MATERIALS AND METHODS
Materials And Methods

The seed material:

The seed of moth bean (*Vigna aconitifolia* (Jacq.) Marechel) was obtained from the cytogenetics and plant breeding laboratory, Department of Botany, Dr.Babasaheb Ambedkar Marathwada University, Aurangabad.

Mutagens used:

Two mutagens, namely Ethyl methanesulphonate (EMS) and Sodium Azide (SA) were used during the present study.

Ethyl methanesulphonate (EMS):

Ethyl methanesulphonate (CH$_3$SO$_2$OC$_2$H$_5$) a monofunctional-alkylating agent, with molecular weight of 124.16, manufactured by Sisco Research Laboratory, Mumbai, was used in the present work.

Sodium Azide (SA):

Sodium Azide (NaN$_3$) with molecular weight 65.01, manufactured by Spectrochem, Pvt. Ltd., Mumbai was used in the present work.

Mode of treatment with the mutagenic agents:

To begin with, the pilot experiments were conducted for determining the suitable concentrations of mutagens for further studies.
**Preparation of mutagenic solution:**

The chemical mutagenic treatments were carried out at room temperature of 25±2°C. The fresh aqueous solutions of the mutagens were prepared prior to their treatments. The concentrations of solutions were 0.05%, 0.10% and 0.15% for EMS and 0.01%, 0.02% and 0.03% for SA. Seeds of uniform size were selected and immersed in distilled water for 6 hours to initiate presoaking. Such presoaked seeds were later on immersed in the mutagenic solution for 4 hours for EMS and 6 hours for SA treatments, with intermittent shaking. The volume of the mutagenic solution used was three times as that of the seeds so as to facilitate uniform absorption. Post soaking was performed for 2 hours. The seeds soaked in distilled water for 12 hours served as control.

For each treatment, 250 seeds were used. 50 seeds from each treatment were kept on moist blotting paper mounted in petriplate to record germination percentage. Another 50 seeds were sown in pots to record the seedling height. The remaining 150 seeds of each treatment were sown in the field following randomized block design (RBD) with three replications each consisting of 50 seeds along with control for raising the M₁ generation. The seeds were sown at a distance of 20cm between the plants and 45cm between the rows.

**Observations in M₁ generation:**

1. **Germination percentage:**

   Germination counts were taken from the seeds sown in the petriplates on the moist blotting paper when the root and shoot emerged out. Germination was expressed as percentage of control and lethality was also estimated as percentage from the count.
2. Seedling height:

Seedling height was recorded on 10th day after germination of seeds, which were earlier sown in pots, by randomly measuring the height of 10 seedlings. The reduction in the mean seedling length of the treated seedlings as compared to control was regarded as seedling injury and expressed as the percentage.

3. Leaf morphological changes:

The frequency of leaf morphological changes was recorded by counting the number of plants carrying leaf abnormalities from the plants of each treatment.

4. Pollen sterility:

Pollen sterility was determined in 10 randomly selected plants in the field by staining the pollen grains with 2% acetocarmine. Stained pollen grains were considered as fertile, while empty, partially stained and shrivelled ones were considered as sterile. The values were expressed as percentage.

5. Survival of plants at maturity:

Survival of plants was recorded at the time of maturity in the field and expressed as the percentage of control.

Studies in M₂ and M₃ generations:

All plants from each treatment in M₁ generation were harvested separately and seeds were collected. From these, seeds of 30 normal looking plants of M₁ selected at random were collected on individual plant basis from all the treatments and control. They were used for raising the M₂ generation on plant to a row basis. The study of M₂ generation comprised the analysis of following parameters.
I. Chlorophyll mutations:

During the period of 10 to 15 days, when the plants were at the seedling stage, the treated as well as the control plants were screened for the frequency and spectrum of chlorophyll mutations. The pertinent values were calculated and the number of mutations per 100 plants of M2 generation was estimated according to the method of Gaul (1957). The classification and characterization of various chlorophyll mutants was done according to Gustafsson (1940) and Blixt (1961) and the spectrum was recorded as follows:

*Albina:* White in colour  
*Xantha:* Yellow to whitish yellow  
*Chlorina:* Yellow-green  
*Viridis:* Light green or yellow green

II. Mutagenic effectiveness and efficiency:

Mutagenic effectiveness is a measure of the frequency of mutations induced by a unit dose of mutagen (kR or Concentration × time). The mutagenic efficiency depicts the proportion of mutation in relation to biological damage induced. The formulae proposed by Konzak et al. (1965) were followed for the calculations of mutagenic effectiveness and efficiency by incorporating the mutation frequency values recorded for each mutagenic treatment,

\[
\text{Mutation frequency (MF)}
\]

\[
\text{Mutagenic effectiveness} = \frac{\text{Mutation frequency}}{\text{Dose or (Concentration×time)}}
\]
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\[
\text{Mutagenic efficiency} = \frac{\text{Mutation frequency (MF)}}{\text{Biological damage}}
\]

i.e. MF/L, MF/I and MF/S

Where T = duration of treatment with mutagen,
C = dose/concentration of mutagen,
L = lethality,
I = injury,
S = pollen sterility
MF = percentage of plants segregating for chlorophyll mutations.

\textbf{Mutation rate:}

Mutation rate (MR) was calculated by the following formula:

\[
MR = \frac{\text{Sum of values of efficiency or effectiveness of particular mutagen}}{\text{Number of treatments of a particular mutagen}}
\]

This gives the knowledge of mutations induced by a particular mutagen irrespective of dose.

\textbf{III. Quantitative Characters:}

Randomly selected plants from each treatment along with control were studied thoroughly for different quantitative characters. The eight quantitative traits analyzed are given below.
1. **Plant height:**

Plant height was measured at the time of harvesting in centimeters from the base of plant, i.e. soil level to the apex.

2. **Days to flowering:**

Days to flowering were counted as the number of days from the date of sowing to the opening of the first flower on the plant.

3. **Days to maturity:**

Days to maturity were recorded as the number of days from the date of sowing to complete pod maturity.

4. **Number of pods per plant:**

Pods were counted for each plant separately and the data recorded.

5. **Number of seeds per pod:**

Ten pods of each plant were opened and the number of healthy seeds was counted in each pod. The mean was calculated for each plant and for each treatment separately.

6. **100 seed weight:**

The weight of the seeds was recorded in grams from a random sample of 100 seeds from each plant.

All the quantitative characters were analyzed for the S.E. (standard error), mean and coefficient of variation by using the standard formulae based upon the total observations for each variable in M₂ and M₃ generations. Various statistical analyses were performed by using the following formulae:
Genetic improvement of moth bean (Vigna aconitifolia (Jacq.) Marechal) through mutation breeding.

\[ \text{Mean} = \frac{\sum x}{N} \]

\[ \text{Variance} = \frac{\sum x^2}{N} - \bar{x}^2 \]

iii) Standard deviation (S.D.) = \text{variance}

iv) Standard error (S.E.) = \frac{\text{S.D.}}{\sqrt{N}}

v) Coefficient of variation (C.V.) = \frac{\text{S.D.}}{\text{Mean}} \times 100

vi) Critical difference (C.D.) = S.E. (d) \times t_{e.d.f. (error degree of freedom)}

vii) S.E._{(m)} = \sqrt{\frac{\text{Mean error sum of squares}}{r}}

where \( r = \text{Number of replication} \)

The ANOVA was calculated as per Panse and Sukhatme (1976) and the following abbreviations were used.

S.V. = Source of variation.
D.F. = Degree of freedom.
S.S. = Sum of squares.
M.S.S. = Mean sum of squares.
F. = Test value.
IV. Viable Mutants:

The viable mutants or macromutants were carefully screened from the population of M₂ generation and the classification of the mutants was carried out according to Swaminathan (1964). The mutants were harvested separately for further field testing in M₃ generation.

Studies in M₃ generation:

Harvested pods from all treatments of M₂ generation of moth bean were sown in field as M₃ generation. The study of M₃ generation comprised the analysis of the quantitative characters. The procedure adopted was similar to that of M₂ generation studies. The viable mutants of M₂ generation were harvested separately and raised again for the field-testing. Characterization of the mutants was carried out both morphologically and biochemically.

Micromutants:

These are the mutations which cannot be detected visually, but need biometrical analysis for their detection. The parameters for recording micromutations were the same for M₂ and M₃ generations.

Biochemical Studies:

1. Extraction of seed proteins:

Mature seeds were washed with water, dried and ground to make fine powder. The mature seed powder was defatted with hexane, air-dried and stored at 4°C. Seed powder was kept for extraction in 1:6 proportion of distilled water with 1% PVP
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(Polyvinyl Polypyrrolidone). The suspension was centrifuged at 12,000 rpm at 4°C for 20 minutes to remove the particulate matter and clear supernatant was used for protein estimation, the trypsin/chymotrypsin inhibitor assay and the native PAGE.

Similarly, the protein extracted in 10mM Tris-HCl buffer, pH 8.0 containing 1mM EDTA, 1% SDS and 25% Glycerol was used for protein estimation and SDS-PAGE.

2 Protein estimation:

The protein estimation was carried out by the Biuret method (Layne, 1957).

3. Standardization of Biuret assay using BSA:

The stock solution of BSA was prepared in distilled water. The protein was estimated by measuring the absorbance at 280nm and by using a molar absorption coefficient (E) for BSA of 6.6. The above stock solution was diluted to prepare 1mg/ml working solution of BSA in distilled water. Biuret reagent was prepared by dissolving 300mg of CuSO₄ and 1.2g of sodium potassium tartarate in 100ml distilled water. Then 60ml of 10% NaOH was added to it with constant swirling. Later the volume was made to 200ml with distilled water and 0.2g of KI was added to it.

BSA (50μg-500μg) was taken and the volume was made up to 500μl with distilled water. Two ml of Biuret reagent was added and incubated at room temperature for 30 minutes. The absorbance was read at 550nm. A graph of absorbance versus concentration of BSA was plotted and this graph was used for estimating the protein concentration in sample. Suitable quantity of extract was assayed by Biuret method and respective protein value was determined from BSA standard graph and the protein value was expressed in mg/g.
4. Standardization of trypsin assay by using BAPNA:

Trypsin stock solution was prepared by dissolving 10mg of trypsin in 1ml (0.1mM Tris-HCl pH 7.8) buffer A. This stock solution was diluted to prepare 1mg/ml working solution of trypsin in the protease buffer. 10 to 50μl trypsin was taken and the volume was made up to 0.5ml with protease buffer. 1mM BAPNA solution was prepared by dissolving 10g of BAPNA in 0.45ml DMSO (Dimethyl Sulphoxide) and then mixed in 22.5ml of protease buffer. 1ml of BAPNA solution was incubated with 150μl of trypsin in Eppendorf tubes at 37°C for 10 minutes. The reaction was stopped with 0.2ml of 30% acetic acid after 10 minutes. The absorbance was read at 410nm. A graph was plotted with absorbance versus concentration of trypsin and according to it, the optimum trypsin concentration to be used for the assay was determined.

5. Trypsin inhibitor assay:

Trypsin activity was measured by using the synthetic chromogenic substrate BAPNA, as described by Erlanger et al. (1961). For trypsin inhibitor assay 20μg of trypsin was found to be optimum from the earlier standardization. Trypsin inhibitor activity was determined by mixing suitable quantity of protein extract containing inhibitor with 20μg of trypsin in a volume of 30μl of buffer A so that trypsin activity could get inhibited up to 40% to 60%. 1ml of 1mM BAPNA was added to the reaction mixture, the incubation was conducted at room temperature and later the reaction was stopped after 10 minutes by adding 200μl of 30% acetic acid. The residual trypsin activity was measured at 410nm. One inhibitor unit was defined as the amount of inhibitor that inhibited 1 unit of trypsin activity.
6. Analysis of M₂ mutants for trypsin inhibitors:

The 7 macromutants and 78 M₂ lines were analyzed for the trypsin inhibitor study. The seeds were powdered and defatted with hexane. The defatted seed powder was kept for extraction in 1:6 proportion of distilled water with 1% (PVP) for overnight and centrifuged at 12000 rpm at 4°C for 20 minutes to collect the supernatant. This seed extract was used for the analysis of trypsin inhibitors by electrophoretic detection and T1 assay as described above.

7. Neutralization of trypsin inhibitors:

To nullify the trypsin inhibitor activity in moth bean seeds, so as to use it in diet, the heat treatment and germination studies have been tried in some mutant lines of moth bean.

I. Effect of heat on trypsin inhibitor:

The seed extract of moth bean obtained earlier, was kept in boiling water bath for 15, 30, 45 and 60 minutes, respectively. The minimized volume of seed extract (which was reduced during heating) was adjusted by adding distilled water. These extracts were used for trypsin inhibitor assay and for the trypsin inhibitor profile. The seed powder obtained from the above heat treatment was kept for extraction as described earlier.

II. Study of trypsin inhibitor during germination:

78 moth bean lines and seven viable mutants of moth bean were selected for this experiment. To study the inhibitor profile during germination, the seeds were kept on filter paper folds. The germinating seeds were kept at 28°C to 30°C. Water was sprayed
on the seeds regularly whenever required. The germinating seeds were examined critically and the contaminated seedlings were discarded. Cotyledons of seedlings were harvested at different stages of germination from 2-10 days and immediately acetone powdered. The dry powder was extracted in 15 volumes of distilled water with 1% PVP, kept overnight at 10°C. The suspension was centrifuged at 12000 rpm at 4°C for 20 minutes and the supernatant was used for electrophoresis and trypsin assay.

8. Electrophoresis:

The water soluble proteins were analyzed by using vertical slab polyacrylamide gel electrophoresis (PAGE) apparatus.

A. Non-denaturing discontinuous PAGE:

It was performed by using Davis (1964) system. The composition of buffer system described in the following protocol pertains to non-denaturing type since it did not contain any detergent/other denaturing agents.

Stock solutions:

i) Monomer solution (29.9% T, 0.9%C):

- Acrylamide 30g
- Bis-acrylamide 1.04g
- Distilled water 100ml

ii) Resolving gel buffer (1.5M Tris-HCl, pH 8.8):

- Tris base 18.16g
- Distilled water 75ml
- pH 8.8

Final volume was made upto 100ml with distilled water.
iii) Stacking gel buffer (1M Tris- HCl, pH 6.8):

- Tris base: 12.12g
- Distilled water: 55ml
- pH: 6.8

Final volume was made upto 100ml with distilled water.

iv) Catalyst (10% APS):

- Ammonium per sulphate: 100mg
- Distilled water: 1000μl

v) TEMED (10% Initiator):

- TEMED: 100μl
- Distilled water: 900μl

vi) Electrode buffer (10X) (0.25 mM Tris, 2 M Glycine, pH 8.3):

- Tris base: 3g
- Glycine: 14.4g
- Distilled water: 1000ml

This solution was diluted 10 times at the time of use.

vi) Sample buffer (0.08M Tris HCl, pH 6.8):

- Stacking gel buffer: 1.6ml
- 87% Glycerol: 2.5ml
- Bromophenol blue: 0.5mg

Final volume was made upto 10ml with distilled water.
vii) **Fixing solution (10%):**

- Trichloro acetic acid 11.4g
- Sulphosalicylic acid 3.4g
- Methanol 30ml
- Distilled water up to 100ml

viii) **Staining solution (0.25% CBBR):**

- CBBR 1.25g
- Methanol 230ml
- Distilled water 230ml
- Acetic acid 40ml

This solution was filtered before use.

ix) **Destaining solution:**

The acetic acid, ethanol and distilled water were taken in 1:3:6 proportions (ratio).

II. **Preparation of gel:**

i) **Resolving gel:**

The working solution of resolving gel was prepared as follows:

<table>
<thead>
<tr>
<th></th>
<th>For 10%</th>
<th>for 12%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monomer solution (i)</td>
<td>10ml</td>
<td>12ml</td>
</tr>
<tr>
<td>Resolving gel buffer (ii)</td>
<td>7.5ml</td>
<td>7.5ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>12.5ml</td>
<td>10.5ml</td>
</tr>
<tr>
<td>Catalyst (IV)</td>
<td>300μl</td>
<td>300μl</td>
</tr>
<tr>
<td>Initiator (v)</td>
<td>20μl</td>
<td>20μl</td>
</tr>
</tbody>
</table>
Resolving gel was prepared in BROVIGA vertical slab gel electrophoresis apparatus. The above working solution of resolving gel was poured quickly into gel mould and over layered with saturated n-Butanol. After polymerization the butanol was poured off and the top of the gel was carefully rinsed with distilled water and later drained with filter paper strip.

ii) **Stacking gel:**

<table>
<thead>
<tr>
<th>Monomer solution (i)</th>
<th>1.7ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stacking gel buffer (ii)</td>
<td>1.2ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>7.1ml</td>
</tr>
<tr>
<td>Catalyst (iv)</td>
<td>100µl</td>
</tr>
<tr>
<td>Initiator</td>
<td>10µl</td>
</tr>
</tbody>
</table>

Stacking gel solution was prepared over resolving gel. The above gel solution was poured on the top of running gel and a comb was inserted to form the sample wells.

iii) **Application of sample:**

Equal protein samples mixed with 2X sample buffer in 1:1 (v/v) ratio were loaded in the sample wells.

iv) **Electrophoresis:**

Electrophoresis was performed at constant voltage (150V) for 4.5 hours. After electrophoresis the gels were fixed in 10% TCA for 30 minutes. Then gels were stained with staining solution (ix) and destained in destaining solution (x).

**B. SDS-PAGE:**

In SDS-PAGE, polypeptides are separated according to their molecular weight, not by intrinsic electrical charge. Sodium dodecyl sulphate (SDS) is an ionic detergent that
denatures proteins by wrapping around the polypeptide backbone. In doing so, the SDS
confers a negative charge to the polypeptide in proportion to its length. When proteins are
treated with SDS and reducing agent 2ME (2-Mercaptoethanol), the polypeptides become
rods of negative charges with equal charge unit per length. Here, denaturing
discontinuous PAGE system was used as described by Laemmli (1970). This system is
almost similar to the native PAGE (Davis, 1964) except for the presence of SDS.

1) **Stock solutions:**

All the stock solutions for resolving, stacking gel and electrode buffer were prepared
similarly as given earlier (Please refer to section 8.A.I.) except the use of SDS and 2-ME.

i) **Sodium dodecyl sulphate (10%):**

SDS 20gm
Distilled water 150ml

Final volume was made up to 200ml with distilled water.

ii) **Preparation of resolving and stacking gel:**

All the composition of resolving gel was similar as described earlier (please refer to
section 8.A.II.) except use of 300µl of 10% SDS solution (8.B.I.i.) and 100µl of 10%
SDS solution used for stacking gel while remaining were similar to above (please refer
to section 8.A.II.i.)

9. **Electrophoretic detection of trypsin inhibitors:**

The seed extracts of moth bean were analyzed by electrophoresis on 10%
polyacrylamide gel by using Davis system (1964), in Broviga vertical slab gel apparatus.
The trypsin inhibitor bands were visualized after electrophoresis by placing the gel in
equilibrium buffer (0.1M Tris-HCl, pH 7.8) for 5-10 minutes. The gel was subsequently
incubated in 0.1mg/ml trypsin solution for 5 minutes, rinsed briefly in buffer and placed on undeveloped X-ray film. The appearance of inhibitor bands on X-ray film was monitored visually after washing the hydrolysed gelatin with warm water (Pichare and Kachole 1994).

10. Extraction of Lectin:

500mg seed was taken and soaked in saline solution for 10 minutes. Then the seed coat was removed and cotyledons were crushed in saline solution to make a fine paste. Then it was centrifuged at 300rpm for 10 minutes and the supernatant was used for the lectin assay and protein estimation.

A. Lectin assay:

Hemaglutination assay was used for measuring the lectin activity as described by Gordon and Marquardt (1974) in round bottomed wells of microtiter plates with erythrocytes (from human blood) suspension in physiological saline. The 50μl seed lectin was serially diluted with 50μl physiological saline solution except first well of the microtiter plate. After this 75μl erythrocytes were mixed (final volume 0.125ml) in microtiter plates. The sedimentation pattern of the erythrocyte suspension was read after 2 hours at room temperature. A positive pattern, indicating agglutination, is a uniform effacement at the bottom of the well by erythrocytes and negative pattern, indicating no agglutination, is a circular clump of erythrocytes surrounded by a concentric, clear zone of the size, equal to the blank. The blank had physiological saline and erythrocytes only; there was no sample in it.
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B. Specific activity:

As mentioned above this parameter was tested by the hemagglutination assay. The specific activity is defined in terms of the minimum concentration of lectin that will show agglutination and was calculated as

\[
\text{Hemagglutination assay units (HAU)} = \frac{\text{Specific activity}}{\text{Amount of protein in the first well}}
\]

The units for specific activity are HAU/mg protein/ml.

C. HAU and its calculation:

HAU is defined as the maximum dilution for which hemagglutination still occurs. Two fold dilution of lectin was carried out through a series of wells, such that each successive well had exactly half the concentration of lectin as compared to the previous well. The 1st well containing lectin was designated as 2^0, the 2nd well containing half the concentration of lectin as compared to the 1st, was designated as 2^1, the third as 2^2 and so on. Thus if activity could be seen upto the 4th well the HAU represented as the reciprocal of 2^3 (1/2^3, which is 8) and this value divided by the concentration of lectin added initially to the 1st well before dilution gave the specific activity.

11. Extraction of Polyphenols:

The defatted seed powder was extracted in 10 volumes of 2 N HCl and kept in boiling water bath for one hour. Then it was allowed to cool at room temperature for few minutes and centrifuged at 3000 rpm for 10 minutes and supernatant was collected. The
above extract was used for further processing. 2 ml of ethyl acetate was added to 2 ml extract, vortexed and allowed to stand for 10 minutes. Ethyl acetate layer was separated in a test tube. Ethyl acetate extraction was repeated thrice and the obtained extracts were kept at 70°C in water bath for evaporation of ethyl acetate. Last traces of ethyl acetate were evaporated to dryness by keeping the test tube in boiling water bath. Dried sample in the test tube was dissolved in minimum amount of ethyl alcohol and centrifuged at 5000 rpm for 10 minutes. Clear supernatant obtained was used for the estimation of polyphenols.

A. Estimation of Polyphenols:

The amount of total polyphenolic compounds in the extracts were estimated as tannic acid equivalent according to the Folin-Denis procedure (Swain and Hills, 1959).

Molecular Studies:

RAPD:

1. Isolation of plant genomic DNA:

The plant genomic DNA was isolated from the fresh leaves of seven viable mutants and control plants of moth bean, using the CTAB method (Doyle and Doyle, 1987) and without liquid N2 procedure (Sharma and Sharma 2003). For isolation of DNA, the cell wall must be broken or digested in order to release cellular constituents, this is carried out by grinding tissue in liquid N2. Cell membrane is disrupted by using detergents such as CTAB (Cetyl Trimethyl Ammonium Bromide). For protection of DNA from endonucleases, EDTA (Ethylene Diamine Tetra Acetic acid) was used. EDTA chelates Mg²⁺ ions, an essential cofactor for most of the nucleases. The ground tissue
mixture is emulsified with chloroform and phenol to denature protein from DNA. Time between thawing of frozen pulverized tissue and its exposure to the extraction buffer should be minimized to avoid nucleolytic degradation of DNA. Leaves were also treated with fixing solutions for 30 and 60 minutes to denature enzymes. The fixing solutions were absolute alcohol, alcohol-chloroform (70:30) and alcohol-0.5M EDTA, P<sup>2+</sup>-8 (70:30). Treated tissue was removed from solution and homogenized with mortar and pestle. Homogenized material was handled as follows (Mohapatra et al. 1992):

- Submerged 1g of tissue in 5ml of alcohol for 30 minutes.
- Allowed alcohol to evaporate. Ground the tissue with mortar and pestle.
- Tissue (1gm) was also homogenized in liquid N2.
- Transferred the homogenized tissue to prewarmed 2X CTAB DNA extraction buffer [100 mM Tris (P<sup>2+</sup>-8), 20Mm EDTA (P<sup>2+</sup>-8), 1.4M NaCl, 2% CTAB, 2 μl/ml β-mercaptoethanol].
- Incubated for 1 hour at 60°C water bath, occasionally mixing by gentle swirling.
- Removed from water bath. Added 0.6 volume of chloroform-isoamylalcohol (24:1). Mixed by inversion for 15 minutes.
- Spun at 12,000 rpm for 10 minutes.
- Transferred the aqueous phase to another tube.
- Added twice the volume of absolute alcohol or 0.6 volume of isopropanol to precipitate the DNA.
- Spooled out or centrifuged briefly to pellet the DNA.
- Washed with 70% alcohol. Inverted the tubes and drained on a paper towel for approximately 1 hour. Dried overnight (covered with parafilm with tiny holes).
- Dissolved the dried DNA in T_{10}E_{1} buffer (P^{*}-80).
- Added 2.5 μl of RNase to 0.5ml of crude DNA (2.5 μl of RNase=25 μg of RNase, thus treatment was at 50 μg/ml of DNA preparation).
- Mixed thoroughly but gently and incubated at 37°C for 1 hour.
- Added 0.3-0.4 ml of chloroform-isoamylalcohol (24:1). Mixed thoroughly for 15 minutes.
- Centrifuged for 15 minutes at 12,000 rpm.
- Removed the supernatant (avoided the whitish interface layer).
- Reprecipitated the DNA by using double quantity of absolute alcohol.
- Removed the DNA with a pasture pipette/micropipette or centrifuged the tube to pellet the DNA.
- Washed the pellet with 70% alcohol. Dried overnight.
- Redissolved the DNA in T_{10}E_{1} buffer (P^{*}-80).

2. Quality check of isolated DNA:

For checking the quality of isolated genomic DNA, 2μl of DNA sample was diluted with 7 μl of T_{10}E_{1} buffer. After adding 1 μl of 6X loading dye, 10 μl solution was loaded in a slot of 1% agarose gel containing 0.05μg/ml of ethidium bromide. Hind III digested lambda DNA served as marker. The agarose gel electrophoresis was carried out for nearly 1 hour at 50Ma. The gel was visualized and photographed on UV light transilluminator or by gel documentation. The intact double stranded DNA forming a thick single band of high molecular weight confirmed the good quality of genomic DNA extracted.
3. Quantity check of isolated DNA:

The concentration of DNA in the extracted sample was measured spectrophotometrically as a function of its optical density. In quartz cuvette 5µl of DNA sample was dissolved in 1ml of T<sub>10</sub>E<sub>1</sub> buffer and the optical density reading was taken at 260 nm against a blank (Control without DNA) having only T<sub>10</sub>E<sub>1</sub> buffer in Sistronic UV-VIS spectrophotometer. The DNA concentration was calculated by the following methods.

50µg of double stranded DNA = OD 260 of 1.0

OD at 260 nm X 50 X dilution factor

\[
\frac{\text{OD at 260 nm} \times 50 \times \text{dilution factor}}{1000}
\]

\[
\text{Total DNA (µg/ml)} = \frac{\text{Volume of sample}}{\text{Volume of the aliquot}}
\]

Optical density values were also taken at 280nm (corresponding to protein) to check the DNA purity by the ratio of the values at OD 260nm: OD 280nm. The pure DNA at 260/280 will be 1.8.

4. PCR amplification:

Amplification reactions (25µl final volume) contained 40ng of DNA, 1mM dNTP 0.3µm Primer OPA-14 (5'-TCTGTGCTGG-3') and OPA-17 (5'GACCGCTTGT-3'), Operon Technologies, Almenda. 1X Taq. Polymerase buffer, 1 unit of Taq polymerase buffer was overlaid with 30µl of mineral oil. DNA amplification was performed in a DNA thermal cycler (Twinblock, Ericomp) programmed to 1 cycle of 4
minute at 94°C (initial strand separation); followed by 35 cycles of 1 minute at 94°C (denaturation), 1 minute at 35°C (annealing) and 2 minutes at 72°C (primer extension).

5. Agarose gel electrophoresis:

The amplification products were size separated by agarose gel electrophoresis and visualized by staining with ethidium bromide under UV light.

The gel tray was prepared by tapping the ends of the tray. The comb was placed and the tray was leveled. About 200ml of 1.4% agarose gel was prepared in 1X TBE buffer [54gm Tris base, 27.5gm Boric Acid, 20ml 0.5M EDTA (P^+ 8.8)/liter (5X)], 20μl of 10mg/ml ethidium bromide was added. Then the gel was carefully poured into the tray without allowing the air bubbles and was allowed to solidify. After solidification the comb was removed carefully without damaging the well and adhesive tapes were removed. The gel tray was placed in electrophoresis tank and the tank was filled with 1X TBE buffer, till the gel was completely immersed.

To the amplified DNA samples, 3μl of 6X gel loading buffer (0.25% bromophenol blue, 0.25% of Xylene cyanol FF, 20% glycerol in deionized water) was added and spun for 2-5 seconds. Samples were gently loaded into the wells using a 20μl micropipette. Lambda DNA Hind III double digest was used as molecular size marker. The leads were connected and the electrophoresis was conducted at a constant 100V current, until the bromophenol blue dye travelled 2/3 length of the gel. After agarose gel electrophoresis the gel was observed under UV light transilluminator (Sambrook et al. 1989) and photographed in the gel documentation.
6. Analysis of amplification profile:

Amplification profile of mutants was compared with each other and with control of moth bean. Each reaction was repeated twice. Variation in the intensity of bands was not taken into account. The bands of DNA fragments were scored as present (1) or absent (0). The data were used to distinguish mutants and control of moth bean on the basis of the number of monomorphic and polymorphic bands.