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^{60}Co gamma ray induced mutants of cowpea and assessment of genetic variability by SCoT marker

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Abstract

Mutagenesis is a well-known technique for introducing new variants into crop plants. In the present study, M_2 populations were generated in the cowpea (*Vigna unguiculata* (L.) Walp.) variety CO7 using gamma irradiation. The M_2 progeny were used to investigate the effectiveness of the gamma irradiation doses and examined for the agronomic traits. The variation present in the mutants and their parent were analysed using five SCoT markers. Marker analysis revealed a total of 87 amplicons and among these, 20 amplicons showed polymorphism. The highest numbers of amplicons were observed at SCoT10 (39), while the lowest number of amplicons was produced by SCoT09 (07). The percentage of polymorphism ranged from 18.18% to 28.57%, with an average of 21.12%. Polymorphic information content (PIC) values ranged from 0.197 to 0.345. Analysis of Molecular Variation (AMOVA) showed 12% and 88% between the genotypes and within the genotypes respectively. The constructions of 4 clusters were identified through Unweighted Pair Group Method with Arithmetic Mean (UPGMA) dendrogram tree based on the genetic distance deduced from SCoT marker analysis. Analysis of the genetic relatedness between parent and mutants through Principal Coordinate Analysis (PCoA) revealed two main groups. The present study concluded that the genetic variability induced by gamma irradiation and inherited in the next generations. This research investigation supports that gamma irradiation alters the growth and yield traits, which is helpful for generating the cowpea improvement.

Keywords

Cowpea, Mutant, SCoT Marker, Genetic variation, Polymorphism

Introduction

Cowpea (*Vigna unguiculata* (L.) Walp.) is one of the important legume crops in tropical and sub-tropical ecosystems and also it is a good source of dietary protein. The seed contains 25% of protein, 64% of carbohydrates and other sources of minerals, vitamins, micronutrients (1-3). It is a need for both humans and cattle. The leaves, immature pods and dried seeds were edible (4). It has several health advantages for humans, including anti-diabetic, anti-cancer, anti-hyperlipidaemic, anti-inflammatory and antihypertensive effects (5). Cowpea may thrive in drought-stressed settings and improve soil fertility by introducing nitrogen-fixing bacteria into the soil. It has low yields in underdeveloped nations due to a lack of better cultivars, limited input utilization and inadequate management (6). However, since

past years, there was no much attention to improve this variety, hence, we taken for consideration to induced mutation for improving the traits and genetic variation. The availability of genetic diversity is the basis for crop development, but since cowpea are predominantly self-pollinated, there is a dearth of genetic variability. Induced mutations offer a means of generating unique genetic variations for cowpea breeding programmes (7).

The increasing world population and climate changes have increased concerns over food security and the projected target is to double the food production by 2050 (8, 9). Current crop science strives towards the development of new crop varieties with improved growth, development and high yielding potential. Even though many technologies are being introduced in the crop sciences to improve the crop performance to attain the highest yield with limited inputs, there are still mysterious gaps in the crop science to achieve the goal. In this context, the creation of new variations in crop plants to screen the best lines with improved yield is the prime objective of crop breeding programmes. Mutagenesis is one of the inevitable techniques to generate novel variations in crop plants. Since the occurrence of mutations spontaneously is too low (10^{-5} to 10^{-8}) (10), the induced mutation is the best way to generate variations in plants. Gamma irradiation is an effective method of physical mutagenesis, to create genetic variation in crops to modify the traits or generate new traits for selection. Apart from the creation of new variations, induced mutagenesis may cause genetic changes in organisms to break the gene linkage, resulting in the producing of new promising traits by removing undesirable traits (11). There have been 3364 varieties officially released by International Atomic Energy Agency/Mutant Variety Database (IAEA/MVD); 2610 mutant varieties released by physical mutagens, among these 1703 varieties are released only through gamma irradiation (12).

Mutation detection in plants may be investigated using both phenotypic and genomic methodologies. There are various limitations and non-fixed properties in phenotypic techniques. Genomic tools offer a more reliable procedure and are more suited for detecting mutations earlier (13). Molecular markers assist breeders in estimating genetic variation among genotypes for various agronomic characteristics (14). In recent years, molecular marker-based approaches in genetic research, such as estimating genetic diversity and population structure, have evolved dramatically (15). Molecular markers are valuable for assessing genetic diversity based on agronomic, morphological and biochemical traits (16). PCR-based markers are useful tools for plant breeding and estimating genetic diversity at the species or sub-species level (17). Cowpea has employed a variety of DNA markers, including restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNAs (RAPD), amplified fragment length polymorphisms (AFLPs), and inter simple sequence repeats (ISSRs) (18-20). Recently, a simple, innovative DNA marker technology known as start codon-targeted polymorphism (SCoT) has been employed for genetic research of agricultural plants, particularly cowpea (21, 22). The SCoT approach is a kind of targeted marker technique in which the ATG context is one of the functional genes and is tied to functional genes and their associated features (23). The SCoT marker was created by combining a short conserved area bordering the translation beginning codon, ATG in plant gene, with a long primer length, which annealed at higher temperatures and produced repeatability than RAPD markers (24-26). Hence, the SCoT markers were employed to investigate the genetic variants of gamma irradiation-induced cowpea mutants and their parent.

Materials and Methods

Seed material and Mutagenic treatment

Cowpea seeds (CO7) were obtained from the National Pulse Research Centre (NPRC), Vamban, Pudukkottai, Tamil Nadu, India. A total of six batches and each containing 100 seeds were irradiated in the gamma chamber available at the Indira Gandhi Centre for Atomic Research (IGCAR), Kalpakam, Tamil Nadu, India. The doses used for gamma irradiation were 200, 400, 600, 800, 1000 and 1200 Gy. After irradiation, seeds were sown in the field along with control. The experiment was laid out as a randomised block design with three replications. Untreated, healthy seeds were used as a control. The space between rows and plants were adopted 45 and 15 cm respectively. All the cultural practices such as, weeding, irrigation, using pesticides and insecticides for crop protection were practiced at regular intervals. The M_1 seeds were collected from the respective doses and control plants.

Screening of Mutants

The M_2 generation was raised from the M_1 seeds of each treatment with a randomised block design with three replications. Screening for the mutants in each treatment was carried out by scoring the M_2 plants for any change in phenotype observed compared with the parent plant (control) in the field from germination to maturity. The selection of mutants was progressively from the seedling to maturity stage in the field based on their phenotype. A wide range of mutants were selected in the M_2 generation of different doses namely, xantha 400 Gy, three primary leaves 400 Gy, dwarf 400 Gy, dwarf 600 Gy, first flowering 400 Gy, first flowering 600 Gy, sessile 600 Gy, bold seed 400 Gy, small seed 200 Gy, constricted pod 200 Gy and high yield 200 Gy along with the control plant (Fig. 1).

Genomic DNA Extraction and SCoT Amplification

The 200 mg of leaf tissue (control and mutants) was used for extraction of genomic DNA with the HipurA™ Super Plant DNA purification kit (Himedia, Code: MB571; Mumbai, India). Five SCoT primers were custom synthesised by Sigma Aldrich (Bangalore, India), and they consists of GC content in the middle of 50 and 61% (Table 1). The PCR reaction was performed in a Thermal Cycler (Cyberlab, Smart PCR) with GoTaq G2 Green PCR master mix (Promega, Cat: M7822; Madison, USA) which consisted of 1x concentration of 12 µl reaction mixture. The PCR program was: 3 minutes 94°C, 40 cycles of 1 min at 94°C, 1 min at 50°C, 2 min 72°C, followed by 5 minutes of final extension at 72°C. The PCR

Fig. 1. Spectrum of viable mutants induced by γ irradiation in the cowpea showed various morphometric changes with agronomic traits. **a**) Xantha 400 Gy, **b**) Three primary leaves, **c**) Dwarf 400 Gy, **d**) First flowering 400 Gy, **e**) Sessile 600 Gy, **f**) Bold seed 400 Gy, **g**) Small seed 200 Gy, **h**) Constricted pod 200 Gy, **i**) High yield 200 Gy, **j**) Control.

reactions mixture (12µl) was checked on 1.5% agarose (Himedia, Mumbai) in a 1x TAE buffer gel. The amplified profiles were visualised under a UV - transilluminator.

Table 1. List of SCoT primers used in the study

Sl. No.	Name of primer	Primer sequence (5'-3')
1.	SCoT03	CAACAATGGCTACCACGC
2.	SCoT07	ACGACATGGCGACCATCG
3.	SCoT08	ACCATGGCTACCACCGAC
4.	SCoT09	ACCATGGCTACCACCGAG
5.	SCoT10	ACGACATGGCGACCCACA

Data Collection and Statistical Analysis

The amplicons of SCoT were converted to the binary matrix as presence (1) or absence (0) and entered in to the Microsoft Excel sheet. The matric was assessed by Free Tree software, ver. 9.1 using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) construction method and similarity coefficient (27). Principal Coordinate Analysis (PCoA) and Analysis of Molecular Variance (AMOVA) were performed in the software-GenAlEx v.6.1 (28). Polymorphism information content (PIC) is determined as https://gene-calc.pl/pic**.** The percentage of polymorphic variation was calculated by using the following formula.

$$\text{Percentage of polymorphism} = \frac{\text{Number of polymorphic bands}}{\text{Total number of bands}} \times 100$$

Results

In this experiment, SCoT markers were used to study the genetic variation in mutants induced by gamma irradiation including, xantha (400 Gy), three primary leaves (400 Gy), dwarf (400 Gy), dwarf (600 Gy), first flowering (400 Gy), first flowering (600 Gy), sessile (600 Gy), bold seed (400 Gy), small seed (200 Gy), constricted pod (200 Gy) and high yield (200 Gy) of cowpea.

Genetic Variation Analysis by SCoT marker

SCoT markers amplification and data scoring results were analysed to identify the genetic diversity in mutant plants (Table 2). The highest number of polymorphic bands was observed with SCoT10 (11 polymorphic bands), while the lowest number of polymorphic bands was scored by SCoT08 (only one polymorphic band). The number of bands varied from mutant to mutant samples. A total of 87 bands were amplified, 20 bands were polymorphic bands with an average a 4 bands for each marker. The highest number of total bands was observed with SCoT10 (39), while the least number of total bands was scored by SCoT09 (07). The percentage of polymorphism from 18.18% to 28.57% with an average of 21.12%. The polymorphic information content (PIC) values are ranged from 0.197 to 0.345, with an average value of 0.281 per primer. SCoT10 marker produced the highest number of bands and also polymorphic bands. SCoT09 marker produced the lowest number of bands. In control samples, SCoT10 marker was only amplified, among the 5 markers. All markers produced bands in the three primary leaves mutant; there was no amplification in the small seed mutant. In comparison to the parent genotype, mutants showed different banding pattern during the marker analysis.

Table 2. SCoT primers used in mutant samples of cowpea under the effects of gamma irradiation

No. of primers	Mutant Samples												Total no. of bands	No. of polymorphic bands	% of polymorphism	Polymorphic Information Content (PIC)
	1	2	3	4	5	6	7	8	9	10	11	12				
SCOT03	0	1	2	1	0	3	0	4	0	0	0	0	11	2	18.18	0.2604
SCOT07	0	1	3	2	3	3	2	2	2	0	3	1	22	4	18.18	0.2604
SCOT08	0	0	2	1	0	1	0	3	0	0	1	0	8	1	12.50	0.197
SCOT09	0	0	2	0	1	0	0	1	1	0	2	0	7	2	28.57	0.3457
SCOT10	5	5	5	5	5	5	0	0	6	0	3	0	39	11	28.20	0.3432
Total	5	7	14	9	9	12	2	10	9	0	9	1	87	20	105.63	1.406
Average													17.4	4.00	21.12	0.281

1- Control, **2**- Xantha 400Gy, **3**- Three primary leaves 400 Gy, **4**- Dwarf 400 Gy, **5-** Dwarf 600 Gy, **6**- First flowering 400 Gy, **7-**First Flowering 600 Gy, **8-**Sessile 600 Gy, **9**- Bold seed 400 Gy, **10**-Small seed 200 Gy, **11**-Constricted pod 200 Gy, **12**-High yield 200 Gy

Thus, it was confirmed that the gamma irradiation induced genetic variability in cowpea.

Distance Matrix and Analysis of Molecular Variation (AMOVA)

The genetic distance matrix or similarity analysis based on the SCoT revealed the distance ranges of 0.133 to 0.941 (Table 3). The highest distance was observed in between the dwarf 600 Gy and bold seed 400 Gy (0.941) mutants, while the lowest distance was observed between three primary leaves and the high yield 200 Gy (0.133) mutants. Here, some of the mutants showed no genetic distance between the mutant genotypes. These variations were caused by changes in DNA banding patterns due to mutations induced by gamma irradiation. The AMOVA analysis revealed significant variations among the groups and within the groups (Table 4). In that, the genetic variation among genotypes was 12%, whereas within the genotypes was 88%.

Table 3. Distance matrix revealed by SCoT marker

Mutant	1	2	3	4	5	6	7	8	9	10	11	12
1	1	0.833	0.526	0.769	0.714	0.588	0	0	0.7692	0	0.461	0
2		1	0.571	0.666	0.75	0.631	0.222	0	0.8	0	0.4	0
3			1	0.727	0.782	0.846	0.25	0.521	0.727	0	0.545	0.133
4				1	0.705	0.7	0.2	0.352	0.75	0	0.5	0
5					1	0.761	0.363	0.222	0.941	0	0.588	0.2
6						1	0.285	0.476	0.7	0	0.5	0.153
7							1	0.181	0.4	0	0.2	0
8								1	0.235	0	0.470	0
9									1	0	0.625	0
10										1	0	0
11											1	0
12												1

1- Control, **2**- Xantha 400Gy, **3**- Three primary leaves 400 Gy, **4**- Dwarf 400 Gy, **5**- Dwarf 600 Gy, **6-** First flowering 400 Gy, **7**-First Flowering 600 Gy, **8**-Sessile 600 Gy, **9**- Bold seed 400 Gy, **10**-Small seed 200 Gy, **11**-Constricted pod 200 Gy, **12**-High yield 200 Gy.

Table 4. Analysis of molecular variation (AMOVA) in mutant samples of cowpea by SCoT marker

Source	df	SS	MS	Est.Var.	% of variation
Among population	11	13.677	1.243	0.083	12%
Within population	84	48.750	0.580	0.580	88%
Total	95	62.427		0.663	100%

df- Degree of freedom, SS-Sum of squares deviation, MS- Mean of squared deviation, Est. Var.- Estimates of variance, %-Percentage of variation.

Dendrogram

A dendrogram was obtained from UPGMA analysis of genetic similarity based on the SCoT marker presented in Fig. 2. The dendrogram was constructed based on the genetic distance between the mutants and parent, which was ranged from 0.1 to 0.500. The dendrogram tree showed 4 main distinct clusters of mutants. The first cluster comprised two mutants such as, small seed 200 Gy and high yield 200 Gy. These are the mutant to operational taxonomic units (OTU) for other mutants. The second and third clusters share a common branch. The second cluster involved sessile 600 Gy. The first flowering 600 Gy shared a common node because characters are almost identical. The third cluster contains xantha 400 Gy and control, while the constricted pod is a common ancestor for control and xantha 400 Gy. The fourth cluster divided into two inter-

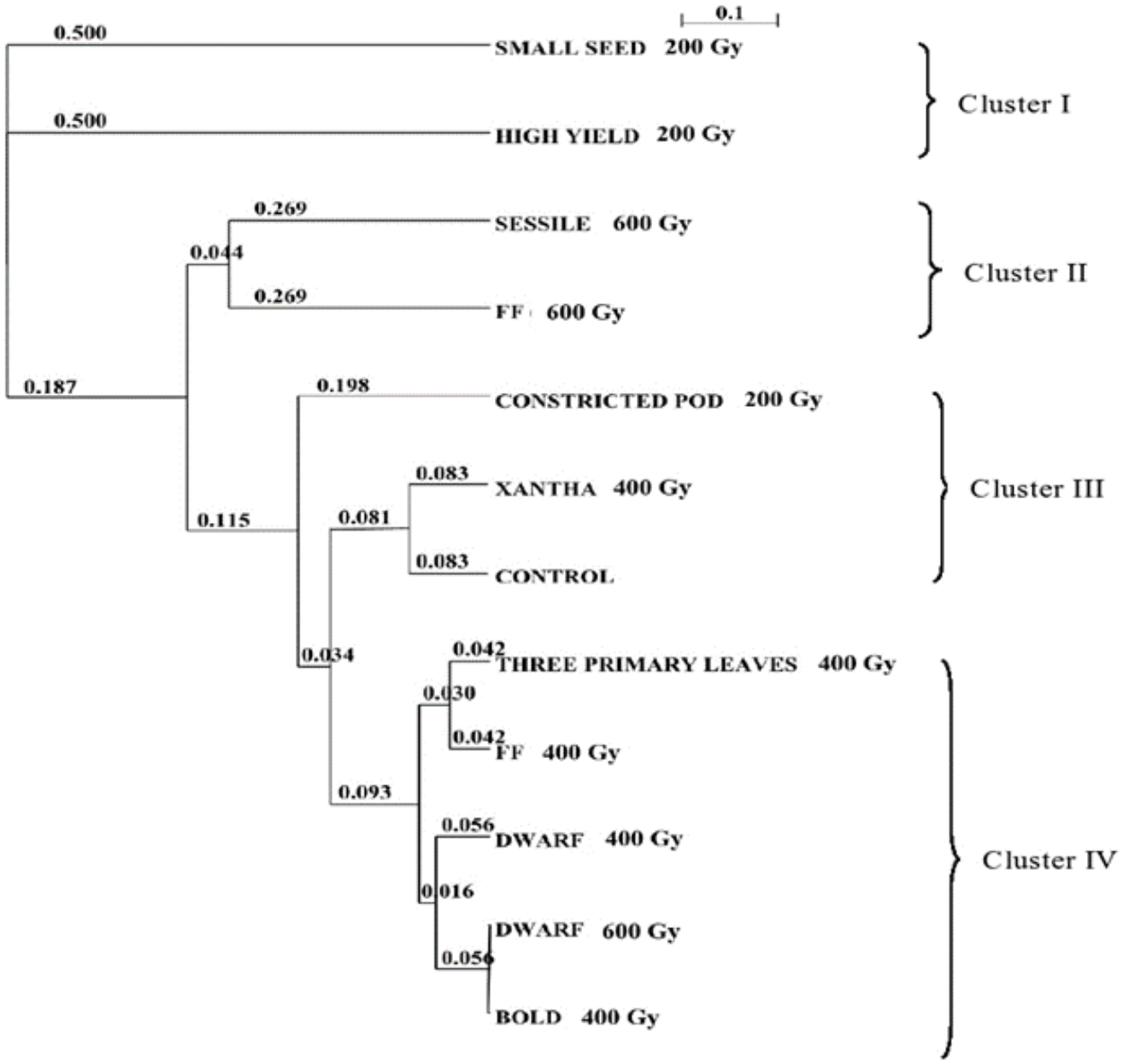

Fig. 2. Dendrogram representing the morphological variation mutants based on genetic relationships between the mutant and control sample. The neighbor-joining method (Free Tree ware) was applied for the dendrogram tree.

node groups. Group I comprise three primary leaves 400 Gy and the first flowering 400 Gy share a common node. Group II contains dwarf 600 Gy and bold seed 400 Gy, which sharing common node. The dwarf 400 Gy is the common ancestor of the dwarf 600 Gy and bold seed 400 Gy. According to the dendrogram, the generated mutants are more distinct from control genotypes.

The Principal Coordinate Analysis (PCoA)

PCoA analysis was carried out to elucidate the genetic relationship between the mutants and parent genotypes based on the SCoT data. PCoA analysis revealed two main groups (Fig. 3). Group I consists of 10 genotypes, and Group II consist of six genotypes. The remaining genotypes are separately or closely positioned. The first, second and third axes represented 45.41%, 69.03% and 84.69% of the cumulative variation.

Discussion

Gamma irradiation increases genetic diversity and yields novel varieties with favourable traits, which aids in advancing agronomic and agricultural improvement (29). Except for days to first flowering, gamma irradiation suppressed changes in morphological and quantitative features in the M_1 generation of cowpea. In our previous studies, the quantitative traits such as plant height, number of branches per plant, number of leaves per plant, number of fruit clusters per plant, number of pods per plant, pod length, number of seeds per pod, hundred seed weight and seed yield per plant were gradually decreased in all concentrations compared to the control (7). In M_2 generation, gamma radiation induced phenotypic alteration of traits leads to obtained new mutants. Screened mutants were categorized based on the phenotypic appearance such as xantha (400 Gy), three primary leaves (400 Gy), dwarf (400 Gy), dwarf (600 Gy), first flowering (400 Gy), first

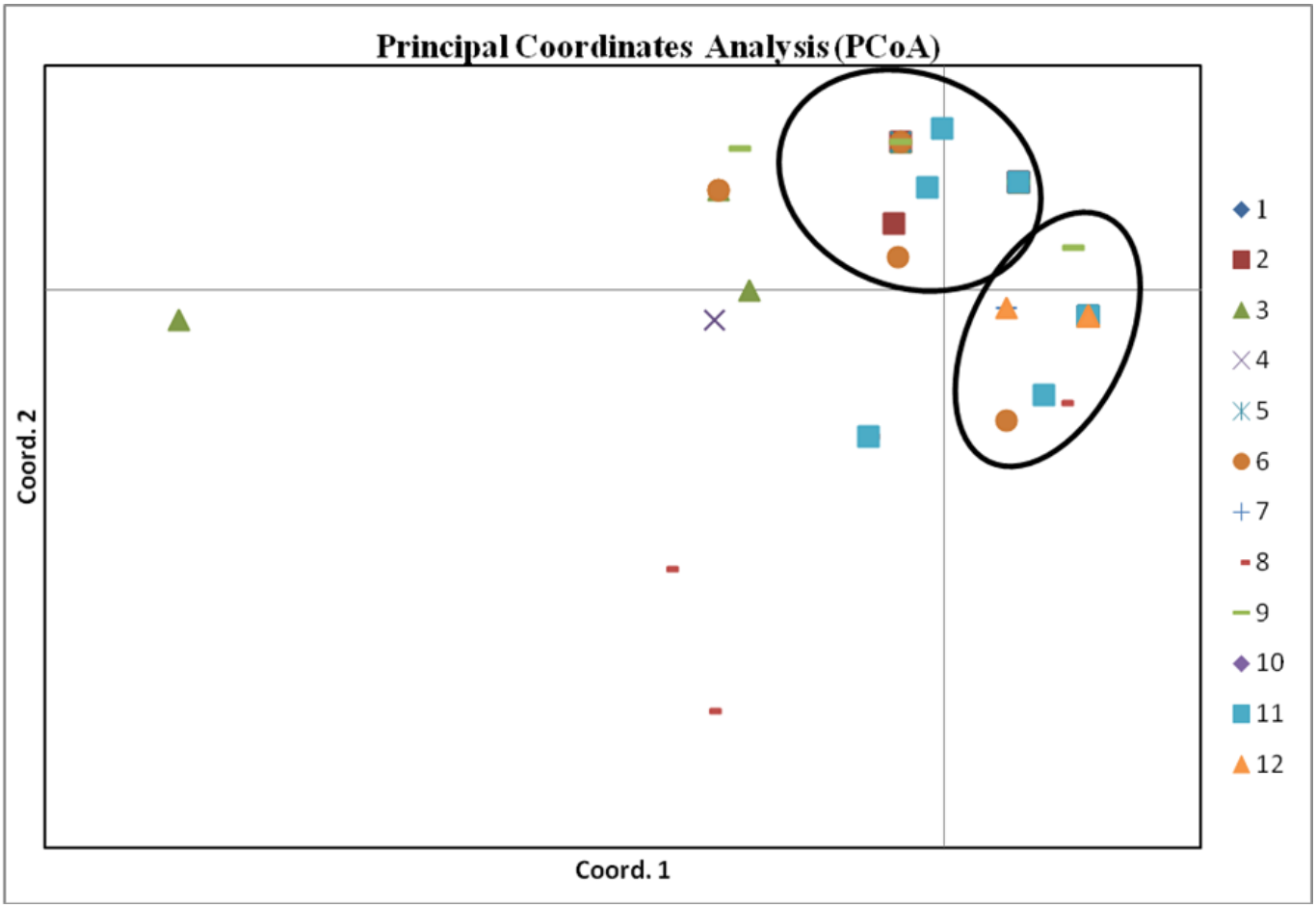

Fig. 3. PCoA analysis genetic variation in the mutant and control plant of cowpea. **1**- Control, **2**- Xantha 400Gy, **3**- Three primary leaves 400 Gy, **4**- Dwarf 400 Gy, **5**- Dwarf 600 Gy, **6**- First flowering 400 Gy, **7**-First Flowering 600 Gy, **8**-Sessile 600 Gy, **9**- Bold seed 400 Gy, **10**-Small seed 200 Gy, **11**-Constricted pod 200 Gy, **12**-High yield 200 Gy.

flowering (600 Gy), sessile (600 Gy), bold seed (400 Gy), small seed (200 Gy), constricted pod (200 Gy) and high yield (200 Gy) was noted in M_2 generation. All these mutants varied when compared to control. Both viable and pod mutants were reported in black gram induced by gamma rays (30). Gamma irradiation induced mutants could help breeders to create new variations in the selection of new varieties (31, 32).

DNA markers are used to identify the diversity in germplasm resources, to determine the genetic diversity and percentage, genetic distance, gene map and marker-assisted selection (33-36) in crop improvement programmes. In the current investigation, the five SCoT markers were used to assess the genetic variability of the mutants and control plant. The five primers, showed amplification of 87 bands, in which 20 were polymorphic, with an average of 4 bands for each marker. Compared to control samples, the present study shows the appearance and disappearance of DNA bands in mutant samples. SCoT maker analysis showed polymorphism variation in gamma -irradiated mutant plants. Earlier findings (37) claimed that mutagens caused polymorphisms in mutants detected by the RAPD marker in black gram. The emergence or disappearance of DNA bands were identified in soybean after gamma irradiation using an ISSR marker (38). Compared to the parent and other mutants indicated by RAPD and ISSR, our results coincided with black gram caused diverse DNA polymorphism in young chlorina and smooth pod mutants. Plant breeders and molecular scientists may further use these mutants to investigate the functional impact of mutations (39).

On the other hand, SCoT maker exposed the polymorphic variation in mutant plants induced by gamma irradiation. The previous report of *Jatropha curcas* samples showed appearance of new bands might be changes in the oligonucleotide priming site due to mutation, deletion, and homolog recombination (40). In other case, disappearance of a fragment in the mutant plants due to DNA damages such as single or double strand breaks, bulky adducts, oxidized bases and modifications of base site, point mutations, DNA protein, cross links and complex chromosomal arrangement and also disassociation of the enzyme complex during the Taq DNA polymerase reaction by the effect of gamma irradiation (41, 42). It may be occurred due to the addition, deletion and transition of a DNA banding influenced by gamma irradiation.

Polymorphism information content (PIC) was considered to estimate the discriminatory power of the marker and determined by the ability of the marker to generate polymorphism on their distribution of frequency (43). The dendrogram and Principal Coordinate analysis (PCoA) is very useful to know about the similarities and dissimilarities between the mutants and control genotypes. Genetic similarity and clustering analysis were helpful to create different genotypes with different genetic backgrounds and can use the development of variety with high produc-

tivity (44). The previous report of genetic distance obtained was 0.78-0.97 and the dendrogram showed four main clusters in *Typhonium flagelliforme* mutants (45). The genetic distance ranged from 0.05 to 0.30 based on AFLP marker and 0.13 to 0.44 ranges from RAPD marker observed in 10 mutant lines of cowpea (46). The genetic matrix was calculated to analyse the mutant and control genotype. In this study, gamma irradiation caused the formation of new bands in mutant plants compared to controls. SCoT marker technology is vital in identifying genetic variation between control and mutant plants. It was shown that gamma irradiation causes phenotypic and genetic level alterations in cowpea, which will be helpful for crop development.

Conclusion

The present study concluded that gamma irradiation caused genomic sequence variations in the cowpea plant. In the cowpea mutant genotypes, the mutations resulted in substantial phenotypic diversity. The development and disappearance of amplicons in mutant genotypes compared to their control type demonstrated that gamma irradiation influenced genetic variability. The difference in the banding pattern of the SCoT markers, clustering pattern, and genetic distance findings in the mutant genotypes further corroborate the usefulness of gamma irradiation and its mutagenesis efficiency in the cowpea crop. Since the primary goals of crop improvement programmes are to create variations and identify variations at both the phenotypic and genotypic levels in crop plants. The combination of gamma mutagenesis and SCoT marker analysis will aid in the development of new cowpea varieties with improved performance in terms of biotic and abiotic stress tolerance, yield and yield contributing traits.

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Authors contributions

Designing of Experiments was made by DAB, Fieldwork and data collection by SV, Laboratory experiment, analysis of data, interpretation and statistical analysis by DAB, SV, DE and SG, Preparation of manuscript by DAB, SV and SG

Compliance with ethical standards

Conflict of interest: The authors declare no conflict of interest.

Ethical issues: None.

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