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Enhancing Moringa (*Moringa oleifera)* leaf biomass using chemical induced mutagenesis and validating the mutagenesis using simple sequence repeats

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Abstract
Moringa (*Moringa oleifera* Lam.), leaves have a significant value for its immense nutritive, therapeutic and other industrial properties, which lead to a unique demand for a cultivar with increased leaf biomass. In this study, the seeds of PKM-1 were chemically mutagenized with 0.25% EMS and desirable leafy mutant lines were selected using leaf morphological traits as well as the genetic variation revealed by SSR markers linked to leafy traits. Assessment of the selected lines showed that 66 to 68% increase in leaf surface area and fresh weight than the wild type, PKM-1. Further, molecular characterization of selected mutant lines by employing GenicSSR890, GenicSSR796, GenicSSR778, GenicSSR859 and GenicSSR983 that are linked to leaf traits such as leaf width, leaf length and fresh weight also revealed the existence of polymorphism when compared with wild type. Improved leaf biomass mutant lines (especially Mutant #22 and Mutant #23) identified in this study were considered as useful lines for further genetic advancement in Moringa through *in-vivo* and *in-vitro* techniques, as it may assure a promising way for the release of leafy biomass cultivar that can offer a healthy food supplement and ensure food security.

Keywords: Moringa. mutation, EMS, leaf biomass, SSR markers

1. Introduction
Vegetable farming has become an essential part of food and nutritional security, due to its role in sustaining economic well-being of the farmers. Farmers always prefer to cultivate vegetables at least once a year in order to maintain their family's income and provide sufficient and nutrient-rich foods to their family. *Moringa oleifera*, a single genus of the Moringaceae family is regarded as an embodiment of nutritional treasure because almost all the parts of the tree (root, bark, leaf, gum, fruit, pods, flowers, seed, and seed oil) have been used for its economically valuable properties in a wide range of industrial sectors, including health-care, pharmaceutical, water treatment and cosmetics. To be particular, Moringa seeds that acts as good source of natural coagulant, possessing a high value of protein-5.97g, fat-38.67g, Vit E-751 mg, Mg-635mg, Cu-520 mg per 100 g [1]. Moringa seeds also have drawn scientific attention for its rich source of oil content in kernel as it contains high quality fatty acid composition such as 71.60% oleic acid and up to 6.45% palmitic acid and several healthy metabolites such as β –sitosterol, stigmasterol and campesterol, which make it suitable for use as a substitute for groundnut and olive oil [2]. Both seeds and pods are said to be endowed with numerous anti-inflammatory qualities in relation to chronic illnesses [3] and known to treat for joint pain, liver and spleen problems [4]. Though different parts of moringa has known for their respective nutritive contents and different medical and therapeutic uses, moringa leaves has been used as a highly commercialized product due to its i) discernible dense load of nutritive contents such as Ca, Fe, K, Zn, folate, zinc, vitamins, bioactive components, natural anti-oxidants and essential amino acids, ii) their protective biological functions including anti-inflammatory, anti-tumour, anti-infertility, anti-depression and iii) also due to its easy preparation for ready-to-use formulation in food sectors [5]. The global moringa product market was worth US$ 5000 million in 2019, and it is predicted to reach USD 8400 Million by 2026 [6]. Despite of this huge demand and its utility in health and industrial sector, there is no cultivar specific to improved leaf biomass has been developed. Rapid progress in the development of molecular markers and next generation sequencing, has attracted researchers in the direction of molecular breeding programmes to develop new cultivar with desired variation rather than using conventional breeding strategies.

Hence, mutation breeding integrated with molecular markers especially with the help of breeder-friendly simple sequence repeat (SSR) markers that easily enumerate the genetic variation that are generated by the mutagenesis is thought to be a good technique to improve the efficiency and frequency of genetic variation [7]. Therefore, this study has attempted to generate novel leafy type variation in Moringa using induced chemical mutagenesis and differentiate the generated mutant plants at molecular level using SSR markers.

2. Materials and Methods

2.1 Plant Materials

The seeds of an annual Moringa variety, PKM 1, released by TNAU in 1989, were collected from Department of Vegetable Sciences, HC&RI, TNAU, Periyakulam, Tamil Nadu, India. This variety was developed by pure line selection and is known for its one-year duration within which it can grow to a height of 4-6 m, flowering comes in 5-6 months and harvesting can be done in 7-8 months (peak period is March – August). It produces pinnate leaves in a branch that are about 40 cm long with small leaflets [8]. It can thrive well in any hardy environment and hence, widely cultivated all over the world. Therefore, this cultivar has been selected to genetically improve their leaf biomass.

2.2 Mutagenic treatment

Mutagenesis was attempted by using 0.25% Ethyl Methane Sulphonate (EMS:$CH_3SO_3C_2H_5$, an alkylating agent with a chemical property of 124.16 Molecular weight and boiling point 80 / 100 mm Hg and density =1.203 g/ml) to treat 200 well filled, mature and disease-free dry seeds of PKM-1 and 10 untreated seeds were used for control. When compared to other concentrations, the LD50 value of 0.25 percent was proved to be very efficient [9]. The seeds were overnight soaked in 100mM Sodium Buffer in a beaker at 4 ºC as presoaking of seeds in buffer solution enhance the absorption of mutagens through cell permeability and also helps in germination process. Those presoaked seeds were placed in beakers that has 0.25% EMS and the mixture was incubated for 8 hours at room temperature with continuous stirring using table top stirrer. After 8 hours, the seeds were washed thoroughly for 50 times in order to nullify the residual effect of the mutagen. The initial five washes were saved for disposal and were detoxified with 6% sodium hypochlorite solution. After EMS mutagenesis, seeds were planted immediately in the pot which has a diameter of 7 inches and having soil mixture of red, sand and FYM in the ratio of 1:1:1 and in parallel10 untreated seeds were sown as control. The pots were watered daily and NPK @19:19:19 was added to each pot once in two weeks and 1% chlorpyriphos was sprayed as and when leaf infestation with spider was notice.

2.3 Phenotyping for Leaf Biomass Traits

Observations on germination percentage on the 14th day (using the emergence of a cotyledonary leaf as an indication of germination) and survival percentage on the 30th days after sowing were computed as described below:

$$\text{Germination percentage} = \frac{\text{Number of germinated seeds on 14th day}}{\text{Total number of seeds}} \text{ x } 100$$

$$\text{Survival percentage} = \frac{\text{Number of plants survived seeds on 30th day}}{\text{Total number of seeds}} \times 100$$

On 45th days after sowing, mutant plants had been selected based on their improved leaf biomass component traits in comparison with control plant. Leaf morphological features including average leaf length (cm), average leaf breath (cm), number of leaves, leaf fresh weight (g), and leaf dry weight (g)) from the third rachis of each mutant line have been calculated.

2.4 Genotyping with SSRs

According to Doyle and Doyle (1987) [10], genomic DNA of selected mutated lines and control plant were extracted and quantified using a Nanodrop Spectrophotometer (Genova Nano 69357), and DNA integrity was assessed using 0.8% (w/v) agarose gel electrophoresis. All the DNA samples were uniformly diluted to 50 ng/µl with sterile water and used as templates for PCR amplification with selected SSR marker. A total of 10 inhouse generated [11] SSR markers (of 7 genic and 3 genomic SSRs including GenicSSR778, GenicSSR796, GenicSSR983, GenicSSR859, GenicSSR890, GenicSSR1081, GenicSSR1204, SSR2927, SSR6561, SSR25987) were used for PCR amplification in accordance with the method established by Mgendi *et al*., [12] with the modest change using smART Prime master mix instead of individual components of PCR chemical components. List of SSR Markers used in this study is given in the Table 1.

Table 1: Details of Moringa specific genomic and genic SSR used in this study

SSR ID	SSR Motif	Number of repeats	Forward primer	Reverse Primer	Annealing temperature (ºC)	Expected PCR product size (bp)
GenicSSR778	TCAGTC	4	CGTCAACTACCATTGAATCGGC	GGCAATTCATGAAGTCGGAGC	57	113
GenicSSR796	GGCTCC	4	TCTTAACTTCCTCGCCTGCC	AGAAAGGCATAGAGGTCGCG	57	176
GenicSSR983	ACCAGG	4	TGGGCATCATCTTGTTGGGC	TGTGAAATTCAACTTGCCTGAGG	57	209
GenicSSR859	TGCCAG	4	TCCGTCCCATAGGTACCTCC	CCCGATGCAGAAGGAGATGG	59	148
GenicSSR890	TGTGAC	4	ACTTGCCAGGGTTATGGTGG	ACTCCACCTTGCAAAATGGC	55	150
GenicSSR1081	TGATCG	4	GACAGACCTAGACCCACTGG	GGAGTTCTTCGAGGCCTACG	59	229
GenicSSR1204	TTACCG	4	CATGGCCTCTTCCTGACTCC	AAATCACACGCTTTTGCCGG	55	167
SSR2927	CTTTT	6	CCAGGAGGCAATGCTTAGC	TTTTGCTCAATGGCACCTGC	55	289
SSR6561	GAAGG	6	AGAAAGCATGCAAGTGTGGC	CAAAACGACAACACCACCCC	55	207
SSR25987	ATGT	15	GGAGTATAGGGTTTAGGGCTAAGC	TAGATTCAGCCTGGCCTTGC	56	261

3. Results and Discussion

Though numerous breeding methods are accessible to induce new variants in plants, mutation breeding also serves well as it can produce new variant randomly without disrupting the entire genotype and its recessive allelic nature encourages breeders to create novel varieties within a relatively shorter period of time [13]. This study was performed to evolve novel leafy biomass types in Moringa using chemical induced

mutagenesis and examine the morphological and genetic variance to select the desirable mutants.

3.1 Impact of EMS on germination and survival rate

EMS concentration efficiency was considered as an important factor in the mutagenic studies for the development of stable and high quality mutants [14]. In this study, it was observed that the germination and survival rate of 0.25% EMS treated PKM-1 seeds was 83.5% and 79.1%. Respectively which is closely related to the previous reports in vegetable crops such as chilli, (Hasan *et al*., 2020) [15], Moringa (Udayakumar *et al*., 2020) [9] and ornamental crop such as snapdragon, *Antirrhinum majus* (Heffron *et al* 2022) [16].

3.2 Selection of mutant plants

Among the mutant population, mutants with improved yield component traits for leaf biomass were selected and the top seven mutant lines that were recorded superior trait values compared with the wild type moringa PKM-1 were described in the Table 2.

Table 2: Morphological Data of mutant lines

Sample	Total No. of Leaves	Avg., length of leaves (cm)	Avg., breadth of Leaves (cm)	Fresh weight of leaves (g)	Dry weight of leaves (g)
Control	23	1.81	1.25	0.506	0.119
Mutant 3	22	2.19	1.60	0.554	0.124
Mutant 5	66	1.43	0.94	0.754	0.225
Mutant 9	20	2.77	1.51	0.720	0.168
Mutant 17	21	2.35	1.62	0.543	0.132
Mutant 19	31	1.92	1.37	0.613	0.133
Mutant 22	20	2.73	1.81	0.740	0.190
Mutant 23	24	2.15	1.54	0.674	0.160

From the above table, it is observed that the selected mutant plant's leaf traits have increased value compared to the wild type PKM-1. For example, total number of leaves were more in Mutant #5 (66) followed by Mutant #19 (31). Similarly, average length of leaves was maximum in Mutant #9 (2.77cm) and breadth was high in Mutant #22 (1.81cm) followed by Mutant #17 (1.62cm). On considering the trait, fresh weight and dry weight, Mutant#5 and Mutant #22 considerably differ a very little and recorded the highest value followed by Mutant #23 . Intriguingly, it was found that the dry weight of all the mutant plants had been increased in the range from 1.1 and 1.8 percent in comparison to the control. As these mutant plants show significant variation for all the investigated traits (Figure 1), they were further subjected to molecular characterization using SSR markers.

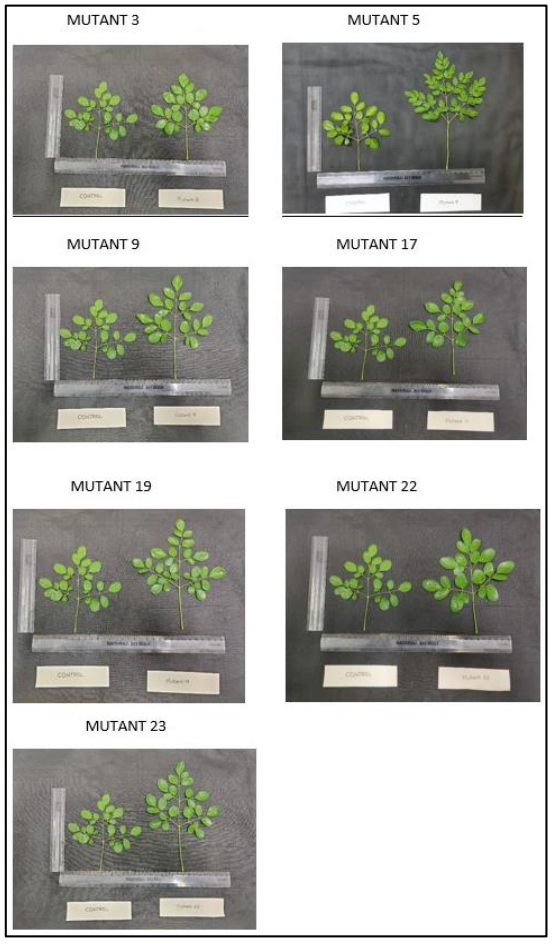

Fig 1: Phenotypic variance of leaf traits in selected mutant lines

3.3 Analysis of genotypic variability

For better understanding of phenotypic variation of selected lines, it is also imperative to confirm the presence of variation at the molecular level. Though different kind of molecular markers have been available to analyse the genetic variation in whole genome sequence, SSR markers has been proposed to be utilized due to its breeder friendly nature [17]. In this study, genetic variability analysis with 10 SSR markers, showed that five SSR markers were found to be polymorphic when compared those two selected mutant lines (*viz*., Mutant#22 and Mutant#23 among the seven selected mutant lines) with the wild type, PKM-1 (Figure 2). In earlier studies, it was reported that these polymorphic markers were linked to the specific leaf trait such as GenicSSR778 (which was linked to longer leaf length), GenicSSR796 (which was linked to leaf length and width), GenicSSR983 (linked to Fresh weight), GenicSSR859 (linked to longer leaf length) and GenicSSR890 (associated with leaf morphology) (Boopathi *et al* 2021; Paul *et al* manuscript submitted). Thus, with polymorphic information on these functional markers and observed phenotypical variation, it has been proven that Mutant#22 and Mutant#23 can be used as potential new leafy type lines in Moringa as they have shown increased longer leaf length, leaf width and fresh weight that PKM-1 and differ from the wild type at molecular level, at least for the above mentioned SSR motifs. Consequently, it was concluded that these two lines, Mutant #22 and Mutant #23, could be used for further screening in successive generation for inheritance of these desirable leafy traits and they can also be used as potential donors in several other breeding techniques and crop improvement programmes.

4. Conclusion

Moringa leaves play a predominant role in the human health supplement and thus the global moringa market is in thrive of leafy cultivar for its commercial production of increased moringa products. In this present study, an apparent phenotypic variation was observed in all seven desirable mutant lines generated with 0.25% EMS, which confirms its efficiency in creation of genetic variation and desired mutants. Mutant#22 and Mutant#23 identified in this study possess higher leaf biomass characteristics besides showing genetic variation when compared with the wild type (which also illustrated the effectiveness of functional SSR markers for this purpose). Hence the use of these two prospective lines in a genetic advancement program will lead to generate a novel Moringa cultivar with high leafy biomass.

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