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Full Length Research Paper

Genetic analysis among selected vernonia lines through seed oil content, fatty acids and RAPD DNA markers

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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 and select vernonia lines in Limpopo province through seed oil content, fatty acid content and RAPD DNA markers. Significant differences were observed for the content 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 the 13 RAPD DNA primers screened, primer OPA_{10} amplified DNA samples and resulted in 4 distinct groupings among tested lines. Four promising lines were selected; Vge-16, Vge-20, Vge-27 and Vge-32 using seed oil content, fatty acids and RAPD markers. The lines will be used for strategic breeding of vernonia as an alternative industrial oil crop in Limpopo province of South Africa or other similar environments.

Key words: Limpopo province, fatty acid, RAPD, seed oil, vernonia.

INTRODUCTION

Vernonia (*Vernonia galamensis;* 2n=18) is potentially novel industrial oilseed crop. It is an annual member of the asteraceae widely distributed in Africa. According to Gilbert (1986) the species *galamensis* is recognized to include six subspecies including *galamensis*, *mutomoensis*, *nairobensis*, *afromomntana, gibbosa*, and *lushotoensis*. Subspecies *galamensis* is the most diverse and widely distributed with 4 botanical varieties viz. *galamensis*, *petitiana*, *australis and ethiopica* (Gilbert, 1986). The subspecies *galamensis* and *mutomoensis* are found in areas that receives as little as 200 mm rainfall per year. Higher elevations and areas of high rainfall are the suitable regions for the subspecies *afromontana* and *lushotoensis* (Perdue et al., 1986).

Seeds of vernonia produce triglyceride oil rich in vernolic acid, a naturally epoxidised fatty acid which is environment friendly, less expensive and less viscous compared to other artificial epoxy oils (Thompson et al., 1994a). Vernolic acid composes 72 - 80% of the acids present in the seed oil. Vernonia oil also contains other fatty acids such as linoleic acid (12 - 14%), oleic acid (4 - 6%), stearic acid (2 - 3%), palmitic acid (2 - 3%) and a trace amount of arachidic acid (Carlson et al., 1981).

V. galamensis subsp. *galamensis* variety *ethiopica* M.G. Gilbert was first identified by Perdue in 1964 in eastern Ethiopia along the Harar-Jijiga road at 9°14' N and 42°35' E, 1700 m above sea level (Perdue et al., 1986). Later south and south-eastern Ethiopia was described as a natural habitat of this botanical variety (Gilbert, 1986). A number of studies (Thompson et al., 1994b; Baye et al., 2001; Shimelis et al., 2006) demonstrated the presence of considerable variability in oil and vernolic acid contents in this botanical variety.

Molecular markers are increasingly being used in marker assisted breeding and to determine genetic diversity and relatedness among species and varieties. The

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Table 1. List of test lines, geographic locations and coordinates.

Line	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)

most widely used DNA based molecular markers include restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD), DNA amplification fingerprinting (DAF), amplified fragment length polymerphism (AFLP) and microsatellites or simple sequence repeats (SSR). Unlike morphological markers molecular markers are not influenced by endogenous and exogenous factors (Tanksley et al., 1989). RAPD DNA analysis is widely used for studying the taxonomy of various genera, species, for differentiation of intra-species variation and to study the genetic diversity of various cultivars and lines (Williams et al., 1990).

Production of vernonia as an alternative industrial crop in marginal tropical and subtropical areas by small-scale low input farming systems or commercial farmers could have various advantages. It may help as a source of raw material for agro-processing industries and in the diversification of existing crop husbandry practice, thereby reducing a potential crop failure. The Limpopo province of South Africa is characterized to have semi-arid climate with mean annual rainfall ranging from 300 - 600 mm and a predominantly sandy loam soil with reduced fertility (Thomas, 2003). Thus, climatic and edaphic situations have significant and foreseeable advantages of domesticating vernonia in the province. However, detailed studies are necessary before large scale production of the crop in the province will be possible. The objective of this study was to evaluate selected vernonia lines in Limpopo province through seed oil content and RAPD DNA markers to select promising lines for quality and quantity vernolic acid. Selected lines may be utilized for further strategic breeding of the crop as an alternative industrial oil crop in Limpopo province of South Africa or other similar environments.

MATERIALS AND METHODS

Plant materials

Ten selected vernonia (*V. galamensis* subsp. *galamensis* variety *ethiopica*) lines were used in the study. Lines and their geographic location and coordination of places of original collections are listed in Table 1. The lines were collected from southern and eastern Ethiopia, where it is believed to be the centre of diversity for this botanical variety. Lines were kept homogenous through five cycles of continued selfing and selection.

Study site and experimental design

10 selected vernonia lines were planted for seed oil analysis at Syferkuil, the university of Limpopo's experimental farm. The farm is characterized by hot dry summers and cool dry winters and situated at 23° south and 29° east with 1261.6 m altitude. The mean average day temperature varies from 28 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 field experiment was laid out as a randomised complete block design with 5 replications. The plot size was 3 x 2.4 m with 60 cm inter-row and 60 cm intra-row spacings.

Determination of seed oil content

Oil content was determined on the basis of dry seed weight and 2 replications per entry were analysed. 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. 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 then the original weight. The OC was calculated as a ratio of weight of the oil to its respective sample mass expressed in %.

Determination of fatty acids

The fatty acid composition was determined after transesterification by the addition of tri-methyl sulphonium hydroxide (TMSOH) (Butte, 1983). Fatty acids were quantified using a varian GX 3400 flame ionisation gas chromatograph, with a fused silica capillary column, chrompack CPSIL 88 (100 m length, 0.25 µm ID, 0.2 µm film thicknesses). Column temperature ranged from 40 - 230°C (hold 2 min; 4°C min^{-1}; hold 10 min). Fatty acid methyl esters in hexane (1 µl) were injected into the column using a varian 8200 CX autosampler with a split ratio of 100:1. The injection port and detector were both maintained at 250°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 varian star chromatography software version 4 and relative % composition of fatty acids quantitated as ratios of peak areas.

DNA extraction, quantification and RAPD amplification

A modified CTAB method was used to isolate the DNA according to Doyle (1990). To determine the quality and amount of isolated DNA, samples of isolated DNA 5 µl were ran 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). For RAPD amplification, 13 arbitrary nucleotide primers were initially screened for amplification and polymorphism and all the primers used were random sequences with GC contents of 60 and 70% (Table 2). Primers that gave reproducible and distinct banding patterns were selected.

Table 2. List of 13 RAPD primers screened for amplification and polymorphism.

Primer code	Nucleotide Sequence (5' to 3')	% G+C	Melting/annealing temperature (T_m)
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

$T_m = 4(G+C) + 2(A+T)$.

RAPD PCR reactions were carried out with varying concentrations of $MgCl_2$ (1.5 mM, 2 mM and 4 mM), Taq DNA polymerase (1 , 1.25 and 2 U) and DNA template (1 and 2 µl) to optimise PCR conditions. 2 different brands of Taq were used, namely Ex Taq, Takara and recombinant Taq polymerase, Fermentas. To reduce the PCR artifact or possibility of cross contamination and pipetting errors for each primer, a reaction master mixture of all the reagents except the genomic DNA was used and then aliquoted.

The best concentrations of $MgCl_2$ for further analyses were selected based on the number, sharpness and intensity of bands. Different annealing temperatures (30, 37 and 40°C) and alternations of the program were made until both 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 s, followed by 30 cycles denaturation (94°C for 15 s), annealing (40°C for 30 s) and extension (72°C for 80 s) with final extension (72°C for 420 s). 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.

Agarose gel electrophoresis

Agarose gel electrophoresis was made by combining 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 1 kb mass ruler DNA ladder was used to estimate the size of RAPD bands. Electrophoresis was conducted at a voltage of 90 V for 40 min. A photograph of the gel was taken with a polaroid camera under ultraviolet (UV) light on UV transilluminator.

Data Analysis

Data on seed oil content and various fatty acids were subjected to an analysis of variance (ANOVA) procedure of the SAS statistical program (SAS, 1989). When found significant from the ANOVA, traits were subjected to the least significant difference (LSD) test procedure to compare means at 5% level of significance. DNA polymorphism was analysed using RAPD bands. Reproducible, polymorphic and monomorphic bands were visually scored as present or absent.

RESULTS AND DISCUSSION

Seed oil content and fatty acids

Tested lines showed no significant differences with respect to seed oil content (Table 3). The average seed oil content was 27.24% that ranged from 22.40 to 33.11% (Table 4). Vge-32 and Vge-20 were the best lines displaying higher seed oil content at 33 and 29%, respectively. This variation was also reported by Angelini et al. (1997) in another study using *V. galamensis* accessions. Seed oil content varied between 22.10 and 31.20% with the mean of 26.70% which is just lower than 27.24% realised in the present study. However, Baye et al. (2001) in other studies of *V. galamensis* var. *ethiopica* accessions sown at Alemaya, Harar and Babile, reported seed oil content averaged 38.70, 39.50 and 34.70% for each respective location.

There were no significant variations between lines for vernolic and linoleic acid (Table 3 and 4). The average vernolic acid content was 74.97% that varied from 73.09 to 76.38% between lines, whereas linoleic acid averaged at 13.31% and varied from 13.02 to 14.05%. The lack of variation in vernolic acid can be explained by the little difference between the best and the lowest lines which are 3.74% as compared to 6.2% reported by Angelini et al. (1997). However, these researchers found lower average vernolic acid content of 68.7 as compared to the present study. Baye and Becker (2005b) reported supporting results when reporting an average vernolic acid content of 74%. On the other hand Baye and Becker

Table 3. Analysis of variance for seed oil content and fatty acids among ten selected vernonia lines when tested from 2 replicates.

Source of variation	DF	Traits[a]											
		SOC		VA		LA		OA		PA		SA	
		MS	F-value[b]	MS	F-value	MS	F-value	MS	F- value	MS	F-value	MS	F-value
Replication	1	2.3	0.20^{ns}	4.9	3.75^{ns}	0.0	0.03^{ns}	0.61	6.22*	0.01	1.24^{ns}	0.00	0.00^{ns}
Line	9	6	1.43^{ns}	2	1.76^{ns}	1	0.58^{ns}	0.46	8.25*	0.06	7.37**	02	2.90^{ns}
Error	9	17		2.3		0.2				0.01		0.07	
Total	19	18		1		3							
		12		1.3		0.4							
		02		1		0							

[a]SOC = Seed oil content; VA = vernolic acid; LA = linoleic acid; OA = Oleic acid; PA = Palmitic acid; SA = stearic acid
[b]Ns = non significant; * = significant at $p < 0.05$; ** = significant at $p < 0.01$.

Table 4. Mean responses and ranks for seed oil content and fatty acid among ten selected vernonia lines.

Lines	Traits[a]											
	SOC		VA		LA		OA		PA		SA	
	Mean	R	Mean	R	Mean	R	Mean	R	Mean	R	Mean	R
Vge-1	28.12	4	75.51	4	13.62	2	4.44	5	2.92	3	2.64	3
Vge-5	27.31	6	75.90	3	13.06	8	4.10	7	2.81	7	2.75	1
Vge-9	22.40	10	76.83	1	13.08	7	3.83	9	2.48	10	2.26	9
Vge-14	23.81	9	74.34	8	13.02	10	4.51	4	2.92	4	2.61	4
Vge-16	26.44	7	74.66	6	13.20	5	4.86	2	2.93	2	2.59	5
Vge-20	29.05	2	73.09	10	13.05	9	5.28	1	2.83	6	2.52	6
Vge-23	27.77	5	74.17	9	14.05	1	4.69	3	2.81	8	2.74	2
Vge-27	26.04	8	74.65	7	13.59	3	4.40	6	2.55	9	2.30	8
Vge-32	33.11	1	75.92	2	13.37	4	3.99	8	2.98	1	2.26	10
Vge-36	28.38	3	74.67	5	13.13	6	3.77	10	2.85	5	2.47	7
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, R-rank.

(2005a) reported lower average vernolic acid content 68.28% and better average linoleic acid content of 16.33%.

Significant variations were found in this study for oleic and palmitic acid between the tested lines (Tables 3 and 4). This study found an average oleic acid content of 4.38 and 2.81% for palmitic acid. The line which had the best oleic content was Vge-20 with 5.28% followed by Vge-16 with 4.86% and Vge-23 with 4.69% for oleic acid. The lowest line was Vge-36 with 3.77% followed by Vge-9 with 3.83% and Vge-32 with 3.99%. For palmitic acid this study found an average of 2.81% which ranged from 2.48 to 2.98%. The best line was Vge-32 with 2.98% followed by Vge-16 and Vge-1 with 2.93 and 2.92%, respectively. Comparable results were reported by Baye et al. (2005) after evaluating 41 *V. galamensis* accession and reported an average oleic acid content of 4.42% which ranged from 2.10 to 7.20% and they reported no traces of palmitic acid unlike the present study. In another study by Baye and Becker (2005b) palmitic acid content had an average palmitic acid content of 3.32% with ranges between 2.50 and 4.01% which are higher than the present study.

No statistical differences were found between the lines with respect to stearic acid composition (Table 3). This study found an average stearic acid content of 2.51% which ranged from 2.26 to 2.75% (Table 4). These results are comparable to that of Baye et al. (2005) as they had mean values ranging from 2.10 to 3.50%. The grand mean of 2.75% of stearic acid composition found in this study together with mean ranges of 2.26 and 2.75% obtained in this study are lower than the grand mean of 4.02% with mean ranges of 3.33 and 5.16% *V. galamensis* averaged from 2 different locations (Baye and Becker, 2005a). Even more corresponding are the mean and ranges for stearic acid of 2.55% (1.4 – 6.5%) from different vernonia accessions collected in Ethiopia (Baye and Becker, 2005b).

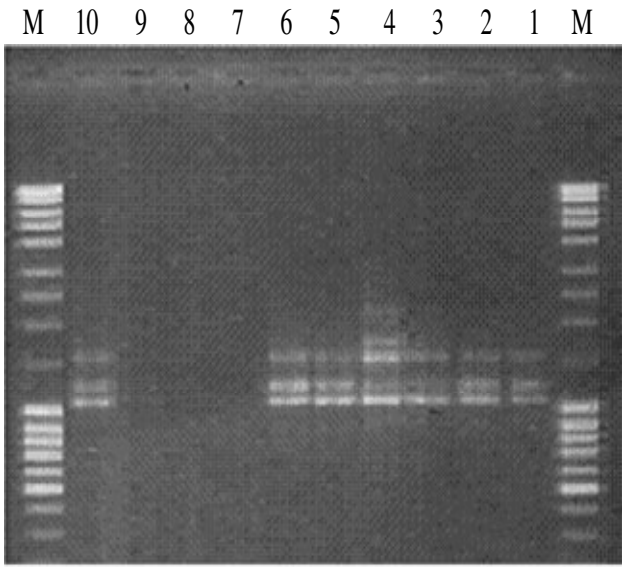

Figure 1. RAPD assays conducted with the DNA samples isolated from ten selected vernonia lines by CTAB method of genomic DNA isolation, where M stands for the molecular mass ruler. Lanes: 1: Vge-5; 2: Vge-9; 3: Vge-14; 4: Vge-16; 5: Vge-1; 6: Vge-20; 7: Vge-23; 8: Vge-27; 9: Vge-32; 10: Vge-36.

RAPD analysis

From the 13 primers screened, only 4 (OPA 09, OPA 10, OPA 11 and OPA 12) produced RAPD amplifications with Vge-16. OPA 10 showed 3 bright and clear bands compared to other primers and it was then selected as the optimum primer to amplify vernonia lines. Only 3 DNA samples did not amplify (Lines 7, 8 and 9) using OPA 10 and the remaining 7 DNA samples amplified (Figure 1). A total of 22 RAPD bands were produced by OPA 10 across all the 10 selected vernonia lines including faint ones averaging at 3.14 per line. DNA sample isolated from Vge-16 produced 3 bright and 2 faint RAPD bands totaling 5, whereas Vge-5, Vge-9, Vge-1, Vge-20 and Vge-36 produced 1 faint upper band and 2 brighter bands at the bottom. On the other hand Vge-14 produced a total of 3 bands with the middle one fainted unlike the latter accessions.

Results showed that there were some degrees of genetic variations among different vernonia lines, as supported by 4 genetically varying groups from 10 selected lines. 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 meaning that the nucleotides pattern of the lines differed slightly from line to line except for Vge-16, which was totally different from other lines. The majority of lines would therefore display slightly the same characteristics or behaviours. Baye (2005a) reported the overall genetic diversity index for all traits of 0.76 using Shannon- Weaver diversity index (H′), which means that there is high polymorphism among different vernonia lines. 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.

Less genetic diversity was observed among different vernonia lines for seed oil content and fatty acid profiles. This suggests that seed oil content and fatty acid profiles would not make good markers for characterisation of different vernonia lines due to the lack of genetic variation. However, RAPD analysis showed that there is a moderate genetic diversity in *V. 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 show polymorphism or genetic variations.

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