I. MATERIAL

Seeds of *Andrographis paniculata* (Burm. F.) Nees (moisture content 11.55 %, seed size 1.403 mm ± 0.34 x 0.970 mm ± 0.25; 100 seed weight 0.117 gm ± 0.04, seed color- Golden brown, code 41332, British Atlas of color, 9th edition, 2007) were obtained from Medicinal Plant Garden, Narendrapur, Ramkrishna Mission, Govt. of West Bengal.

II. METHODS

A. Cultivation Processes

The plant species was raised from seeds directly. The seeds of *A. paniculata* were sown in the Experimental field Plots of Department of Botany, University of Kalyani (latitude 22°50' to 24°11' N, longitude 88°09' to 88°48' E; soil nature: 9.91% sand, 2.89% silt, 87.20% clay, nitrogen 0.76%, carbon 3.25% and hydrogen 0.56% - Chowdhury et al. 2009; soil pH 6.85- Bhattacharya and Datta 2010), during late (34° ± 1° to 36°C ± 1°C) April (2008-2012). The first flowering of the plant species (untreated control and mutants) was noted during the months of October and November and maximum flowering was studied at mid- November. Leaf coloration (green to purple) changed during late November and in general the harvesting process continued from mid- January to late February. The plants were raised at the spacing of 30.0 cm between plants and 30.0 cm between rows. No fertilizer applications were made at any stage of growth of the plant species. Three irrigations (1 – early stage; 2 – vegetative; 3 – prior to flowering stage) were provided to the plant species.

After harvesting the plants were sundried (22°C ± 1°C to 24°C ± 1°C) under field conditions for 3 to 4 days and subsequently the seeds were collected and stored under dessication.

B. Morphological Assessment of the species

Vaucher specimen of the plant species is deposited (AP-1) in Botany Department, Kalyani University. The plant specimens were examined with Olympus binocular dissecting
microscope under 10X. The study included detailed description of every part of the specimens. The flowers were dissected and examined. Measurements of the leaves and floral parts were also made. Measurements of seeds were made from an average of 15 seeds in a stereo dissecting microscope. Seed weight (100 seeds) and moisture content were also determined.

C. Anatomical Studies

Transverse sections (hand sections) of the stem from the basal region (5.0 to 6.0 cm from base at maturity) and root (4.0 to 4.5 cm below base at maturity) were made from fully matured untreated control plants (at fruit ripening stage: 110-125 days from sowing), and the sections were double stained using 1.0% safranine (Merck, AR) dissolved in 50.0% alcohol and 1.0% Light green (Merck, AR) dissolved in 90.0% alcohol (Johansen 1940).

D. Determination of Moisture Content

Mother seed stock of *A. paniculata* was analyzed for moisture content. Moisture content of seeds of the species was estimated by drying seeds (3 replicates) in a hot air oven at 100°C for nearly 3 hours. The dried seeds were weighed again (until the weight becomes constant) and the loss in weight represented the weight of water loss due to drying. The moisture content was determined from the following formula:

\[
\text{Moisture content (\%) } = \left( W_1 - W_2 \right) \times 100/W_1, \quad \text{where } W_1 \text{ is the weight of seed sample before drying and } W_2 \text{ is the weight of seed sample after drying.}
\]

E. Studies of Induced Mutagenesis

a. Mutagens

Chemical mutagens namely, EMS (Ethyl Methane Sulphonate: empirical formula- \( \text{C}_2\text{H}_5\text{SO}_2\text{CH}_3 \); Sigma, USA) and dES (Diethyl sulphate: empirical formula- \( \text{SO}_2(\text{OC}_2\text{H}_5)_2 \); Sigma, USA) were used in the present investigation.
b. Treatments

Dry seeds of *A. paniculata* (Burm. F.) Nees were treated with different doses (0.25%, 0.50%, and 1.00% for 2 and 4 hour durations) of EMS (dilutions were made in 0.2 M phosphate buffer; pH adjusted to 6.8) and dES (dissolved in 50% alcohol and diluted in deionised water; pH 6.8). Treatments were performed at 37°C ± 1°C. The samples were intermittently shaken during treatments. Doses were administered after pilot trial. The treated seed (100 seeds in each lot) were thoroughly washed in running water (3-4 hours) and dried on blotting paper.

c. Studies on M₁ generation

i. Raising M₁ plant population

Treated and control seeds were sown (50 seeds from each lot of treatment as well as from control) in the Experimental Field plots of Department of Botany, University of Kalyani in late April at the spacing of 30 cm between plant as well as between lines to raise M₁ generation (2008-09).

ii. Assessment of germination and seedling growth from petriplates

Percentage of seed germination and seedling growth were assessed from treated and untreated seeds (50 seeds in each case) from petriplates under controlled conditions (35°C ± 1°C). Growth of radicle following bursting of seed-coat was taken as an index for germination in petriplates (seeds were allowed to germinate in petriplates lined with moist filter papers). Seedling growth was studied from randomly taken 15 germinating seedlings and measured on a millimeter graph paper after 15 days from the date of treatment. Biological damages like lethality and injury were assessed from seed germination frequency and seedling growth respectively as was suggested by Konzak *et al.* (1965). The extent of lethality and injury were determined from the relative reduction of seed germination frequency and seedling growth in the treated samples respectively as compared to control (per cent of control). Enhancement in seed germination frequency and seedling growth over respective control
values were studied in some mutagen doses and consequently lethality and injury could not be analyzed in those treatments.

iii. **Studies on seed germination, survivability, pollen fertility and seed yield under field conditions**

Emergence of cotyledonary leaves above the soil surface was taken as an index for seed germination under field conditions. Survivability percentage was determined from harvested plants. Seed yield [seeds of 10 pods were assessed from each plant sequentially from first flowering and weighed] per plant was studied from each surviving M1 plant. Seed sterility was represented as percentage of reduction in seed weight in treatments in relation to control. Pollen fertility was observed in treatments as well as in control following staining of pollen grains in 1% acetocarmine solution and the fully stained pollen grains were considered fertile in accordance to Marks (1954).

d. **Studies on M2 generation**

i. **Raising M2 generation**

Randomly selected 50 selfed (2-3 inflorescences were selfed by polythene bags which were perforated by fine needle for aeration) seeds from each M1 plant along with control were sown in the Experimental field plots in plant to row keeping 30 cm between plants as well as between lines to raise M2 generation (2009-10).

ii. **Detection of macromutants**

Macromutants (including the chlorophyll mutant) were screened throughout the growth period (germination to harvest) of M2 mutagenized plants. The chlorophyll mutant scored was classified after Blixt (1961). Mutation frequency was determined as per hundred plants (Gaul 1964). Color of leaves and stems were confirmed from British Atlas of Color (7th edition 2007).
The mutant trait(s) of viable M_2 macromutants were confirmed from self segregating progenies raised from M_2 mutant seeds at M_3 (seeds of similar mutant types were bulked and sown at M_3). Morphological data as well as leaf chlorophyll content (in the chlorophyll mutant) were assessed in M_2 macromutants in comparison to control plants.

iii. Efficiency and effectiveness of the mutagens

The efficiency and effectiveness of the chemical mutagens were calculated from viable mutation frequency (Walther 1969) using the formulae proposed by Konzak _et al._ (1965). The mutagenic efficiency was calculated as Mf/L, Mf/I, and Mf/S and the effectiveness as Mf/cxt (Mf= mutation frequency, L= lethality, I= injury, S= sterility, c= concentration of the mutagens, t= time of treatment).

e. Studies on M_3 generation

i. Raising of M_3 plant population

Selfed seed of M_2 macromutants along with control were sown at M_3 (30 cm between plants and lines) in the year 2010-11.

ii. Mode of inheritance of macromutants

Inheritance pattern of the mutant trait(s) was only studied from selfed seed segregation of the mutants in M_3 generation. The mutants segregated into normal and mutant(s) and the data obtained was computed following $\chi^2$- test analysis.

f. Studies on M_4 generation

i. Raising of M_4 generation

Selfed seeds of the macromutants were randomly sown in 3 lines along with control (30 cm between plants and lines) during the year 2011-2012. No fertilizer application was made during growth period of the plants.

ii. Studies on the phenotypic variables of control and mutant plant types
Morphological attributes like plant height (cm), primary branches/plant, root length/plant (cm), root yield/plant (gm), leaf yield (gm), capsule length (mm), seed/capsule, 100 seed weight/plant (gm) were studied in the mutants namely, viridis, lax branching, bushv, unbranced 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 plants at maturity (condition when leaves turned purplish in color) in comparison to control. Observations were made from 5 randomly selected plants from each line of each mutant plant type as well as in control. Critical difference (C.D) at 5% level was estimated for each parameter to assess significant variations between/among the plant types.

Heritability (%) in broad sense was calculated from the following formula:

Heritability (%) = \( \frac{\sigma^2 g}{\sigma^2 p} \times 100 \), where \( \sigma^2 g \) = genotypic variance and \( \sigma^2 p \) = phenotypic variance. Genotypic variance = Mean Sum of Square (MS) due to genotypes- MSe (error)/No. of replications. Phenotypic variance = \( \sigma^2 g + \sigma^2 e \) (variance due to error = MSe).

iii. Cytological studies

Meiotic analysis: Meiotic analysis was performed in control and in 14 viable mutant plant types at M₄. Cytological data was pooled over M₄ plants for each plant type. Meiosis was studied from 2-3 suitable sized flower buds (3 to 4 plants for each type was scored) from each plant type, which were fixed between 6.30 A.M. to 7.00 A.M. in carnoy's fluid (3 changes were given in the fixative at an interval of 48 hours) and preserved in 70% alcohol under refrigeration. Anthers were squashed in a drop of 4% propionocarmine solution (9 drops of 4% propionocarmine mixed with 1 drop of 1% of ferric chloride) on a glass slide. The slide was then heated slightly on a alcohol lamp and pressure was applied over the cover slip with thumb through several folds of blotting papers to scatter and flatten the chromosomes in pollen mother cells. Chromosome association were studied from metaphase I plates (MI). Anaphase I (AI) segregation of chromosomes was also noted.
All the observations were made from temporary slides and photomicrographs were taken from suitable meiotic plates and later on they were enlarged suitably during printing.

iv. Determination of pollen fertility

Pollen fertility was estimated from control and viable macromutant at M₄. Pollen grains from mature anthers were squeezed out in a drop of 1% acetocarmine solution on a glass slide. The debris were removed and a cover slip was placed on it. The excess stain was removed by a blotting paper. After 10-15 minutes the slide was observed under the microscope. The stained pollen grains with regular shape were scored as fertile ones; while, shrunken, deformed and partially stained pollen grains were considered sterile in accordance to Marks (1954).

v. Pollen viability

Pollen viability was assessed in control and in mutant plants of M₄ generation using different stains namely Lugal’s Iodine (detects presence of starch, viable pollen turns blue- Bengtsson 2006), aniline blue (detects presence of callose in pollen wall, viable pollen turns blue- Bengtsson), X-gal-5-bromo-4-chloro-3-indolyl-β-galactoside (detects presence of β galactosidase, viable pollen turns blue- Atyaksheva et al. 2000), amido black (detects presence of protein in pollen wall, viable pollen turns black- Regan and Maffiatt 1990), TTC-2-3-5-triphyenyl tetrazolium chloride (detects presence of enzyme dehydrogenase, viable pollen turns deep pink- Redriguez - Riano and Dafni 2000), neutral red and methylene blue (detects presence of cytoplasm, viable pollen turns red and blue respectively - Horn and Clark 1992).

vi. DAPI staining of pollen grains

Mutants namely dark green leaf, broad leaf I and drooping leaf II showed considerably low pollen fertility and viability in relation to 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 of pollen grain was assessed by staining with DAPI-4',6-diamino-
vii. Pollen morphology

**Acetolysis method:** Pollen grains were collected from fully open flowers of *A. paniculata* and were analyzed morphologically (surface ornamentation) following acetolysis method of Erdtman (1952). The pollen grains were treated in a mixture of acetic anhydride and concentration H$_2$SO$_4$ (9 : 1) and the suspension was heated to boiling. The acetolysis mixture was removed and the pollen grains were rinsed first in glacial acetic acid and then in water and mounted in glycerine. They were examined under compound microscopic (15X × 100X). Ten pollen grains from each species were examined for determination of pollen sizes. Shape and surface ornamentations were also recorded. Photomicrographs were taken from suitable preparations.

**SEM analysis:** For SEM (Scanning Electron Microscopy) study, the pollen grains of control and mutants (*broad leaf I, dark green leaf and drooping leaf II* – these mutants showed very low pollen fertility) were put into 70% ethanol for 2 days in microfuge tubes and cleaned in an ultrasonic vibrator (Bran Sonic 221) for 6 minutes. Pollen grains of each samples were fixed in glass plates and then mounted to specimen stubs with the double sided adhesive tape and silver painted. Pollen grains, mounted on the respective stubs, were placed on the resolving disc and coated with 200-300 Å thick gold in vacuum evaporator of (Polaron) sputter coating system. The specimen stubs were then observed under SEM (Model- Zeiss EVO® HD, Germany) at 15 kV accelerating voltage at GSI (Geological Survey of India, Kolkata). On an average 10 pollen grains from each sample were analyzed for assessment of morphological parameters. Photomicrographs were taken from suitable preparations.
viii. Estimation of total andrographolide content

Mutants and control (M₄ plants, true breeding) were assessed for their total andrographolide contents from matured (initiation of purplish coloration of green leaves) leaves. About 1gm of leaf powder (1 gm in each replica for each plant type) accurately weighed in a 100 ml round-bottomed flask, add 50 ml of ethanol (85%), reflux in a water bath for 2 hours, and filtered. Wash the marc with sufficient amount of ethanol (85%) until the last washing is almost colorless. Combine the washing and the filtrate and allow to cool. Add 1 ml of basic lead acetate solution, set aside for 15 min, filter and wash the precipitate with ethanol until the last washing is no longer green. Combine the washings and the titrate, add drop wise with swirling 1 ml of a 25% w/v solution of sodium sulphate and mix well. Set aside for 1 hour, add 500 mg of decolorizing charcoal, and reflux in water-bath for 10 min. Filter through the Buchner funnel containing 500 mg of decolorizing charcoal and wash with three 2 ml portions of hot ethanol. Combine the washings and the filtrate, add 20 ml distilled water, allow to cool, and neutralize with 0.1 M sodium hydroxide, using phenolphthalein TS as indicator. Add 5 ml of 0.1 M sodium hydroxide Vs, reflux in a water-bath for 30 minutes, allow to cool, and titrate with 0.05 M hydrochloric acid Vs perform a blank determination. Each ml of 0.1 M sodium hydroxide Vs is equivalent to 35.05 mg of andrographolide, C₂₀H₃₀O₅.

Preparation of Basic lead acetate solution: Triturate 14 gm of lead (II) oxide with 10 ml of water, add 100 ml of water and transfer into a 100 ml volumetric flask. Add a solution prepared by dissolving 22 gm of lead (II) acetate in 70 ml of water, shake vigorously for 5 min, set aside for 1 week, filter and add sufficient previously boiled water to produce 100 ml.

ix. Methodology for quantification of andrographolide

Sample preparation: 1.5 gm of sun dried powdered leaf sample (reproductive stage- 95 to 100 days from sowing, leaves showing complete purple coloration; 3 replica in each case)
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 to 25 ml in volumetric flask. Only in control, leaf samples at vegetative stage (61 to 65 days from sowing, green in color) were considered for quantification of andrographolide content.

**Standard preparation:** 1000 ppm of stock solution was prepared by dissolving 10±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. MerckKgaA, 20X20 cm² 0.3 mm thick) was developed in camag TLC twin through glass chamber presaturated (30 mins. prior to development in ambient temperature) with chloroform:methanol (7 : 1) v/v as mobile phase. Methanolic extracts of samples (4, 6, 8 µl volume) and andrographolide standard solution (2, 4, 6 µl of 1000 ppm) 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 0.01µl/sec.; number of tracts 13). After development the plates were dried and quantified with Camag TLC scanner- 3 equipped (slit dimension 6.00X0.45 mm, scanning speed 20 nm/sec., absorption reflection scan mode) with winCATS software at a wavelength of 220 nm with D2 lamp. Rf of standard was 0.59 and it matched with that of the extract there by confirming that they were the same substance.

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 – $Y = 1.087 + 46.79x$, $r = 0.9985$, $sdv = 2.49$). 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 Pawar et al. (2010) and...
the mean recovery was 95.98% of the compound (close to 100.0%) suggesting the accuracy of the method.

x. Quantitative studies of seed protein

Extraction of soluble protein: Quantitative analysis of protein content from seed samples (control and 11 macromutants) were performed. Extraction of seed protein from the plant types was done following Osborne (1962). For extraction of total seed proteins, 0.5 gm of dry seeds from each sample (3 replicas in each case) were allowed to imbibe water for 18 hours at 18°C in dark and the imbibed seeds were homogenized in a chilled mortar and pestle using suitable volumes of 5% TCA (trichloroacetic acid, E. Merck, Germany) at 0°C-4°C. The homogenate was centrifuged at 10,000 r.p.m. for 30 minutes in a cold refrigerated centrifuge (REMI). The supernatant was discarded and the process was repeated twice. The residues were then extracted once with cold absolute alcohol and twice with hot ethanol: diethyl ether (3:1). Supernatant was discarded each time after centrifugation and the residue was dissolved in 50 ml of 1 (N) NaOH.

Estimation of protein: The protein was estimated using the method of Lowry et al. (1951). Reaction mixture was prepared by adding 2.5 ml of reagent A [2% sodium carbonate in 0.1 (N) NaOH] and reagent B [0.5% CuSO4, 5H2O in Na-K-tartarate] mixed in proportion of 50:1 (freshly prepared before use) to 0.5 ml of the sample (allow to stand for 20 mins.) followed by addition 0.25 ml of Folin-Ciocalteu reagent. The reaction mixture was mixed thoroughly and kept for 30 minutes for development of color. Similarly a blank was prepared using 0.5 ml of 1 (N) NaOH. The optical density of the blue color developed was recorded in a Shimadzu-double beam spectro UV-180 at 660 nm. against the blank. Protein content was estimated by referring to a standard curve prepared with known concentration of Bovin Serum Albumin Fraction V (Sigma chemical)
xi. Qualitative estimation of protein

Preparation of seed protein for polyacrylamide gel electrophoresis: Seed sample (selfed control lines and true breeding M₄ mutants) were imbibed in water (0.25 gms of seeds in each case) for 18 hours at 18°C followed by extraction (crushing of the samples were done in 0°C-4°C in a chilled mortar and pestle) in 0.4 ml 0.2 M Tris – HCL buffer (pH- 8.5; containing 0.056M 2-mercaptoethanol and 1M sucrose). The crushed samples were suspended (0°C-4°C) for 3-4 hours and centrifuged (-4°C to 0°C) 3 times at 12,000 r.p.m. for 20 minutes (each time). The clear supernatant was collected and used as protein source for polyacrylamide gel electrophoresis (SDS-PAGE). To study seed protein polymorphism, one dimensional SDS-PAGE (10%) was carried out following Laemmli (1970) in a vertical gel system (BIOTECH).

xii. Procedure for SDS-PAGE

Assembly of the glassplates: Thoroughly cleaned (chromic acid, liquid soap and running water) and dried (air dried followed by absolute alcohol) glass plates with spacers (1.5 mm) were assembled properly with the help of steel grip tape and clamps.

Preparation of Separating gel: Separating gel (10.0%) of 45.2 ml was prepared by the addition of following ingredients-

Stock acrylamide solution (30%) – 15 ml

1.875 (M) Tris – HCL (pH- 8.8) – 11.25 ml (SDS is mixed in the proportion of 4 gm/100 ml)

Water – 18.75 ml

The solution was degassed on a vacuum pump for 10-15 mins and then TEMED (N, N, N’, N’ – tetramethyl diamine) – 25 µl and ammonium persulphate – 0.2 ml were added to the separating gel.
Carefully the separating gel solution (40ml) was poured in the chamber between the glass plates. Drops of distilled water were given on the top of the gel (to prevent evaporation) and the gel was left to set (polymerization) for 30-45 minutes.

**Preparation of Stacking gel**: Stacking gel (4.5%) of about 12 ml was prepared by addition of the following solutions-

Stock acrylamide solution (30%) – 1.8 ml

0.6 (M) Tris – HCL (pH- 6.8) – 3.0 ml (SDS is mixed in water 6.8 ml the proportion of 4 gm/100ml)

Water – 6.8 ml

The solution was degassed for 10 minutes and then TEMED – 20 µl and ammonium persulphate – 40 µl was added.

After decanting of the water layer from the top of the separating gel and washing with a little stacking gel solution, the stacking gel mixture was poured on the top of the separating gel and immediately the combs (1.5 mm) was placed carefully in the stacking gel and the gel was allowed to set for 30 minutes.

**Installation of the gel in the electrophoresis apparatus**: After the stacking gel has polymerized, the comb was removed carefully without distorting the shape of the well. Carefully the steel grip tape and clamps were removed and the gel was installed in the electrophoresis apparatus.

**Loading of protein samples**: The extracted protein samples prepared for electrophoresis was adjusted by using one strength sample buffer (Tris-HCL buffer, pH- 6.8 – 0.5 ml; sucrose – 500 mg, SDS – 50 mg, bromophenol blue 0.1 ml and water upto 1 ml) in each lot of sample in such a way that the same amount of protein is present per unit volume. The protein samples were dipped in boiling water 10 times (each dip – 8 seconds) to ensure complete interaction between proteins and SDS. The samples were cooled to 0-4°C and 7 µl of each
sample was appropriately (marker protein was also added in the same way) loaded in each well after the wells were thoroughly washed with distilled water and running buffer. The sequence of the samples loaded in the wells was maintained through specific adjustment.

**Preparation of running buffer (pH- 8.2-8.4):**

Tris – 2.4 gm.
Glycine – 11.52 gm.
SDS – 800 mg.
Water upto – 800 ml.

**Electrophoresis:** The gel apparatus was filled with running buffer and any trapped air bubbles at the bottom of the gel was removed. The cathode and the anode were connected to the gel apparatus and to the DC – power pack to complete the electric circuit. The entire gel unit (gel apparatus and the glass plates) was kept in cooling condition so that heat generated during the run of the protein sample is dissipated and does not affect the gel. A constant current of 3 mA/slot (voltage depends on the total current applied) for 3 – 4 hours was given for complete run of the protein samples as indicated by the bromophenol blue dye marker (current was stopped when dye marker reaches almost at the bottom of the gel).

**Staining of the gel:** After complete run of the protein samples in the gel, the gel was carefully removed from between the plates and immersed in staining solution (250 mg. coomassie brilliant blue + 40 ml methanol + 10 ml glacial acetic acid + 50 ml water) for overnight with uniform shaking. The proteins absorb the coomassie brilliant blue.

**Destaining of the gel:** The gel was then transferred in a suitable container with at least 50 – 70 ml destaining solution (methanol : glacial acetic acid : water – 25:7:68). The dye that was not bound to proteins was removed. Frequent changes in the destainer was made until the background of the gel was colorless. The proteins fractioned were observed as
blue colored bands. Destaining process was carefully conducted so that all faint bands could be appropriately visualized.

**Recording of data:** The position of the protein bands in the gel was detected and expressed as relative mobilities (Rm) by measuring the distance migrated by the particular protein band to that of bromophenol blue. The protein bands were evaluated against the protein molecular weight marker for SDS gel electrophoresis (mol. weight range: 14,000 to 100,000) obtained from Bangalore Geni Pvt., India (Cat No. PMW - M). Molecular weight of different bands was studied using the software (TotalLab™ Quant v 11; website: http://www.totallab.com/).

**Photography:** The gel with stained protein bands was placed inglow box to take appropriate photographs.

xiii. Extraction and estimation of chlorophyll content

Chlorophyll was extracted and estimated from leaf tissues (of identical maturity) of control and two M₄ (*viridis* and *dark green leaf*) mutants following the method of Arnon (1949). For chlorophyll extraction, samples of 0.1 gm fresh lamina were crushed in 25 ml of 80% acetone and centrifuged at 8000 r.p.m. in REMI cold centrifuge and the supernatants were collected and volumes were made up to 100 ml by the addition of 80% acetone.

For densitometric reading, 5 ml of the extract was transferred to a 50 ml volumetric flask and made up to the volume with 80% acetone. Readings of optical density values were recorded in a Shimadzu-double beam spectra UV-180 model at 663 nm and 645 nm for chlorophyll-a and chlorophyll-b respectively against 80% acetone.

Estimation of chlorophyll-a, b and total chlorophyll was made by using the following formula:

\[
\text{mg chlorophyll-a/gm of tissue} = [12.7 (A663) - 2.69 (A645)] \times \frac{V}{1000 \times W}
\]

\[
\text{mg chlorophyll-b/gm of tissue} = [22.9 (A645) - 4.68 (A663)] \times \frac{V}{1000 \times W}
\]

\[
\text{mg total chlorophyll/gm of tissue} = [20.2 (A645) + 8.02 (A663)] \times \frac{V}{1000 \times W}
\]
Where, A = absorbance at specific wavelength, V = final volume of chlorophyll extract in 80% acetone and W = fresh weight of tissue extracted. Final quantitative determination of chlorophyll content was performed by averaging the values of the replicas in each plant type.

xiv. Molecular analysis

Total genomic DNA of 11 plant types (seeds samples of selfed control lines and harvested M₁ seeds of 10 macromutant) was isolated from 0.2 g of germinated seedlings, raised in Petriplate condition (27°C±1°C), using DNeasy Plant Mini Kit of Qiagen, USA. The mucilage and polyphenolic compounds are removed by passing DNA through an Au-Prep purification column (Life Technologies, Rockville, MD, USA). Subsequently, quality and quantity of extracted DNA are checked by running the dissolved DNA in 0.8% agarose gel by comparison with standard lambda DNA marker of known concentration. The DNA has been diluted to 30 ng/μl for RAPD analysis.

xv. RAPD analysis

Ten 10-base primers (OPB 1-10; Operon Technologies, Alameda, USA) were used for Polymerase Chain Reaction (PCR).

Amplification reactions are performed in volumes 25 μl containing 2.5 μl of 10X assay buffer (100 mM Tris-HCl; pH 8.3, 500 mM KCl, 15 mM MgCl₂), 100 mM of each dNTPs (dATP, dCTP, dGTP and dTTP) (MB1 Ferment Inc., Maryland, USA) 5 pg of primer, 1.0 unit of Taq DNA polymerase (Bangalore Genei, India) and 30 ng of template DNA.

The amplification reaction has been carried out in a DNA Thermal Cycler (Eppendorf AG, Hamburg, Germany) programmed for 44 cycles as follows: 1st cycle of 5 min at 94°C followed by 43 cycles each of 1 min at 92°C, 1 min at 37°C, 2 min at 72°C. The final step consisted of one cycle of 7 min at 72°C for complete polymerization. After completion of the PCR, 2.5 μl of 6X loading dye (MB1 Ferment Inc., Maryland, USA) has been added to the amplified products and were electrophorized in a 1.5% (m/v) agarose (Bangalore Genei,
India) gels with 1X TAE buffer, stained with ethidium bromide and documented by a gel documentation system (Syngene, Cambridge, UK).

RAPD bands were scored as present (1) or absent (0) in the studied species and entered in a binary data matrix. Based on the results on bands spectra, proximity matrix has been generated for all possible pairs from Euclidean Distance and used to construct the dendrogram by Unweighted Pair Group Method with Arithmetic Mean (UPGMA). The data has been analyzed using STATISTICA version 7.1 (www.statsoft.com).

xvi. Test of viability of seeds

Seeds of control and mutants of M4 plants were tested for their viability on harvest. Viability of seeds was tested following tetrazolium method. This method determines the percentage of viable seeds which may be expected to germinate. The chemical tetrazolium chloride is colorless, but it develops intense red color when it is reduced by living cells. This phenomenon is used to determine the percentage of viable seeds in a seed sample. Test seeds were soaked in tap water for overnight and were split longitudinally with the help of a blade so that a portion of embryo was attached with each half of seeds. One half of each seed was placed in a crucible and dipped in 1% aqueous solutions of tetrazolium chloride for 24 hours. The seeds were then washed on tap water and the number of seeds in which the embryo was stained red was determined. The percent of viable seed was computed as follows:

\[
\text{Viable seed (\%) = (Number of half seed stained red) \times 100/Total number of half seed}
\]