INTRODUCTION

Abelmoschus moschatus (L.) Medik (synonym: Hibiscus abelmoschus; Family: Malvaceae; common name: Ambrette; English name: Musk mallow) is an annual or biennial, erect, hirsute herb/ undershrub\(^1\,^2\) or shrub\(^3\,^4\) yielding ambrette oil (essential oil obtained from outer layer of seed coat - Nee et al.\(^5\) possessing characteristic musk like odour – Rout et al.\(^6\) of commerce which finds application both in flavour and fragrance formulations\(^6\) not withstanding the potential significance of the species (roots/leaves/rarely seeds) in traditional system of medicine\(^7\,^8\). Further, ambrette seed oil is reported to be edible\(^9\,^10\), and therefore may be considered as an alternative resource to the traditional oil crops. However, commercial cultivation of the species is restricted in Java, India (mainly in the Deccan and Carnatic, Madagascar and in parts of Central and South America) on a small scale\(^11\). Considering the potential significance of ambrette seed oil it would be of utmost importance to keep the species under cultivation for sustainable use in regions conducive for growth. Further, genetic variations in the species (the species being self-pollinated and offer little scope for variations) for betterment (yield of essential oil content and value added plant products) exploring the existing germplasm(s) may also be recommendable. Induction of mutation widens the gene pool and offers scope for suitable selection. The objective of the present work is to raise plant type mutation in \(A.\) moschatus, which may be important genetic resources for exploration in human benefits. The present authors have undertaken a research programme on induced mutagenesis (ethyl methane sulphonate and hydroxylamine) in \(A.\) moschatus (in two cultivars) and this communication sheds light on mutagenic sensitivity, mutation types and frequency, mutagenic effectiveness and mutagenic efficiency and cytological behavior of the mutants.

MATERIALS AND METHODS

Plant materials

Seeds of cultivar I (cv.I: moisture content 3.5%, seed size 3.47 mm ± 0.08 × 3.20 mm ± 0.06) and cultivar II (cv.II: moisture content 1.5%, seed size 2.92 mm ± 0.07 × 3.33 mm ± 0.03) of \(A.\) moschatus (L.) Medik were collected from Medicinal Plant Garden, Narendrapur, Ramkrishna Mission, Govt. of West Bengal. Cultivar I is recommended as tall, branched whereas cultivar II as dwarf, unbranched types\(^12\).

Mutagenic treatment

Dry seeds of cv.I and II were treated with different concentrations (0.25, 0.50 and 1.00% for 3 and 6h durations) of ethyl methane sulphonate (EMS - Sigma, USA; dilutions made in 0.2M Phosphate buffer, pH 6.8) and hydroxylamine (NH\(_2\)OH - aqueous solution) at 36°C ± 1°C. The dose were applied after pilot trials.

Assessment of mutagenic sensitivity

Fifty seeds from each treatment along with control were given in petriplates (lined with moist filter paper) to assess germination (growth of radicle following bursting of seed coat was taken as an index of germination) and seedling growth (measured on a millimeter graph paper on the 8\(^{th}\) day from treatment) under uniform condition(s). Biological damages like lethality and injury were assessed from seed germination frequency and seedling growth (as percent of control) respectively as was suggested by Konzak et al.\(^13\). LD\(_{50}\) was also ascertained from reduction in germination frequency.

Raising of \(M_1\), \(M_2\) and \(M_3\) generations

Fifty seeds from each treatment were sown in the Experimental field plots of Department of Botany, University of Kalyani (West Bengal plains, Nadia, latitude 22°50’ N to 24°11’ N, longitude 88°09’ E to 88°48’ E, elevation 48 ft above sea level, sandy loamy soil, soil pH 6.85) in late April to raise \(M_1\) generation (spacing 25 cm between plants and 30 cm between rows). \(M_1\) plants were harvested from late November to December. Seed sterility was assessed in each treatment (seeds of each plant was weighed on harvest) and was represented as percentage of reduction in seed weight in treatments in relation to control. Selfed seeds of each surviving \(M_1\) plants (2 to 3 flowers were bagged) were harvested separately and were used to raise \(M_2\) (plant to row) generation (late April to December, 2010). Control lines were also grown. Macromutants were carefully screened at \(M_2\) throughout the growth period and the frequency of the mutants was estimated as per 100 plants\(^4\). Selfed seeds of \(M_2\) macromutants along with control were sown at \(M_3\) (April 2011) for confirmation of the mutant trait(s) from segregating progenies.
The efficiency and effectiveness of EMS and hydroxylamine were calculated from viable (total) mutation frequency as proposed by Konzak et al. The mutagenic efficiency was calculated as $Mf/L$, $Mf/I$ and $Mf/S$ and the effectiveness as $Mf/c$ (Mf = mutation frequency, L = lethality, I = injury, S = sterility, c = concentration of EMS and hydroxylamine, t = duration of treatments).

**Meiotic analysis**

Meiotic analysis was performed in the cultivars as well as in $M_2$ mutant plants. For the purpose, suitable sized flower buds (2 to 3 plants were scored for each plant type) were fixed between 6 am to 7 am in Carnoy's fluid (three changes were given at an interval of 24h) and preserved under refrigeration in 70% alcohol. Anthers were squashed in 2% propinocarnine solution and meiotic data (pooled over the plants in each plant type) were scored at metaphase I (MI) and anaphase I (AI). Photomicrographs were taken from temporary squash preparation.

**Pollen grains viability**

Pollen grains viability (Lugol's iodine--detect the presence of starch, viable pollen grains turns black - Bengtsson) was also assessed in the cultivars as well as in mutants.

**Anatomical studies**

Transverse hand sections of the stem (10 cm from base) were made in dwarf cultivar and in thick stem mutant from fully matured plants on harvest, and were doubled stained using 1% safranin dissolved in 50% alcohol and 1% light green dissolved in 90% alcohol.

**RESULTS AND DISCUSSION**

**Mutagenic sensitivity**

Germination frequency was 24.0% in cv.I and 64.0% in cv.II. In treatments (cv.I: EMS: 4.0 to 32.0%, hydroxylamine: 4.0 to 16.0%); cv.II: EMS: 28.0 to 68.0%, hydroxylamine: 4.0 to 60.0%) germination frequency was reduced (exception: cv.I: 0.25 and 0.50% EMS, 3h – 32.0 and 24.0% respectively; cv.II: 0.25%, 3h EMS - 68.0%) mostly than respective controls. Frequency of germination was not dose dependent. Six hour EMS treatments have shown higher reduction in germination frequency than 3 hour treatments at comparable doses; however, such trend was not observed in NH$_2$OH treated plants. Lethality was 16.67 to 83.33% in EMS and 33.33 to 83.33% in NH$_2$OH treatments of cv.I, and 6.25 to 56.25% in EMS and 6.25 to 37.50% in NH$_2$OH of cv.II. LDS could only be ascertained in EMS (cv.I: 0.50%, 6h; cv.II – prior to 1.0%, 6h); while, in NH$_2$OH treatments lethality was variable among the doses of cv.I and 50% reduction in germination was not attained in cv.II.

Seedling length was 21.0 cm (range: 9.0 to 38.0 cm) in cv.I and 76.2 cm (3.0 to 110.0 cm) in cv.II and in treatments it enhanced as well as reduced (cv.I: EMS – 4.0 to 59.6 cm, NH$_2$OH – 10.5 to 73.3 cm; cv.II: EMS – 49.6 to 96.1 cm, NH$_2$OH – 50.2 to 81.5 cm) in comparison to controls. However, reduction (cv.II: 0.50%, 6h EMS – 49.6 cm, p<0.05; 0.50%, 6h NH$_2$OH – 50.2 cm, p<0.05) or enhancement (cv.I: 0.25%, 3h NH$_2$OH – 59.0 cm, p>0.001; 0.25%, 6h NH$_2$OH – 73.3 cm, p<0.001) were significant only in few doses. Injury varied from 0.0 to 79.95% (cv.I) and 65.13 to 95.81% (cv.II) in EMS treatments; while, it ranged from 0.0 to 79.95% (cv.I) and 0.0 to 59.29% (cv.II) in NH$_2$OH treatments. Reduced as well as enhanced percentage of germination and seedling growth noted under similar conditions in petriplates accentuates the assumption that treatments have affected the physiological phenomena. Evans considered cessation of growth resulted due to blockage of cells into mitosis. Gunckel and Sparrow attributed inhibition of growth in higher doses of mutagens due to destruction of auxin of meristematic cells as well as due to other biochemical disturbances.

**Mutation frequency and types**

Spectrum of mutation at $M_2$ was low in both the cultivars (cv.I: 5; cv.II – 3). Macromutants (all viable) namely pigmented stem (Fig. 1E-b), lax branching (Fig. 1B), long petiole (Fig. 1D), large flower (Fig. 1C-b) and early flowering were isolated in cv.I; while, funnel (Fig. 2B), thick stem (Fig. 2C-b) and late flowering mutants were scored in cv.II. Mutant traits were confirmed at $M_2$. EMS has induced relatively higher frequency (cv.I – 3.31%, plant scored 1089, range – 2.04 to 3.92%; cv.II – 1.75%, 1945 plants scored, range – 0.48 to 3.66%) of mutation than NH$_2$OH (cv.I – 2.90%, 517 plants scored, range – 1.82 to 4.35%; cv.II – 1.27%, 2838 plants scored, range – 0.35 to 2.51%). Mutation frequency was not dose dependent. Mutation frequency over $M_2$ population was higher in cv.I (3.11%, 1606 plants scored) than cv.II (1.46%, 4783 plants scored) and the mutants occurred in the following order: cv.I – pigmented stem (1.68%) > long petiole (0.75%) > lax branching (0.56%) > early flowering (0.06%) = large flower (0.06%); cv.II – late flowering (1.02%) > funnel (0.27%) > thick stem (0.17%). In both the cultivars, maximum frequency of mutation was recorded in 0.25% 6h EMS (cv.I – 6.29%, cv.II – 3.66%) and 0.50% 3h NH$_2$OH (cv.I – 4.35%, cv.II – 2.51%) treatments. Quantitative traits of the mutants in relation to respective control cultivars (Fisher's t-test was performed at 8 DF to assess significant variation, if any) were presented in table1. Long petiole was associated with large broader leaves; while, large flower mutant was with short pedicel and larger ovary although the number of ovules were alike as to control (10 ovules). Thick stem mutant showed profuse secondary activity both inter and extrastral in stem as compared to control (Fig. 2D-E) and the branching pattern was characteristic (mutant in cv.II which was characterized as non branched type) in funnel mutant plant type. Late flowering mutant plants were dwarf in nature (mutant: 72.0 to 94.0 cm, 85.8 cm ± 3.90; control: 124.0 to 176.0 cm, 147.6 cm ± 9.51; t = 5.42, 8 DF, p<0.01). Stem coloration (consistent) in pigmented stem mutant was signal brown (Code: RAL 8002) in comparison to normal stem colour- fern green (RAL 6025) in cv.I.

**Mutagenic effectiveness and efficiency**

The mutagenic effectiveness which relates doses to mutation events was found to be higher in 0.25% mostly (exception – NH$_2$OH: 2.51 in 0.50% 3h in cv.II) irrespective of the mutagens, duration of treatments and cultivars (cv.I: EMS- 4.15 – 3h, 4.19 – 6h; NH$_2$OH- 3.31 – 3h, 2.15 – 6h; cv.II: EMS- 1.47 – 3h, 3.66– 6h) and subsequently decreased in higher concentrations. Assessment of comparable doses of the mutagens it seems that EMS was more effective than NH$_2$OH. The mutagenic efficiency defined as the relation of number of mutational events to the mutagens population was higher in cv.I (3.11%, 1606 plants scored) than cv.II (1.46%, 4783 plants scored) and the mutants were isolated in cv.I; while, cv.II (0.06%) > pigmented stem (0.75%) > lax branching (0.56%) > early flowering (0.06%) = large flower (0.06%); cv.II – late flowering (1.02%) > funnel (0.27%) > thick stem (0.17%). In both the cultivars, maximum frequency of mutation was recorded in 0.25% 6h EMS (cv.I – 6.29%, cv.II – 3.66%) and 0.50% 3h NH$_2$OH (cv.I – 4.35%, cv.II – 2.51%) treatments. Quantitative traits of the mutants in relation to respective control cultivars (Fisher’s t-test was performed at 8 DF to assess significant variation, if any) were presented in table1. Long petiole was associated with large broader leaves; while, large flower mutant was with short pedicel and larger ovary although the number of ovules were alike as to control (10 ovules). Thick stem mutant showed profuse secondary activity both inter and extrastral in stem as compared to control (Fig. 2D-E) and the branching pattern was characteristic (mutant in cv.II which was characterized as non branched type) in funnel mutant plant type. Late flowering mutant plants were dwarf in nature (mutant: 72.0 to 94.0 cm, 85.8 cm ± 3.90; control: 124.0 to 176.0 cm, 147.6 cm ± 9.51; t = 5.42, 8 DF, p<0.01). Stem coloration (consistent) in pigmented stem mutant was signal brown (Code: RAL 8002) in comparison to normal stem colour- fern green (RAL 6025) in cv.I.

**Meiotic analysis and pollen viability**

Meiotic chromosome behaviour was nearly normal and comparable in both cultivars as well as in most mutant plant types. The chromosome number was 2n=72 always. Mean chromosome association at MI was 35.81 I + 0.39 I to 36II in cv.I and 35.8 II + 0.34 I to 36II in cv.II. Most of the mutants of both cultivars showed normal pairing (36II...
formation) at MI; however, early flowering mutant showed mean association of 32.5 II + 6.92 I (PMC scored 13; association like – 19 II + 34 I – Fig. 3A, 21 II + 30 I – Fig. 3B along with 36II were noted). AI chromosome segregation was always balanced (36/36). Pollen viability was recorded to be higher in mutants (cv.I: control 42.09%, mutants 46.34 to 57.14%, however, early flowering showed 31.76% viability; cv.II: control 38.79%, mutants 44.39 to 48.07%) than controls.

Macromutants evolved as the consequence of gene mutation (cytologically the mutants are normal) were viable and therefore may be important genetic resources in the species for future productive exploration. Variations induced in A. moschatus through mutational approach have widened the gene pool and enriched genetic diversity. The two-cultivars responded differentially to mutagenic treatments, thereby providing evidences for their characterization.

ACKNOWLEDGEMENT

The authors are thankful to Aninda Mandal, Research Scholar for his sincere help in many ways during preparation of the manuscript. The research is grant aided by DST-PURSE, University of Kalyani.

REFERENCES


Table 1. Quantitative traits in mutants and respective control of A. moschatus

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Mutants</th>
<th>Control</th>
<th>Probability</th>
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<tbody>
<tr>
<td>Lax branching</td>
<td>Angle of divergence to main axis</td>
<td>87.2±2.50</td>
<td>68.4±3.30</td>
</tr>
<tr>
<td>Long petiole</td>
<td>Petiole length (cm)</td>
<td>40.7±0.68</td>
<td>32.4±0.61</td>
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<tr>
<td></td>
<td>Leaf length (cm)</td>
<td>23.8±0.46</td>
<td>23.8±0.22</td>
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<tr>
<td></td>
<td>Leaf breadth (cm)</td>
<td>39.8±1.56</td>
<td>34.3±0.59</td>
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<tr>
<td></td>
<td>Leaf area (cm²)</td>
<td>638.5±20.55</td>
<td>488.7±10.04</td>
</tr>
<tr>
<td>Large flower</td>
<td>Flower length (cm)</td>
<td>6.3±0.11</td>
<td>4.8±0.18</td>
</tr>
<tr>
<td></td>
<td>Flower breadth (cm)</td>
<td>5.1±0.34</td>
<td>3.7±0.11</td>
</tr>
<tr>
<td></td>
<td>Petal length (cm)</td>
<td>5.8±0.11</td>
<td>4.7±0.11</td>
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<tr>
<td></td>
<td>Petals breadth (cm)</td>
<td>4.5±0.55</td>
<td>3.5±0.02</td>
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<tr>
<td></td>
<td>Pedicel length (cm)</td>
<td>3.1±0.17</td>
<td>4.0±0.20</td>
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<td></td>
<td>Ovary length (cm)</td>
<td>0.6±0.03</td>
<td>0.4±0.02</td>
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<tr>
<td>Early flowering</td>
<td>Days from sowing</td>
<td>124</td>
<td>135±147</td>
</tr>
<tr>
<td>Late flowering</td>
<td>Days from sowing</td>
<td>193±210</td>
<td>130±145</td>
</tr>
<tr>
<td>Thick stem</td>
<td>Diameter 6 cm above base</td>
<td>2.96±0.07</td>
<td>1.52±0.13</td>
</tr>
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</table>
Fig. 1. A-E. Normal (A) and mutants of cv. I (tall) of *A. moschatus*. A. Normal plant type. B. *Lax* branching. C. Petals of large flower mutant (b) with that of normal (a) flower. D. *Long petiole*. E. Pigmented (b) and normal (a) stem.

Fig. 2. A-E. cv. II (dwarf) of *A. moschatus*. A. Normal plant type. B. *Funnel* mutant. C. Thick (b) and normal (a) stem. D. T.S. of normal stem. E. T.S. of thick stem showing profuse secondary activity. Scale bar (D-E) = 2.5 µm.

Fig. 3. Meiotic configurations at MI in early flowering mutant. A. 19II+34I (bivalents are marked). B. 21II+30I. Scale bar = 2.5 µm.

Source of support: Nil, Conflict of interest: None Declared