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Molecular, morphological, and biomolecular characterization of ethyl methanesulfonate-induced mutations in *Aerides odoratum*, an orchid

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ABSTRACT

Aerides odoratum L. is a medicinal and ornamental orchid of the Eastern Himalayas, India. Its uses in pharmacology are nought because of its rare occurrence, longer life cycle, and lack of artificial methods for large-scale propagation. Protocorms (60 days old) were treated with various concentrations of ethyl methanesulfonate ranging from 0.025% to 0.5% for different durations (2, 5, 7, and 10 days) during M_0 (treatment) *in vitro* generation. The survival of these protocorms and the generation of shoots and roots were noted. 10–20 plantlets of each treatment showing variation in the number and weight of shoots in M_0 generation (treatment generation) were maintained for three subsequent generations. M_3 (third generation) axenic plantlets were hardened in the orchidarium with a 75% survival frequency. Four mutant lines (M_3V1–M_3V4) were established based on RAPD analysis of the leaf DNA of 300-day-old plants. M_3V-1 was noted as the best among all mutant lines based on morphological analysis, whereas M_3V-2 was noted as the most superior in terms of having the highest quantity of osmolytes, pigments, stomata, and phenol. M_3V-3 had maximum root length, leaf breadth, and carotenoids. M_3V-4 showed the viridis chlorophyll spectrum as the most distinguishing factor. Hence, this standardized protocol can be used for the genetic improvement of *A. odoratum*.

1. INTRODUCTION

Orchids are a unique group of plants whose beautiful and fragrant flowers have fascinated humans, and their medicinal properties have cured and saved human lives from several acute and chronic sufferings [1]. *Aerides odoratum* L. is a medicinal and ornamental orchid of the Eastern Himalayas found at altitudes of 800 m–1200 m. The leaf juice has antimicrobial properties and is used to cure boils in the ears and nose by tribal people [2,3]. Unfortunately, use of this orchid in pharmacology is nought because of its rare occurrence, slow growth, longer life cycle, and lack of artificial methods for large-scale propagation of superior variants [4].

Chemical-induced mutagenesis is one of the major breeding approaches for plant improvement. Ethyl Methane Sulfonate (EMS) has been recommended to improve cultivars of vegetatively propagated plants, especially ornamental plants. *In vitro* mutation induction in plants provides an efficient method for its rapid mass propagation and selection of better stress-tolerant morphological variants with improved phytochemical and osmolite contents [5]. The application of mutation techniques for improving both seed and vegetatively propagated plants has been made easier by the advent of effective *in vitro* culture methods [6]. Mutation induction, combined with *in vitro* culture techniques, has become the only viable option for plant regeneration and improvement in many vegetatively propagated crops, notably slow-growing ornamental plants. Effective use of low-dose mutagens on a large number of propagules and the ease of handling plantlets are a few benefits of *in vitro*-induced mutation [7].

Using a potent chemical mutagen, like EMS, is one method of causing mutation. It is a highly recommended chemical mutagen due to its low cost and easy application methods; however, the temperature of the solution, treatment period, and concentration are the three most crucial factors when using EMS to induce mutation [8]. EMS typically results in point mutations in plants, but it can also cause chromosome loss or deletion. Consequently, EMS may be able to modify loci of specific interest without causing a significant number of closely related alterations. However, it can cause cell death either instantly or by mitotic death that occurs when the cell divides [9]. Research has shown that EMS can be used to produce mutants in a variety of crop plants [9-12] However, very few studies are available claiming the

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success of EMS-induced mutations in orchids [13,14]. Therefore, this study aimed to establish superior *A. odoratum* mutant lines in terms of morphology and osmolytes, pigments, and phenol contents.

2. MATERIALS AND METHODS

2.1. Seed Source and Establishment of *in vitro* Culture

The study design is illustrated in Figure 1. Mature pods of *A. odoratum* were collected from their natural habitat. They were surface sterilized by liquid detergent for 5–10 min and then washed thoroughly in distilled water and subsequently double-distilled water to remove all the traces of detergent from the pod. Further, pods were disinfected using mercuric chloride solution (0.5% w/v) and 70% ethanol. The sterilized pods were split and opened in a laminar air flow unit (LAF) with a sterile surgical blade [15]. Powdery seeds were kept on nutrient media MSBM (Murashige and Skoog, 1962) fortified with a combination of coconut water (10%), kinetin (4.65 µM), and casein hydrolysate (250 mg/l) to obtain optimum seed germination and protocorm formation. The cultures were incubated at standard *in vitro* culture conditions.

2.2. *In vitro* Induction of Mutation

Doses and duration of treatment were decided based on a trial-and-error method. Ethyl methylsulfonate (SIGMA-Aldrich) stock solution (200 mg/mL) was prepared by adding 1 mL of sterile water to 200 mg of EMS and sterilized with a millipore filter (pore size: 0.01 µm) under LAF. In the sterilized culture media, the required volume of EMS was added to get different concentrations of mutagenic agents ranging from 0.025% to 5%. Further media with EMS were dispensed into the tubes or bottles containing nutrient media. Young protocorms (30 days old) were transferred to 120 bottles having different concentrations of EMS in mutagenic media [Figure 2]. 10 bottles of each EMS concentration to be studied were kept for different time intervals (2, 5, 7, and 10 days) to induce mutation. After the treatment of chemical mutagens, the protocorms were subcultured in the differentiation and multiplication media, MSBM in combination with Kn (4.65 µM) and TDZ (4.54 µM), and produced multiple shoots. The effect of mutagen on protocorms was recorded in terms of the percent survival of protocorms the increase or decrease in the number and weight of shoot bud initials. Further, half-strength MSBM supplemented with NAA (5.46 µM) was used to develop root initials. 10–20 plantlets of each treatment showing variation in the number and weight of shoots in M_0 generation (treatment generation) were maintained for three subsequent generations: M_1 (first generation after treatment), M_2 (second generation after treatment), and M_3 (third generation after treatment) under *in vitro* conditions. Leaf segments of M_0, M_1, and M_2 generation seedlings were excised and subcultured on regeneration media in subsequent generations to obtain multiple shoots and roots. M_3 generation mutants having strong and stout roots and 3–6 leaflets were hardened and acclimatized in the Orchidarium [7,10].

2.3. Evaluation of Mutants at M_0 Generation

M_0 generation-treated protocorms were evaluated for mutagenic effects based on variation in survival frequency and fresh weight (FW).

2.3.1. Percent survival of protocorms

Mutagen-treated protocorms were observed under a stereo microscope to find the effect of different doses of mutagen in terms of shrinkage, death, and/or shoot initiation, and percent survival was calculated.

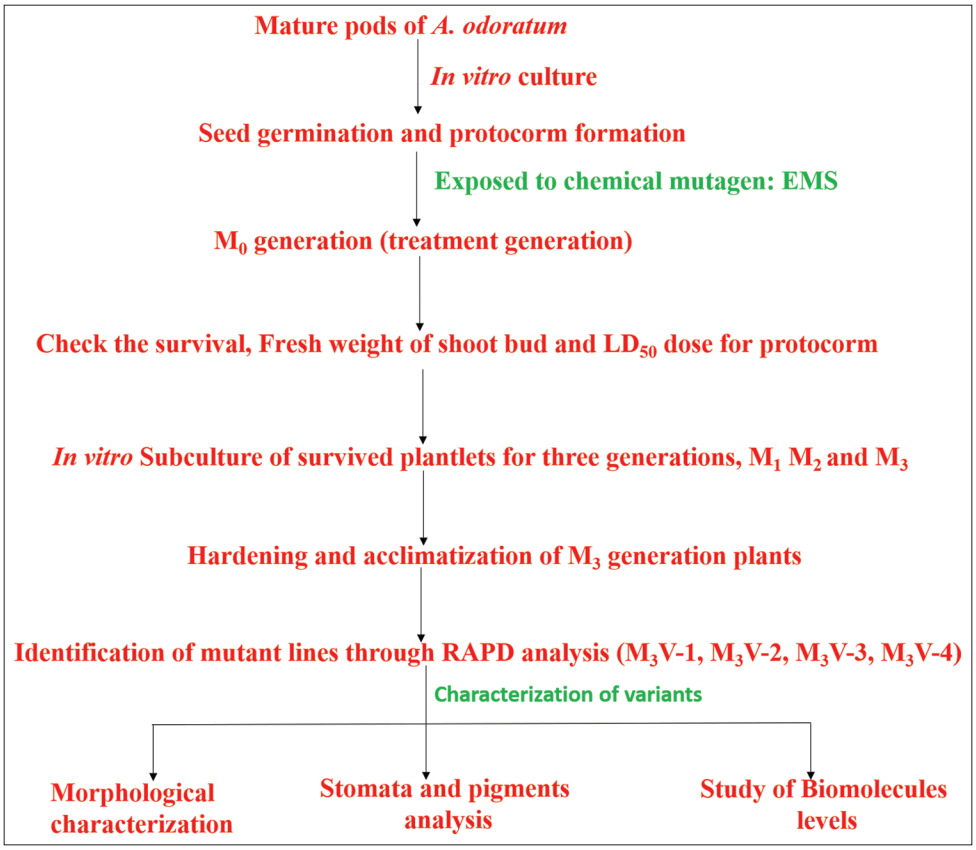

Figure 1: A schematic diagram of experimental design for *in vitro* mutation breeding in *A. odoratum*.

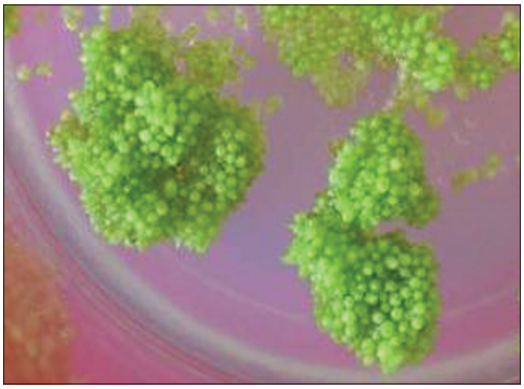

Figure 2: Protocorms subjected to mutagen treatment.

2.3.2. FW

The treated protocorms were taken out of the culture bottles when they attained maximum growth, and the moisture was gently removed by blotting with filter paper and transferred to pre-weighed aluminum foil. The weight of the protocorm was determined with the help of an electronic balance.

2.4. Identification of Mutants through RAPD Analysis in M_3 Generation

M_3-generation 18-month-old plants were screened for molecular characterization. A total of 50 M_3-generation plants, who were apparently looking morphologically different than the control, were used for molecular analysis. DNA was isolated from a hundred-day-old hardened mutant plant leaf using the standard CTAB method. Universal RAPD genetic marker: OPA-18 forward and reverse primers were used to amplify the DNA of control and mutant plants. Mutants were identified by looking for unique bands on the DNA gel picture.

2.5. Study of Morphological Parameters at the M_3 Generation

Variations in morphological parameters such as plant height and weight, number of leaves, leaf length, breadth, and root length were studied among control and mutated plants in the M_3 generation.

2.5.1. Mutation spectrum of chlorophyll at M_3 generation

Mutant plantlets from the M3 generation were screened for changes in the chlorophyll spectrum caused by mutation. Chlorophyll mutants were categorized as follows [16]:

1. Albino: Leaves are white, devoid of chlorophyll and carotenoids, and typically have few plastids
2. Xantha: Yellow leaves with carotenoid pigments but little to no chlorophyll
3. Alboviridis: The rate at which plastids develop at the base and tips of leaves varies for each leaf and displays various hues in these sections.
4. Viridis: Their color is a pale green.
5. Tigrina: Display alternating narrow bands of green, yellow, or brown transverse stripes along the leaf in areas where pigment degradation has taken place.
6. Striata: These contain longitudinal stripes that alternate between green and yellow or white.
7. Maculata: Contains areas where carotene and/or chlorophyll have been damaged.

2.6. Stomatal Length (SL) and Density at M_3 Generation

SL, µm was measured with the help of a scale present in the software from 30 stomata selected randomly. Stomatal density is a simple calculation of the number of stomata seen in a millimeter square of tissue. Grids of 100 μm^2 were composed on the microscopic image to count the number of stomata and epidermal cells (E) within that. Stomatal density was calculated using the following formula: Stomatal Density = Number of Stomata present in 1 grid (S) X 100.

2.7. Estimation of Chlorophyll and Carotenoids at M_3 Generation

Chlorophyll and carotenoids were estimated using the modified Holm method [17]. The concentration of chlorophyll was calculated by Porra's (2002) equation [18]. Carotenoid concentration was calculated using Lichtenthaler and Wellburn's equation [19].

2.8. Extraction of Sugars and Amino Acids at M_3 Generation

Leaf samples that had been oven-dried at 60° C were blended with 80% hot ethanol, centrifuged for 10 min at 2000 rpm, and the supernatant was poured off. The residue mixture was then treated with 3 mL of 80% ethanol, and recentrifugation was performed. To guarantee that all sugars and amino acids were recovered, the extraction process was done twice. The residue was saved to estimate starch. A boiling-water bath at 45°C was used to cool and evaporate the supernatant, leaving a Petri dish-like residue that was eluted with 5 mL of 20% ethanol before being subjected to analysis for sugars and amino acids.

2.8.1. Quantification of total sugars at M_3 generation

Quantification of total sugars was made by following the "anthrone method." Using a standard curve that was plotted using a known quantity of glucose, the concentration of total sugars was determined [20].

2.8.2. Reducing sugar estimation at M_3 generation

The DNSA method was used to estimate the amount of reduced sugars. Based on a standard curve that was produced with a known quantity of glucose, the amount of reducing sugars was determined [21].

2.8.3. Estimation of amino acids at M_3 generation

Amino acid content was estimated by the "Ninhydrin method". The concentration of free amino acids was calculated from a standard curve with a known concentration of glycine [22].

2.8.4. Estimation of total phenols at M_3 generation

Concentrations of total phenol were estimated in M_3 generation plant tissues using the modified method proposed by Waterman and Mole (1994) and expressed in terms of gallic acid equivalent (mg g^{-1} of dry mass) [23].

2.9. Statistical Analysis

The percent of data was calculated in MS Excel (2019) software. The data obtained were subjected to a one-way ANOVA in SPSS software, Version 26. The mean values, standard deviation, and level of significance were calculated.

3. RESULTS AND DISCUSSION

3.1. Effect of EMS Treatment on M_0 Generation

The effect of EMS on survival frequency was scored based on shoot bud initiation after 60–80 days of treatment. It was found that the survival percentage of protocorms decreased with increasing concentration and time duration of EMS exposure. Protocorms [Figure 2] exposed to different concentrations of EMS for 2 days showed more than 50% survival frequency, ranging from 89 ± 7% to 55 ± 8%, and

the maximum FW of shoot buds ranged between 40 ± 7 mg and 20 ± 5 mg. There was no significant difference in survival frequency or FW between treated and control plantlets. Hence, this duration was found ineffective in changing the survival frequency and weight of the shoot bud because shorter exposure is usually unproductive for the uptake and penetration of EMS into protocorms and seeds under *in vitro* conditions [24,25].

Treatment of protocorms with different doses of EMS for 5 days was found to be most effective in causing mutagenic effects. The frequency of protocorm survival ranged between 81 ± 2% and 2 ± 2% at doses of 0.025% and 0.5%, respectively. EMS concentrations of 0.3% showed an approximate 50% survival frequency at 5 and 7 days of treatment, whereas a drastic reduction in survival was noted with further increases in EMS concentrations. EMS (0.5%) was found to be lethal and showed only 2% survival frequency for 5-day treatment and zero survival for longer-term treatment. Radical variations in the FW of shoot buds on EMS treatments for 5 and 7 days of exposure were noted, which may be due to the mutagenic effect. The effect of EMS on *A. odoratum* is evident by the increasing concentration and duration of treatment. Similar doses were noted as effective in other studies on orchids as well [7,14]. Maximum FW of 60 ± 7 mg was attained at EMS (0.1%) and minimum 4 ± 3 mg at EMS (0.5%) for 5 days of treatment, whereas it varied from 37 ± 7 mg to 0 for 7 days of treatment [Table 1]. Variation in FW as an effect of different doses of EMS was also observed in orchids [7] and other species [26].

EMS doses of 0.3% and 0.5% were noted as LD50 and lethal doses, respectively [Table 1]. EMS treatment for 10 days showed deleterious effects of mutagenesis, resulting in a decrease in FW of shoot buds and <50% protocorm survival except at doses of 0.025% to 0.15% [Figures 3-5]. A similar genotoxic effect of EMS was noted in a wide range of plant species, including *Eclipta alba* [27], rose [28], citrus [29], and mungbean [30]. Further, the time required for shoot bud formation was exponentially increased with increasing the concentration of EMS in the present study. The delay in seed germination as well as the reduction in seed germination percentage with increasing ethyl methanesulfonate doses were also reported in crop plants such as lentil and wheat [31,32].

Surviving plantlets were maintained for three generations. M_3-generation plantlets [Figure 6] were maintained at culture conditions for 100 days. Finally, 501 axenic plantlets [Figure 7] were obtained by treatment with different EMS doses for various time durations in M_3 generation. These axenic plantlets were hardened and acclimatized to a potting mixture containing broken brick pieces, peat, and small pieces of charcoal (1:1:1 w/v) and maintained in Orchidarium for 18 months with a 75% survival frequency [Figure 8].

3.2. Identification of Mutant Lines Using RAPD Analysis

RAPD analysis is one of the simplest and most accurate methods to identify epigenetic and somaclonal variations among variants. It has been used for the identification of mutations in *Allium sativum* L [33] and genetic stability in *Rhynchostylis retusa* [34]. Four mutant lines were established among variants of *A. odoratum,* which showed unique RAPD bands [Figure 9]. These mutant lines were denoted as M_3V-1-M_3V-4. Mutant line M_3V-1 was obtained from 0.05% EMS treatment for 5 days, whereas M_3V-2 was produced from 0.075% EMS

Table 1: Effect of different doses and time duration of EMS treatment on the survival frequency and fresh weight of shoot buds.

EMS doses (%)	Treatment for 2 days		Treatment for 5 days		Treatment for 7 days		Treatment for 10 days	
	Survival frequency (%)	Fresh weight of shoot bud	Survival frequency (%)	Fresh weight of shoot bud	Survival frequency (%)	Fresh weight of shoot bud	Survival frequency (%)	Fresh weight of shoot bud
Control	85±5	32±3	85±5	32±3	85±5	32±3	85±5	32±3
0.025	89±7	33±5	81±2	43±5	85±9	37±7	50±3	31±7
0.05	82±8	37±7	78±9	52±6	81±7	48±8	74±9	38±9
0.75	79±11	34±4	72±11	56±6	79±7	41±7	88±6	36±5
0.1	75±9	36±9	75±10	60±7	72±11	43±2	67±3	30±4
0.125	82±8	38±5	78±9	46±9	66±8	40±8	57±3	26±7
0.15	85±9	32±6	67±14	50±11	62±6	33±2	51±7	21±9
0175	80±12	39±9	68±9	38±6	60±11	31±6	48±5	21±3
0.2	88±14	40±7	69±8	34±4	56±13	28±5	40±3	17±5
0.225	92±8	36±8	61±9	33±5	52±6	31±7	41±4	14±3
0.25	85±9	30±4	58±9	42±6	36±3	38±9	29±6	12±8
0.275	87±12	25±4	55±11	36±8	46±4	36±5	6±2	11±6
0.3	88±11	36±4	50±12	30±9	48±4	30±4	18±3	17±3
0.325	82±8	39±4	40±12	33±5	33±4	29±5	14±9	16±5
0.35	85±12	36±8	38±9	32±6	32±3	26±5	11±3	13±8
0.375	88±3	33±8	32±11	26±6	27±1	19±5	14±3	16±4
0.4	81±11	34±16	30±6	20±5	21±9	31±7	10±4	15±4
0.425	70±7	36±5	25±8	13±8	13±2	26±5	5±4	7±3
0.45	64±9	24±6	10±4	19±6	11±4	23±8	0	0
0.475	55±8	20±5	8±3	10±9	1±1	11±3	0	0
0.5	60±7	23±8	2±2	4±3	0	0	0	0

* All the data is mean±SD.

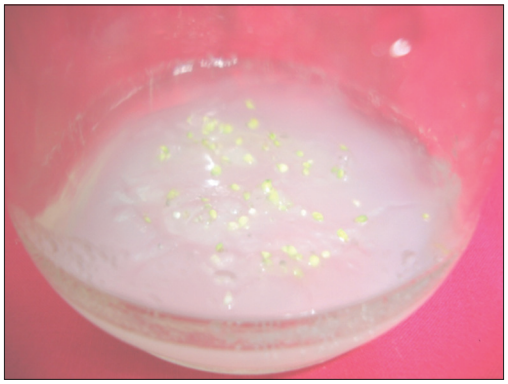

Figure 3: Survival of protocorms at LD_{50} Dose, EMS (0.3%).

Figure 6: Development of axenic plantlet at M_3 generation.

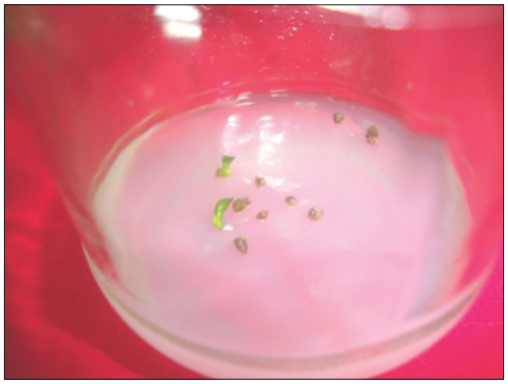

Figure 4: Shoot bud formation at Lethal Dose, EMS (0.5%).

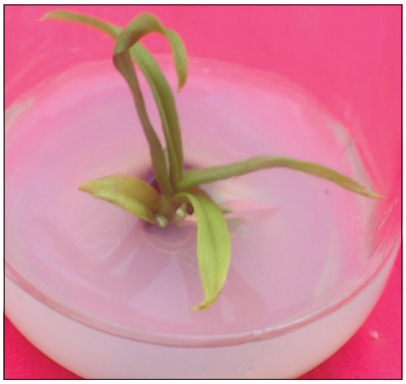

Figure 7: M_3 generation plantlet ready for hardening.

Figure 5: Development of axenic plantlet at M_0 generation.

Figure 8: M_3 generation, 300 days old hardened plants.

treatment for 5 days. M_3V-3 was formed from 0.1% EMS treatment for 5 days, and the M_3V-4 mutant was obtained using EMS (0.05%) for 7 days. Similar results were noted in a study by Srivastava *et al*. [7].

3.3. Evaluation of Mutants in M_3 Generation

Among four mutant lines (M_3V-1-M_3V-4) isolated from EMS treatment in M_3 generation, superior morphological mutants M_3V-1, M_3V-2, and M_3V-3 evolved from EMS 0.05%, 0.075%, and 0.1% treatments for 5 days, respectively, showed superior morphological, stomatal osmolytes, pigments, and phenol contents than control and were significantly diverse in morphology among them also [Table 2]. Among the three mutant lines, M_3V-1 showed the least plant height (3.49 ± 0.03 cm), weight (549.83 ± 6.76 mg), root length (1.93 ± 0.09 cm), and number of leaves (3.39 ± 0.09) but the longest (3.99 ± 0.04 cm) and thinnest (1.27 ± 0.03 cm) leaf compared to the control [Figures 10 and 11]. It can also be distinguished by having maximum chlorophyll contents (4.42 ± 0.11 µg/mg) and total phenol (42.6 ± 0.6 mgGAE/g DW) [Table 2]. The variation among three morphologically superior mutants (M_3V-1, M_3V-2, and M_3V-3) can be explained as mutations of genes, the breaking of tightly linked regions and crossing over within these regions, enhanced recombination, or an individual or a combination of two or more such effects [35,36].

Mutant line M_3V-2 showed maximum plant height 4.48 ± 0.08 cm, weight (648.47 ± 3.97 mg), number of leaves (3.99 ± 0.11), total soluble sugar (265.6 ± 5.6 µg/gDW), reducing sugar (228.5 ± 3.7 µg/gDW), and amino acids (161.2 ± 3.2 µg/gDW) [Table 3]. M_3V-3 showed a maximum root length of 2.95 ± 0.13 cm and the broadest leaf (1.67 ± 0.13 cm) as significantly distinguishable morphological characters. Mutation by EMS is known to increase resistance to abiotic stress through the accumulation of osmolytes such as carbohydrates and amino acids. These compounds serve to maintain turgor pressure

Table 2: Effect of EMS on the morphology of M_3 generation plants in *Aerides odoratum*.

EMS doses (%)	Plant height (cm)	Plant weight (mg)	Number of leaves	Root length (cm)	Leaf length (cm)	Leaf breadth (cm)	Length of stomata (µm)	Stomatal density
Control	3.20±0.04	520.60±9.24	2.94±0.06	1.47±0.07	2.89±0.07	1.36±0.05	24.8±0.9	99.4±7.9
0.05% for 5 days (M_3V-1)	3.49±0.03	549.83±6.76	3.39±0.09	1.93±0.09	3.99±0.04	1.27±0.03	18.8±1.9	118.9±1.5
0.075% for 5 days (M_3V-2)	4.48±0.08	648.47±3.97	3.99±0.11	2.45±0.08	3.47±0.12	1.50±0.07	21.4±1.6	128.5±3.7
0.1% for 5 days (M_3V-3)	3.96±0.02	595.03±9.50	3.67±0.13	2.95±0.13	3.69±0.07	1.67±0.13	20.0±1.4	136.0±5.9
0.05% for 7 days (M_3V-4)	3.27±0.04	511.52±2.52	2.19±0.09	1.20±0.08	2.81±0.04	1.28±0.04	23.8±1.6	122.5±6.5

*All the data is mean±SD, $P<0.05$.

Table 3: Effect of EMS on osmolytes, pigments, and total phenol concentration of M_3 generation leaf in *Aerides odoratum*.

EMS doses (%)	Total soluble sugar (µg/gDW)	Reducing sugar (µg/gDW)	Amino acids (µg/gDW)	Total Chlorophyll (µg/mg)	Carotenoid (µg/mg)	Total phenol (mgGAE/g DW)
Control	174.5±4.4	123.2±6.7	88.4±5.5	3.22±0.05	1.50±0.09	20.5±0.8
M_3V-1	199.7±3.8	145.1±5.1	139.1±8.4	4.42±0.11	2.32±0.15	42.6±0.6
M_3V-2	265.6±5.6	228.5±3.7	161.2±3.2	3.91±0.14	2.62±0.10	35.5±0.3
M_3V-3	223.4±6.2	180.7±4.8	107.4±4.7	4.19±0.17	1.91±0.09	29.5±1.4
M_3V-4	188.0±3.4	131.3±2.3	89.1±6.4	3.02±0.18	1.19±0.07	21.8±3.2

*All the data is mean±SD, $P<0.05$.

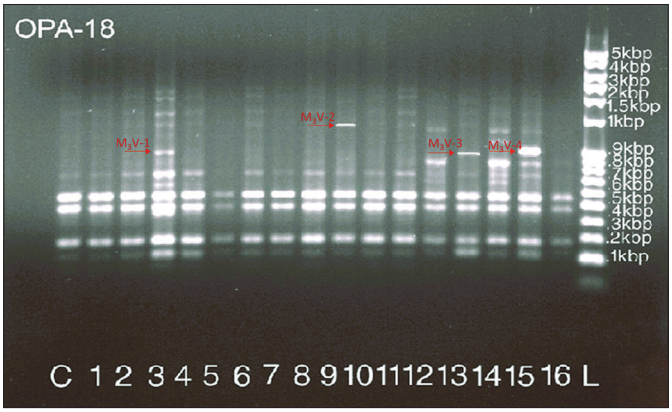

Figure 9: RAPD analysis of hardened mutant plants.

Figure 10: Leaf of control *A. odoratum*.

Figure 11: Longer and thinner mutant leaf.

Figure 12: Thinner mutant leaf with viridis chlorophyll spectrum.

in the form of organic and inorganic compounds such as amino acids, sucrose, soluble sugars, reducing sugars, and various other solutes in the cytoplasm to increase the absorption of water from the soil [37,38]. In the present study, all morphological mutants showed an enhanced level of these osmolytes, which may be considered stress-resistant mutant lines for further studies.

The length of stomata in M_3V-1, M_3V-2, and M_3V-3 was shorter, but the stomatal density was significantly greater than the control. For M_3V-4, although the length of the stomata was shorter than that of the control, the difference was not significant. However, the stomatal density was significantly higher than the control in M_3V-4. Further, an increase in chlorophyll, carotenoid, and total phenolic contents was noted as an important impact of mutation in all species. Similar effects were noticed in many stress-resistant crops, including orchids [7,17,39,40].

The M_3V-4 mutant line showed the most distinguishing feature, the viridis chlorophyll spectrum in leaves. The leaf was thickest, having spots where chlorophyll and/or carotene had been destroyed. Less quantity of chlorophyll (3.02 ± 0.18 µg/mg) and carotenoid (1.19 ± 0.07 µg/mg) compared to the control estimation confirmed the morphological mutation in the M_3V-4 mutant line [Table 3]. The chlorophyll variegation in leaves after EMS treatment is common to change and seen in different ornamentals, which may be due to the high specificity of EMS for mitochondrial and plastid genome mutations [7,41,42]. Our investigation of improved stomatal density, chlorophyll, and carotenoid contents in the M_3V-4 mutant line also supports the formation of viridis variegation in the leaf [Figure 12].

4. CONCLUSION

The use of low EMS doses on protocorms of orchids can generate superior variants of orchids in terms of morphological and biomolecular characteristics, which may be considered stress-resistant mutant lines for further studies. Four mutant lines (M_3V1-M_3V4) were established based on RAPD analysis of the leaf DNA of 300-day-old hardened plants. M_3V-1 (developed from 0.05% EMS for 5 days of treatment) was noted as the best among all mutant lines based on morphological analysis, whereas M_3V-2 (generated from 0.075% EMS for 5 days treatment) was noted as the most superior in terms of having the highest quantity of osmolytes, pigments, stomata, and phenol. M_3V-4 (0.05% EMS for 7 days) showed the viridis chlorophyll spectrum as the most distinguishing factor. EMS (0.1%) for 5 days was responsible for the creation of M_3V-3, which had maximum root length, leaf breadth, and carotenoids. The present study developed the first method for the *in vitro* mutation of *A. odoratum,* which provides a framework for implementing mutation breeding and improving the orchid species. Future studies on identifying and encoding genes responsible for these mutations can help in directly introducing agronomically important features into orchid plants.

5. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agreed to be accountable for all aspects of the work. All the authors are eligible to be authors as per the International Committee of Medical Journal Editors (ICMJE) requirements and guidelines.

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The authors report no financial or any other conflicts of interest in this work.

8. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

9. DATA AVAILABILITY

The authors confirm that the data supporting the findings of this study are available within the article.

10. USE OF ARTIFICIAL INTELLIGENCE (AI)-ASSISTED TECHNOLOGY

The authors declares that they have not used artificial intelligence (AI)-tools for writing and editing of the manuscript, and no images were manipulated using AI.

11. PUBLISHER'S NOTE

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