African Journal of Life Sciences Research (AJLSR)
E-ISSN 2978-3496 (Online); ISSN 2978-3488 (Print)

Indexed by SABINET

Volume 1, Number 1, June 2025

Pp 37-53

Assessing Genetic Variation in Lima Bean Breeding Lines Through Cytological and Molecular Marker Analysis

DOI: https://doi.org/10.31920/2978-3496/2025/v1n1a2

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Abstract

Lima bean (*Phaseolus lunatus*), the second most important edible legume in the Fabaceae family, has received limited attention in mutation breeding despite its potential for genetic improvement and poverty alleviation in Nigeria. This study assesses the genetic variation of Lima bean mutant lines induced by fast neutron irradiation (FNI), colchicine (COLCH), and ethyl methanesulfonate (EMS) using Cytological and Random Amplified Polymorphic DNA (RAPD) markers. Chromosomal aberrations such as fragmentation, chromosome unorientation, and C-metaphase were observed in Lima bean mutants subjected to fast neutron irradiation. Mutants treated with colchicine exhibited abnormalities including sticky chromosomes, unorientation, fragmentation, and chromosome bridge formation. Similarly, mutants derived from ethyl methanesulfonate (EMS) treatment displayed sticky chromosomes and chromosome unorientation. Genetic diversity analysis revealed major allele frequencies ranging from 23 to 46%, with a mean of 36.5%. Primers OPB04 and OPH04 exhibited the highest frequency (46%), while OPT05 recorded the lowest (23%). A total of 27 alleles were detected, averaging 6.75 per primer. The polymorphic information content (PIC) values indicated high allele diversity, with OPT20 and OPT05 showing the highest PIC (81%), and OPH04 at 66%. A dendrogram based on genetic distance (0.1) identified three clusters: (1) EMS 0.3%, EMS 0.4%, and FNI 0.32 Sv (similarity = 0.11); (2) the control and five mutants (FNI 0.65 Sv, COLCH 0.4 mM, EMS 0.1%, COLCH 0.6 mM, and COLCH 0.8 mM) with a similarity of 0.05; and (3) COLCH 0.2 mM, EMS 0.2%, FNI 0.49 Sv, and FNI 0.16 Sv (similarity = 0.02). The findings confirm that the mutagens were effective at inducing chromosomal aberrations and RAPD markers are effective tools for detecting genetic diversity among mutant lines, demonstrating the potential of induced mutagenesis to expand the genetic base of Lima bean for breeding programs in Nigeria.

Keywords: *Cytology, Gene pool, Mutation, Variability, Genetic diversity, RAPD*

Introduction

Lima bean (*Phaseolus lunatus* L.) is the second most important legume within the *Phaseolus* genus globally (Castillo *et al*., 2023) and serves as a crucial protein source for rural populations in South America and Africa (Lioi *et al*., 1998). It belongs to the Fabaceae family (Palupi *et al*., 2021) and is well adapted to tropical environments, where it is widely cultivated for food (Diniyah *et al*., 2020). In Nigeria, it is known as *Ukpa* in Igbo (Ikechukwu, 2010), *Papala* in Yoruba (Adegbehingbe, 2013), and *Wake rumpa* in Hausa (Ikani *et al*., 2017). Previous studies and field reports

indicate that while phenotypic diversity exists among landraces cultivated by smallholder farmers across different agroecological zones, comprehensive molecular data on their genetic diversity are lacking. This gap has necessitated the importance of employing molecular markers to establish baseline genetic information. This research thus contributes to filling this critical knowledge gap and supports future breeding efforts for Lima bean improvement and conservation in the region.

Mutation is a heritable change in genetic material independent of recombination or segregation (Lamo *et al*., 2017), leading to the formation of mutant individuals with genetic alterations that can be identified through molecular analysis or phenotyping (Shu *et al*., 2012). Mutations may occur spontaneously or be induced by biological, chemical, or physical mutagens, resulting in irreversible genetic modifications (Udage, 2021). Mutation breeding plays a pivotal role in crop genetic improvement by creating artificial genetic variation, thereby expanding the gene pool of a species. It uses a plant's genetic resources and mimics the natural process of spontaneous mutation, the motor of evolution. By using mutagens, scientists can significantly shorten the time it takes to breed new and improved plant varieties compared to the conventional breeding method. Induced mutations offer numerous benefits to crop improvement, especially when conventional breeding techniques fail due to a lack of appropriate genetic variation (FAO/IAEA, 2018).

Mutagenic treatments alter the structure and arrangement of chromosomes. Chromosomes are distinct subunits of an organism's nuclear genome, and any alteration in their structure serves as a strong indicator of the effectiveness of mutagenic treatments. Grover and Tejpaul (1982) observed chromosomal abnormalities such as sticky chromosomes, fragments, and ring chromosomes at metaphase, as well as laggards and bridges at anaphase in *Vigna radiata* treated with gamma rays, maleic hydrazide, and their combinations. These aberrations were found to be significantly dose-dependent. Vandana and Dubey (1996) reported meiotic anomalies in *Vicia faba* induced by EMS and DES, noting that the frequency of these abnormalities increased with higher mutagen concentrations. Interestingly, EMS treatments led to a greater incidence of pairing anomalies, while DES predominantly caused disjunction anomalies during anaphase. Similarly, Khan and Tyagi (2009) reported the occurrence of chromosome bridges and laggards in soybean following treatment with EMS, gamma rays, and their combination.

Polymerase Chain Reaction (PCR)-based markers have been extensively utilized to assess genetic diversity in plants. In particular, the Random

Amplified Polymorphic DNA (RAPD) technique has been effective in evaluating genetic variation and relationships within plant populations (Prakash and Abhilsha, 2016). Despite its nutritional and economic potential in Nigeria, Lima bean remains under-researched (Ikani *et al*., 2024), largely due to its introduction as an alien crop and its limited genetic diversity.

This research sought to assess the level of genetic variability of mutant lines induced by fast neutron irradiation, colchicine and ethyl methanesulfonate using Random Amplified Polymorphic DNA (RAPD) marker due to their efficiency, simplicity, and ability to detect a wide range of DNA polymorphisms without prior genomic information which are valuable for understanding mutation induced diversity and guiding molecular breeding efforts for improved Lima bean cultivars in Nigeria.

Materials and Methods

Source of Germplasm and Sowing of Seeds

Second mutant generation (M_2) seeds (breeders' line) harvested from plants which were treated with different doses of Fast Neutron Irradiation (0.16, 0.32, 0.49 and 0.65 Sv), Colchicine (0.2, 0.4, 0.6, 0.8 mM) and Ethyl methanesulfonate (0.1 0.2, 0.3, 0.4%) as well as the control seeds (untreated) were obtained from the gene bank of Modibbo Adama University. Treated and untreated (control) Lima bean seeds were sown in plastic pots, each filled with sterilised sand-loam soil to raise third mutant generation (M_3) seedling.

Cytological Studies of Lima Bean Root Tip

About 1cm of the root tips from different germinating mutant plant in vermiculite were cut and fixed in Carnoy's fluid (3 parts absolute alcohol and 1 part Glacial Acetic Acid) for 24 hours. Then preserved in 70% alcohol and stored in the refrigerator at 4°C until when needed for laboratory analysis. The root tips were hydrolysed in 1NHCl at 60°C for 10-12 minutes in a water bath; washed twice in two quick changes of distilled water to rinse off the HCl. Hydrolysed root tips were placed on a clean glass slide, and 1-2 drops of aceto-orcein stain were added and covered gently with a cover slip. The cover slip was tapped gently till the stain gradually spread and a smear formed; after which the slide was placed between the folds of a blotting paper and pressed gently with the thumb,

this pressure squeezed out excess stain (Adelanwa *et al.*, 2014). Prepared slides were viewed with the aid of a light microscope at a magnification of X100 and photomicrographs were taken with a Panasonic digital camera DMC-FX77.

Collection of Leaf Samples

Young leaves were collected from two (2) weeks old M_3 seedlings, washed with distilled water, and placed in a white polythene bag. They were properly labelled, stored in a plastic container at -80 °C.

Genomic DNA Extraction

Total genomic Deoxyribonucleic Acid (DNA) for each mutant line was isolated from a bulked leaf tissue sample of one-week-old plants from five randomly selected plants per accession using the Cetyltrimethyl Ammonium Bromide (CTAB) method (Doyle and Doyle, 1987). Three grams of young leaf tissue was ground with liquid nitrogen and 15 ml of preheated CTAB buffer (65°C) was added to the powder. It was then incubated at 65°C in a water bath for one hour. After bringing the tubes to room temperature, an equal volume (15 ml) of chloroform: isoamyl alcohol (24:1) was added and the contents were mixed well for 10 minutes to form an emulsion, centrifuged at 10,000 rpm for 15 minutes at 15°C. The supernatant was transferred to a fresh tube, and the chloroform: isoamyl alcohol step was performed with ice-cold isopropanol added and incubated in a freezer overnight. The contents were then centrifuged at 10,000 rpm for 20 minutes at 16°C. The pellet was saved by discarding the solution and was washed with 70% ethanol by centrifuging the contents at 10,000 rpm for 10 minutes. The alcohol was discarded, and the pellet was air-dried. The pellet was dissolved in 3 mL of double-distilled water. Then, 1 µl of RNase was added and incubated at 37°C for 30 minutes. DNA was precipitated by adding 50 µl of 3M sodium acetate and 7.5 ml of 100% ethanol, and the contents were again centrifuged at 10,000 rpm for 10 minutes. The supernatant was discarded. The pellet was washed with 70% ethanol and air-dried. It was finally dissolved in TE buffer (150 µl) and stored at -20°C.

DNA Quality and Quantity Check

To check the quality and quantity of the extracted genomic DNA, gel electrophoresis was carried out on a 0.8% agarose gel. For Polymerase Chain Reaction (PCR) amplification, DNA concentration was estimated by comparing the band intensity of a sample with known dilutions that gave good amplifications (Selvarasu and Kandhasamy, 2017).

Polymerase Chain Reaction (PCR)

Using four (4) RAPD primers, PCR amplifications were performed in 20 µl reaction volumes containing 5X GoTaq Green Master Mix, 10 µM of each of the forward and reverse primers, 50 ng template DNA, and nuclease-free water up to 20 µl. All the PCR reactions were carried out in 200 µl thin-walled PCR tubes using a Gene Amp 9700 PCR System Thermal cycler (Applied Biosystem Inc., USA). Amplifications was performed in a Thermal Cycler as follows: initial denaturation at 94 °C for 5 minutes, followed by 30 cycles of denaturation at 94 °C for 30 seconds, annealing at 46 – 61 °C (depending on the primer pair) for 60 seconds, extension at 72 °C for 2 minutes with a final extension at 72 °C for 7 minutes. The samples were maintained at 10 °C. The PCR reaction for each RAPD primer was performed at least twice using DNA from independent extractions, and only clear and reproducible bands were used in data evaluation (Gyang *et al.*, 2017).

Separation Of Amplified PCR Products by Agarose Gel Electrophoresis

The separation of bands as produced by each primer was done in a 1.5 % Agarose gel. The buffer (1XTAE buffer) was prepared and subsequently used to prepare a 1.5 % agarose gel. The suspension was boiled in a microwave for 5 minutes. The molten agarose was allowed to cool to 60 °C and stained with 3 µl of 0.5 g/ml ethidium bromide (which absorbs invisible UV light and transmits the energy as visible orange light). A comb was inserted into the slots of the casting tray and the molten agarose was poured into the tray. The gel was allowed to solidify for 20 minutes to form the wells. The 1XTAE buffer was poured into the gel tank to barely submerge the gel. Seven µl of each PCR product was loaded into the wells after the 100 bp DNA ladder was loaded into the wells. The gel was electrophoresed at 120 V for 45 minutes, visualized by ultraviolet

transillumination, and photographed. The sizes of the PCR products were estimated by comparison with the mobility of a 100bp molecular weight ladder that was run alongside experimental samples in the gel.

Scoring And Analysis of Microsatellite Data

The PCR reaction for each rapd primer was performed at least twice. only clear and reproducible bands were used in data evaluation. The alleles/bands were scored as present (1) and absent (0) and were recorded in a data matrix table as discrete variables.

Data Analysis

Binary data matrix was analysed and used to generate the genetic diversity parameters such as major allele frequency, gene diversity, and polymorphic information content using the PowerMarker software (v2.35). The genetic relationship among mutant lines was also estimated by constructing a dendrogram through the Unweighted Pair Group with Arithmetic Mean method (UPGMA), and genetic distances were computed using the Mega6 software.

Results and Discussion

Cytological Abnormalities of Lim Bean Mutant Lines

Various types of chromosomal abnormalities were observed at different doses of fast neutron irradiation. These included asynchronized anaphase (0.32 Sv), diagonal telophase (0.32 Sv), curved bridge at anaphase (0.32 Sv), chromosome fragmentation at metaphase (0.49 Sv), precocious chromosome movement at metaphase (0.49 Sv), scattered chromosomes (0.49 Sv), sticky metaphase (0.65 Sv), C-metaphase (0.65 Sv), unoriented sticky anaphase (0.65 Sv), chromosome bridge at anaphase (0.65 Sv), and unoriented telophase (0.65 Sv).

Colchicine treatment also induced chromosomal anomalies, including sticky chromosomes at early anaphase (0.2 mM), laggard chromosomes (0.2 and 0.4 mM), sticky metaphase (0.8 mM), disturbed anaphase (0.2 mM), scattered chromosomes (0.4 mM), chromosome fragmentation at anaphase (0.4 mM), and chromosome bridges at anaphase (0.4 mM).

Similarly, ethyl methanesulfonate (EMS) caused chromosomal changes such as sticky chromosomes at metaphase (0.2%), scattered metaphase (0.2%), and diagonal telophase (0.4%).

Metaphases with sticky chromosomes lose their typical morphology, appearing with a "sticky" surface that leads to chromosome agglomeration. This phenomenon may result from mutagen-induced alterations in the physicochemical properties of DNA, proteins, or both. It may also be due to the formation of complexes with DNA phosphate groups, impaired DNA condensation, or the creation of inter- and intra-chromatid cross-links (Turkoglu, 2007). Chidambaram *et al.* (2009) suggest that such aberrations could arise from disruptions in nucleic acid metabolism. According to Gaulden (1987), chemically induced stickiness may stem from direct mutagenic action on histone proteins, causing improper DNA folding a finding supported by Pessim *et al.* (2015) in Panicum maximum.

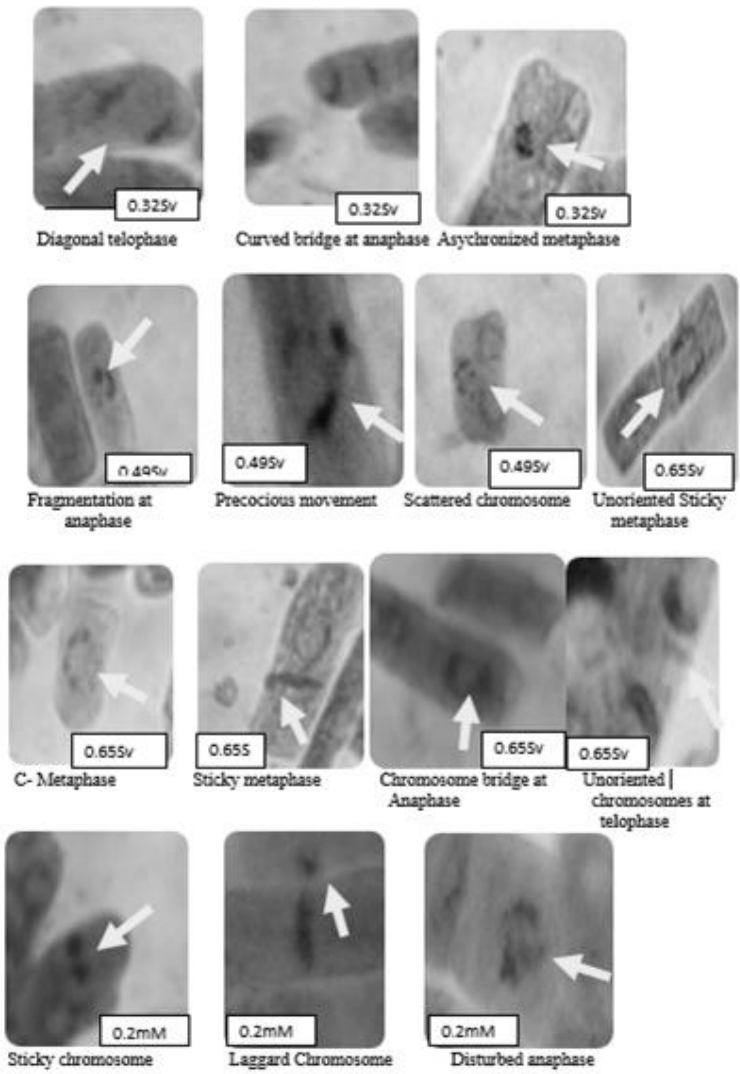
Precocious chromosome movement and disturbed polarity may indicate malfunctioning of the spindle apparatus. These anomalies result from asymmetric spindle action, where certain chromosome arms are abnormally pulled toward one pole. Similar observations were reported by Sri Devi and Mullainathan (2011) in Capsicum annuum, Kumar and Shikha (2012) in *Cyamopsis tetragonoloba*, and Kumar and Dwivedi (2013) in *Trachyspermum ammi*.

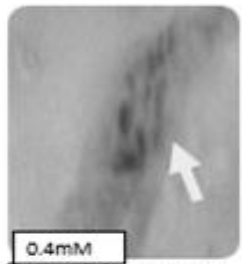
Chromosomal bridges may occur due to stickiness that impedes normal anaphase separation or result from unequal translocation of chromosomal segments. Saylor and Smith (1996) proposed that such bridges arise from the failure of chiasmata to resolve in terminal bivalents, causing chromosomes to stretch between poles. Bridge formation during telophase may also be linked to paracentric inversions. Comparable findings were reported by Kumar and Chaudhary (2015) in *Phaseolus vulgaris* and by Srivastava and Kumar (2014) in *Sesbania cannabina*.

Lagging chromosomes, which fail to attach to spindle fibers, are excluded from normal segregation, leading to genetic imbalance between daughter cells (Truta *et al.*, 2011). This lagging may result from defective spindle apparatus function (Patil and Bhat, 1992). It may also occur due to weak or absent spindle fiber attachment, or from acentric fragments created during chromosomal breaks or exchanges. Similar results were reported by Mahakhode and Somkuwar (2013) in *Psoralea corylifolia* and Bhatta and Sakya (2008) in *Allium cepa*.

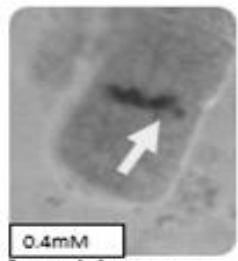
Chromosome scattering and unorientation may result from inhibition of spindle formation or the destruction of already-formed spindle fibers. These findings are consistent with those of Kumar and Yadav (2010) in *Sesamum indicum*, Suthakar *et al.* (2015) in *Sorghum*, and Bharathi *et al.* (2015) in *Withania somnifera*.

Abnormal C-metaphase configurations may arise due to complete spindle inactivation (Fiskesjö, 1993), as also reported by Kumar and Shikha (2012) in *Cyamopsis tetragonoloba*

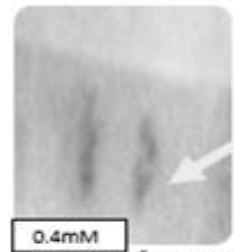




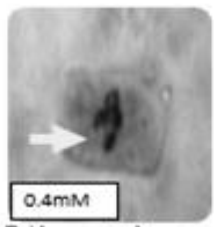
Scattered chromosomes



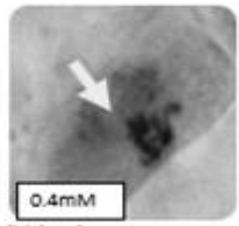
Laggard chromosome



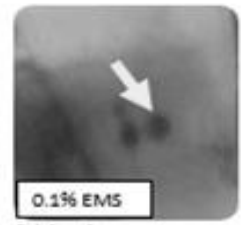
Chromosome fragmentation



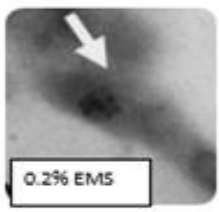
Bridge at anaphase



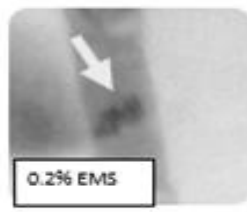
Sticky chromosome



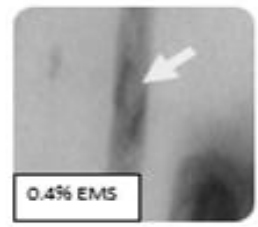
Sticky chromosome



Scattered metaphase



Stickiness at metaphase



Diagonal telophase

Genetic diversity statistic of *P. lunatus* induced by the mutagens based on four (4) RAPD markers

Table 1 shows the genetic diversity statistics of the M_3 mutant lines of Lima bean based on four RAPD markers. The major allele frequency ranged from 23 - 46% with a mean of 36.5%. Primer OPB04 and OPH04 showed the highest major allele frequency of 46% while OPT05 had the lowest major allele frequency of 23%. A total of 27 alleles were detected with an average of 6.75 alleles per primer. Primer OPT20 detected the highest allele number of 8. Average genetic diversity was 77% and ranged from 70 - 83%. The Polymorphic information content (PIC) value, which is a reflection of allele diversity and frequency among the mutants, was found to be high among the mutants with the primers utilized. The highest PIC value of 81% was obtained in OPT20 and OPT05 while the lowest (66%) was recorded in OPH04.

The high PIC values and gene diversity of the primers are indicative that the primers are informative to assess the genetic variability among closely related mutants (Chen *et al.*, 2011). The high polymorphism despite a wide range of genetic similarity implies variations in genes that code for the same character, hence the mutants are thought to have a broad genetic base. High detectable diversity with primer OPT20 and OPT05 within the mutants maybe due to possession of high G-C and C-A repeat by the primers and is an indication that both primers were highly informative in the detection of genetic diversity induced by the mutagens. High PIC value of RAPD markers ranging between 0.34 (OPA-11) and 0.79 (OPG-14 and OPE-1), with a mean of 0.54, have also been reported by Bukhari *et al.* (2015) in the assessment of genetic diversity in common bean.

Table 1: Genetic diversity statistic of *P. lunatus* induced by the mutagens based on four (4) RAPD markers

Makers	Sequences (Forward)	Sequences (Reverse)	Major Allele (%)	Allele No.	Gene Diversity (%)	PIC
OPB04	GGACTGGAGT	ACTCCAGTCC	46.00	7.00	73.00	71.00
OPT20	GACCAATGCC	GGCATTGGTC	31.00	8.00	83.00	81.00
OPH04	GGAAGTCGCC	GGCGACTTCC	46.00	5.00	70.00	66.00
OPT05	GGGTTTGGCA	TGCCAAACCC	23.00	7.00	83.00	81.00
Mean			36.50	6.75	77.00	77775.00

Genetic variability of *P. lunatus* mutants induced by fast neutron irradiation, colchicine and EMS

Dendrogram constructed with PowerMarker software grouped the mutants and the control into 3 groups (Fig. 1). At a genetic distance of 0.1, three clusters were identified. The first cluster comprises three Lima bean mutants (EMS 0.3%, EMS 0.4% and FNI 0.32 Sv). Members of cluster 1 had 0.11 similarities. Cluster 2 comprised the control and five Lima bean mutants (FNI 0.65Sv, control, COLCH. 0.4 mM, EMS 0.1%, COLCH. 0.6mM, and COLCH. 0.8 mM). Members of cluster 2 had 0.05 similarities. Cluster 3 comprised four Lima bean mutants (COLCH. 0.2 mM, EMS 0.2|%, FNI 0.49 Sv, and FNI 0.16 Sv). Members of this cluster had 0.02 similarities. The low genetic similarity between mutants of different clusters and high genetic similarity between mutants within the same cluster, suggests a wide variation of DNA exists between mutants from different clusters and smaller within mutants of the same clusters, similar findings have been reported by Adesoye and Ojobo (2012) in the assessment of genetic diversity of *Phaseolus vulgaris* landraces using RAPD markers. It is worth noting that the mutant lines were not clustered based on the mutagen treatment, which could signify a high level of induced divergence or diversity existing among the mutant lines.

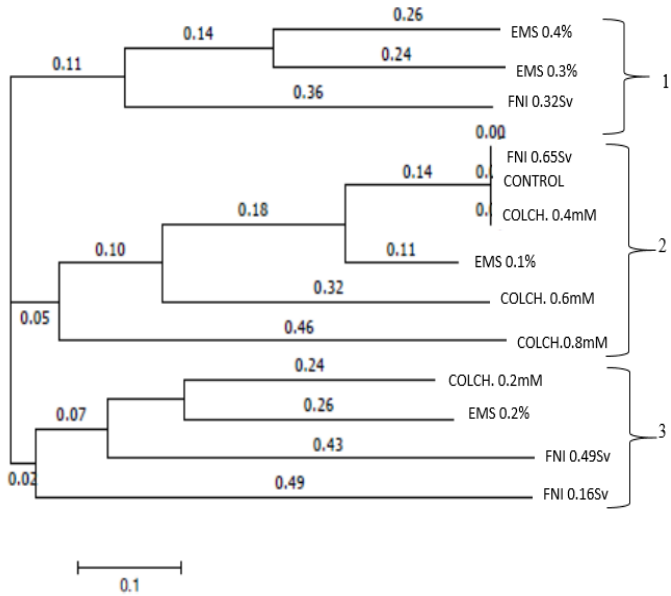


Figure 1: *Dendrogram of Phaseolus lunatus mutants induced by fast neutron irradiation, colchicine and ethyl methanesulfonate based*

Key: FNI- Fast neutron irradiation, COLCH - Colchicine, EMS- Ethyl methanesulfonate

Conclusion

Chromosomal aberrations such as stickiness, laggards, unorientation, fragmentation, and chromosomal bridges were observed in Lima bean mutant lines. RAPD markers were highly polymorphic in the detection of existing genetic diversity in the Lima bean mutant lines, OPT20 and OPT05 were the most polymorphic. The mutant lines were highly diverse, which indicates the effectiveness of fast neutron irradiation, colchicine, and ethyl methanesulfonate in the creation of artificial genetic variability in Lima bean, thus broadening its gene pool for further breeding of the crop in Nigeria.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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