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# Estimation of Morphological and Molecular Variability in Induced Mutant Fennel Lines

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## ABSTRACT

In the present study, $M_3$ mutant lines generated from gamma radiation of widely adopted fennel cultivar Rajasthan Fennel-125 (RF- 125) was used. High variability was recorded for time of flowering, number of primary and secondary branches, number of umbels and umbellate/plant, number of seeds/umbel, plant height, days to maturity and seed yield/plant. Total of 24 diverse mutants were further selected for molecular characterization based on morphological diversity. The Random Amplified Polymorphic DNA (RAPD) analysis showed broad genetic diversity among the mutant lines. Total 73 loci were amplified in all 24 mutants and 23 loci were found polymorphic. Dendrogram showed similarity coefficient value in range of 0.00 to 0.88. The dendrogram divided the 24 mutants into four main clusters. The morphological and molecular study revealed that the mutants selected from the $M_2$ generation showed variability at the genetic level for different essential traits of fennel $M_3$ generation.

*KEYWORDS*

Fennel, Gamma rays, Genetic diversity, Molecular marker, Morphological characterization, Mutant lines, PCR

## INTRODUCTION

Fennel (*Foeniculum vulgare* Mill.) is a member of Apiaceae family with a diploid chromosome number of 2n=22. The nature of the crop is annual herbaceous and cross-pollinated. The crop is successfully cultivated in moderately cool and dry climate during *Rabi* season in an area free from severe frost during flowering. Fruit usually recognized as seed and contains essential oil in its seed. It is also used to provide aroma in several food products (Zoubiri *et al.* 2014), and a constituent of several cosmetics and medicine products (Telci *et al.*, 2009; Kooti *et al.*, 2015). The extract of fennel is an enormous source of medicinal value and has high free radical scavenging ability that can be used for therapeutic purposes (Choudhary *et al.*, 2017; Lucinewton *et al.*, 2005). The average plant height is very high and generally grows more than 2 metres, hence, it is susceptible to lodging, thus reducing the yield and quality of produce. It has been documented that fennel crop is lacking in its genetic base for such characters and to generate such variability in fennel, the induced mutation is one of the viable methods (Verma *et al.*, 2017; 2018). Limited information is available on the improvement of the fennel crop by non-conventional breeding approaches. The induced mutation has been widely used in different crops and species to generate a broad spectrum of genetic variability (Tanaka *et al.*, 2010). Mutagens created variability in $M_2$ generation and confirmation of mutants can be done in $M_3$ generation. Confirmation can be done based on molecular and or morphological characterization (Çagirgan 2001; Forster and Shu, 2011; Laskar *et al.*, 2018), DUS guidelines are essential for variety registration as followed informed by Verma *et al.* (2015) in charsanthemum. In the morphological characterization of plants, traits which are inherited, simple to score and express every time in all conditions are vigilantly described (Lopes *et al.*, 2010). In the breeding program of crop plants, to address the improvement of different characters, it is very important to estimate the quantity of genetic variability present in the inhabitants. Therefore, the use of morphological characterization along with molecular markers would provide better result to understand the level of genetic variability present in the fennel mutants.

Molecular tools are the advanced method for diversity analysis in fennel as these tools are time-saving, reliable and reproducible (Choudhary *et al.*, 2018). To morphologically confirm and validate the extent of genetic variability among induced mutants population requires number of years. Hence, the variability created through induced mutation can be confirmed in $M_3$ generation based on morphological and molecular characterization (Çagirgan, 2001). Among the molecular marker techniques, RAPD is the most widely used, as results obtained quickly and are fairly less expensive to generate as compared to other advanced molecular techniques. Furthermore, RAPD markers are easy to generate and allow analysis of very small amount of sample and require no prior genetic information. Molecular analysis, along with morphological data, can help in the registration and certification of advanced breeding lines as commercial varieties with diverse genetic base. Molecular marker coupled with phenotype marker give more reliable information for characterization of the mutants. Therefore, an investigation was carried out to characterize the fennel mutant population based on morphological and molecular markers.

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## MATERIAL AND METHODS

### Plant materials

The mutant lines used were generated from different doses gamma rays, i.e. 150 Gy, 175 Gy, 200 Gy, 225 Gy and 250 Gy. The plant materials handled in this study was 99 mutant lines of $M_3$ generation of fennel cv. Rajasthan Fennel-125 (RF-125). These fennel mutant lines were produced from the selfing of gamma rays induced mutant lines of $M_2$ generation.

### Field experiment

The experiment was conducted in the *Rabi* season of the year 2016-2017at ICAR- National Research Centre on Seed Spices, Ajmer, Rajasthan, India. To confirm mutation and to know the stability of selected mutants for different characters, $M_3$ population was raised from $M_2$ seeds. Ninety-nine $M_3$ mutant lines together with the parent (RF-125) were evaluated under field condition during cropping seasons. The selected mutants in $M_2$ generation were sown plant to progeny row in 2x3 m size plot. The selected mutant lines were covered with a muslin cloth to prevent out-crossing at flowering stage. All recommended agronomical practices for fennel crop were carried out.

### Morphological analysis

All the mutant lines were morphologically discriminated by fennel descriptors at the various growth phases i.e. days taken for flower initiation, primary and secondary branches/plant, number of umbel/plant, number of umbellate/umbel, number of seed/umbel, plant height (cm) at the time of harvesting, days to maturity, seed yield/plant and test weight (g).

### DNA isolation, PCR amplification and data analysis

The young and healthy leaves were harvested after 8 weeks of sowing. The whole genomic DNA was extracted from the fresh leaves by means of CTAB method (Doyle and Doyle, 1990) with minor modifications (Choudhary *et al.*, 2015). Twenty RAPD primers were used for PCR amplification. Scoring of bands and analysis were done using method described by Choudhary *et al.* (2018).

### Statistical analysis

The scoring of RAPD marker was done based on the presence (1) or absence (0) for every marker locus. The recorded data analysis was carried out using the NTSYSpc version 2.0 (Rohlf, 1998). Data were entered in a binary data matrix as discrete variables and pair-wise similarities were acquired using the Jaccard's coefficients (Jaccard, 1908). The matrices of similarity coefficients were subjected to unweighted pair group method with arithmetic average (UPGMA) for measurement of the genetic similarities among the mutant lines and generate the dendrogram. The Principal component analysis (PCA) was determined to underscore the resolving power of the ordination.

## RESULTS AND DISCUSSION

### Morphological diversity

A wide range of variation was observed among the 99 mutants of fennel (M-2 to M-100) with regard to different morphological characters (Table 1). Based on the morphological characterization of 99 mutants (Fig. 1a), 24 more diverse and representative of each group from 99 mutants were selected for molecular characterization.

**Table 1:** Morphological characterization of fennel mutant lines

| Traits | Parent variety (RF-125) | Novel mutant lines with specific traits |
|---|---|---|
| Flower appearance (day) | 56 | M-36 (54 days), M -39 (55 days), M-33 (56) |
| Number of primary branches/plant | 13 | M-4 (20), M-32 & M -72 (18), M -35, M -47 & M -49 (16), M -22, M -23, M -34, M -55, M -84& M -85 (15) |
| Number of secondary branches/plant | 46 | M-72 (58), M -22 (55), M -17, M -70, M-77 & M -88 (52), M-55 (50), M-2, M -4, M -58, M -84& M -97 (48) |
| Number of umbels/ plant | 86 | M-90 (260), M-7 (242), M -84 (215), M -4 (205), M -47 & M -98, (186), M -88 (178), M -14 & M -77 (176), M-2 (175) |
| Number of umbellate/umbel | 26 | M-85 (42), M -13 (35), M -22 &M -52 (31), M-32, M-47 & M-84 (30), M-19, M-73 & M-98 (29) |
| Number of seeds/ umbel | 676 | M-85 (980), M -52 ( 961), M -13 (945), M-84 (930), M-19 (899), M-47 (870), M -92 (840), M -23 (806), M-39 (812) |
| Plant height at the time of maturity (cm) | 157 | M-36 (60 cm), M -40&M-61 (67.5 cm), M-76 (72 cm), M -69& M -69 (75 cm), M-74 (78 cm), M-62, M -27, M -82 & M-99 (82.5 cm), M-60 (84), M -3,M-33 & M -38 (90), M-30, M-51&M-71 (97.5 cm) |
| Days to maturity (days) | 179 | M-38 (155 days), M -36 (161 days) |
| Seed yield/plant (g) | 121 | M-4 (307.1 g), M -35 (270.99 g), M-55 (275.42 g) |
| Test weight (g) | 4.2 | M-70 & M -97 (10 g), M -73 (8.6g), M - 63 (8.52 g), M-91 (8.3 g) |

All 99 mutants of fennel showed a wide range of variability for characters like days to appearance of flower (54-107), primary branches/plant (07-20), secondary branches/plant (16-58), number of umbel/plant (24-260), number of umbellate/umbel (10-42), number of seed/umbel (120-980), plant height at the time of maturity (60-195 cm), days to maturity (155-187), seed yield/plant (04-307g) and test weight (3.23-10g). The flower initiation in mutants varied from a minimum of 54 days (M-36) to a maximum of 107 days (M-32) compared to 56 days for parent cultivar- RF-125 (Table 1). The primary branch is one of the important characters of yield attributing traits of fennel crop that directly related to the yield. Mutants line M-4 (20) recorded maximum number of primary branches followed by M-32 & M-72 (18), M-35, M-47& M-49 (16), whereas least (7) primary branches were recorded in M-27, M-36, M-45, M-62 and M-67. Control (RF-125) was recorded 13 numbers of primary branches. Likewise, M-72 (58) had the highest secondary branches followed by M-22 (55), M-17, M-70, M-77 and M-88 (52) whereas lowest secondary branches were recorded in M-78 (16). Forty-six numbers of secondary

branches were recorded in parent. Highest umbels per plant were found in mutant line M-90 (260) umbels followed by M-7 (242), M-84 (215), M-4 (205), M-47& M-98 (186). Lowest umbels per plant were found in mutant M-27 (24) while control (RF-125) was recorded with 86 number of umbel/plant. Highest number of umbellate/umbel was registered in mutant M-85 (42) followed by M-13 (35), M-22 &M-52 (31). The lowest (10) umbellate per umbel was recorded in mutants M-38, M-40 & M-62 (10) and control recorded 26 number of umbellate per umbel. The maximum number of seeds was recorded in M-85 (980) followed by M-52 (961), M-13 (945), M-84 (930) and RF-125 recorded 676 seed per umbel. The dwarf mutants were also identified in the study with height ranging from 60 cm (M-36) to 78 cm (M-74) compared to 157.5 cm height of control plant at the time of maturity. Mutant M-38 (155 days) took minimum days to mature followed by M-36 (161), which was early than parent plant (179 days). Among the different mutant lines, seed yield significant increased as compared to the control plant. Maximum seed yield/plant was registered in M-4 (307.1 g) followed by M-55 (275.4 g) and M-35 (270.9 g) whereas 121 g seed yield per plant was recorded in parent plant. In mutant lines M-70& M- 97, highest test weight (10 g) was found followed by M-73 (8.6 g), M-63 (8.52 g) and M-91 (8.3 g) as compared to control (4.2 g). The huge variation was recorded in all the 99 fennel mutants as compared to parental line (RF-125) with regards to all the characters studied.

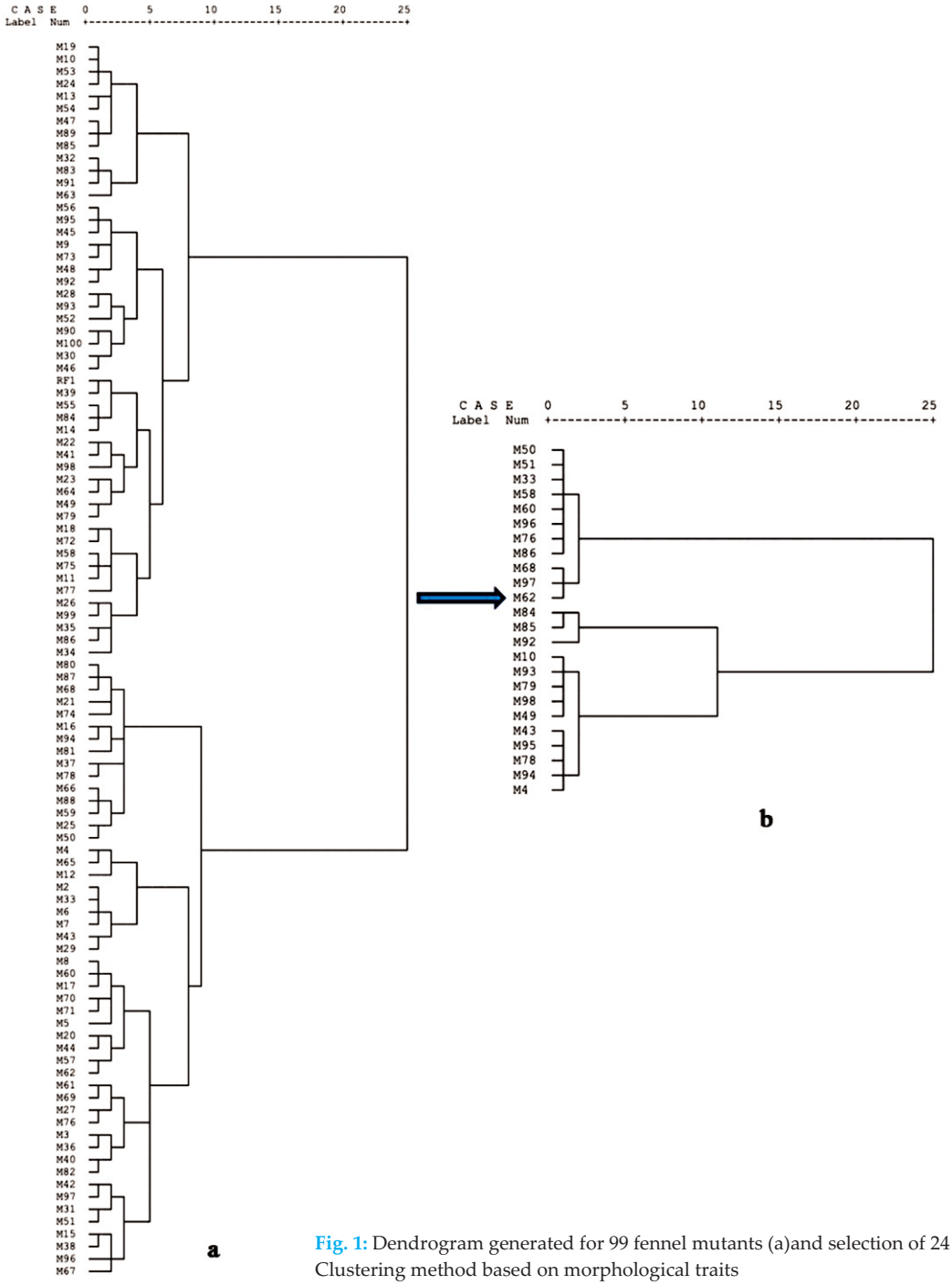


**Fig. 1:** Dendrogram generated for 99 fennel mutants (a)and selection of 24 fennel mutants (b) using Ward's Clustering method based on morphological traits

**Cluster analysis based on morphological characters**

Ward's hierarchical cluster analysis was carried out for 28 morphological characters studied to measure genetic distance between 99 fennel mutants and parent. A dendrogram was constructed using Ward's cluster analysis (Fig. 1a). All the mutants had a wide variability at morphological level for main characters. Based on Ward's hierarchical cluster analysis of 99 mutants and its parent, we have selected diverse 24 mutants and 15 important characters for molecular characte-rization to save time and lobour (Fig. 1b). Cluster analysis grouped the mutants into 2 clusters. Cluster I having 11 mutants and cluster II included 13 mutants. Cluster I and cluster II were apart at 25% recalled values. Which are representative samples from each cluster generated through morpho-logical data.

In the present investigation, efforts were made to characterize the fennel mutants ($M_3$ generation) based on morphological and genetic markers to explore the presence of induced genetic variability. During present investigation, $M_3$ lines of fennel derived from Rajasthan Fennel–125 after treatment with 150, 175, 200, 225 and 250 Gy doses of gamma radiation and selection of the desired mutant in the $M_2$ generation. The improved characters like early flowering, primary and secondary branches/plant, number of umbel/plant, number of umbellate/umbel, number of seed/umbel, plant height, seed yield/plant and test weight were recorded in the mutant lines. We have found that some mutant lines showed early flowering as compared to control.

Datta and Sengupta, (2002) found mutants that showed early flowering in coriander by using gamma rays (10,20,40,80 and 100 Gray) and EMS (0.25, 0.5 and 1%).With regards to improvement in the number of branches in mutant lines as compared to parent plant. It was due to the change in the genetic constitution of the mutant lines. These characters significantly contributed in yield of the crop. A wide range of variability for the number of primary branches followed by secondary branches was observed in mutant lines of black cumin (Datta *et al.*, 2003). The number of umbel/plant, number of umbellate/umbel and number of seed/umbel was improved when compared with parent plant. It was reported that number of umbellate/umbel and umbels/plant increased in mutant lines of coriander (Punia and Ramkrishna, 2002). Plant height is one of the important parameters which need to be addressed in fennel crop. The fennel germplasm is deficient in short height type. In the mutant population, it was found that some of the plants showed dwarf stature. In a study, Mahla and Ramkrishna (2002a&b) reported that many fennel mutants showed dwarfism as compared to control variety in$M_2$ progenies. Arisha *et al.* (2015) induced genetic variability in pepper and selected dwarf mutants, which is a commercially desirable trait. They also reported that days to harvesting in the case of physical mutagenic treated populations were changed towards early maturity. It is very important to get varieties associated with escape from biotic and abiotic stresses that happened in the late growing winter season.

Among the 99 mutant lines many mutants showed improvement in the seed yield per plant as compared to control. These results are similar to the findings of Jeliazkova *et al.* (1997) on coriander by irradiated the seed using gamma irradiation. High yielding progenies resulting from mutation breeding had already been reported by researcher in fennel (Mahla *et al.*. 1999; Paul and Datt, 2016; Verma *et al.*, 2017), coriander (Datta and Sengupta, 2002; Punia and Ramkrishna, 2002) and black cumin (Datta and Rang, 2000). The results on plant morphometrics showed that gamma rays induced the plenty of genetic variability in the $M_3$ generation that can be utilized for the selection of ideal plant phenotype for further improvement.

**Molecular marker characterization**

All the 24 mutants of fennel cultivar were examined for DNA polymorphism using 20 RAPD primers. Out of 20 RAPD primers, 5 primers were found polymorphic (Table 2). Out of 24 mutants, 21 mutants showed amplification with primer OPE-16 and OPE-18, 10 mutants with primer OPE-19, 22 mutants with primer OPE-20 and all the 24 mutants were amplified with the primer OPE-15. A total no. of 73 bands were recorded in RAPD out of which 23 were polymorphic. Percent polymorphism exhibited by primers ranged from 24.4% (OPE-20) to 37.2% (OPE-15). PIC (Polymorphism Information Content) value is a feature of a primer therefore; PIC values were calculated for all the primers. The primer OPE-20 gave maximum PIC value with 0.83 which was at par with primer OPE-18 (0.81) whereas, minimum PIC value was obtained with primer OPE-19 (0.51) and average PIC value all 5 primers was 0.70. Average PIC value 0.70 showed a good efficiency of the used primers in discrimination of the individuals.

**Table 2 :** Details of amplified RAPD Primers in fennel mutant lines

| Primer Name | Sequence | Mol. Wt | GC content | TM | TGA | TB | PB | PP | PIC |
|---|---|---|---|---|---|---|---|---|---|
| OPE -15 | ACG CAC AAC C | 2966.0 | 60 | 36.9 | 24 | 43 | 16 | 37.2 | 0.67 |
| OPE -16 | GGT GAC TGT G | 3099.1 | 60 | 31.8 | 21 | 30 | 09 | 30.0 | 0.69 |
| OPE -18 | GGA CTG CAG A | 3077.1 | 60 | 33.7 | 21 | 68 | 21 | 30.8 | 0.81 |
| OPE -19 | ACG GCG TAT G | 3068.0 | 60 | 35.6 | 10 | 15 | 05 | 33.3 | 0.51 |
| OPE _20 | AAC GGT GAC C | 3037.0 | 60 | 34.9 | 22 | 90 | 22 | 24.4 | 0.83 |

*TGA = Total Number of Genotype Amplified, TB = Total Number of Bands, PB = Polymorphic Bands, MB = Monomorphic Bands, PP = Percent Polymorphism, PIC = Polymorphism Information Content.

**Genetic relationship and cluster analysis among fennel mutant lines based on RAPD**

The 23 polymorphic loci were used to calculate the genetic similarity among the 24 fennel mutants. Based on RAPD banding patterns, genetic similarity estimation were calculated using method of Jaccard's Coefficient Analysis. The cluster analysis divided the 24 mutant lines into 4 main clusters with similarity coefficient ranged from 0.00 to 0.88 (Fig. 2). The average similarity across all the mutants was 0.12 indicating a low genetic similarity among the mutants. Cluster I had 14 mutants, cluster II carried 7 mutants and Cluster III had 2 mutants. The mutant line M-93 was most dissimilar among the mutant lines and formed the separate cluster IV.

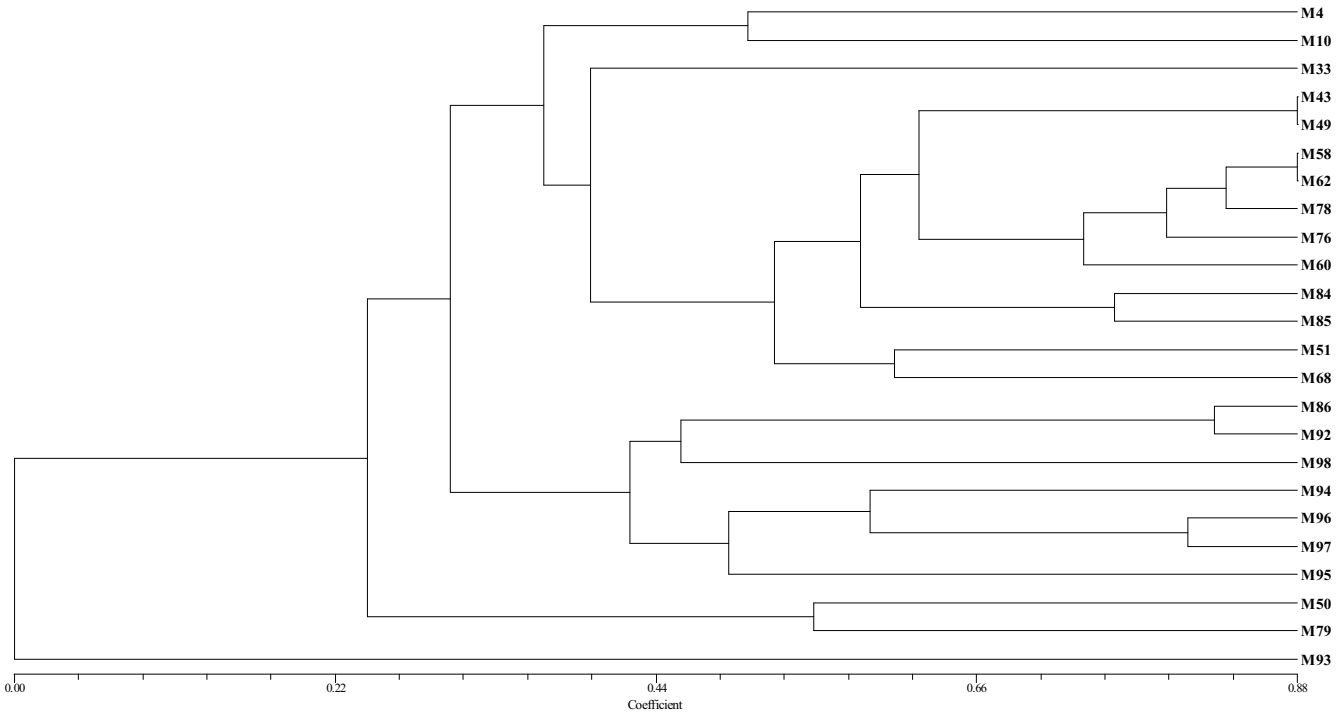


**Fig. 2:** Dendrogram generated for 24 promising fennel mutants using UPGMA cluster analysis based on RAPD data

**Principal component analysis (PCA)**

Three-dimensional principal component analysis based on RAPD data (Fig. 3) showed similar clustering pattern of 24 mutants as evident from cluster tree analysis. Most of the mutants were in two major clusters. The first cluster had maximum mutants having 5 sub-clusters totaling 23 mutants.

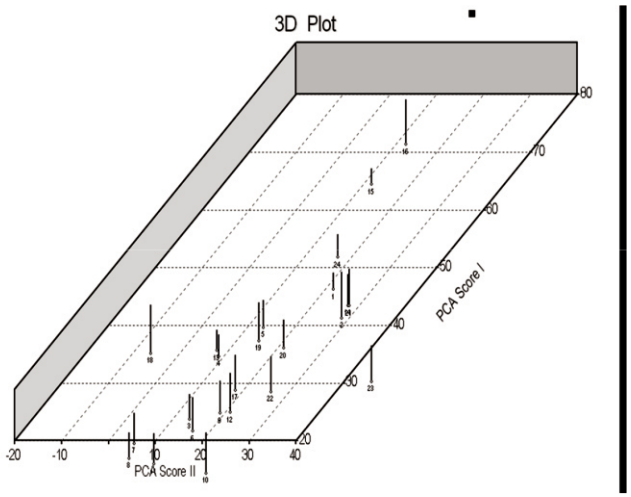


**Fig. 3:** Three dimensional PCA (Principal Component Analysis) scaling of 24 mutants of fennel using RAPD markers

The present experiment was carried out in order to find out the genetic diversity in the fennel mutant lines. The combination of phenotypic and molecular markers has been successfully applied to study the diversity and its repercussion for breeding and conservation of fennel (Choudhary *et al.*, 2018). DNA markers are the most widely used type of marker predominantly due to their abundance and ease of use. The genetic diversity is analyzed by using phenotypic as well as molecular tools like DNA markers (Bennici *et al.*, 2003) and sophisticated molecular PCR amplification techniques (Shiran *et al.*, 2007). Variability using molecular markers has been examined in fennel by various investigators (Zahid *et al.*, 2009; Bahmani *et al.*, 2013; Kelardashti *et al.*, 2015).

Molecular diversity by RAPD assay was carried out on selected mutants with 20 oligonucleotide primers. Out of 20 primers, five primers produced good amplification and were polymorphic. The average Percent polymorphism exhibited by RAPD primers was 29.6. Paul *et al.* (2012) in ginger used 27 random primers out of which only 5 primers successfully produced good amplification with polymorphism percent ranged from 0 to 33.75%. Similarly, Solouki *et al.* (2012) in dill (*Anethum graveolens* L.) cultivar, obtained 39.8% polymorphism. The PIC value for RAPD primers ranged from 0.51 to 0.83 with an average of 0.70. The genetic similarity coefficients of the mutants based on 23 polymorphic loci ranged from 0.00 to 0.88 with an average of 0.12 indicating a good source of diversity which could be due to highly genetic difference among mutant lines. Choudhaey *et al.* (2018) also found similarity coefficients range from 0.34 to 0.76 and 0.36 to 0.87 using RAPD markers in fennel. Mostafa *et al.* (2015) identified salt tolerance mutant in fennel using molecular markers. The molecular techniques such as genetic markers are becoming more and more significant as efficient tools in crop improvement programmes, but their use in fennel is lagging due to limited research on the use of DNA markers in the mutant lines. The observed polymorphism may be useful for the development of molecular markers in the genetic enhancement of the fennel crop. The dendrogram was constructed by cluster analysis, based on the genetic

similarity matrices produced based on different coefficients. Cluster analysis of 15 morphological traits divided mutant lines into the separate groups. Both molecular and morphological dendrogram having 24 mutants, were divided into 2 clusters. In morphological dendrogram cluster I included 11 mutants. This cluster was further divided into two sub-clusters IA and IB. In molecular dendrogram cluster I included 23 mutants. This cluster was divided into 4 sub-clusters namely IA, IB, IC and ID. In morphological dendrogram cluster II included 13 mutants. This cluster was further divided into 3 sub-clusters IIA, IIB and IIC whereas, in molecular dendrogram cluster II had only one mutant namely M-93. At molecular level, M-93 showed zero similarity coefficients which indicate that this mutant was very diverging and did not show any similarity with all other 23 mutants. The dissimilarities among the two cluster groups were statistically significant. It can be inferred from the obtained results that gamma irradiation has induced substantial genetic variability in fennel mutant lines with regard to morphological characters. Choudhary *et al.* (2013) used RAPD markers to estimate genetic variability in fenugreek wherein fifteen polymorphic primers showed a 57.66% polymorphism in fenugreek varieties. This study indicated the existence of adequate variability for various yield attributing characters, which can be exploited for further breeding programme of this crop. It was also recommended that to improve seed yield in fennel, more emphasis should be given to plant height, total branches /plant and effective umbels/plants (Patel *et al.,* 2008). El-Mahrouk *et al.* (2015) studied the mutagenic treatments induced various mutants in nigella. The electrophoretic pattern of protein analysis produced bands between 21 to 24. The percentage of polymorphism detected by markers reached 68.9% and 12 out of 45 ISSR markers were mutant specific. The DNA polymorphism obtained by RAPD analysis offered a useful molecular marker for the identification of mutant plants in the populations induced by gamma rays (Dhakshanamoorthy *et al.,* 2011; Dhillon *et al.,* 2014).

## CONCLUSION

The present study suggests that induced mutagenesis is an important crop improvement technique to generate a spectrum of genetic variability for different essential traits. When genetic variation for the trait of interest is inadequate or absent, gamma rays can be employed to induce mutations and thereby generate genetic variability from which desired mutant lines may be selected. A considerable number of mutants were found significant that was superior in agronomical traits. The morphological and molecular study revealed that the mutants selected in $M_2$ generation showed variability at genetic level for different essential traits of fennel in $M_3$ generation. The variability created through induced mutation and mutants identified for different traits can be further evaluated and use in breeding programmes of fennel improvement. The morphological assessment in combination with molecular marker data reveals more accurate diversity among the mutants instead of using morphological data alone.

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