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RESEARCH ARTICLE

Genetic improvement through chemical and physical mutagenesis for enhanced leaf production in moringa (*Moringa oleifera* Lam.)

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Abstract

Creation of variation and selection of novel moringa (*Moringa oleifera* Lam.) cultivars with the desirable qualities demanded by farmers, industrialists and consumers have long been the breeding objective at HC and RI, Periyakulam, TNAU. Breeding for new leafy type moringa cultivar through induced mutagenesis approach was taken as an objective for this experiment. Several desirable Ethyl methane sulfonate induced mutant lines that were identified from moringa cultivar PKM-1. These mutant lines were recorded more than four times (0.200 g.) of leaf dry weight production than PKM-1. It was also observed that the percentage of germination was almost affected during physical mutagenesis (Gamma irradiation). This reveals that the percentage of germination is reduced with an increased mutagen dose. Considerable phenotypic changes in the mutant population were recorded during the investigation. The prospects of exploring the identified mutant lines and applications of tools of modern genomics assisted breeding in genetic improvement of moringa with enhanced nutritionally, medicinally and industrially essential traits.

Key words: Ethyl methanesulfonate, moringa, gamma irradiation, mutagenesis, leafy type

Introduction

Moringa oleifera Lam., belongs to mono-generic family of shrubs and trees, the *Moringaceae*, has recently been prime focus due to its rich affordable nutrients that are obligatory to the poor people living in resource-limited environments, malnourished children, pregnant and lactating mother. Among the 13 species identified within Moringa, *M. oleifera* is evergreen, fast-growing, deciduous and widely cultivated species. It has some common names (such as moringa, drumstick tree, horseradish tree, ben oil tree) Though it is indigenous to northeast India, now *M. oleifera* is cultivated in almost the whole tropical and sub-tropical belt (Anwar *et al.,* 2007). Currently, moringa plants are widely cultivated in the Middle East, Africa and Southern Asia as a multipurpose crop (Sánchez *et al.*,2006; Nouman *et al.,* 2014), using a production system characterized by high biomass yield and fast re-growth after pruning (Foidl *et al.,* 2001). Moringa crops can produce approximately 580 t ha^{-1} of moringa fresh shoot biomass annually. In India, particularly in South India, Moringa is mainly grown for its pods, which is routinely placed as the key component in vegetable basket, recently the nutritional importance of the moringa leaf attracted many minds due to their cheap and round the year availability with high nutrient content.

In the past few years, seven report on its nutritional and medicinal properties have been published in peer-reviewed journals. Its utility as a non-food product (e.g. lumber, charcoal, fencing, water clarification, lubricating oil) has also been extensively described (Fahey, 2005). Featured by increased richness in proteins, minerals, antioxidants and vitamins compared with other food stuffs, the leaves of *M. oleifera* are suggested as a highly nutrient vegetable (Mughal *et. al.*, 1999).

Though the above said varieties are widely cultivated, they are mainly being grown for pods production. There is no specific moringa variety that suits for high leaf biomass production. To date, moringa leaves are used by agro-food and biochemical industries as a food, fodder, bio-pesticide, green manure, natural coagulant for turbid water, and plant growth enhancer (Nouman *et al.*, 2014). Since, moringa leaves have been investigated as a valuable source of dietary proteins and essential aminoacids, over the last several years their use as an ingredient in livestock and human's nutrition has been encouraged (Amaglo *et al.*, 2010). Besides their use as an ingredient for foods and feeds, an impressive range of intrinsic bioactive phytochemicals including glucosinolates, isothiocyanates, carotenoids, and phenolic compounds of moringa leaves, has allowed to envisage their potential applications as a functional food and nutraceutical health promoter. To date, three major strategies are followed for genetic improvement of crop plants: conventional hybridization, mutagenesis and molecular breeding. Mutation breeding is considered as an appropriate strategy to enhance the breeding efficiency of evolving novel moringa leafy types because this method offer simple protocol to induce novel and heritable genetic variation within a short-span of time, easily create new variation not found in nature, generate novel alleles in orders of magnitude faster than that occur spontaneously and produce variation in organisms where traditional introgression is impeded (such as linkage drag or asexual propagation). Physical (e.g. Gamma rays, X-rays, fast neutron, ion beam), chemical (e.g. Ethyl methanesulfonate (EMS), Sodium azide) and biological (e.g. transposable elements) mutagens are being explored to induce genetic variation in crop plants. Majority of the mutant crop varieties were released using physical mutagen (87%) followed by chemical mutagen (13%) (http://mvgs.iaea.org/) since these two types of mutagens has potential to induce mutations irrespective of crop plants and ploidy level. Hence, this experiment is formulated with the specific long-term goal of genetic improvement of moringa with increased leaf biomass production through chemical and physical mutagenesis.

Materials and methods

Plant material

In this experiment, newly harvested seeds from the annual Moringa cultivar PKM-1 were used to create the mutants in the population, as such seed material ensures high levels of germination and avoids seed loss. It is a pureline selection developed by continuous selfing for six generations, collected from Eppothumvendran of Tirunelveli region and collections were maintained at the Horticultural Research Station, Periyakulam. Indeed it is phenotypically stable annual genotype with wide adaptation to fertile soil to resource limited environments. It has fleshy, thick 70 cm long pods with a mean annual yield of 33.24 kg per tree. It is suitable for growing as an intercrop and/or border crop and also fits well for ratoon cropping. However, this cultivar is mainly grown for pod production and not evolved for leafy biomass production. Hence, this cultivar was selected to evolve new leafy mutant with enhanced green biomass production.

Gamma irradiation

Since there was no literature on physical (Gamma irradiation) or chemical (EMS) mutagenesis protocol, it was initially proposed to perform kill curve analysis to identify the appropriate dose that provide indication of the quantity of recognizable effects of mutagen on the mutated seeds (M_1). Totally, 20 numbers of air dried PKM-1 moringa seeds (M0) for each treatment group were placed in the labelled covers and irradiated at 20, 40, 60, 80, and 100 Gy in the Gamma Chamber 1200 (BRIT, DAE, Mumbai, India) under Co-60 source that was housed at Center for Plant Breeding and Genetics, Tamil Nadu Agricultural University, Coimbatore by scrupulously following the procedure provided by the manufacturer. On the same day, the irradiated seeds (M1) were soaked for 6 hours in water and sown in polybag containing 1:2 ratio FYM and red soil mixture together with a control of 20 non-irradiated seeds and were regularly irrigated under the shade net.

EMS treatment

Similarly, different concentrations viz., 0.05%, 0.1%, 0.15%, and 0.2%v/v EMS in double distilled water, were tried with two different incubation periods (3 and 24 hours). As a first step, 20 seeds (M_0) for each treatment were hand-picked the wings were manually removed and then soaked in water for 1 hour. The imbibed seeds were air dried to remove the excess water and incubated with the above mentioned conditions of EMS (0.05%, 0.1%, 0.15%, and 0.2%v/v) and duration (3 and 24 hours) at room temperature (it was around 32^{o}C during the experiment), with intermittent gentle shaking. On the other hand, 50 untreated seeds were used as control. Seeds were then thoroughly washed under running tap water for more than 3 hours and air dried. All the used plastic ware was immersed in 1M NaOH for 24 hours and discarded as regular waste. The mutagenized seeds (M_1) were sown in polybag containing mixture of FYM and red soil in the ratio of 1:2 along with control under shade net and standard cultural practices such as irrigation, weeding and crop protection were carried-out thereafter.

Results and discussion

Impact of mutagen on seed germination

In general, PKM-1 requires 7 to 10 days to germinate and produce first compound leaf branch depending on the season and soil characteristics. It was observed in this experiment that the wild type PKM-1 took 6-7 days to germinate with a mean value of 6.38. In the experiment both Gamma and EMS mutagens have drastically increased the time required for germination and number of mutated alleles also been increased. whereas in the wild type (PKM-1) seeds with 0.1% EMS for 24 hours had shown tremendous variation in time required for germination (minimum of 6 days to maximum of 17 days) and 26% of the seed were not germinated (Figure 1 and 2; Table 1). Reduction in germination percentage might be due to Oxidative damage in mRNA results in inhibition of protein synthesis and protein degradation that caused protein function disruption due to modification of the enzymatic and binding properties (Awatif, *et. al.,* 2017) and Multiple causes, such as Mitochondrial damage, destruction / decrease GAs, auxins or cytokine level.It was also noticed during the physical mutagenesis germination percentage was almost affected. However, few mutated moringa lines were recovered from in dose 80 GY and 100 GY. (Table 1; Figure 3). This shows that there is a reduction in the percentage of germination with an increased dose of mutagen. (Singh, *et. al.*, 2010; Nyla Jabeen and Bushra Mirza 2002).

Table 1: Impact of mutagens on percentage of seed germination (Lethal Dose) and survival in the PKM-1 moringa mutagenized population

Exp. No.	Dosage (duration of treatment, if any)	Number of seeds treated	Number of seeds germinated (M_1 seedlings in %)	Lethal Dose (LD)
GAMMA RAYS				
1	0 Gy	20	20	Control
2	20 GY	20	0 (0)	100%
3	40 GY	20	0 (0)	100%
4	60 GY	20	0 (0)	100%
5	80 GY	20	9 (45%)	55%
6	100 GY	20	1 (5%)	95%
EMS				
7	0% (3 hours)	20	20	Control
8	0.05% (3 hours)	50	47 (94%)	6%
9	0.1% (3 hours)	50	47 (94%)	6%
10	0.15% (3 hours)	50	44 (88%)	12%
11	0.2% (3 hours)	50	46 (92%)	8 %
12	0% (24 hours)	20	18	Control
13	0.05% (24 hours)	50	49 (98%)	2%
14	0.1% (24 hours)	50	37 (74%)	26%
15	0.15% (24 hours)	50	5 (10%)	95%
16	0.2% (24 hours)	50	0 (0%)	100%

Figure 1: Impact of 0.1% EMS on PKM1 seed germination

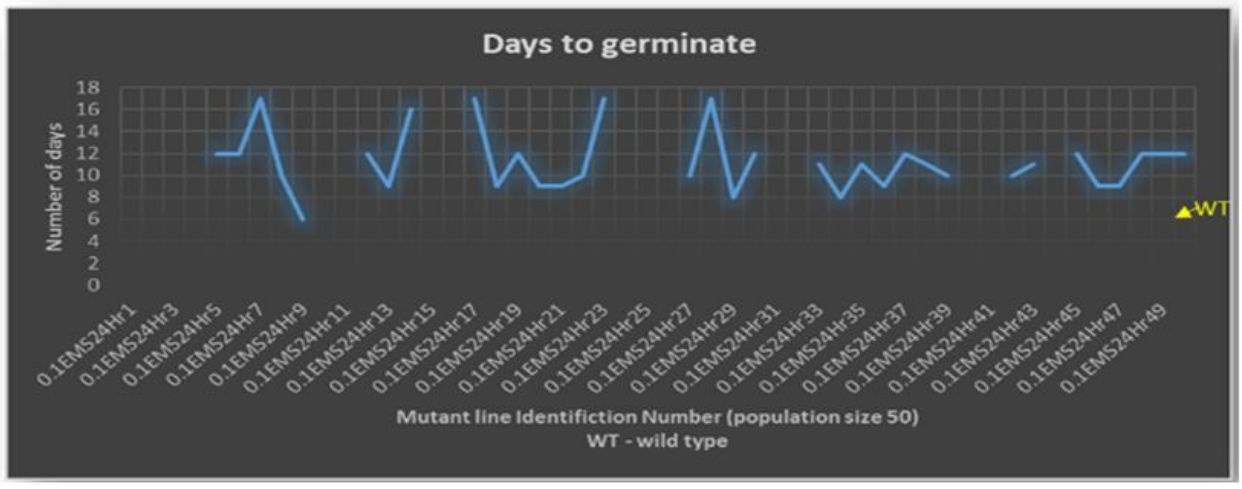
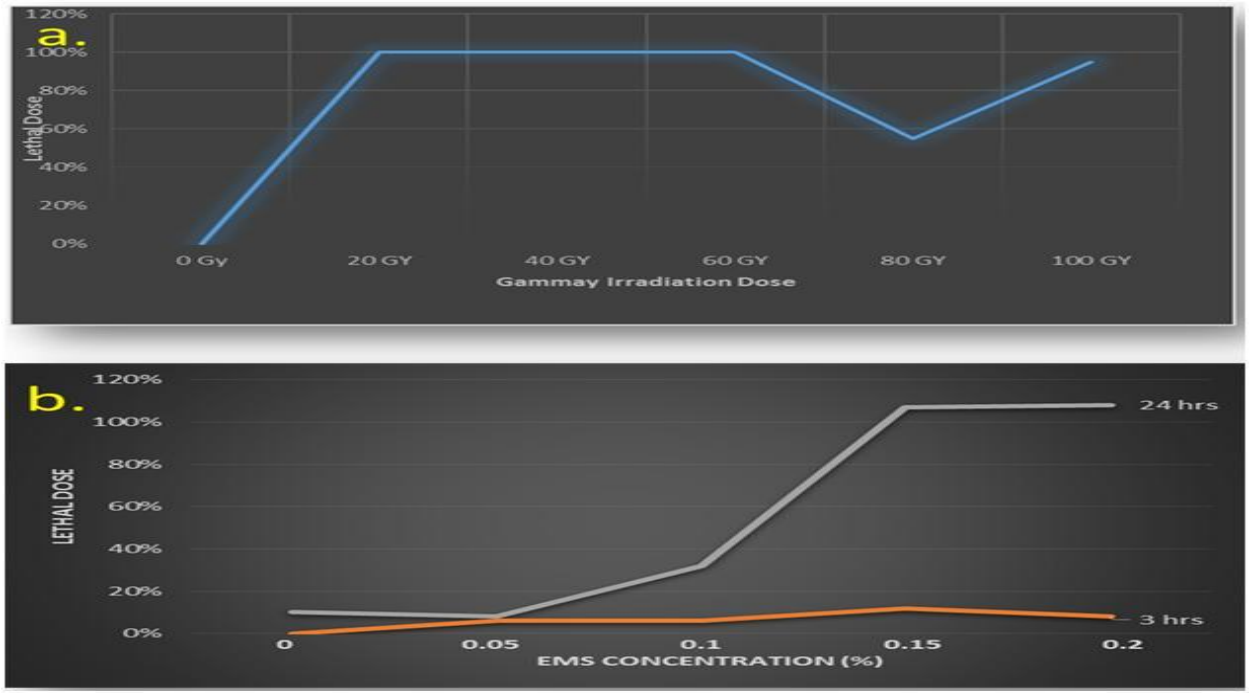


Figure 2: Impact of mutagens (a. gammy rays and b. EMS) on percentage of seed germination (Lethal Dose) and survival in the PKM-1 moringa mutagenized population



A population with high density of mutations is a pre-requisite for mutagenesis breeding to screen for mutations in desired genes. Concentration of mutagen and duration of treatment influence mutation frequency. Toxicity of EMS was determined by scoring germination of seeds treated at varied concentrations. None of the seeds germinated treated at a concentration equivalent to 0.2% for 24hrs. Therefore, one effective concentration with acceptable plant survival rate was proposed to be used for seed treatment in order to raise the M_1 generation. From the kill curve analysis (Table 1), it is concluded that incubation with 0.1% EMS for 3 hours would provide maximum number of desirable mutants in moringa (PKM-1). whereas in Gamma mutagen there may be likelihood to induce mutants with higher doses.

Evaluation of mutant lines for desirable phenotypes

Chlorotic clonal sectors, developmental abnormalities and embryo lethality in the mutant population were known as indicators for scoring efficacy of a mutagen. Occurrence of chimeric phenotypes (white or yellow patches) at seedling stage (Figure 3) clearly indicated the impact of mutagen on expression of moringa phenotypes and it was speculated that there could be a desirable leafy mutant among the mutagenized population. As an end route for the goal of identifying desirable leafy type, phenotypic data on plant height, number of branches, number of leaves in the 3rdcompound leaf from the top, internode length, fresh and dry weight of the leaves were collected from all the experimental materials on regular interval (30^{th} day after germination).

Figure 3: Chimeric phenotypes in mutagenized moringa population

Selection of beneficial mutants and trait diversity in the mutant population

Mutants with improved yield component traits for leaf biomass such as more and broader leaves with enhanced fresh and dry weight production were selected from the M_1 population and the top ten mutant lines that were recorded superior trait values compared with wild type moringa (Table 2). In case of plant ID# 0.05EMS24Hr34, plant height and average internode length were found to be shorter when compared with wild type. In contrast, the leaf fresh and dry biomass were significantly higher in this mutant line (0.238 g). Similarly, another mutant line #0.15EMS3Hr16 also produced increased dry weight 0.224 g).

Table 2: Trait values of superior moringa mutant lines for the investigated characters compared with wild type

Plant Identification number	Number of compound leaves on 30th Day	Number of leaves in 3rd compound leaf from the top 30th Day	Plant height on 30th Day (cm)	Average internode length on 30th Day (cm)	Leaf fresh weight on 30th day (g)*	Leaf dry weight on 30th day (50°C; 48 hours) (g)*
Wild Type	8	20	41	6.5	0.106	0.049
0.15EMS3Hr16	6	9	35	2.5	0.442	0.224
0.15EMS3Hr37	7	9	27	3.5	0.508	0.214
0.15EMS3Hr39	8	12	38	4.5	0.403	0.217
0.15EMS3Hr46	8	20	56	3.0	0.401	0.207
0.15EMS3Hr48	6	11	31	3.5	0.409	0.216
0.2EMS3Hr4	7	11	39	4.5	0.405	0.222
0.2EMS3Hr16	8	12	42.5	2.5	0.412	0.211
0.2EMS3Hr27	9	37	50	7.0	0.465	0.208
0.2EMS3Hr34	7	12	47	3.5	0.373	0.213
0.05EMS24Hr34	8	11	31	4.5	0.402	0.238
C.D.	0.385	0.446	1.248	0.227	0.021	0.011
SE(m)	0.13	0.151	0.423	0.077	0.007	0.004
SE(d)	0.185	0.214	0.598	0.109	0.01	0.005
C.V.	3.032	1.754	1.842	3.226	3.198	3.068

10th, 20th, and 30th Day – 10th, 20th and 30th days after sowing; * Leaf fresh and dry weight were recorded from all the leaves obtained from the 3rd compound leaf from the top on 30th days after sowing

Interestingly, all the selected mutant lines, described in this section, were recorded higher than 0.200 g of dry weight from a single compound leaf which was more than four times higher dry weight than the wild type (0.049 g; Figure 4), though the identified mutant lines have shown tremendous variations (sometimes, inferior to wild type!) for other investigated leaf biomass component traits (Table 2; Figure 5). These ten mutants are ideal candidates for breeding moringa for higher leaf yield. Thus, it was concluded that the ten mutant lines shown in this section would have great potential in future breeding program. Further, the identified mutant line would be an excellent resource material for reverse genetics approaches such as targeted induced local lesions in genome (TILLING) and they could be an asset for mining novel alleles for leaf architecture and genes that governs nutrient and mineral content in leaves.

Figure 4: Variations in leaf fresh and dry weight among the selected mutant lines

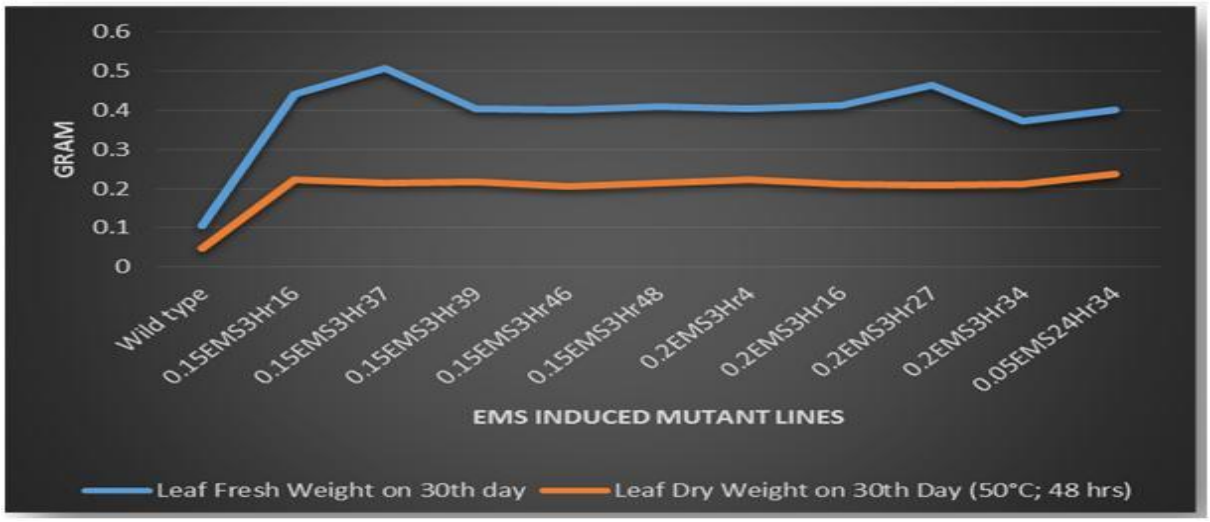
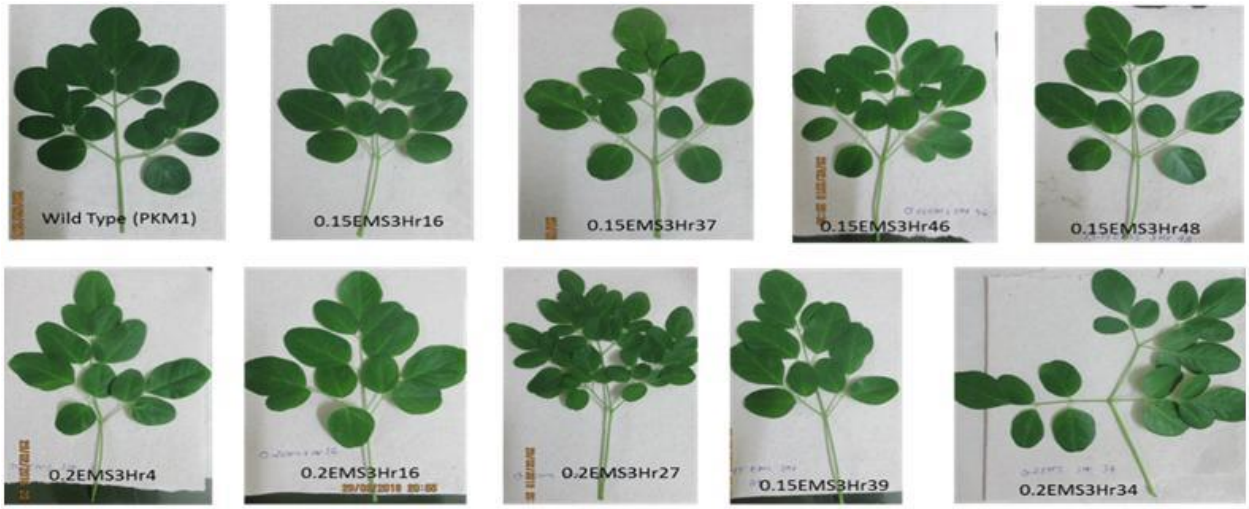


Figure 5: Different leaf phenotypes observed in EMS induced moringa mutant population



Conclusion

Present investigation indicated the existence of considerable phenotypic changes in the mutant population that were created by the EMS and such diversity could be utilized for future breeding program. In conclusion incubation of moringa seeds in 0.1% EMS for 3 hours would provide maximum number of desirable mutants at least in the variety PKM-1. Also, though there were several lines, top ten mutant lines with superior leaf biomass production were identified and proposed that would have great potential in future breeding program or to be directly released as moringa leafy type varieties, provided they have similar nutrient and mineral content as that of PKM-1.However, the genetic basis of morphometric variations observed in this study should be investigated by analyzing the segregation of phenotypes within family and their inheritance from M_2 to M_3 generation. Selected mutants with agronomically superior traits such as more leaves with increased dry weight are being integrated in ongoing breeding program for higher leaf yield.

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