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Research Article

Molecular characterization and yield analysis of EMS-treated accessions of *Vigna subterranea* using RAPD markers

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Vigna subterranea, EMS mutagenesis, genetic diversity, RAPD markers, yield enhancement, molecular variation.

Abstract

Mutations produce raw materials for the genetic improvement of economically important crops. Genetic improvement of underutilized legumes requires the generation of novel variability that exceeds the limitations of natural recombination. *Vigna subterranea* (L.) Verdc., commonly known as Bambara groundnut, is valued for its drought resilience and nutritional quality, yet breeding progress has been slow due to its narrow genetic base and self-pollinating nature. This study investigated the capacity of ethyl methanesulfonate (EMS) to induce heritable variation and improve yield performance in two varieties, brown and cream. Seeds were exposed to six EMS concentrations (0.00–0.5%) and evaluated across the M1 and M2 generations using agronomic traits and RAPD-based molecular analysis. The mutagen treatments generated substantial polymorphisms with polymorphic information content values between 0.5025 and 0.9103 and gene diversity estimates ranging from 0.5278 to 0.9167. Phylogenetic clustering separated the treated lines into two principal groups with multiple sub-clusters, indicating a clear genetic divergence from the untreated controls. Yield components responded positively to moderate EMS doses. In M_1, peak yields occurred at 0.3% for brown (129.33 g/plant) and 0.4% for cream (125.00 g/plant). Enhanced performance was more evident in M_2, where brown at 0.3% produced 138.67 g/plant and cream at 0.5% yielded 135.67 g/plant. Improvements in 100-seed dry weight, reaching 5.09 g, further demonstrated mutagen effectiveness. The findings confirm that carefully optimized EMS application can broaden the genetic base of Bambara groundnut and generate stable, high-performing lines suitable for future breeding programs and adaptation to diverse agro-ecological conditions.

1. Introduction

Bambara groundnut (*Vigna subterranea* [L.] Verdc.) is a drought-tolerant African legume cultivated mainly by smallholder farmers using low-input systems. Its nutritional value and adaptability to poor soils make

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it important for food security in semi-arid regions [1–3]. In addition, crops contribute to soil fertility through biological nitrogen fixation [4, 5].

The species is believed to have originated in West Africa, particularly between northeastern Nigeria and northern Cameroon, where both cultivated and wild forms exist [2, 6]. Despite its importance, genetic improvement has been slow due to its self-pollinating nature and limited genetic variability, which restrict the effectiveness of conventional breeding methods [7, 8].

Induced mutagenesis offers an alternative approach for generating novel variations. Ethyl methanesulfonate (EMS) is widely used because it produces heritable point mutations through base substitutions, thereby altering gene function [9, 10]. It is relatively inexpensive, non-transgenic, and suitable for diverse plant materials [11, 12]. However, its effectiveness depends on the careful optimization of treatment conditions to avoid excessive damage [13, 14].

Molecular markers, such as RAPD, provide a rapid means of detecting induced genetic variation and assessing relationships among genotypes [15, 16]. This study evaluated the impact of EMS on genetic diversity and yield traits in *Vigna subterranea*, with the aim of supporting its genetic improvement.

2. Materials and methods

The study employed a Randomized Complete Block Design (RCBD) with three replicates. For each treatment, 10 plants were maintained per replicate under open field conditions in Item Bende Local Government Area, Abia State, Nigeria. Routine agronomic management practices were implemented uniformly throughout the experimental period.

2.1. Seed materials and treatments

Seeds were treated with ethyl methanesulfonate (EMS) at concentrations of 0.00%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% (w/v). After soaking in the EMS solution for a defined period, the seeds were thoroughly rinsed with sterile distilled water to eliminate chemical residues [17–19].

2.1.1. Seed sterilization

To minimize contamination, seeds were surface-sterilized in 70% ethanol for 1 min, followed by immersion in 5% sodium hypochlorite for 5 min and rinsed three times with sterile distilled water [20].

2.1.2. Germination setup

Germination was carried out in Petri dishes lined with moistened filter paper. Each dish contained 10 seeds per treatment group, and 5 mL of distilled water was added to maintain moisture. The dishes were incubated at 25 28 °C with a 12-hour light/dark cycle for 7 days [20].

2.1.3. Seed treatment

Chemical mutagenesis was performed using ethyl methanesulfonate (EMS), with dimethyl sulfoxide ($DMSO_4$) as a solvent to enhance mutagen absorption through the seed coat. This combination is commonly used to increase the mutation efficiency.

2.1.4. Pre-soaking

Seeds were soaked in 200 mL of distilled water for 6 h to soften the seed coats and improve mutagen uptake. After soaking, the samples were air-dried for 20 min to avoid oversaturation.

2.1.5. Preparation of EMS-$DMSO_4$ solutions

EMS solutions at 0.00% (control), 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% (w/v) were prepared in buffer solution 7 [21]. $DMSO_4$ was added to enhance EMS penetration. Solutions were freshly prepared before use to maintain their effectiveness.

2.1.6. Mutagenic treatment

Pre-soaked seeds were immersed in EMS-$DMSO_4$ solutions for 6 h. The beakers were gently agitated periodically to ensure even exposure. $DMSO_4$ improved EMS uptake and promoted uniform mutagenesis.

2.1.7. Post-treatment washing

After treatment, the seeds were rinsed under running tap water and washed five times to eliminate residual chemicals. They were then air-dried for 30 min before sowing [22].

2.2. Data collection

2.2.1. Yield assessment

Data were collected when the plants reached physiological maturity to evaluate the effects of each treatment on yield. The following parameters were recorded:

2.2.2. Number of pods per plant

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Pods from each plant were individually counted to determine pod production.

2.2.3. Dry weight per plant

Harvested pods were dried in an oven at 60 °C for 72 h to remove moisture. The dried pods were then weighed to determine the dry matter per plant, which reflects both the yield quantity and quality.

2.2.4. 100-Seed weight

A sample of 100 seeds was randomly selected from each treatment group and weighed to assess seed size and uniformity.

2.2.5. Yield per plant (g)

After threshing, the total seed weight per plant was measured in grams to quantify the overall productivity.

These measurements provided a comprehensive evaluation of plant yield performance under the experimental treatments.

2.2.6. Genomic DNA extraction from Vigna subterranea

The extraction of genomic DNA from *Vigna subterranea* was performed using the CTAB (cetyltrimethylammonium Bromide) method, as described by [23]. This method is widely recognized for its effectiveness in obtaining high-quality DNA suitable for molecular analyses, such as polymerase chain reaction (PCR) and genetic fingerprinting.

2.2.7. Sample collection and DNA extraction

Fresh plant tissue was ground with liquid nitrogen, and incubated in CTAB buffer at 65°C for cell lysis. DNA was separated using chloroform: isoamyl alcohol (24:1) and centrifugation. The aqueous phase was-collected, and DNA was precipitated with cold isopropanol or ethanol, followed by centrifugation. The DNA pellet was washed with 70 % ethanol, air-dried, and resuspended in TE buffer or distilled water. If, required RNase is used to remove RNA. The extracted DNA was stored at -20 °C for molecular applications [23].

2.2.8. Agarose gel electrophoresis

The quality of the DNA was checked by running it on a 1.5 % agarose gel stained with ethidium bromide. The stained gel was photographed using a gel documentation system (Kodak EDAS 290). DNA concentration and purity were further quantified using a spectrophotometer at 260 and 280 nm, and appropriate dilutions were made for subsequent SSR analyses.

2.2.9. DNA amplification via PCR

Polymerase chain reaction (PCR) was carried out using five RAPD primers. The 15 μL reaction mixture consisted of 20 ng genomic DNA, 1.5 μL of 10X reaction buffer, 1.5 μL of 10X dNTPs, 0.5 μL of magnesium chloride (25 mM), 1 μL of primer, 1 μL of Taq polymerase, and 8.5 μL of nanopure water. Amplification was performed using a programmable thermal cycler under the following conditions: an initial denaturation of 1 cycle at 35°C for 1 min, 72 °C for 2 min, and 92 °C for 3 min; followed by 44 cycles of denaturation at 92 °C for 1 min, annealing at 35 °C for 1 min, and extension at 72 °C for 2 min. A final extension was performed at 15 °C. The PCR products were resolved on a 1.5% agarose gel in TAE buffer, stained with ethidium bromide, and visualized under UV light using a Kodak EDAS 290 system.

2.3. Molecular data analysis

For RAPD-PCR analysis, only distinct and reproducible amplification bands were scored, assigning a presence (1) or absence (0) to each band as appropriate. To ensure consistency, the bands were scored independently by two researchers across repeated PCR runs. Polymorphism Information Content (PIC) was calculated to assess marker informativeness using the formula: where Pi represents the frequency of the i-th band. Cluster analysis and Principal Component Analysis (PCA) were performed using NTSYS (Numerical Taxonomy and Multivariate Analysis System) Version 2.01i, applying Jaccard's similarity coefficient to assess genetic relationships. A dendrogram was constructed using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) based on Nei's genetic distance to classify individuals into distinct clusters. Molecular analysis was conducted using PowerMarker Software Version 3.25 [39] for genetic diversity estimates and allele frequency calculations. Additionally, Darwin 6 software [16] was used for cluster validation and PCA visualization.

3. Results

Table 1 shows the effect of the difference in mutant levels on the yield performance of the two varieties of

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Table 1. Analysis of variance test on the effect of difference in levels of mutants on yield performance of two varieties of Bambara in M_1 generation.

Variety	Treatments (%)	DWPS	DW(100 seeds)	Yield (g/plant)
Brown	0	3.62±0.49	4.89±0.69	126.67±8.33
	0.1	3.05±0.23	3.64±0.56	111.67±7.64
	0.2	3.22±0.49	3.28±0.61	112.33±7.57
	0.3	2.90±0.64	4.21±1.68	129.33±17.93
	0.4	3.11±0.63	3.69±0.50	117.33±13.43
	0.5	3.38±1.11	4.09±1.98	124.67±25.48
	Total	3.21±0.60	3.97±1.11	120.33±14.42
Cream	0	2.67±0.58	3.25±0.92	116.33±13.58
	0.1	2.89±0.56	2.99±0.68	110.67±10.07
	0.2	2.94±0.17	3.03±0.08	111.33±7.02
	0.3	3.12±0.76	3.38±0.76	122.33±12.58
	0.4	3.96±1.84	4.24±1.88	125.00±20.52
	0.5	2.92±0.58	3.37±0.91	115.33±10.60
	Total	3.08±0.88	3.37±0.96	116.83±12.26
Totalmean	0	3.14±0.71	$4.07±1.16^{a}$	121.50±11.55
	0.1	2.97±0.39	$3.32±0.66^{ab}$	111.17±8.01
	0.2	3.08±0.36	$3.15±0.41^{b}$	111.83±6.56
	0.3	3.01±0.64	$3.79±1.25^{ab}$	125.83±14.37
	0.4	3.53±1.31	$3.96±1.26^{ab}$	121.17±16.07
	0.5	3.15±0.83	$3.73±1.43^{ab}$	120.00±18.19
	Total	3.15±0.74	$3.67±1.07^{ab}$	118.58±13.31
LSD_{trt}		0.7291066	0.8500172	15.94574
CV		19.34366	9.33846	11.23051
V. Effect		0.526567	0.02023*	0.4389
T. Effect		0.657050	0.21479	0.3633
InterEffect		0.224385	0.21885	0.8507
Block		0.001158**	0.000***	0.1217

Signif.codes: 0 ‘***’ 0.001 ‘**’ 0.01 ‘*’ 0.05

Bambara in the M_1 generation. For the Brown variety in the M_1 generation, the highest yield was observed at 0.3% EMS treatment (129.33 ± 17.93), whereas the lowest yield occurred at 0.1% EMS treatment (111.67 ± 7.64). The highest dry weight per seed (DWPS) was recorded in the 0% EMS treatment (3.62 ± 0.49), and the lowest was at 0.1% EMS treatment (3.05 ± 0.23). The dry weight per 100 seeds (DW) was highest in the 0% EMS treatment (4.89 ± 0.69) and lowest at the 0.1% EMS treatment (3.28 ± 0.61).

For the Cream variety in the M_1 generation, the highest yield was observed at 0.4% EMS treatment (125.00 ± 20.52), while the lowest was at 0.1% EMS treatment (110.67 ± 10.07). The highest DWPS was observed at 0.4% EMS treatment (3.96 ± 1.84), and the lowest DWPS was observed at 0% EMS treatment (2.67 ± 0.58). The highest DW was observed at 0.4% EMS treatment (4.24 ± 1.88), and the lowest was observed at 0.1% EMS treatment (2.99 ± 0.68).

Overall, the findings from the M_1 generation indicated that higher EMS concentrations (0.3% and 0.4%) had a more favourable impact on yield and dry weight, particularly in the Cream variety, whereas lower EMS concentrations (0.1%) negatively affected growth parameters in both varieties. Table 2 shows the effect of the differences in mutant levels on the yield performance of the two Bambara varieties in the M_2 generation. The harvest data for M_2 showed results for two varieties (Brown and Cream), across six treatments (0, 0.1, 0.2, 0.3, 0.4, and 0.5) for three parameters: yield, dry weight (DW), and dry weight per unit (DWPS).

For the Brown variety, yield started at 129.33 and increased to a high of 138.67 at 0.3, then remained

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Table 2. Analysis of variance test on the effect of difference in levels of mutants on yield performance of two varieties of Bambara in M_2 generation.

Variety	Treatments (%)	Yield	DW	DWPS (g)
Brown	0	129.33±11.02	4.53±0.94	3.82±0.61
	0.1	117.33±16.65	3.55±0.39	3.17±0.13
	0.2	128.67±1.53	4.16±0.68	3.64±0.40
	0.3	138.67±4.16	5.09±0.07	4.28±0.27
	0.4	133.67±20.79	4.97±1.10	4.40±0.79
	0.5	137.33±11.37	5.07±1.13	3.78±0.60
	Total	130.83±12.98	4.56±0.90	3.85±0.60
Cream	0	118.00±15.10	3.55±0.93	3.36±0.66
	0.1	124.00±18.33	4.03±1.09	3.82±1.03
	0.2	118.00±10.00	3.54±0.34	3.22±0.06
	0.3	127.00±23.00	4.33±1.40	3.80±1.17
	0.4	134.00±25.06	4.40±1.49	3.82±1.26
	0.5	135.67±4.04	3.77±0.33	3.27±0.47
	Total	126.11±16.35	3.93±0.94	3.55±0.79
Totalmean	0	123.67±13.35	4.04±1.00	3.59±0.62
	0.1	120.67±16.08	3.79±0.78	3.50±0.75
	0.2	123.33±8.66	3.85±0.59	3.43±0.35
	0.3	132.83±16.11	4.71±0.98	4.04±0.80
	0.4	133.83±20.60	4.69±1.21	4.11±0.99
	0.5	136.50±7.69	4.42±1.03	3.53±0.56
	Total	128.47±14.74	4.25±0.96	3.70±0.71
LSD_{trt}		18.37978	1.087269	0.8537574
CV		11.9484	21.37623	19.27912
V. Effect		0.3661	0.05028	0.2144
T. Effect		0.3721	0.33031	0.4178
InterEffect		0.8626	0.65427	0.6681
Block		0.3997	0.21149	0.2767

Signi^7f.codes: 0 '***' 0.001 '**' 0.01 '*' 0.05

137.33 at 0.5. The dry weight for Brown reached its maximum of 5.09 at treatment 0.3, while DWPS was the highest at treatment 0.4, reaching 4.40.

For the Cream variety, the yield increased steadily from 118.00 at treatment 0 to a maximum of 135.67 at treatment 0.5. However, Cream's DW values were lower than Brown's, with the highest DW of 4.40 at treatment 0.4. Its DWPS peaks at around 3.82 for treatments 0.1 and 0.4. When the data for both varieties were combined (Total Mean), the yield showed an increasing trend from 123.67 at treatment 0, up to 136.50 at treatment 0.5. Overall, the highest combined Dry Weight is observed at treatment 0.3 (4.71), and the highest overall DWPS is at treatment 0.4 (4.11). The LSD values (18.38 for yield, 1.09 for DW, and 0.85 for DWPS) indicate that differences exceeding these thresholds were statistically significant.

Fig. 1 shows the molecular analysis of two accessions of *Vigna subterranea* treated with at different concentrations of EMS (0.00%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5 w/v), which were analyzed using five RAPD markers (OPT-05, OPT-07, OPH-05, OPB-04, and OPB-17) as presented in Table 3, to assess their ability to detect polymorphisms. The DNA quality and RAPD amplification profiles illustrating polymorphic banding patterns across treatments are presented in Plates 1–4. The integrity of extracted genomic DNA is shown in Plate 1.

Considerable genetic diversity was found among the treated *Vigna subterranea* accessions. Cluster analysis was carried out to determine the relationship among the treated accessions. The dendrogram showed two main clusters and a total of 11 clusters. RAPDs

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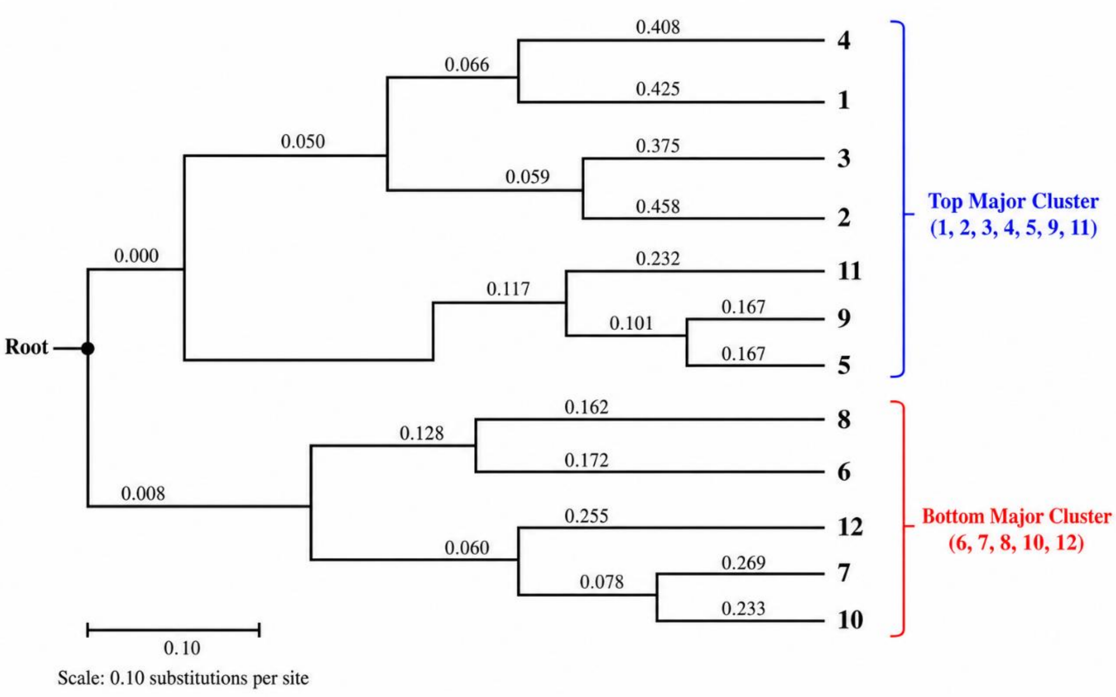

Figure 1. Phylogenetic tree of *Vigna subteranea* accessions studied.

Table 3. Primer sequences.

OPT–05 -5^1–GGG	TTT	GGC	A–3^1	
OPT–07 -5^1–GGC	AGG	CTG	T–3^1	
OPH–05	-5^1–AGT	CGT	CCC	C–3^1
OPB–04 5^1–GGA	CTG	GAG	T–3^1	
OPB–17 5^1–AGG	GAA	CGA	G–3^1	

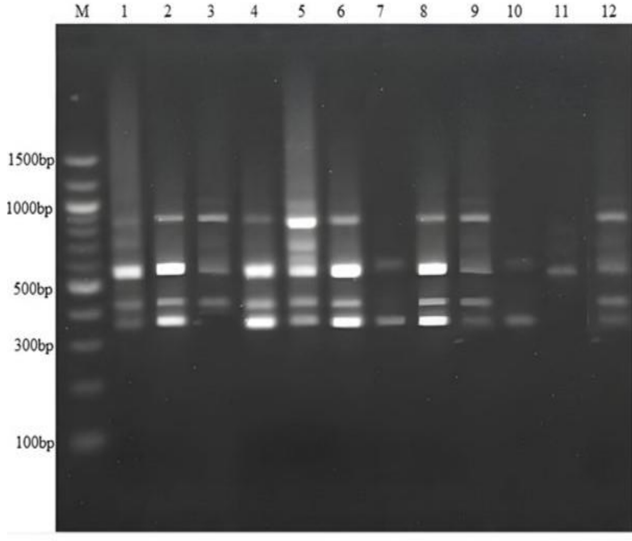

Plate 1. Molecular weight DNA extraxed from the samples.
Sample codes: 1. BcControl, 2. 0.1%, 3. 0.2%, 4. 0.3%, 5. 0.4%, 6. 0.5%, 7.C. CControl (0.0), 8. C.C0.1%, 9. C.C0.2%,10. C.C0.3%, 11. C.C0.4%, 12. C.C0.5%.

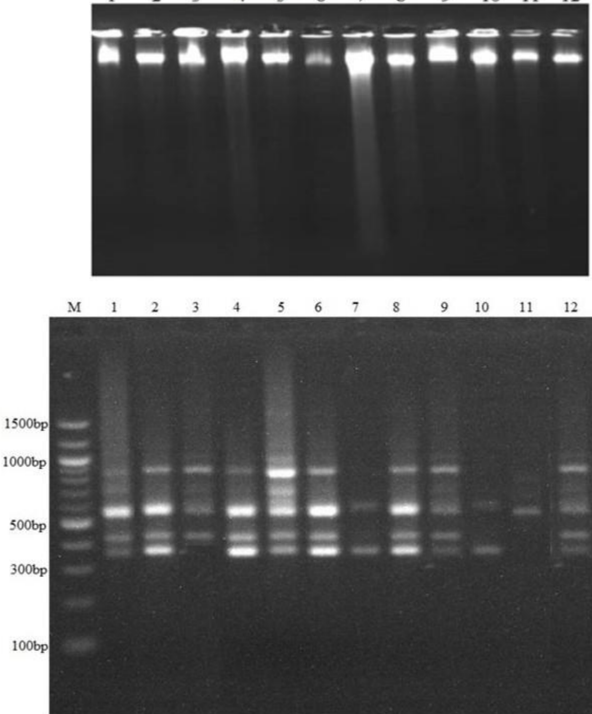

Plate 2. RAPD amplification profile using primer OPT-05 showing polymorphic bands among EMS-treated accessions. Lane M represents 100 bp DNA ladder.
Sample codes: *1. BcControl, 2. 0.1%, 3. 0.2%, 4. 0.3%, 5. 0.4%, 6. 0.5%, 7.C. CControl (0.0), 8. C.C0.1%, 9. C.C0.2%,10. C.C0.3%, 11. C.C0.4%, 12. C.C0.5%.*

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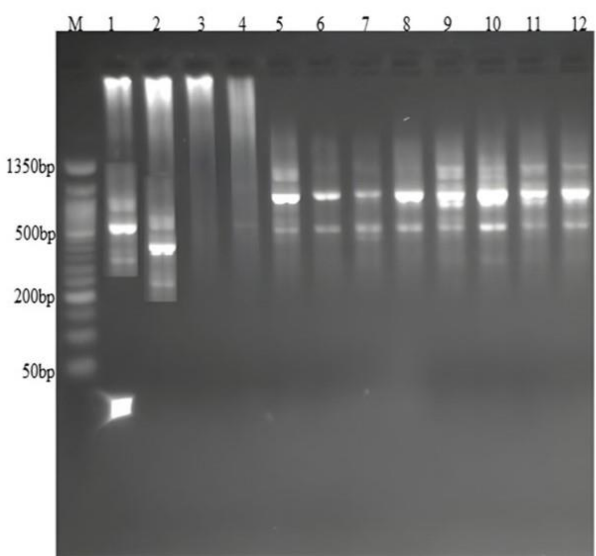

Plate 3. RAPD amplification profile using primer OPB04 showing polymorphic bands among EMS-treated accessions. Lane M represents 100 bp DNA ladder.
Sample codes: 1. BcControl, 2. 0.1%, 3. 0.2%, 4. 0.3%, 5. 0.4%, 6. 0.5%, 7.C. CControl (0.0), 8. C.C0.1%, 9. C.C0.2%,10. C.C0.3%, 11. C.C0.4%, 12. C.C0.5%.

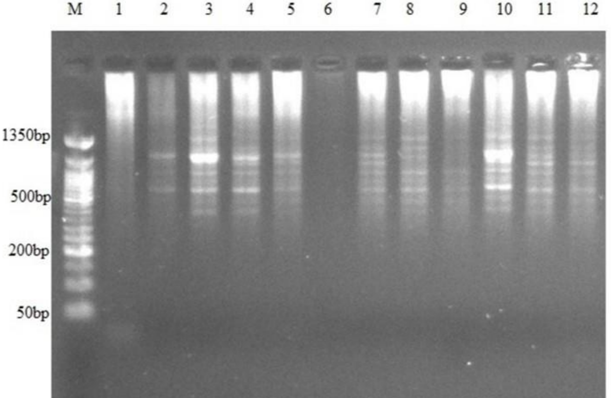

Plate 4. RAPD amplification profile using primer OPr07 showing polymorphic bands among EMS-treated accessions. Lane M represents 50 bp DNA ladder.
Sample codes: 1. BcControl, 2. 0.1%, 3. 0.2%, 4. 0.3%, 5. 0.4%, 6. 0.5%, 7.C. CControl (0.0), 8. C.C0.1%, 9. C.C0.2%,10. C.C0.3%, 11. C.C0.4%, 12. C.C0.5%.

revealed a high level of polymorphism among the treated accessions.

Table 4 shows the polymorphic information content of the studied accessions, ranging from 0.5025 to 0.9103. The gene diversity ranged from 0.5278 to 0.9167, indicating sufficient genetic diversity among the genotypes of the treated accessions. The phylogenetic tree showed the genetic relatedness of the accessions studied. RAPD markers indicated that the accessions were monophyletic. The morphological characteristics and seed colour variations of the studied accessions are illustrated in Plates 5 and 6.

4. Discussion

EMS mutagenesis effectively generates genetic variability in *Vigna subterranea* (Bambara groundnut). Improved performance in the M_2 generation indicates the stabilization of beneficial mutations, with moderate EMS doses (0.3–0.5%) enhancing yield traits without harming plant viability. The Brown variety outperformed Cream, consistent with [24], whereas higher EMS concentrations caused sterility and malformed pods [25, 26]. Yield parameters, including dry weight per seed (DWPS), 100-seed weight (DW), and yield per plant, increased in M_2, supporting previous findings [27]. Genotype-specific responses to EMS reflect differences in DNA repair and stress tolerance [28-30].

RAPD analysis revealed substantial genetic variation among the treated accessions. The minor allele frequency ranged from 0.0833 (GENOTYPE) to 0.6667 (OPB04), and gene diversity ranged from 0.5278 (OPB04) to 0.9167 (GENOTYPE), exceeding untreated germplasm [31] and typical crop ranges [32]. PIC values (0.1527–0.5000) indicated moderate marker informativeness [33-35], with OPB17, OPH05, and OPT07 being the most informative.

RAPD markers revealed genetic variation among EMS-treated accessions, but are limited by dominant inheritance and poor reproducibility [15, 16]. Although cost-effective and useful for detecting mutation-induced variations [36, 37], they provide limited resolution. SSR and SNP markers offer higher accuracy, co-dominant inheritance, and broader genome coverage [38-44]. Their integration would improve genetic analysis and Bambara groundnut breeding. Distinct polymorphic banding patterns generated by different primers are illustrated in Plates 2–4, confirming the effectiveness of RAPD markers in detecting EMS-induced variation.

Genetic clustering showed two main clusters with sub-clusters, reflecting divergence among mutants while some remained similar to the controls [45, 31]. Selecting genotypes from both distant and parental

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Table 4. Genetic diversity and polymorphic information content.

Marker	Major.Allele.Frequency	Sample size	No. of obs.	Allele No	Availability	Gene diversity	PIC
GENOTYPE	0.0833	12.0000	12.0000	12.0000	1.0000	0.9167	0.9103
OPB04	0.6667	12.0000	12.0000	5.0000	1.0000	0.5278	0.5025
OPB17	0.5000	12.0000	12.0000	6.0000	1.0000	0.6944	0.6645
OPH05	0.4167	12.0000	12.0000	5.0000	1.0000	0.7222	0.6800
OPT05	0.3333	12.0000	12.0000	5.0000	1.0000	0.7361	0.69201
OPT07	0.4167	12.0000	12.0000	5.0000	1.0000	0.7222	0.6800
Mean	0.4028	12.0000	12.0000	6.3333	1.0000	0.7199	0.6882

Plate 5. Vigna subterranea leaf, stem and seeds.

Plate 6. *Vigna subterranea* cream and brown color and seeds.

clusters can maximize the identification of elite mutants [46, 47]. Phylogenetic analysis confirmed the EMS-induced genotypes were monophyletic but genetically differentiated, underscoring EMS as a tool for generating heritable variation and novel alleles for yield improvement, stress tolerance, and breeding [48-51].

5. Conclusions

These findings reinforce the effectiveness of EMS in enhancing genetic variability and highlight its value as a strategic tool in mutagenesis breeding programs. The alignment of our results with those of previous studies further confirms EMS as a reliable mutagen for broadening the genetic base of *Vigna subterranea* and improving key agronomic traits. Notably, the improved performance observed in the M_2 generation, particularly in yield-related parameters, underscores the heritability of beneficial mutations. This supports the use of moderate EMS concentrations (0.3–0.5%) to induce stable and advantageous genetic changes. Future research incorporating advanced molecular markers and genomic tools could provide deeper insights into the genetic architecture of *Vigna subterranea*, ultimately facilitating its improvement and sustainable cultivation across diverse agro-ecological zones.

Disclaimer (artificial intelligence)

Author(s) hereby state that no generative AI tools such as Large Language Models (ChatGPT, Copilot, etc.) and text-to-image generators were utilized in the preparation or editing of this manuscript.

Authors' contributions

Investigation, project administered, data curation, visualization and original draft preparation, N.N.N.; data curation, visualization, and original draft preparation, C.L.O.; suagwu, conceptualized,

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supervised, and validation, G.G.E.O.; performed the formal analysis, methodology and software, N.C.E. contributed to data curation, J.O.E.; assisted in investigation, F.E.A.; contributed to conceptualized, supervised, and methodology, G.O.; performed the formal analysis and methodology, B.N.U.

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Availability of data and materials

All data will be made available on request according to the journal policy

Conflicts of interest

The authors affirm that there are no conflicts of interest, financial, academic, or personal associated with the preparation or publication of this paper. All authors contributed objectively and hold no competing interests.

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