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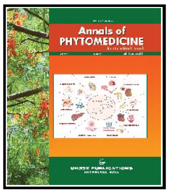

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Phytonutrients of M_4 moringa (*Moringa oleifera* Lam.) influenced by ethyl methane sulphonate

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Abstract

The objective of the current study is to identify viable moringa mutants to meet food and nutritional security. Therefore, without compromising nutrition or quality, this investigation was conducted using varying dosages (0.10%, 0.15%, 0.20%) of ethyl methane sulphonate (EMS), followed by selection from M_1 to M_4 generation based on yield. To verify the effectiveness of the chosen mutant, nutritional and quality analysis were conducted on the M_4 generation plants. The results showed that the mutants 35-1-68-43 and 35-1-68-39 had the highest levels of vitamin A (9.49 mg/100 g) and protein content (39.0 mg/100 g). The mutant 35-1-68-35 had the highest crude fibre (10.35%), vitamin C (216.67 mg/100 g), chlorophyll (48.1) and carbon content (48.18%). The highest calcium (2.33 mg/100 g) and nitrogen concentration (6.35%) was found in the mutant 35-1-68- 07. The highest iron content (0.87 mg/100 g) was found in the mutant 35-1-68-49. These mutants are all the result of 0.15% of EMS treatment. This suggests that the EMS treatment may bring favourable modifications in moringa. Because of this, these mutants have the potential to be introduced as a variety and can be passed on to subsequent generations for assessment of their performance stability to combat food and nutritional security.

1. Introduction

The Tarai region of Uttarakhand is home to the *Moringa oleifera* Lam. tree, commonly referred to as the Miracle tree, Drumstick tree, and Horse radish tree (Joshi *et al*., 2023). Every component of the moringa tree-leaves, bark, blossoms, fruits, seeds, and roots are used medicinally or eaten raw to provide a range of health advantages (Gandhi, 2018). According to biochemical study, *M. oleifera* is rich in vitamin A and C and includes a variety of phytoconstituents, including polyphenolics, tannins, alkaloids, flavonoids, steroids, and terpenes. About 180-200 mg of vitamin C can be found in 100 g of plant extracts from moringa leaves, which is more than from conventional sources (Mbikay, 2012). In addition to protecting the liver, kidney, and stomach from gastric ulcers, it possesses a variety of essential biological properties, including anti-inflammatory, antidiabetic, antioxidant, antihypotensive, and antibacterial properties (Prajapati *et al*., 2022).

Phytochemicals are organic molecules that occur naturally in vegetation and have biological action. They serve as a defense mechanism for host plants and have also been employed as agrochemicals, fragrances, dyes, and pharmaceuticals in the past (Rani *et al*., 2023). Products made from the moringa plant have been linked to decreased blood sugar, blood cholesterol, and remarkable antioxidant properties in both large ruminants and humans (Ali, 2017). Antioxidants are critical in maintaining the human health. Free radicals can be neutralized by other substances such as vitamins, minerals, proteins, or natural antioxidants. The trend towards a change in lifestyle includes the growing interest in herbs. The underlying tenet of this movement is the extensive potential of plants as complementary and alternative treatments (Malik *et al*., 2020). Farmers can improve the physiological health of their badri cattle by substituting dried *M. oleifera* leaves in their concentrate feed at rates of 10% and 20% in both their homes and on their fields which gives improved haematological protein quality (Joshi *et al*., 2023).

Furthermore, the vitamin A content of moringa leaves is high, and it is essential for humoral and cellular immunity, antibody synthesis, and phagocytic cell activity. Subsequent amounts of essential minerals, such as calcium, are also present in moringa that can improve immune function (Verma *et al*., 2022). According to UNICEF, India accounts for one in three malnourished children globally (Singh, 2020). Malnutrition results from poor household's inability to buy nutrient-rich food because of its high cost. Increased consumption of foods high in vitamin C and dark green leafy vegetables (GLVs) is advised by the National Nutrition Strategy (2017). The World Health Organization (WHO) states that the most common nutritional deficit is iron deficiency anaemia (IDA), and one of the six goals of the organization's comprehensive implementation plan on infant, young child and maternal nutrition is to reduce IDA (Gupta and Sarwat, 2022). The best and most lasting way to avoid IDA is to encourage the consumption of foods rich in iron (Taneja *et al*., 2020). Although, eating green leafy vegetables can be a rich source of nutrients, their low biomass, brief duration, and less available nature make their availability difficult. Nowadays, it presents a problem for vegetarians

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because a lot of plant-based proteins are deficient in all of the essential amino acid profiles. Under such circumstances, moringa leaf which is especially high in critical amino acids, emerges as a competitive substitute protein source. In comparison to yoghurt, fresh leaf of moringa has two times more protein content (Singh *et al*., 2018). As such, it is acknowledged as a substitute source of protein that can meet the on-going nutritional requirements of malnourished people (Islam *et al*., 2021). With respect to the crop improvement strategies, conventional breeding might take more time due to the long duration of the moringa crop. A kind of non-transgenic chemical mutagen is called ethyl methane sulphonate (EMS), and EMS mutagenesis is a crucial method for producing mutations and finding novel genes for plants. For many plant species, specific guidelines have been developed (Unan *et al*., 2021). This study was conducted to develop a moringa variety with high yield without compromising the nutrient content using induced mutagenesis with EMS.

2. Materials and Methods

2.1 Materials

Moringa variety PKM 1 seeds were gathered, and then immersed in 0.15%, 0.20%, and 0.25% EMS for 4 h and washed with water to remove the residue. Subsequently, the seeds were planted, grown, and assessed for characteristics related to leaf yield and nutrient content. Udhayakumar *et al.* (2019) reported that the best performing mutants are selected and raised as the second generation (M_2). Mutants with increased yield and nutrition were transferred to the third generation (M_3) from the M_2 generation. The best performing mutant 35-1-68 from the moringa M_3 generation were chosen and forwarded to the M_4 generation (Hari *et al.,* 2022). The mutant family 35-1-68 was generated by treating the PKM 1 moringa seed to 0.15% of ethyl methane sulphonate (EMS) and then going through a selection process to move from M_1 to M_3. Every seed was grown to produce unique plant progeny without replication. Based on their leaf yield and other relevant features, the top-performing progenies from the M_4 generation were chosen, and their nutritional contents were analysed.

2.2 Methods

2.2.1 Ascorbic acid

A gram of powdered, dried moringa leaf was dissolved in four per cent oxalic acid, added to a 100 ml volume, and centrifuged at 5000 rpm for thirty min. Next, 5 ml of the supernatant was removed and combined with 100 ml of 4% oxalic acid. This mixture was then titrated against a dye solution that contained 52 mg of 2, 6-dichlorophenolindophenol and 42 mg of sodium bicarbonate, which was made up to 200 ml. The titre value achieved was noted as V_2. The titre value has been recorded as V_1 ml once the standard was prepared (Sadasivam and Manickam, 2018).

Using the following formula, the ascorbic acid content (mg/100 mg) was calculated:

Ascorbic acid (mg/100 mg) = $(0.5 \times V_2 \times 100) / (V_1 \times 5 \times \text{Weight of the sample}) \times 100$

2.2.2 Vitamin A

Grounded 1 to 5 g of moringa leaf into a paste and 1 ml of 2N potassium hydroxide in 90% alcohol was added and kept at 60ºC for 20 min. After cooling the tubes, 20 milliliters of water was added. Using 10 milliliter of petroleum ether in a separating funnel containing leaf extract, the procedure was performed twice to extract vitamin A. After adding the sodium sulfate, the mixture was left to settle for 30 to 60 min. The ether extract was then allowed to evaporate to 5 ml aliquot at 60ºC until it is completely dry. Finally, 1.0 milliliter of chloroform was used to dissolve the dried residue. A solution of trichloroacetic acid (2.0 ml) was then added. Using a spectrophotometer, the absorbance at 620 nm was immediately measured. Similar procedure was followed for the standard tubes. The amount of vitamin A per gram of tissue in the samples was then determined using a standard graph (Bayfield and Cole, 1980).

2.2.3 Protein

First, one g of moringa leaf was homogenized in 10 ml of phosphate buffer, and the supernatant was collected by centrifugation. Then, using buffer solution, it is diluted to 100 ml. The sample extract (0.1 and 0.2 milliliters) was obtained and combined with five milliliters of reagent C (alkaline copper solution) to each test tube, and they were left for ten min. After that, 30 min were spent in the dark with 0.5 ml of reagent D, also known as Folin Ciocalteau reagent. At 660 nm, the absorbance was measured in relation to a blank. Using the usual graphical method, the protein content in 100 grams of leaves was determined (Lowry, 1951).

2.2.4 Crude fibre

Two grams of dried moringa leaf powder was boiled in 200 ml of sulfuric acid for 30 min. Following that, the sample was cleaned with boiling water and filtered through muslin cloth. It was once again cooked for around 30 min in a 200 ml solution of sodium hydroxide, filtered through muslin fabric, and then cleaned with a 1.25% sulphuric acid solution. After removal, the residues were placed in an ash dish that had been previously weighed (W1), then cooled in a desiccator for two hours at 130 ± 2°C, after which it was weighed (W2). The leftovers were subsequently ignited for 15 min at 600 ± 15°C, cooled, and then weighed again (W3) (Maynard, 1970). The crude fibre percentage was estimated based on the formula:

Crude fibre % = Loss in weight on ignition (W2 – W1) – (W3 – W1) / Weight of the sample × 100.

2.2.5 Calcium

The triple acid extract (25 ml) was mixed with 10% sodium hydroxide drop by drop. A little amount of murexide indicator is added, and the mixture is titrated against 0.02 N EDTA until the red colour changes from pinkish red to violet or purple (Jackson, 1973). The sample's calcium content was estimated with the formula, 1 ml of 0.02 N EDTA = 0.0004 g of calcium.

2.2.6 Iron

Ten millilitres of the plant extract (made as a triple acid extract) and 0.5 ml of strong hydrochloric acid was filled in 250 ml beaker. The volume was then increased to 25 ml by adding two milliliters of hydroxylamine hydrochloride and one milliliter of orthophenanthroline. At 490 nm, the resultant colour development was measured and compared to a reference (Jackson, 1973).

2.2.7 Chlorophyll content

The chlorophyll content of the mutants was recorded using the SPAD meter.

2.2.8 Carbon

A dry crucible (Wc) was weighed, a plant sample (1-5 g) was ground, and the plant sample was weighed with a crucible (WPC). After that, the material was heated to 550°C for an hour in a muffle furnace. Removed the components and allowed it to cool. Calculated the final weight (WF) for the plant sample chemical analysis. It used a dry combustion process (Baruah and Barthakur, 1997). The ash percentage was calculated first by dividing WF-WC by WPC-WC. Then the carbon content was calculated by the formula:

Carbon % = 100-Ash % -Total nitrogen % -Sulphur %/1.724.

2.2.9 Nitrogen

A dried leaf sample was dissolved in a diacid mixture, digested in a sand bath, and then transferred to a volumetric flask. The liquid was then distilled using a beaker containing 2% boric acid and 40% NaOH. The beaker's contents were collected and tested for completion using red litmus paper. The colour shift from green to pink indicated the conclusion point (Rangana, 1979).

2.2.10 Statistical analysis

Using the TNAUSTAT program, a progeny-wise analysis of the unreplicated trial was conducted for the statistical analysis.

3. Results

3.1 Performance of mutants for different nutrient and biochemical parameters

In this study, the biochemical and nutrient parameters *viz.*, vitamin A, ascorbic acid, protein, crude fibre, chlorophyll, carbon, nitrogen, calcium and iron content were analysed using standard protocols. As shown in the Figure 1, among the 15 mutants studied the mutant 35-1-68-43 had the highest vitamin A content (9.49 mg/100 g), followed by the mutant 35-1-68-79 (8.68 mg/100 g). The mutant 35-1-68-21 had the lowest vitamin A content (4.58 mg/100 g), compared to the control plant (6.72 mg/100 g) and the vitamin A content of the mutants showed variation.

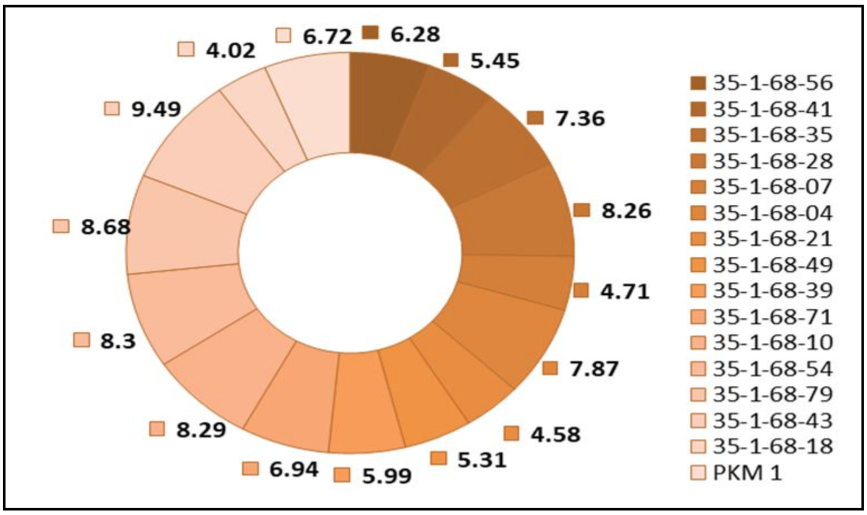

Figure 1: Vitamin A content of moringa mutants with variety PKM 1.

The mutant 35-1-68-35 had the greatest ascorbic acid concentration (216.67 mg/100 g), followed by 35-1-68-21 (216.67 mg/100 g) in the 15 mutants analysed. In contrast, 35-1-68-49 had the lowest ascorbic acid concentration of 121 mg/100 g when compared to the control variety PKM 1 (213.62 mg/100 g) (Figure 2). Both increase and decrease in ascorbic acid concentration were observed among the M_4 generation mutants.

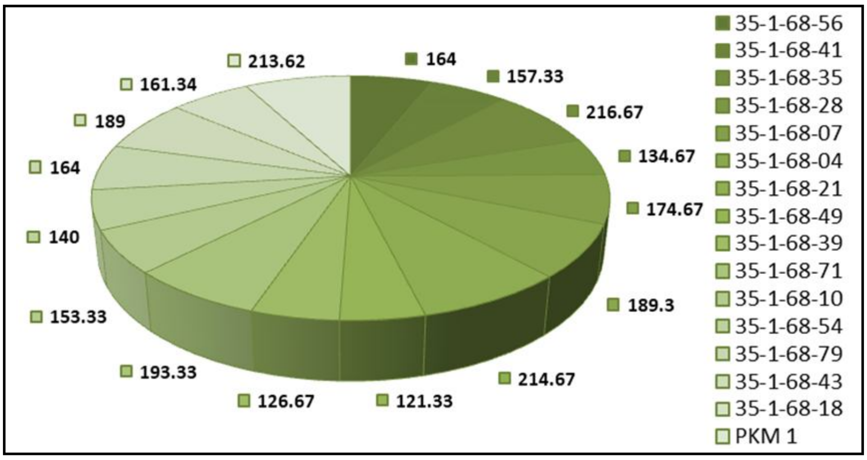

Figure 2: Ascorbic acid content of moringa mutants with variety PKM 1.

The mutant 35-1-68-35 (10.35%) had the highest crude fibre content, followed by 35-1-68-43 (9.05%), and the mutant 35-1-68-10 registered the lowest (4.1%). Very few mutants, *viz.,* 35-1-58-35 (10.35%), 35-1-68-07 (7.35%), 35-1-68-39 (8.3%), 35-1-68-79 (7.65%) and 35-1-68-43 (9.05%) had a higher percentage of crude fibre than the PKM 1 moringa (20.8%) (Figure 3).

Out of all the 15 mutants, 35-1-68-39 had the maximum protein content (39.0 mg/100 g), followed by 35-1-68-28 and 35-1-8-10 (37.9 mg/100 g). The mutant 35-1-68-56 exhibited the minimum protein level of 18.45 mg/100 g than all the other mutants (Figure 3). When compared to the control, the mutants displayed both increasing and decreasing trend in protein content. This suggested that the amount of protein in plants may have positive or negative impact by mutation, either slightly or significantly.

3.2 Performance of mutants for micronutrient content

The highest calcium content of 2.33 mg/100 g was observed in the mutant 35-1-68-07, followed by 2.30 mg/100 g in the mutant 35-1-68-56, whereas the mutant 35-1-68-10 posses the lowest calcium content of 1.70 mg/100 g (Figure 4).With respect to the iron content, the mutant 35-1-68-49 showed the highest (0.87 mg/100 g) value, followed by the mutant 35-1-68-10 (0.78 mg/100 g) (Figure 4). The iron content was only 0.44 mg/100 g in the mutant 35-1-68-04, whereas in PKM 1 it was 0.62 mg/100 g.

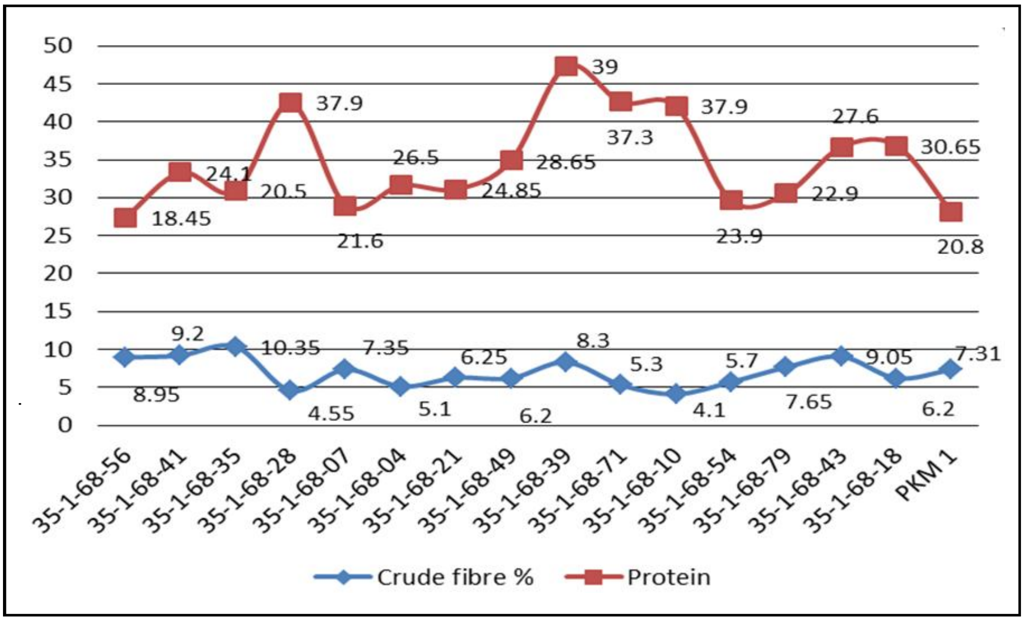

Figure 3: Protein and crude fibre content of moringa mutants with variety PKM 1.

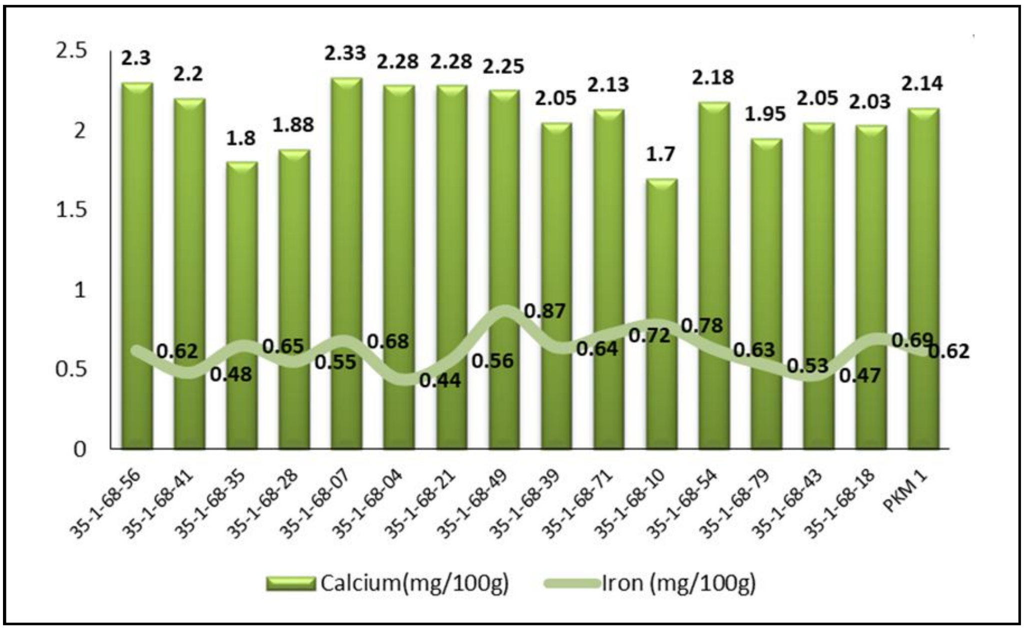

Figure 4: Calcium and iron content of moringa mutants with variety PKM 1.

3.3 Performance of mutants for chlorophyll, carbon and nitrogen content

The chlorophyll content was high in the mutant 35-1-68-35 (46.1), followed by the mutant 35-1-68-07 (40.8) compared to the control variety PKM-1 (38.5). The lowest chlorophyll content was observed in the mutant 35-1-68-49 (24.4) (Table 1).

Table 1: Chlorophyll content of moringa mutants with variety PKM 1.

S.No.	Mutants	Chlorophyll (SPAD)
1.	35-1-68-56	34.8
2.	35-1-68-41	26.5
3.	35-1-68-35	46.1
4.	35-1-68-28	37.6
5.	35-1-68-07	40.8
6.	35-1-68-04	36.7
7.	35-1-68-21	35.4
8.	35-1-68-49	24.4
9.	35-1-68-39	32.6
10.	35-1-68-71	34.4
11.	35-1-68-10	31.9
12.	35-1-68-54	31.3
13.	35-1-68-79	40.7
14.	35-1-68-43	37.4
15.	35-1-68-18	35.5
16.	PKM 1	38.5
	SE	2.57
	CD (5%)	7.43
	CV %	2.63

Note: SE - Standard error; CD - Critical difference; CV- Coefficient of variation

The carbon percentage was high in the mutant 35-1-68-35 (46.18%) followed by 35-1-68-07 (45.72%) whereas it was low (40.34%) in the mutant 35-1-68-43 compare to the control PKM 1 (41.85%). Carbon content was both increased and decreased in case of mutants comparing to the control (Figure 5). This implied that the EMS treatment had significant effect on plant carbon content. With respect to nitrogen, the mutant 35-1-68-07 (6.35%) recorded the highest followed by the mutant 35-1-68-22 (6.22%). The lowest nitrogen content was recorded in 35-1-68-56 (5.57%) comparing to the control (6.15%).

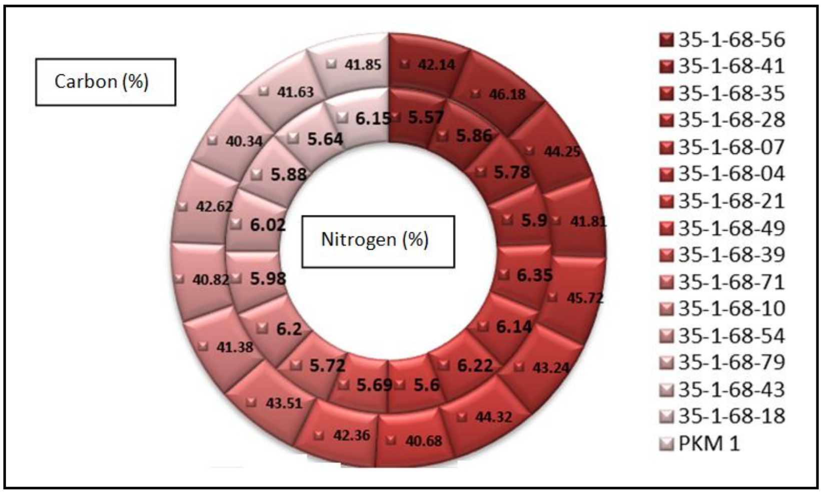

Figure 5: Carbon and nitrogen content of moringa mutants with variety PKM 1.

4. Discussion

Moringa is indigenous leafy vegetable which is being utilized for its rich source of digestible proteins, calcium, ascorbic acid, iron, vitamins K and A. The fresh leaf of moringa contains four times more vitamin A than carrot and 10 times in case of dried leaf powder (Singh *et al*., 2018). Mostly, it is used in fresh form by most of the people in India. The vitamin A content was in positive direction in the mutants (EMS treated) even after selection through four generations. EMS induction tended to lower the amount of carotenoids in the mature fruit and to raise the amount in the leaves of all genotypes. In young chilli fruits, carotenoids content was unaffected by EMS induction (Arumingtyas *et al*., 2018). In another study, significantly higher levels of carotenoid (1.57 mg/g FW) were found in the green pod of the control population of the local french bean cultivar. The results of the biophysiological investigation demonstrated a dose-dependent rise in inhibitions of mutagens, with a maximum in higher dosage (0.4%) in french bean (Laskar *et al*., 2024).

The fresh leaf of moringa contains seven times higher vitamin C content than oranges (Singh *et al*., 2018). In the present study, EMS treatment had greater influence on ascorbic acid content of moringa even in the M_4 generation. Also, in chilli, the ascorbic acid content in the M_1 generation decreased in the mutagenic treatments with an increase in the dose of mutagens. The lower dose of EMS treatment (0.2%) registered higher ascorbic acid content (116.03 mg/100 g) but lower than control (118.13 mg/100 g). On the other hand, the increased EMS treatment dose (0.4%) resulted in the lowest ascorbic acid level (113.43 mg/100 g) (Soyam, 2021). Also, in another study the analysis of variance revealed that the potassium and EMS treatment had a highly significant impact on vitamin C content of tomato sample (Slameto *et al*., 2023).

The EMS treatment at 0.15% had both positive and negative influence on the crude fibre content of M_4 moringa mutants. The mutants *viz.,* 35-1-68-35, 35-1-68-43, 35-1-68-56, 35-1-68-39, 35-1-68-79 and 35-1-68-07 showed higher crude fibre content than control PKM 1. In cowpea, the maximum crude fibre content for both the local and early white varieties were found at 0.40 per cent EMS concentration (Muhammad, 2023). In relation to this, in bhendi cultivar Arka nikita, the EMS treatments T11 - 0.4 % EMS, T12 - 0.5 % EMS (12.71), T10 - 0.3 % EMS (12.62), and T13 - 0.6% EMS (12.47) reported a substantial maximum crude fibre content (13.49%), while treatment T1 (control) recorded the minimum crude fibre content of 11.2 % (Sourab, 2023).

In comparison to yogurt, fresh leaf of moringa has two times more protein content (Singh *et al*., 2018). Significant rise in protein content was also observed in the mutants, *viz.,* 35-1-68-39, 35-1-68-28, 35-1-68-10, 35-1-68-71, 35-1-68-18, 35-1-68-43, 35-1-68-04, 35-1-68-21, 35-1-68-41 and 35-1-68-79 with respect to EMS treatment (0.15%). In groundnut, four EMS concentrations of 0.3%, 0.6%, 0.9%, and 1.2%, each paired with four treatment durations (1, 3, 5, and 7 h) comprised of 16 treatments were tested at 25°C. In this study, the M_2 population of HY22 and YY45 showed the peak protein content occurred in the groups which range from 24 to 24.99 and 25 to 25.99, respectively. In contrast, the protein content of their control plants was 23.1% and 23.5% respectively. Overall, 164 (57.14%) and 65 (22.65%) HY22 families, had protein content that was greater and lower than the control, respectively. In the meantime, 244 (73.05) M_2 families of YY45 showed high protein content, whereas 41 (12.28%) families showed low protein content (Chen *et al*., 2020). Also, two distinct concentrations of mutagen EMS (0.5% and 1%) were given to the grass pea cultivar for four hours in a study. The mutant GPM37 showed one of the highest values (>35%) for crude protein content in grass pea. But the protein level of GPM4 was 9.08% approximately three times lower than that of the control plant (Prasanth *et al*., 2020).

Dairy products are the rich source of calcium. The calcium content was four times higher in moinga fresh leaf when compared to milk leaf (Singh *et al*., 2018). Positive influence on calcium content can was noticed in the present study with the moringa mutants due to the influence of 0.15% EMS treatment. In sunflower, the cultivar Çetin bey showed the highest Ca ratio, which was found at 1.0% EMS dose (0.33%) and 1.25 g/l NaCl dose (0.32%), based on the bilateral interaction under *in vitro* conditions. In light of the trilateral discussion, the cultivar Çetin bey had the highest Ca ratio of 0.36% when administered as follows: 0g/l NaCl + 1.0% EMS doses; 1.25 g/l NaCl + 1.5% EMS doses; and 2.5 g/l NaCl + 0% EMS doses. In the cultivar Palanci I, the maximum Ca ratio was 0.24 % in the doses of 0g/l NaCl + 1% EMS (Altindal, 2019). Similarly in a study conducted by Kumar *et al*. (2022), EMS mutant of wheat lines showed huge variation in iron content in the grains (23.90-55.50 ppm) where set of more than 1200 mutants were studied.

A natural plant based pigment chlorophyll which is available in large quantity in leafy vegetables like moringa are utilized in different forms like tablet, capsules, powder and leaf extract. These chlorophylls act as anticancerous, antimutagenic and anti-inflammatory agent (Kumar *et al*., 2020). The EMS treatment had significant influence on chlorophyll content in most of the mutants (35-1-68-35, 35-1-68-07 and 35-1-68-79). In chilli, the maximum chlorophyll 'a' (4.3 mg/g) was obtained at the highest dose of EMS treatment (0.4%), which was comparable to the lower doses of EMS and control treatment. The maximum chlorophyll 'b' (0.82 mg/g) and maximum total chlorophyll (1.08 and 1.03 mg/g) was found in the higher dosages of EMS (0.4% and 0.3%) (Soyam, 2021). In the local french bean cultivar, the green pod of the control population had notably greater levels of chlorophyll content (10.02 mg/g FW). The results of biophysiological study indicated that the inhibitions increased in response to mutagen dose strength, with the highest inhibitions (0.4%) occurring in higher dose. In populations that had been mutagenized, there was a decrease in the average amounts of chlorophyll content (Laskar *et al*., 2024).

The mutagenic treatment with EMS had positive influence on carbon content of the leaves. During the anthesis stage in rice, the mutants O8, X48, X53, X57, and X63 showed a considerable increase in biomass and total N content as compared to wild type. However, there was no significant change in total N concentration between wild type, O8, and EMS mutants. At the maturation stage, in O8 and EMS mutants, the biomass and total N content greatly increased in comparison to wild type, while their total N concentration dramatically reduced in leaves in comparison to wild type (Chen *et al*., 2022).

5. Conclusion

Regarding the percentage of crude fibre, vitamin C, chlorophyll and carbon content, the mutants 35-1-68-35 (10.35%, 216.67 mg/100 g, 46.1 and 46.18%) performed well when compared to the other mutants and control. The results indicated that the maximum protein content (39.0 mg/100 g) and vitamin A (9.49 mg/100 g) were found in the mutants 35-1-68-43 and 35-1-68-39. The mutant 35-1-68-07 has the greatest calcium (2.33 mg/100 g) and nitrogen content (6.35%) and then the mutant 35-1-68-49 has the greatest iron concentration (0.87 mg/100 g). Consequently, further observation warranted to select the plant with high leaf biomass without compromising the quality and nutrition. Also, forwarding the best performing mutant based on both yield and quality helps in developing a variety suitable to meet both the food and nutritional security. However, subsequent generations of these mutants have to be studied to determine the best and stable expressing mutants based on the desired characters.

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Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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