bands at the bottom. On the other hand Vge-14 produced a total of three bands with the middle one fainted unlike the latter lines.

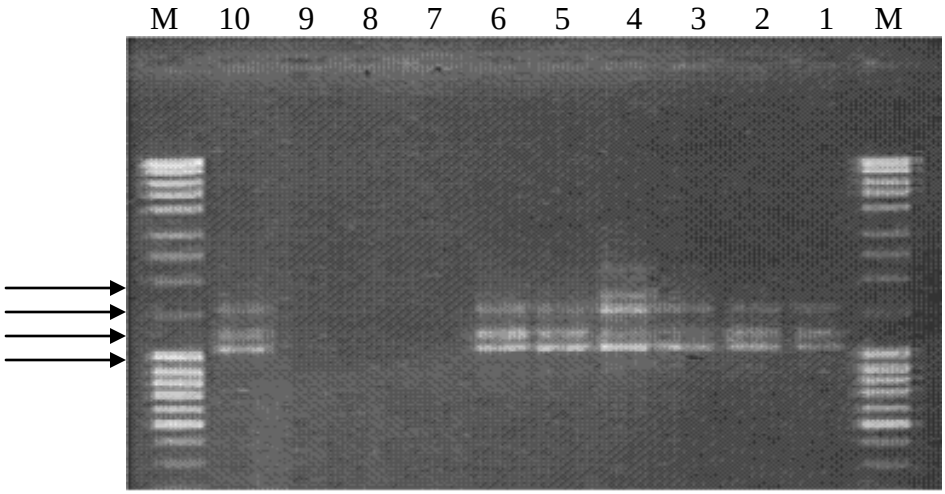

Figure 5.3: RAPD assays conducted with the DNA samples isolated from ten selected vernonia lines by CTAB method of genomic DNA isolation, where M represents the molecular mass ruler. Lanes: 1: Vge-5; 2: Vge-9; 3: Vge-14; 4: Vge-16; 5: Vge-1; 6: Vge-20; 7: Vge-23; 8: Vge-27; 9: Vge-32; 10: Vge-36.

5.4 DISCUSSON

The modified CTAB method of DNA isolation was able to extract or isolate enough DNA that can be involved in the RAPD analysis and this was supported by Aras *et al.* (2003) as they reported this method as the best for high quality DNA for PCR reactions compared to other methods of DNA isolation from dry plant material. This study showed that there is little genetic variation among ten selected vernonia lines, as shown by four genetically varying groups from ten selected lines. OPA 10 revealed polymorphism for only one line (Vge-16) while others displayed non-polymorphism. The results therefore suggest that there is polymorphism among these lines by RAPD analysis. Most of the lines tested produced equal number of bands on average but the bands are of different brightness, which might be due to the DNA quality, meaning that the CTAB method of DNA extraction does not yield DNA samples of the same quality. The majority of lines would therefore display slightly the same characteristics or behaviours. Baye (2004)

reported the overall genetic diversity index for all traits of 0.76 using Shannon-Weaver Diversity Index (H′), which means that there is high genetic diversity among different vernonia accessions. It was further reported that the majority of the genetic diversity, 89 and 95 %, was observed within the region of origin and altitudinal group, respectively. The lack of high polymorphism in the present study might have been as a result of methods and selection of primers employed in determining the genetic diversity for this crop. For instance, method of DNA isolation i.e. CTAB, even though it proved that it was good for other crops, might have not been the best method for vernonia DNA isolation. Other contributing factors could be the handling and storing of DNA samples as well as PCR products according to Terzia *et al.* (2005). The set of primers used in this study might have also been inappropriate for amplifying DNA isolated from *V. galamensis.*

5.5 CONCLUSIONS

This analysis showed that there is less genetic diversity in *Vernonia galamensis* lines as shown by the resulting four different groupings from ten selected lines. This suggests that for a given number of vernonia lines tested for polymorphism about 40 % would result in polymorphism or genetic variations. The DNA isolated by the CTAB method could still be exploited for further manipulations by much improved or modified RAPD analysis. This study shows that among the primers tested only one gave a satisfactory amplification product. However, these primers could not satisfactorily differentiate between the different lines. The vernonia lines used in this study might been genetically close that the RAPD was not a suitable technique to find polymorphism.

6. SUMMARY OF THE STUDY

The results from the morphological diversity analysis showed considerable variations among the studied traits. This is a positive indication to explore the crop for improvements and also to select the best line for a given agro-ecological zone. Four promising lines were selected viz. Vge-16, Vge-20, Vge-27 and Vge-32 using morphological, seed oil content and RAPD markers. However, vernonia seeds displayed less diversity in seed oil content and various fatty acid profiles. Most of this characters showed that their degree of relatedness were insignificant at $P < 0.01$ except for vernolic acid and palmitic acid. The higher percentage composition of vernolic acid makes this crop even more adoptable to the this region because small farmers and commercial industries can be able to produce more earnings by taking advantage of the growing demand of renewable resource as the reliance on fossil fuel is declining due to its negative impact on the environment. Results from RAPD analysis showed that there is less genetic diversity among the selected ten vernonia lines. The results from the morphological, the seed composition and the RAPD analyses suggest that there is enough genetic diversity among different *Vernonia galamensis* lines that can be exploited in marker assisted selection for production of well adapted lines or accessions.

7. FUTURE RESEARCH

There is a need for further research to evaluate morphological variation in *Vernonia galamensis* across different locations in Limpopo province, extending to other parts or provinces of South Africa. Varying cultural practices should also be assessed in the evaluation of this crop in order to come up with a better practice which is cost effective and environment friendly. This is because only one location was considered in the present study and Limpopo province is characterised by varying climatic conditions ranging from dry lands to wet lands and therefore evaluation of this crop from one location would not be sufficient to conclude about the its adaptability in this province. In-depth genetic diversity analysis should be done to find out about the genetic variation in different vernonia lines and accessions, and also to develop DNA markers for this crop. These can be accomplished by employing more complex molecular DNA techniques such as ALFP and SSR primers. Working conditions should also be well chosen so that more reliable genetic information is made available for better manipulation of *Vernonia galamensis* as these will lead to improved selection process, which is the first step in every breeding programme.

8. REFERENCES

Abbott TP, Dierig DA, Foster M, Nelson JM, Coates W, Frykman HB, Carlson KD and Arquette JD, 1997. Status of lesquerella as an industrial crop. Information 8:1169-1175.

Angelini LG, Moscheni E, Clonna G and Belloni P, 1997. Variation in agronomic characteristics and seed oil composition of new oilseed crops in central Italy. Industrial Crops Production 6:313–323.

AOCS, 1999. Physical and Chemical Characteristics of Oils, Fats and Waxes. In: Firestone D. (Ed.), American Oil Chemist's Society Press, Champaign, Illinois pp 84–85.

Aras S, Duran A and Yenilmez G, 2003. Isolation of DNA for RAPD analysis from dry leaf material of some *Hesperis* L. specimens. International Society for Plant Molecular Biology 21**:**461a–461f.

Archak S, Gaikwad B, Gautam D, Rao V, Swamy M and Karihaloo L, 2003. Comparative assessment of DNA fingerprinting techniques (RAPD, ISSR and AFLP) for genetic analysis of cashew (*Anacardium occidentale* L.) accessions of India. Genome 46:362-369.

Ayorinde FO, Osman, JG, Shepard RL and Powers FT, 1988. Synthesis of azelaic acid and suberic acid from *Vernonia galamensis* oil. Journal of American Oil Chemist's Society 65:1774–1776.

Ayorinde FO, Power FT, Shepard LD and Tabir DN, 1989. Synthesis of dodecanedioc acid from *Vernonia galamensis* oil. Journal of American Oil Chemist's Society 66:690–692.

Ayorinde FO, Butler BD and Clayton MT, 1990a. *Vernonia galamensis*: a rich source of epoxy acid. Journal of American Oil Chemist's Society 67:844–845.

Ayorinde FO, Carlson KD, Pavlik RP and McVety J, 1990b. Pilot plant extraction of oil from *Vernonia galamensis* seed. Journal of American Oil Chemist's Society 67:512–518.

Ayorinde FO, Nwaonicha CP, Parchment VN, Bryant KA, Massan M and Clayton MT, 1993. Enzymatic synthesis and spectroscopy characterisation of 1.3-divernoloylglycerol from *Vernonia galamensis* seed oil. Journal of American Oil Chemist's Society 70:129–132.

Bafor ML, Jonsson AK, Stobart and Stymne S, 1990. Regulation of triacylglycerol biosynthesis in embryos and microsomal preparations from the developing seeds of *Cuphea lanceolata*. Biochemistry Journal 272:31–38.

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, 1997. Vernonia galamensis, a new industrial oilseed crop for Ethiopia, Bursting with Potentials. Alemaya University of Agriculture, Newsletter 2:15–18.

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, United Kingdom pp 49–53.

Baye T, Kebede H and Belete K, 2001. Agronomic evaluation of *Vernonia galamensis* germplasm collected from Eastern Ethiopia. Industrial Crops Production 14:179–190.

Baye T and Gudeta S, 2002. Reprinted from: Trends in new crops and new uses. Janick J and Whipkey A (eds.). Pest Survey of *Vernonia galamensis* in Ethiopia. American Society of Horticultural Science Press, Alexandria, Virginia.

Baye T, 2004. Exploration, Genetic Diversity and Seed Quality Analyses in Ethiopian Populations of Vernonia galamensis. Cuvillier Verla. Intec Broadband Communication Networks 3-89873-970-8.

Baye T and Ling W, 2004. Natural outcrossing rate in *Vernonia galamensis*. In Plant Breeding 123:398-399.

Baye T, Witzke-Ehbrecht SV and Becker HC, 2004. Genetic diversity in *Vernonia galamensis*, a new potential crop for dry regions. Vorträge für Pflanzen-züchtung 64:181-183.

Baye T and Becker H, 2005a. Genetic variability and interrelationship of traits in the industrial oil crop *Vernonia galamensis.* Euphytica 142:119–129.

Baye T and Becker H, 2005b. Exploration of *Vernonia galamensis* in Ethiopia, and variation in fatty acid composition of seed oil. Genetic Resources and Crop Evolution 52:805–811.

Baye T, Becker H and Witske-Ehbrecht S, 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.

Bhardwaj HL, Hamama AA, Rangappa M and Dierig DA, 2000. Vernonia oilseed production in the mid-Atlantic region of the United States. Industrial Crops and Products 12:119–124.

Breyne P, Boerjan W, Gerats T, Van Montagu M and van Gysel A, 1997. Applications of AFLP in plant breeding, molecular biology and genetics. Belgium Journal of Botany 129:107–117.

Brown S, Hopkins M, Mitchell S, Senior L, Wang T, Duncan R, Gonzalez-Candelas F and Kresovich S, 1996. Multiple methods for the identification of polymorphic simple sequence repeats (SSRs) in sorghum [*Sorghum bicolor* (L.) Moench]. Theoretical and Applied Genetics 93:190–198.

Bulle B, Millon L, Bart LM, Gallego M, Gambarelli F, Portus M, Schnur L, Jaffe CL, Fernandez-Barredo S, Alunda JM and Piarroux R, (2002). Practical Approach for Typing Strains of *Leishmania infantum* by Microsatellite Analysis. Journal of Clinical Microbiology 40 (9):3391–3397.

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.

Caetano-Anolles BG, Bassam BJ and Gresshof PM, 1991. DNA amplification fingerprinting using very short arbitrary oligonucleotide premers. Bio/Technology 9:553-557.

Carlson KD, Schneider WJ, Chang SP and Princen LH, 1981. *Vernonia galamensis* seed oil: A new source for epoxy coatings. p 297-318. In: Pryde EH, Princen LH and Mukherjee KD (eds.). New sources of fats and oils. American Oil Chememist's Society Champaign, Illinois.

Chague V, Mercier JC, Guenard M, de Courcel A and Vedel F, 1996. Identification and mapping on chromosome 9 of RAPD markers linked to Sw-5 in tomato by bulked segregant analysis. Theorical and Applied Genetics 92:1045-1051.

Chao SC, Sharp PJ, Worldland AJ, Warham EJ, Koebner RMD and Gale MD, 1989. RFLP-based genetic maps of wheat homologous group 7 chromosomes. Genetics. 78:495–504.

Cheng J, Fu JL and Zhou ZC, 2003. The inhibitory effects of manganese on steroidogenesis in rat primary Leydig cells by disrupting steroidogenic acute regulatory (StAR) protein expression. Toxicology 187:139–148.

Choumane W and Heizmann P, 1988. Structure and variability of nuclear ribosomal genes in the genus *Helianthus*. Theoretical and Applied Genetics 76:481-489.

Corazza-Munnes M, Machado M, Nunes W, Cristofani M and Targon M, 2002. Assessment of genetic variability in grapefruits (*Citrus paradise* Macf.) and Pummelos (*C. maxima* (Burn) Merr) using RAPD and SSR markers. Euphytica 126:169-176.

Coulibaly S, Pasquet RS, Papa R and Gepts P, 2002. AFLP analysis of the phenetic organisation and genetic diversity of Vigna unguiculata L. Walp. reveals extensive gene flow between wild and domesticated types. Theoretical and Applied Genetics 104:358–366.

Cuperus FP and Derksen JTP, 1996. High Value-Added Applications from Vernolic Acid. In: Janick J (ed.), Progress in new crops. American Society of Horticultural Science, Press, Alexandria, Virginia pp 354-356.

Dasardhi P, Neelakantan P, Rao SJ, and Bhalerao UT, 1991. The oxidation of bombykol to bombykal. Synthetic Communication 21:183.

Devas KM and Gale MD, 1992. The use of RAPD markers in wheat. Theoretical and Applied Genetics 84:567-572.

Dierig DA and Thompson AE, 1993. *Vernonia* and *Lesquerella* potential for commercialisation. In: Janick J and Simon JE (eds.). New crops. Wiley, New York p 362-367.

Dierig DA, Thompson AE, Rebman JP, Kleiman R, and Phillips BS, 1996a. Collections and evaluation of new *Lesquerella* and *Physaria* germplasm. Industrial Crops Production 5:53-63.

Dierig DA, Coffelt TA, Nakayama FS, and Thompson AE, 1996b. Lesquerella and vernonia: oilseeds for arid lands. In: Janick J (ed.), Progress in new crops. American Society for Horticultural Science Press, Alexandria, Virginia pp 347-354.

Dirlikov SK, Frischingr I, Islam MS and Graham J, 1990. *Vernonia* oil: A new reactive monomer. Polymer Material Science and Engineering 62:217–221.

Doyle JJ and Doyle JI, 1990. Isolation of plant DNA from fresh tissue. Focus 12:13-15.

Earle FR, 1970. Epoxy oils from plant seeds. Journal of American Oil Chemist's Society 47:510–513.

Ehlers JD and Hall AE, 1997. Cowpea (*Vigna unguiculata* L. Walp.). Field Crops Research 53:187–204.

Erasmus U, 1993. Fats that heal fats that kill. Canada. Alive Books.

Erickson DR, Pryde EH, Brekke OL, Mounts TL and Falb RA, 1980. Handbook of soy oil processing and utilisation. American Soybean Association and the American Oil Chemist's Society. St. Louis, Missouri and Champaign, Illinois.

Fall L, Diouf D, Fall-Ndiaye MA, Badiane FA and Gueye M, 2003. Genetic diversity in cowpea [*Vigna unguiculata (L.)*Walp.] varieties determined by ARA and RAPD techniques. African Journal of Biotechnology 2:48–50.

Faroog S, Shah TM and Arif M, 1995. Utilisation of RAPD markers for the identification of cultivated and wild rice species. Pakistan Journal of Botany 27:127-138.

Fatokun CA, Danesh D, Young ND and Stewart E, 1993. Molecular taxonomic relationships in the genus *Vigna* based on RFLP analysis. Theoretical and Applied Genetics 86:97-104.

Fatokun CA, Mignouna HD, Knox MR and Ellis THN, 1997. AFLP variation among cowpea varieties. In: Agronomy. Abstracts. American Soybean Association, Madison, Wisconsin p 156.

Fernandez-Martınez J, del Rio M and de Haro A, 1993. Survey of safflower (*Carthamus tinctorius* L.) germplasm for variants in fatty acid composition and other seed characters. Euphytica 69:115–122.

Ferdinandez Y and Coulman B, 2002. Evaluating genetic variations and relationships among two bromegrass species and their hybrid using RAPD and AFLP markers. Euphytica 125:281-291.

Folch J, Lees M, and Sloane-Stanley GH, 1957. A simple method for the isolation and purification of total lipids from animal tissue. Journal of Biological Chemistry 226:497-509.

Frankel OH and Bennett E, 1997. Genetic resources in plantstheir exploration and conservation. International Biological Programme Handbook II. Oxford: Blackwell Scientific Publications.

Gentzbittel L, Perrault A and Nicolas P, 1992. Molecular phylogeny of the *Helianthus* genus, based on nuclear restriction fragment length polymorphism (RFLP). Molecular Biology 9:872-892.

Gilbert MG, 1986. Notes on East African *Vernonieae* (Compositae). A revision on the *Vernonia galamensis* complex. Kew Bulletin 41:19-35.

Gilbert KG, Garton S, Karam MA, Arnold GM, Karp A, Edwards KJ, Cooke DT and Barker JHA, 2002. A high degree of genetic diversity is revealed in *Isatis spp* by amplified fragment length polymorphism (AFLP). Theoretical and Applied Genetics 104:1150-1156.

Godwin ID, Aitken EAB and Smith LW, 1997. Application of inter simple sequence repeat (ISSR) markers to plant genetics. Electrophoresis 18:1524–1528.

Grist SA, Firgaira FA and Morley AA, 1993. Dinucleotide repeat microsatellite loci from *Elymus alaskanus* and length polymorpolymorphisms isolated by the polymerase chain reaction. Bio phism in several *Elymus* species (*Triticeae*: *Poaceae*). Genome 41(15):304–309.

Groot P, 1990. A new industrial crop for developing countries. Oil Crops Newsletter, International Development Research Centre 8:24–26.

Gunstone FD, 1954. Fatty acids, Part II. The nature of the oxygenated acids present in *Vernonia anthelmintica* (Willd.) seed oil. Journal of Oil Chemist's Society, London pp 1611-1616.

Gunstone FD, 1993. The study of natural epoxy oils and epoxidised vegetable oils by 13C nuclear magnetic resonance spectroscopy. Journal of Oil Chemist's Society 70:1139-1144.

Gupta PK, Varshney RK, Sharma PC and Ramesh B, 1999. Molecular markers and their applications in wheat breeding. Plant Breeding 118:369–390.

Harborne JB and Williams CA, 1977. Vernonieae: chemical review. In: Heywood BL and Turner JL (Eds.), The Biology and Chemistry of Compositae. Academic Press, London pp 523–535.

Harlt L, Mohler V, Zeller FJ, Hsam SLK and Schweizer G, 1999. Identification of AFLP markers closely linked to the powdery mildew resistance genes Pm1c and Pm4a in common wheat (*Triticum aestivum*). Genome 42:322–329.

Harrington TC and Wingfield BD, 1995. A PCR-based identification method for species of *Armillaria*. Mycology 87:280–288.

Haumann BF, 1991. Work continues on new oils for industrial use. Information 2:678-692.

Haymer DS, 1994. Arbitrary (RAPD) primer sequences used in insect studies. Insect Molecular Biology 3(3):191.

Higgins JJ, 1968. *Vernonia anthelmintica*: a potential seed oil sources of epoxy acid. I. Phenology of seed yield. Agronomy Journal 60:55–58.

Higgins M, 1984. Synthesis regulation of major protein seeds. Annual Review of Plant Physiology (35):191-221.

Hill J, Becker HC and Tigerstedt PMA, 1998. Quantitative and Ecological Aspects of Plant Breeding. Chapman and Hall, London.

Hiruy B and Getinet A, 1990. Suitable areas of oil crops production in Ethiopia, IAR. Addis Ababa, Ethiopia.

Holland JB, Nyquist WE and Cervantes-Martinez CT, 2002. Estimation and interpreting heritability for plant breeding: An Update.in Plant Breeding Revision 22:9–112.

Igbal MJ, Paden DW and Rybwn AL, 1995. Assessment of genetic relationship among rhododendron species, varieties and hybrids by RAPD analysis. Horticultural Science 63:215-223.

Isaacs SM, Manivannan N and Muralidharan V, 2003. Genetic diversity analysis using rapd marker in inbred lines of sunflower (*Helianthus annuus* L.). HELIA 26(39):59-66.

Jatoi SA, Kikuchi A, Yi SS, Naing KW, Yamanaka S, Watanabe JA and Watanabe KN, 2006. Use of rice SSR markers as RAPD markers for genetic diversity analysis in Zingiberaceae. Research communication, Breeding Science 56:107-111.

Johnson HW, Robinson HF and Comstock RE, 1955. Estimates of genetic and environmental variability in soybeans. Agronomy Journal 47:314–318.

King JW, Mohamed A, Taylor SL, Mebrahtu T and Paul C, 2001. Supercritical fluid extraction of *Vernonia galamensis* seeds. Industrial Crops 14:241-249.

Kleiman R, Smith CR (Jr.), Yates SG and Jones Q, 1965. Search for new industrial oils. XII. 58 Euphorbiaceae oils, including one rich in vernolic acid. Journal of American Oils and Chemistry Society 42:169-172.

Knapp SJ, 1990. New temperature oilseed crops. In: Janick J and Simon JE (Eds), Advances in new crops. Proclaim of the first National Symposium. New Crops: Research, Development, Economics', Timber Press, Portland Oregon, United States of America pp 203-210.

Krawczak M and Schmidtke J, 1994. Introduction in DNA fingerprinting. Bio scientific publishers Ltd, Oxford, United Kingdom pp 1-16.

Li CD, Fatokum CA, Ubi B, Singh BB and Scoles G, 2001. Determining genetic similarities among cowpea breeding lines and cultivars by microsatellite markers. Crop Science 41:189–197.

Liu L, Hammond, EG and Nikolau BJ, 1998. In vivo studies of the biosynthesis of vernolic acid in the seed of *Vernonia galamensis*. Lipids 33:1218–1221.

Mackil DJ, 1995. Classifying Japonica rice cultivars with RAPD markers. Crop Science 35:889-894.

Mandal S, Yadav S, Singh R, Begum G, Suneja P and Singh M, 2002. Correlation studies on oil content and fatty acid profile of some cruciferous species. Genetic Resource Crop Evolution 49:551–556.

Massey JH, 1971. Harvesting *Vernonia anthelmintica* (L.) Willd. to reduce seed shattering losses. Agronomy Journal 63:812.

McGregor C, Lambert C, Geryling M, Louw J and Warnich L, 2000. A comparative assessment of DNA fingerprinting techniques (RAPD, ISSR, AFLP and SSR) in tetraploid potato (*Solanum tuberosum*) germplasm. Euphytica 13:135-144.

Mharapara IM and Mugabe FT, 1991. Development of *Vernonia* in Zimbabwe, Chiredzi Research Station report. Unpublished.

Mignouna HD, Ng HD, Ikca J and Thottapilly G, 1998. Genetic diversity in cowpea as revealed by random amplified polymorphic DNA. Journal of Genetics and Breeding 52:151–159.

Mikolajczak KL, Miwa TK, Earle FR and Wolff IA, 1961. Search for new industrial oils. Part V. Oils of Cruciferae. Journal of American Oils and Chemistry Society 38:678-681.

Milan P, Hayashi H and Appezzato-da-Glória B, 2006. Comparative leaf morphology and anatomy of three asteraceae species. Brazilian Archives of Biology and Technology. An International Journal 49(1):135-144.

Millar AA, Wrischer M and Kunst L, 1998. Accumulation of very-long-chain fatty acids in membrane glycerolipids is associated with dramatic alterations in plant morphology. Plant Cell 10:1889–1902.

Mohan M, Nair S, Bhagwat A, Krishna TG, Yano M, Bhatia CR and Sasaki T, (1997). Genome mapping, molecular markers and marker-assisted selection in crop plants. Molecular Breeding 3:87–103.

Morales MA, Chicharro C, Ares M, Canavate C, Barker DC and Alvar J, 2001. Molecular tracking of infections by *Leishmania infantum*. Transactions of the Royal Society of Tropical Medicine and Hygiene.

Moran GF and Hopper SD, 1987. Conservation of the Genetic Resources of Rare and Widespread Eucalypts in Remnant Vegetation. In: Saunders DA, Arnold GW, Burbridge AA and Hopkins AJ,1987 (eds.). Nature Conservation: The Role of Remnants of Native Vegetation. Surrey Beatty & Sons Pty Ltd and others, Norton, New South Whales.

Muuse BG, Cuperus FP and Derksen JTP, 1992. Composition and physical properties of oils from new oilseed crops. Industrial Crop Production 1:57-65.

Naliaka S, 1990. Study of the wood anatomy of the *Vernonia leopoldii* complex with the implication of its taxonomy. M.Sc Thesis, Alemaya University of Agriculture, Addis Ababa, Ethiopia.

Nyquist WE, 1991. Estimation of heritability and prediction of selection response in plant populations. Critical Review of Plant Science 10:235–322.

Ochsenreither S, Kuhls K, Schaar M, Presber W and Schönian G, 2006. Multilocus microsatellite typing as a new tool for the discrimination of *Leishmania infantum* MON-1 strains, Journal of Clinical Microbiology 44:495–503.

Ologunde MO, Ayorinde FO and Shepard RL, 1990. Chemical evaluation of defatted *Vernonia galamensis* meal. Journal of American Oil Chemist's Society 67:92–94.

Ovesna J, Polakova K, Lisova L (2002). DNA analyses and their applications in plant breeding. Czech Republican Journal of Genetics and Plant Breeding 38:29-40.

Palombi M and Damiano C, 2002. Comparison between RAPD and SSR molecular markers in detecting genetic variations in kiwifruit (*Actinida deliciosa* A. chev.). Plant Cell Reproduction 20(11):1061-1066.

Panella L and Gepts P, 1992. Genetic relationships within *Vigna unguiculata* (L.) Walp. Based on isonzyme analyses. Genetic Research and Crops Evolution 39:71–88.

Pascual-Villalobos MJ, Ortiz JM and Correal E, 1993. Morphometric characterization of seeds of *Euphorbia lagascae*. Seed Science and Technology 21:53–60.

Pasquet RS, 1993. Variation at isoenzyme loci in wild *Vigna unguiculata* (L.) Walp. (Fabaceae, Phaseoleae). Plants System Evolution 186:157–173.

Pasquet RS, 2000. Allozyme diversity of cultivated cowpea *Vigna unguiculata* (L.) Walp. Theoretical and Applied Genetics 101:211–219.

Patterson HBW, 1989. Handling and storage of oilseeds, oils, fats and meal. Elsevier Applied Science, London pp112-113.

Perdue RE, Carlson KD and Gilbert MG, 1986. *Vernonia galamensis* potential new crop source of epoxy acid. Economical Botany 40:54-68.

Perdue RE, 1988. Systematic botany in the development of *Vernonia galamensis* as a new industrial oilseed crop for the semi-arid tropics. Symbolae Botanicae Upsaliensis 28:125–135.

Perdue RE (Jr.), Jones E and Nyati CT, 1989. *Vernonia galamensis*: a Promising New Industrial Crop for the Semi-arid Tropics and Subtropics pp 197-207. In: Wickens GE, Haq N and Day P (eds.), 1986. International Symposium on New Crops for

Food and Industry, University of Southampton, England, Chapman and Hall, New York.

Polunin NUC, 1983. Marine "genetic" resources and the potential role of protected areas in conserving this. *Environmental Conservation* 10(1):31-41.

Princen LH and Rothfus JA, 1984. Development of new crops for industrial raw materials. Journal of American Oils and Chemistry Society 61:281-289.

Rasmuson M, 2002. The genotype-phenotype link. Heredity 136:1-6.

Riesberg LH, Soltis DE and Palmer JD, 1998. A molecular re-examination of introgression between *Helianthus annuus* and *Helianthus bolanderi* (Compositae). Evolution 42:227-238.

Robbelen G, 1987. Development of new industrial crops. Fat Science and Technology 89:563–570.

Robinson H, 1999. Revisions in paleotropical Vernonieae (Asteraceae). Proclaimed by Biology Society, Washington 112:220–246.

Rucker B and Robbelen G, 1995. Development of high oleic acid rapeseed. In: Global Change International Research Center (Ed.), Proclaim 9th International Rapeseed Congress, Cambridge, United Kingdom pp 389-391.

Rumyantseva LG and Lemeshev NK, 1994. Current state of hemp breeding in the C.I.S. Journal of International Horticultural Association 1(2):49-50.

Saker M, Nagchtigall M and Kuehne T, 2005. A comparative assessment of DNA fingerprinting by RAPD, SSR and AFLP in genetic analysis of some barley genotypes. Egyptian Journal of Genetics and Cytology 34: 81-97.

Sambrook J, Fritsh EF and Maniatis T, 1989. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor, Cold Spring Harbor Laboratory Press, New York.

SAS institute, 1989. SAS/IML software: Usage and reference. Version 6.

Seiler GJ and Brothers ME, 1999. Oil concentration and fatty acid composition of achenes of *Helianthus* species (*Asteraceae*) from Canada. Economical Botany 53:273–280.

Sharma AD, Gill PK and Singh P, 2002. DNA isolation from dry and fresh samples of polysaccharide-rich plants. Plant Molecular Biology Reporter 20(4):415a-415f.

Smith JSC and Smith OS, 1992. Fingerprinting crop varieties. Advanced Agronomy 47:85-140.

Soldatov KI, 1976. Chemical mutagenesis in sunflower breeding. In: Proceedings 7th International Sunflower Conference pp352–357.

Spencer MM, Ndiaye MA, Gueye M, Diouf D, Ndiaye M and Gresshoff PM, 2000. DNA-based relatedness of cowpea (*Vigna unguiculata* (L.) Walp.) genotypes using DNA Amplification Fingerprinting. Physiology and Molecular Biology of Plants 6:81–88.

Sperling LH and Manson JA, 1983. Interpenetrating polymer networks from triglyceride oils containing special functional groups: a brief review. Journal of American Oils and Chemistry Society 60:1887-1892.

Stanton MA, Stewart JM, Percival AE and Wendel JF, 1994. Morphological diversity and relationships in A-genome cottons, *Gossypium arboretum* and *G. herbaceum.* Crop Science 34:519-527.

Staub JE, Serquen FC and Gupta M, 1996. Genetic markers, map construction, and their application in plant breeding. Horticultural Science 31:729–741.

Steg A, Hindle VA and Yong-Gang L, 1994. By-products of some novel oil seeds for feeding: laboratory evaluation. Animal Feed Science and Technology 50:87-99.

Sweeny PM and Danneberger TK, 1995. RAPD characterisation of *Poa annua* L. population in golf course greens and fairways. Crop Science 35: 889-894.

Taba S, Diaz J, Franco J and Crossa J, 1998. Evaluation of Caribean maize accessions to develop a core subset. Crop Science 38:1378-1386.

Takagi Y and Rahman SM, 1996. Inheritance of high oleic acid content in the seed oil of soybean mutant M23. Theoretical and Applied Genetics 92:179–182.

Tanksley SD, Young ND, Paterson AH and Bonierbale MW, 1989. RFLP mapping in plant breeding: new tools for an old science. Bio/Technology 7:257–264.

Terzia V, Morciaa C, Gorrinia A, Stancaa AM, Shewryb RP and Facciolia P, 2005. DNA-based methods for identification and quantification of small grain cereal mixtures and fingerprinting of varieties. Journal of Cereal Science 41:213–220.

Tesfaye B, 1996. Characterisation and evaluation of *Vernonia galamensis var*. *ethiopica* germplasm collected from eastern Ethiopia. M.Sc Thesis. Alemaya University of Agriculture, Dire Dawa, Ethiopia.

Theimer R R and Mölleken H, 1995. Analysis of the oil from different hemp cultivars - perspectives for economical utilisation. In *Bioresource Hemp*. Nova-Institute, Cologne, Germany 2:536-543

Thomas CM, Vos P, Zabeau M, Jones DA, Nocott KA, Chadwick BP and Jones JDG, 1995. Identification of amplified fragment length polymorphism (AFLP) markers

tightly linked to the tomato *Cf-9* gene for resistance to *Cladosporium fulvum*. Plant Journal 8:786–794.

Thompson AE, Dierig DA and Kleiman R, 1994a. Characterisation of *Vernonia galamensis* germplasm for seed oil content, fatty acid composition, seed weight, and chromosome number. Industrial Crops Production 2:299–305.

Thompson AE, Dierig DA and Kleiman R, 1994b. Variation in *Vernonia galamensis* flowering characteristics, seed oil and vernolic acid content. Industrial Crops Production 3:175–183.

Tosti N and Negri V, 2002. Efficiency of three PCR-based markers in assessing genetic variation among cowpea (*Vigna unguiculata* subsp. *unguiculata*) landraces. Genome 45:268–275.

USDA, 1986. Vernonia. Growing Industrial Materials Fact Sheet, Office of Critical Materials, Washington, D.C.

Velasco L, Fernández-Martínez JM, De Haro A, 1996 The influence of bulk density on the selection for low glucosinolate content in Ethiopian mustard. Eucarpia Cruciferae Newsletter 18:94-95.

Virk S, Zhu J, Newbury H, Bryan G, Jackson M and Ford-Lloyd B, 2000. Effectiveness of different classes of molecular markers for classifying and revealing variations in rice (*Oryza sativa*) germplasm. Euphytica 112:275-284.

Vos P, Hogers R, Bleeker M, Rijans M, van de Lee T, Hornes M, Frijters A, Pot J, Peleman J, Kuiper M, and Zabeau M, 1995. AFLP: a new technique for DNA fingerprinting. Nucleic Acids Research 23:4407-4414.

Watkins C, 1999. Crambe - ready to be a commercial success. Information 10:828-836.

Weising K, Atkinson G and Gardner C, 1995. Genomic fingerprinting by microsatellite-primed PCR: a critical evaluation. PCR Methods and Applications 4:249-255.

Weiss EA, 1983. Oilseed Crops. Tropical Series, Longman Inc. Leonard Hill Books, New York p603.

Welsh J and McClelland M, 1990. Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Research 18:7213-7218.

Williams JGK, Kubelik AP, Livak KJ and Tingey SV, 1990. DNA polymorphism amplified by arbitrary primers as useful as genetic markers. Nucleic Acids Research 18:6531-6535.

Xiao J, Li J, Yuan L, Mccouch S and Tanksley SD, 1996. Genetic diversity and its relationship to hybrid performance and heterosis in rice as revealed by PCR-based markers. Theoretical and Applied Genetics 92:637-643.

Zemanova E, Jirku M,Mauricio IL, Miles MA and Lukes J, 2004. Genetic polymorphism within the *Leishmania donovani* complex: correlation with geographic origin. American Journal of Tropical Medical Hygiene 70:613–617.

Zhang Y, Rajagopalan M, Brown BA and Jr. Wallace RJ, 1997. Randomly amplified polymorphic DNA PCR for comparison of *Mycobacterium abscessus* strains from nosocomial outbreaks. Journal of Clinical Microbiology 35:3132-3139.

Zhao J, 2002. QTLs for oil content and their relationships to other agronomic traits in an European × Chinese oilseed rape population. Doctoral Dissertation, University of Gottingen, Germany pp 60-74.

9. APPENDICES

APPENDIX 9.1 Analysis of variance for seed yield per plant in grams from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value[a]	Pr > F
Line	9	394.546	43.838	35.22**	<.0001
Rep	2	23.137	11.569	9.30**	0.0017
Error	18	22.402	1.245		
Total	29	440.086			

[a] ** = significant at *p<0.01*.

APPENDIX 9.2 Analysis of variance for days to 50 % flowering from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value[a]	Pr > F
Line	9	4785.867	531.763	59.92**	<.0001
Rep	2	160.267	80.133	9.03**	0.0019
Error	18	159.733	8.874		
Total	29	5105.867			

[a] ** = significant at *p<0.01*.

APPENDIX 9.3 Analysis of variance for plant height in centimeters from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value[a]	Pr > F
Line	9	1846.141	205.127	35.97**	<.0001
Rep	2	45.011	22.505	3.95*	0.0379
Error	18	102.649	5.703		
Total	29	1993.801			

[a] ** = significant at $p<0.01$; * = significant at $p<0.05$.

APPENDIX 9.4 Analysis of variance for number primary heads from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F-Value[a]	Pr > F
Line	9	3290.133	365.570	40.11**	<.0001
Rep	2	80.600	40.300	4.42*	0.0274
Error	18	164.067			
Total	29	3534.800			

[a] ** = significant at $p<0.01$; * = significant at $p<0.05$.

APPENDIX 9.5 Analysis of variance for number secondary heads from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F- Value[a]	Pr > F
Line	9	860.300	95.589	12.52**	<.0001
Rep	2	83.267	41.633	5.45**	0.0141
Error	18	137.400	7.633		
Total	29	1080.967			

[a] ** = significant at *p<0.01*

APPENDIX 9.6 Analysis of variance for 200-seed weight in grams from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value[a]	Pr > F
Line	9	8.325	0.925	107.95**	<.0001
Rep	2	0.228	0.114	13.29**	0.0003
Error	18	0.154	0.009		
Total	29	8.707			

[a] ** = significant at *p<0.01*

APPENDIX 9.7 Analysis of variance for seed yield in kilograms per hectare from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean square	F-Value[a]	Pr > F
Line	9	304433.961	33825.996	35.22**	<.0001
Rep	2	17852.860	8926.430	9.30**	0.0017
Error	18	17285.823	960.324		
Total	29	339572.645			

[a] ** = significant at *p<0.01*

APPENDIX 9.8 The analysis of variance table for seed oil content from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	154.643	17.183	1.43 ns	0.3016
Rep	1	2.361	2.360	0.20 ns	0.6682
Error	9	108.203	12.023		
Total	19	265.206			

[a]ns = non significant

APPENDIX 9.9 The analysis of variance for vernolic acid from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	20.750	2.306	1.76 [ns]	0.2066
Rep	1	4.920	4.920	3.75 [ns]	0.0847
Error	9	11.800	1.311		
Total	19	37.471			

[a]ns = non significant

APPENDIX 9.10 The analysis of variance for linoleic acid from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	2.087	0.232	0.58 [ns]	0.7889
Rep	1	0.013	0.013	0.03[ns]	0.8615
Error	9	3.631	0.403		
Total	19	5.731			

[a]ns = non significant

APPENDIX 9.11 The analysis of variance for oleic acid from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	4.113	0.457	8.25*	0.0060
Rep	1	0.606	0.606	6.22*	0.0184
Error	9	0.660	0.073		
Total	19	5.379			

[a] * = significant at $p<0.05$

APPENDIX 9.12 The analysis of variance for palmitic acid from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	0.495	0.055	7.37**	0.0033
Rep	1	0.009	0.009	1.24 ns	0.2947
Error	9	0.067	0.008		
Total	19	0.572			

[a]ns = non significant; ** = significant at $p<0.05$

APPENDIX 9.13 The analysis of variance for stearic acid from ten selected vernonia lines

Source of variation	Degrees of freedom	Sum of squares	Mean squares	F- Value[a]	Pr > F
Line	9	0.603	0.067	2.90 [ns]	0.9772
Rep	1	0.000	0.000	0.00 [ns]	0.0645
Error	9	0.209	0.023		
Total	19	0.811			

[a]ns = non significant