**INDUCTION OF MACROMUTATION IN ANDROGRAPHIS PANICULATA (BURM. F.) NEES**

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**ABSTRACT**

Fourteen viable macromutants (virdis, lax branching, bushy, unbranched I and II, dark green leaf, broad leaf I and II, narrow leaf I and II, drooping leaf I and II, dwarf and early maturity) were induced in Andrographis paniculata (Burm. F.) Nees (Family: Acanthaceae) at M2 following EMS (Ethyl methanesulfonate) and dES (Diethyl sulfate) treatments (0.25, 0.50 and 1.00% for 2h and 4h durations) to dry seeds (moisture content 11.55%). Mutation frequency over M0 population was 2.82% and lax branching mutant was maximum (0.51%). EMS induced relatively higher (3.12%, spectrum 1 to 4) frequency of mutation than dES (2.46%, spectrum 1 to 3). The mutant trait(s) were monogenic recessive mostly (virdis showed digenic mode of inheritance). All mutants bred true at M1. Meiotic analysis revealed 2n=50 chromosomes always in the plant types (control: 24.92II+0.51I/cell; mutants: 24.80II+0.40I to 25II/cell at MI; AI cells were cytologically balanced in all cases). Pollen fertility (control: 80.63%, mutants: 21.28% to 89.25%) and viability (control: 80.63%, mutants: 27.55% to 59.04%) were also assessed. DAPI staining was performed to assess pollen nuclear composition (3-nucleate stage) in dark green leaf, broad leaf I and drooping leaf II as the mutants showed considerably lower pollen fertility and viability than normal plants. Results indicated that pollen developmental phase may be significant for reproductive outcome. Andrographolide (estimated from matured leaves by HPTLC) content (control: 3.41%, mutants: 0.03 to 3.99%) was significantly higher in bushy and broad leaf I and II than normal plants. The mutants induced were considered to be important genetic resources in the plant species.

**Keywords:** Andrographis paniculata; DAPI staining; genetic segregation; macromutation; meiotic analysis

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**INTRODUCTION**

Andrographis paniculata (Burm. F.) Nees (Family: Acanthaceae; Synonyms: Justiciae latemossa Russ., J. stricta Lam. Ex Steud – Hooker1; Anonymous2; common names: Kalmegh, Kariyat, Green chireta, Krea bitter; English name: King of Bitters) is an annual herb3 as well as reported to be perennial shrub4 possessing immense therapeutic uses (used parts- roots, leaves and aerial parts of mature twig) in Ayurvedic (26 Ayurvedic formulations – Indian Pharmacopoeia; in Traditional Chinese Medicine it is an important “cold property” herb used to release body heat in fever – Bobbara et al.4) and in modern system of medicine. The therapeutic uses of the plant species are mainly due to diterpenoid andrographolide and related compounds.5,6 The species is widely cultivated in tropical and subtropical Asia, South-East Asia and India8 and also reported from different phytogeographical and edaphic zones of China, America, West Indies and Christmas Island in Indian Ocean.9 Medicinal plant species are gift of nature and considering their global significance it would be worthwhile to raise novel plant types which may meet up the up surging demands of value added products in National and International markets. The methodology of induced mutagenesis may be adopted which provides an opportunity to create allelic variation at one or more gene loci of interest in a quick span of time, thereby widening the gene pool thereby offering scope of suitable selection exploring the existing germplasm(s). Kharkwal10 reported that mutation breeding has contributed significantly to the global agriculture by producing more than 3000 mutant varieties with enhanced production and productivity in about 175 plant species. The objective of the present work is to raise macromutant(s) in A. paniculata which would be important genetic resources for exploration in human benefits. The present communication describes EMS and dES induced mutagenesis in A. paniculata and shed light on the types and frequency of macromutation, cytomorphological and genetical aspects of the mutants and andrographolide content in them.

**MATERIALS AND METHODS**

**Plant material**

Seeds of Andrographis paniculata (Burm. F.) Nees (moisture content: 11.55%; seed size: 1.403 mm ± 0.34 × 0.970 mm ± 0.25; 100 seed weight: 0.117 gm ± 0.044; seed colour: Golden brown, code 41332 British Atlas of Colour) were obtained from Medicinal Plant Garden, Narendrapur, Ramkrishna Mission, Govt. of West Bengal, India.

**Mutagenic treatment and raising of M1, M2, M3 and M4 generations**

Dry seeds of A. paniculata were treated with different concentrations (0.25, 0.50 and 1.00%); treatments for 2h and 4h durations) of ethyl methane sulphate (EMS – Sigma, USA; dilution were made in 0.2M Phosphate buffer) and diethyl sulphate (dES - Sigma, USA; aqueous solution) at 36°C ± 1°C at pH 6.8. The doses were applied after pilot trials. Control and treated (50 seeds in each lot) seeds were sown in the experimental field 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, organic carbon 0.76%, soil pH 6.85) to raise M1 generation (spacing 30 cm between plants and 30 cm between lines) during the months of April to January (2008–09). Selfed seeds of each surviving M1 plant (2-3 inflorescences per plant were bagged) were harvested.
separately and M₂ plants were raised as plant to row progenies (2009-10'). Macromutants were carefully screened from M₂ mutagenized plant population throughout the growth period and the frequency of the mutants was assessed as per 100 plants. Selfed M₂ seeds of the mutants along with control were sown at M₃ (2010-11'). The mutants segregating in M₃ generation was noted and inheritance of the mutant trait(s) was estimated by using χ² test analysis. Selfed seeds of M₃ mutants were sown in 3 lines (for each mutant type) randomly along with control lines at M₄ (2011-12'). All the mutants bred true at M₄. Spacing (between lines and plants) and growth period (mid April to late January) of the plants were uniform over the generations. No fertilizers were applied during the growth period of the plants.

Morphometric traits were analyzed from M₄ plants (6 to 9 plants randomly selected from 3 lines in each case) on harvest. The seedling colour of chlorophyll mutant (classified as per Blixt3) and all colour codes were laid with reference to British Atlas of Colour (7th edition 2007). Chlorophyll content (chlorophyll mutant, dark green leaf mutant as well as in normal plants) was estimated from leaf tissue of identical maturity quantitatively as per Arnon11.

Meiotic analysis
Suitable sized inflorescences from mutants as well as from control plants (randomly selected 3 plants for each plant type) at M₄ were fixed (6:30 am to 7:00 am during the months of October to November) in Carnoy’s solution and minimum of 3 changes were given in the fixative at an interval of 48 hours and preserved in 70% alcohol under refrigeration. Anthers were squashed in 3% propinocarmine solution and meiotic data (pooled over the plants for each plant type) were scored from metaphase I (MI) and anaphase I (AI) plates. Photomicrographs were taken from temporary squash preparations.

Pollen characteristics
Pollen fertility (M₄ plants) was assessed following staining of pollen grains in 2% propinocarmine solution and fully stained pollen grains were considered fertile. Pollen grain viability (aniline blue in lactophenol- detects the presence of callose on pollen wall, viable pollen turns blue- Bengtsson13) percentage was also analyzed.

Mutants namely dark green leaf, broad leaf I and drooping leaf II showed considerably lower pollen fertility and viability than control, and therefore in these plant types (M₄ generation) pollen nuclei composition (number of vegetative – v and generative – g nuclei present at 3-nucleate stage per pollen grain was assessed by staining with DAPI - 4',6-diamidino-2-phenylindole - Johnson and McCormick16, under fluorescence microscope Carl Zeiss Axio Fluor 900EX, Carl Zeiss Mag Analytic 10.1, DAPI excitation range 350-360 nm, emission maximum 460 nm) was studied to ascertain the possible cause for sterility, if any.

Methodology for quantification of andrographolide
Sample preparation: 1.5 gm of sun dried powdered leaf sample (reproductive stage - 95 to 100 days from sowing when leaves were showing purple colouration – Brilliant purple 219461; 3 replicas from each plant type; true breeding M₄ mutant plants and selfed control lines were considered; leaves from a single plant constituted single replica) were extracted in 50 ml methanol for overnight, sonicated at 60°C for 3 hours, extracts filtered by Whatman No. 42 filter paper and made up to 25 ml in a volumetric flask.

Standard preparation: 1000 ppm of stock solution was prepared by dissolving 10 mg ± 0.01 of andrographolide (analytical standard, Chroma Dex, California, U.S.A.) in 10 ml of methanol (certified ‘A’ class).

HPTLC analysis: Aluminium TLC plates pre-coated with silica gel 60F₂₅₄ (E. Merck KgaA, 20×20 cm², 0.3 mm thick) was developed in Camag TLC twin through glass chamber pre-saturated (30 mins. prior to development in ambient temperature) with chloroform : methanol (7:1) v/v as mobile phase. Methanolic extracts of samples (1, 2, and 5 µl volume) and andrographolide standard solution (1, 2, 3 and 4 µl of volume) were applied on plate by using Linomat V automatic applicator (nitrogen flow 150 nl/sec. - Peak N₂ Generator, Renfrew, UK) to a 6 mm wide band, positioned 10 mm from the bottom of the plates (syringe delivery speed 0.01 µl/sec.; number of tracts 18). After development, the plates were dried and quantified with Camag TLC scanner- 3 equipped (slit dimension 5.00×0.45 mm, scanning speed 20 mm/sec.; absorption-reflection scan mode) with winCATS software at a wavelength of 220 nm with D₂ lamp. RI of standard was 0.34 and it matched with that of the extract thereby confirming that they were the same substances.

The linearity was examined by applying the calibration working standard solution for 3 consecutive days. The calibration curve, log-transformed peak area versus log-transformed concentration was calculated according to least square method (Y = a + bx) for compound under consideration (linear regression- Y = 162.4 + 0.99557x, r = 0.98069, sdx = 7.51 via height; Y = 196.2 + 2.709x, r = 0.99122, sdx = 6.54 via area). The calibration curve was linear in the range 100 ng to 1 µg. The accuracy and reproducibility of the method was established by means of recovery experiment as per Pawer et al.17 and the mean recovery was 95.98% of the compound (close to 100%) suggesting the accuracy of the method.

RESULTS
Types and frequency of macromutation
Fourteen different (viable) macromutants (mutant traits confirmed at M₃ from selfed segregation; mutants bred true at M₄) were spotted (viridis, lax branching, bushy, unbranched I and II, dark green leaf, broad leaf I and II, narrow leaf I and II, drooping leaf I and II, dwarf and early maturity) at M₃ (Figure 1: B-F) mutagenized population (1168 plants scored). Leaf mutations were predominant. Lax branching, bushy, unbranched I and broad leaf I and II appeared only in EMS treatments; while, narrow leaf I, drooping leaf I and dwarf mutants were recovered only from dES treatments.

Viridis (colour: yellowish green, 125249; normal plants – Figure 1:A possessing leaf colour - leaf green [s.g.3] 15627(3); chlorophyll content mg/gm of tissue in viridis: chlorophyll a 0.16, chlorophyll b 0.17, control: chlorophyll a 0.27, chlorophyll b 0.24) plant type attained
Segregation of the macromutants

Segregation patterns were assessed at M₃ of mutants as either 1:1 or 3:1 (lax branching: normal 24, mutant 23, \( \chi^2 = 0.022 \) for 1:1, p>0.80; bushy: normal 14, mutant 11, \( \chi^2 = 0.36 \) for 1:1, p>0.50; unbranched I: normal 25, mutant 20, \( \chi^2 = 0.556 \) for 1:1, p>0.30; unbranched II: normal 12, mutant 9, \( \chi^2 = 0.046 \) for 1:1, p>0.80; broad leaf I: normal 11, mutant 7, \( \chi^2 = 0.888 \) for 1:1, p>0.30; broad leaf II: normal 16, mutant 14, \( \chi^2 = 0.134 \) for 1:1, p>0.70; dark green leaf: normal 15, mutant 6, \( \chi^2 = 0.143 \) for 3:1, p>0.20; narrow leaf I: normal 30, mutant 26, \( \chi^2 = 0.286 \) for 1:1, p>0.50; narrow leaf II: normal 36, mutant 33, \( \chi^2 = 0.13 \) for 1:1, p>0.70; drooping leaf I: normal 11, mutant 7, \( \chi^2 = 0.888 \) for 1:1, p>0.30; drooping leaf II: normal 18, mutant 17, \( \chi^2 = 0.13 \) for 1:1, p>0.70; early maturity: normal 17, mutant 6, \( \chi^2 = 0.013 \) for 3:1, p>0.90; dwarf: normal 21, mutant 8, \( \chi^2 = 0.336 \) for 3:1, p>0.50) thereby indicating possible monogenic recessive inheritance of the mutant trait(s); however, viridis showed digenic (normal 33, mutant 3, \( \chi^2 = 0.267 \) for 15:1, p>0.50) mode of inheritance.

Meiotic analysis

Meiotic analysis revealed 2n=50 chromosomes always in the plant types (Figure 2: A-B). Mean chromosome association at MI was 24.92 II + 0.15 I in control (52 PMCs studied), 24.85 II + 0.30 I in viridis (27 cells), 24.93 II + 0.15 I in lax branching (40 PMCs), 24.94 II + 0.13 I in bushy (47 meiocytes), 24.87 II + 0.26 I in unbranched I (23 cells), 24.93 II + 0.14 I in unbranched II (44 cells), 24.45 II + 1.10 I in dark green leaf (58 PMCs), 24.95 II + 0.09 I in broad leaf I (44 meiocytes), 24.90 II + 0.21 I in broad leaf II (58 PMCs), 24.80 II + 0.40 I in drooping leaf I (20 PMCs), 24.82 II + 0.37 I in drooping leaf II (38 cells); while, narrow leaf I (44 cells scored) and II (23 PMCs), dwarf (35 cells) and early maturity (23 PMCs) mutants showed 25II formation. Though bivalent frequency per cell was random (p>0.05) over the plant types, univalent per cell was inconsistent (p<0.001) as evidenced from \( \chi^2 \) test of heterogeneity (DF 14). The AI chromosome segregation was always equal (25:25, 52 cells scored in control and 23 to 72 cells in mutants). Pollen fertility and viability assessed in normal plants was 80.63% (888 pollen grains analyzed) and 72.24% (893 pollen assessed) respectively. In mutants pollen fertility ranged from 21.28% (broad leaf I, 1001 pollen studied) to 89.25% (narrow leaf I, 1113 pollen scored) and that of viability varied between 27.55% (broad leaf I, 940 pollen observed) and 59.04% (bushy, 437 pollen estimated). Mostly, pollen viability was less than pollen fertility.

Pollen nuclei composition

Data presented in Table 1 suggested that dark green leaf and broad leaf I mutants were with lower pollen fertility and viability than control but seed set per capsule, seed yield per plant and abnormal (0v+1g, 1v+0g, v dispersed and 1v+2-5g) pollen nuclei composition (Figure 3: A-F) were more or less comparable in the plant types. Drooping leaf II possessing low pollen fertility and viability had low seed set per capsule and seed yield per plant and the percentage (40.04) of abnormal pollen nuclei composition was much higher than control (20.2%).

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Andrographolide content

The yield of andrographolide (Figure 4: A-D) in matured leaves was 3.41% in normal plants and it was noted to be 2.33% in viridis, 3.45% in lax branching, 3.99 in bushy, 0.40% in unbranched I, 0.37% in unbranched II, 0.30% in dark green leaf, 3.76% in broad leaf I, 3.84% in broad leaf II, 0.11% in narrow leaf I, 1.26% in narrow leaf II, 0.64% in drooping leaf I, 0.02% in drooping leaf II, 0.03% in dwarf and 1.28% in early maturity. Bushy (t=26.85, DF=4, p<0.001), broad leaf I (t=14.71, DF=4, p<0.001) and II (t=19.91, DF=4, p<0.001) mutants were with significantly higher amount of andrographolide content than normal plants.

Table 1: Pollen characteristics and reproductive parameters in control and mutant plants of A. paniculata

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Plant types</th>
<th>Control</th>
<th>Dark green leaf</th>
<th>Broad leaf I</th>
<th>Drooping leaf II</th>
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<tbody>
<tr>
<td>Pollen fertility (%)</td>
<td></td>
<td>80.63 (888)</td>
<td>72.74 (735)</td>
<td>59.67 (1168)</td>
<td></td>
</tr>
<tr>
<td>Pollen viability (%)</td>
<td></td>
<td>72.74 (735)</td>
<td>33.49 (618)</td>
<td>27.55 (940)</td>
<td>45.67 (648)</td>
</tr>
<tr>
<td>Pollen nuclei composition (%)</td>
<td></td>
<td>1v+2g</td>
<td>32.32</td>
<td>46.21</td>
<td>26.93</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0v+2g</td>
<td>47.43</td>
<td>30.52</td>
<td>47.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0v+1g</td>
<td>9.16</td>
<td>17.37</td>
<td>16.09</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1v+0g</td>
<td>6.27</td>
<td>1.85</td>
<td>5.75</td>
</tr>
<tr>
<td></td>
<td></td>
<td>v-dispersed</td>
<td>4.50</td>
<td>3.88</td>
<td>2.96</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1v+(2-5)g</td>
<td>0.32</td>
<td>0.17</td>
<td>0.99</td>
</tr>
<tr>
<td>Pollen grain analyzed</td>
<td></td>
<td>622</td>
<td>593</td>
<td>609</td>
<td>597</td>
</tr>
<tr>
<td>Seed per capsule</td>
<td></td>
<td>8.67±1.51</td>
<td>7.50±2.07</td>
<td>8.40±1.34</td>
<td>6.20±1.30</td>
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<tr>
<td>Capsule length (cm)</td>
<td></td>
<td>1.74±0.31</td>
<td>1.64±0.15</td>
<td>1.80±0.14</td>
<td>1.60±0.14</td>
</tr>
<tr>
<td>Total seed weight per plant (gm)</td>
<td></td>
<td>2.05</td>
<td>2.01</td>
<td>2.21</td>
<td>0.45</td>
</tr>
</tbody>
</table>

Data in parenthesis indicate number of pollen grains studied

Figure 1: Normal and mutant (B) plant types of A. paniculata. A- Normal plant; B- Dark green leaf; C- Bushy; D- Drooping leaf II; E- Unbranched I; F- Lax branching
Figure 2: Meiotic chromosome configurations (A and B) in *A. paniculata* (2n=50). Scale bar = 20 μm.

Figure 3: Pollen nuclei composition following DAPI staining in plant types of *A. paniculata*. A- 1v+2g; B- 0v+2g; C- 0v+1g; D- 1v+0g; E- v-dispersed; F- 1v+5g. Scale bar = 20 μm.

Figure 4: Chromatograms reflecting andrographolide content. A- Standard; B- Control; C- Bushy D- Narrow leaf II.

**DISCUSSION**

EMS and des induced mutations have affected various plant parts of *A. paniculata* and brought about gross morphological changes in seedling colour (*viridis*), branching pattern (*lax branching, bushy and unbranched I and II*), growth habit (*dwarf*), leaf morphology (*dark green leaf, broad leaf I and II, narrow leaf I and II and drooping leaf I and II*) and maturity (*early maturity*), resulting into alteration of the plant ideotype. The mutants evolved as the consequence of gene mutation (all the mutants were cytologically balanced as normal plants) seems to be in the direction of the objective underlined (*bushy and broad leaf I and II were with significantly enhanced andrographolide contents*). *Early maturing* mutant type corresponds closely to the ideotype being looked for in the plant species. *Viridis, dark green leaf, narrow leaf I and II, drooping leaf I and II and lax branching* mutants induced were important genetic resources in the plant species and the mutant trait(s) may be explored as marker(s) in efficient breeding. Kharkwal was of opinion that macromutations appearing in the segregating M₂ population were consequences of mutational changes of the ‘major genes’. In the present investigation, mutant traits were mostly (excepting *viridis*...
which showed digenic mode of inheritance) monogenic recessive to normal plants. Concomitant occurrence of certain characteristics with the mutant trait(s) in some mutant lines (broad leaf, narrow leaf, unbranched and drooping leaf) gives an indication of pleiotropism or linkage or possibly the mutants have affected different sites of the same gene; which, however, needs further investigation to derive to a specific conclusion. Emery et al.19 presumed that all induced mutations have varying degree of pleiotropic influence on other characters. Kreft20 reported high lysine genes in barley and genes for the determinant growth in buckwheat (Fagopyrum esculentum) were examples to show that the effects of single mutation could be complex or pleiotropic. Pollen grains, product of male meiosis, play key role in fertility and reproduction. Analysis (pollen fertility and viability, pollen nuclei composition and reproductive parameters) of few mutants namely, dark green leaf and broad leaf I and drooping leaf II in relation to control revealed that pollen fertility and viability (dark green leaf and broad leaf I) were not detrimental in determining the reproductive outcome; however, pollen developmental phases (nuclei composition in relation to germination and fertilization) may also play significant role (as was noted in drooping leaf II) in the reproductive process. Ferri et al.21, Lyra et al.22 amongst others were of opinion that pollen germination is an important criterion for reproductive biology. Sedgley and Griffin23 suggested that pollen germination may be controlled by vegetative nucleus in the absence of viable generative nucleus; however, such condition may not be appropriate for proper fertilization and subsequently seed setting24. Andrographolide content in control was 3.41% and in mutants it varied from 0.02 to 3.99%. Saxena et al.25 demonstrated variations (1.77 to 2.66%) in andrographolide content from leaves using different solvent system for extraction and methanol was found most appropriate and it was also corroborated by Srivastava et al.26. Sanyal et al.27 quantified andrographolide content from matured leaves as 1.40 to 2.14% in New Alluvial zone, 2.28 to 2.91% in Red Lateritic zone and 2.16 to 2.25% in Coastal zone in West Bengal plains following HPTLC methods and the authors were of opinion that soil and climatic factors were important parameters for biosynthesis of secondary metabolites. Vijaykumar et al.28 reported andrographolide content (HPTLC method) from four different herb samples (0.47 to 1.67 mg/100 mg) and different leaf extracts (21.31 to 85.88 mg/100mg). Pawar et al.29 found that the quantity of andrographolide from two samples (whole plant) from Ghaziabad, U.P., India was 0.77133% w/w and 0.75189% w/w (solvent: 5 parts toluene: 4.5 parts ethyl acetate: 0.5 part formic acid v/v as mobile phase). Ghosh et al.20 reported yield of andrographolide to be 0.93% in young leaves and 3.41% in matured leaves under similar agro climatic conditions and were of opinion that possibly some key enzyme(s) may be triggered during reproductive phase of growth. Thus, maturity of the plant, germplasms under consideration, soil and climatic factor(s) seems to be important attributes for the biosynthesis and accumulation of andrographolide. Released variation through induced mutation have widened the gene pool and enriched genetic diversity in A. paniculata, which did not exist previously. Mutational approach to develop important genetic resources in medicinal plant species has been rather meagre30-33 and the present endeavour may provide some insight on the aspect.

ACKNOWLEDGEMENT

The research is Grant aided by DST-PURSE programme of University of Kalyani.

REFERENCES


Source of support: DST-PURSE programme of University of Kalyani, Conflict of interest: None Declared