Materials and Methods
MATERIALS AND METHODS

Biochemical and Nutritive Evaluation

Plant Material

Germplasm seed materials of *Mucuna monosperma* DC. ex. Wight were collected from two different agroclimatic regions in India viz., i) Thirunelly Reserve Forest, Wynaad District (Kerala) and ii) Siruvani Reserve Forest, Coimbatore District, (Tamilnadu). After suitable seed treatments plants were raised in the plots of botanical garden, Department of Botany, Bharathiar University, Coimbatore. The studies on biochemical composition and nutritive evaluation were undertaken for both the germplasm seed materials of *M. monosperma* collected.

Chemicals

The chemicals used were either from BDH (AR grade) or from Sigma Chemical Company, St. Louis, M.O., U.S.A., throughout the study unless and otherwise specified.

Biochemical Composition

Proximate Analysis

Determination of Moisture Content

Fifty transversely cut air-dried mature seeds were taken and incubated in a hot-air-oven at 80°C for 24h. Then the samples were cooled in a desiccator and the weight was taken. The average loss in weight of the samples were calculated as moisture content and expressed on percentage basis.
Preparation of Seed Flour

The air-dried and oven-dried seeds were powdered separately in a Willey Mill to 60 mesh size. The fine seed powder, so obtained, will be referred hereafter as air-dried or oven-dried seed flour. The powdered samples were stored in screw-cap bottles until further use.

Digestion (Humphries, 1956)

Nitrogen content of the seed flour was determined by micro-kjeldahl method (Humphries, 1956). One hundred mg of oven-dried flour was taken into a micro-kjeldahl digestion flask. To this 2 ml of 5% salicylic acid dissolved in conc. $H_2SO_4$ was added and mixed well. After 20 min., 0.3g of sodium thiosulphate was added and heated gently until fumes disappeared. After cooling, the contents of the flask, 60 mg of catalyst (a mixture of 1 g copper sulphate, 8g potassium sulphate and 1 g selenium dioxide) followed by 1 ml of conc. $H_2SO_4$ were added. The contents of the flask were digested until they turned apple green in colour. The flask was cooled and the contents were made up to a known volume.

Distillation

Ten ml aliquot from the volumetric flask was transferred to Paranas micro-kjeldahl distillation flask. To this 10 ml of 40% NaOH solution along with 2 ml of glass distilled water were added. The contents were heated by a bunsen burner. The liberated ammonia was collected in 2% boric acid solution containing a drop of
double indicator (83.3 mg bromocresol green + 16.6 mg of methyl red dissolved in 10 ml of 95% ethanol).

The contents were titrated against N/50 H₂SO₄. A blank was run simultaneously using all the reagents and the value of the blank was deducted from the value of the sample before calculation. One ml of N/50 H₂SO₄ corresponds to 0.00028 g of N which forms the basis for calculation of N content in the sample.

The crude protein content was calculated by multiplying the percent nitrogen content of the sample with the factor 6.25.

**Determination of Ether Extract or Total Crude Lipid Content (AOAC, 1970)**

Two g of air-dried seed flour was extracted with ether in a Soxhlet apparatus for 16 h, according to AOAC (1970) method. The ether was evaporated and the residue was weighed. The average value of triplicate experiments was expressed as percentage of ether extract or total crude lipid content on dry weight basis.

**Determination of Crude Fibre Content (AOAC, 1970)**

After extraction with ether, the left-out residue was successively digested with 0.225 N H₂SO₄ solution and 0.313 N NaOH solution. After digestion, it was washed with boiling water followed by absolute ethanol in a Gooch crucible. The contents of the crucible were dried to constant weight, cooled, weighed and ignited in an electric muffle furance. After cooling, the contents were reweighed. The loss in weight was expressed as percentage of crude fibre on dry weight basis.
Determination of Ash Content (AOAC, 1970)

Two g of oven-dried seed flour were weighed into a pre-weighed porcelain crucible. The crucible with the seed flour was placed in an electric muffle furnace set at 600°C and maintained for 2 h. The contents of the crucible were cooled in a desiccator and weighed immediately. The difference in weight of the crucible was expressed as percentage of ash on dry weight basis.

Determination of Nitrogen Free Extractives (NFE) or Total Crude Carbohydrate Content

% NFE = 100 - (CP% + EE% + CF% + Ash %)

Where

CP = Crude protein    EE = Ether extract
CF = Crude fibre

Determination of Calorific Value

The energy content of the seeds was determined by multiplying the percentage of crude protein, crude fat and total crude carbohydrates by the factors 4, 9 and 4, respectively (Osborne and Voogt, 1978).

Extraction and Estimation of Total Soluble Carbohydrates

Extraction

One g of air-dried seed flour was suspended in 1:5 (W/V) hot 80% ethanol and extracted for 5 min. at 90°C. The pellet was reextracted twice with equal volumes of hot 80% ethanol. The ethanol extracts were clarified by centrifugation, pooled and con-
centrated to 1 - 2 ml by evaporation in vacuo. The concentrated ethanol extract was diluted to 50 ml with glass distilled water and used for estimation of total soluble carbohydrates.

Estimation (Yemm and Willis, 1954)

From suitable aliquots of the extract, total soluble carbohydrates were estimated by the anthrone reagent method using glucose as a standard at 620 nm in Spectronic 20D spectrophotometer. The values were expressed as percentage on dry weight basis.

Extraction and Estimation of Starch (Clegg, 1956)

Extraction

The extraction of starch was carried out at room temperature. Five ml of distilled water was added to the residue which had been left after extraction with alcohol as stated above and to this 6.5 ml of 52% perchloric acid was added. The mixture was stirred continuously for 5 min. with a glass rod and then occasionally for the next 15 min. Then, 20 ml of distilled water was added and centrifuged. The supernatant was collected in a 100 ml volumetric flask. Again to the residue 5 ml water was added and extraction with perchloric acid was repeated, stirring, occasionally for the next 30 min. and centrifuged. Both the supernatants were pooled into a 100 ml volumetric flask and diluted to 100 ml by adding distilled water and filtered. While filtering, first 5 ml of the filtrate was discarded. A suitable aliquot portion was diluted to give a final concentration equal to about 100 μg glucose/ml and used for estimation of starch.
Estimation

0.1 ml of the extract was diluted to 1.0 ml with distilled water. To this 5 ml of anthrone reagent was added and thoroughly mixed. Then the tubes were heated in a boiling water bath for 12 min. and cooled to room temperature. The colour intensity developed was measured at 625 nm in a Spectronic 20D spectrophotometer, using glucose as a standard. The values were expressed as percentage on dry weight basis.

Calculation

The glucose equivalents in the samples were calculated using the optical density obtained for the standard glucose solution. Using the conversion factor 0.9, the glucose content in each sample was converted into starch on the basis that 0.9 g of starch yields approximately 1.0 g of glucose on hydrolysis.

Extraction and Estimation of Total Free Amino Acids

Extraction

Two g of air-dried defatted seed flour was extracted with 1:5 (W/V) 80% ethanol at 55°C for 1 h. The contents were filtered. The residue was extracted again with 1:3 (W/V) ethanol thrice as described above. The supernatants were pooled, cleared by centrifugation at 5000 x g for 10 min. The clear supernatant was evaporated to 2–3 ml of volume. The contents were diluted to 100 ml with distilled water in a standard flask.
Estimation (Rosen, 1957)

From suitable aliquots of the above extract, total free amino acids were estimated by the modified ninhydrin reagent method of Rosen (1957) using leucine as a standard at 540 nm in a Spectronic - 20D spectrophotometer. The values were expressed as percentage on dry weight basis.

Extraction and Estimation of Total Proteins (true protein)

Extraction (Basha et al., 1976, with slight modification)

One g of air-dried seed flour was defatted by macerating with petroleum ether (1 : 10 W/V) for 6 h. The petroleum ether extract was removed by centrifugation at 5000 x g for 10 min. The pellet was washed with petroleum ether twice and the defatted meal was washed with 100 ml of cold 10% trichloroacetic acid (TCA) and centrifuged at 20,000 x g for 15 min. The procedure was repeated, the resulting TCA-washed pellet was suspended in 50 ml of IN NaOH solution and incubated at 45°C for 16 h. The resulting hydrolysate was centrifuged at 20,000 x g for 20 min. The pellet was re-extracted with 20 ml of IN NaOH solution and centrifuged. The supernatants were pooled together.

Protein Determination (Lowry et al., 1951)

The protein from 1 ml of the pooled supernatants was precipitated with equal volume of cold 20% trichloroacetic acid (TCA) for 30 min. at 4°C. After centrifugation the protein pellet was redissolved in 0.1N NaOH and from suitable aliquots,
the protein was measured by the method of Lowry et al. (1951) using bovine serum albumin fraction V (Sigma chemical) as a standard in Spectronic 20D spectrophotometer at 750 nm. The average value of triplicate determinations were expressed as percentage on dry weight basis.

Fraction of Different Solubility Classes of Seed Proteins and Estimation

Extraction of Albumins and Globulins (Basha and Beevers, 1975)

Albumin and globulin fractions of seed proteins were extracted following method of Basha and Beevers (1975) with slight modification. Two g air-dried seed flour was extracted with 40 ml of 1M NaCl, 20mM sodium phosphate buffer, pH 7.0 at 4°C for 24 h. with constant magnetic-stirring and centrifuged at 20,000 x g for 15 min. The resulting pellet was extracted again with the same buffer and centrifuged. The pellet after centrifugation was saved for the extraction of prolamins and glutelins in sequence. The above two supernatants were pooled and made to 70% saturation by adding solid ammonium sulphate. After standing for 1 h, the contents were centrifuged at 20,000 x g for 30 min. The supernatant was discarded and the pellet suspended in 0.2M NaCl, 5mM phosphate buffer, pH 7.0 and dialysed against distilled water for 2 days with five changes of distilled water.

The dialysates were centrifuged at 20,000 x g for 15min. The supernatant fraction was designated as albumin and the pellet as globulin. The pellet was dissolved in 0.2M NaCl, 5mM phosphate buffer, pH 7.0
Extraction of Prolamins

The pellet saved from the above experiment was extracted with 75% ethanol 1:5 (W/V) overnight. The contents were centrifuged at 20,000 x g for 20 min. The supernatant was air-dried at room temperature, ground to a fine powder and dissolved in 0.1N NaOH solution.

Extraction of Glutelins

The pellet (from the above experiment) after extraction of prolamins, was extracted with 0.4N NaOH 1:10 (W/V) overnight and centrifuged at 20,000 x g for 20 min. The supernatant thus obtained was designated as glutelins.

Protein Determination

The protein content of different solubility classes of proteins separated were estimated following the method of Lowry et al (1951) after TCA precipitation, as described earlier. The values were expressed as percentage on dry weight basis.

Polyacrylamide Disc Gel Electrophoretic (PAGE) Separation of Albumin and Globulin Fractions of Seed Proteins

Electrophoresis in 7.5% polyacrylamide gel was performed according to the method of Davis (1964). The following stock solutions were prepared and stored refrigerated.

Solution A

\[ 1N \text{ HCl} = 48.0 \text{ ml} \]

\[ \text{Tris (hydroxymethyl) aminomethane} = 36.6g \]
N, N, N', N' - tetramethyl ethylene diamine = 0.46 ml

The volume was made up to 100 ml with glass distilled water, filtered and stored in amber-coloured bottle.

Solution B

Acrylamide = 30.0 g

Crystallised, N - methylene bis acrylamide (Bis) = 0.8 g were dissolved and the volume was made up to 100 ml with glass distilled water, filtered and stored in amber-coloured bottle.

Solution C

Ammonium persulphate (28 mg) was dissolved and the volume was made up to 20 ml with glass distilled water and filtered. Solution C was freshly prepared prior to use.

Reservoir (Tank) Buffer Solution

Tris = 6.0 g

Glycine = 28.8 g were dissolved and the volume was made up to 1000 ml with glass distilled water. The pH was adjusted to 8.6, filtered and stored at 4°C. 1/10 strength of the above stock buffer was used.

To prepare a gel concentration of 7.5%, the stock solutions were mixed in the proportions given below.

Solution A - 2.5 ml
Solution B - 5.0 ml
Solution C - 12.5 ml
(All the three solutions were mixed thoroughly and the gel tubes were filled immediately followed by water layering).

**Polymerization of the Gel**

Eight open-ended glass tubes (5mm x 110 mm) were stoppered with rubber caps at one end and placed with the open and up. The thoroughly mixed gel solution was pipetted into each tube to a height of 90mm taking care to avoid air bubbles. Glass distilled water was added to a height of 5 mm with a Pasteur pipette. The tip of the pipette was first placed above the surface of the gel solution and water was added slowly to avoid mixing. A sharp refractive boundary was visible between the gel solution and water. This step was found to be critical in obtaining high resolution. The entire set up was allowed to stand undisturbed for 20 min. and the water layer was sucked out with the help of Pasteur pipette after polymerization.

**Electrophoresis**

Electrophoresis was performed in an apparatus containing two perspex containers or tanks (upper tank 6.5/9.7 Cm and lower tank 7.5/9.7 Cm). Each contained a platinum electrode placed vertically in the centre. These two tanks were filled each time with buffer solution. The upper container was provided with 8 holes at its bottom at equal distances along the circumference, 2 Cm from the periphery. Each hole was fixed with a rubber grommet of 7 mm (ID) which permitted a water tight seal when the tubes containing polymerized gels were subsequently inserted.
After polymerization of the gels in the tube, albumin and globulin protein samples 180-200 \( \mu g \) in 10% sucrose were loaded per gel tube studying the protein profiles. The caps were removed from the bottom of the tubes in such a way that suction did not displace the gels from the walls of the tubes. Air bubbles, if any, trapped at base of these tubes were removed and the resultant space filled with tris-glycine buffer. The upper ends of the tubes were inserted into the rubber grommets at the bottom of the upper container. Tank buffer was pipetted into each tube carefully without disturbing the sample solution until the level reached the grommets. The upper and lower containers (tanks) were then filled with 500 ml of ice-cold 1/10 strength of tank buffer (tris-glycine). A few drops of 0.5% (W/V) alcoholic bromophenol blue was added to the upper buffer solution. The power supply was connected, cathode to the top and anode to the bottom. Initially for 10 min. the current supply was 2mA per tube and later increased to 3mA per tube for about 2½ h.or until the tracking dye had migrated to a distance of about 85 mm into the running gel. The entire process was carried out at 4°C.

**Staining and Destaining of the Gel for Proteins**

At the end of electrophoresis, the gel columns were loosened from their tubes by gently rimming them with a blunt needle under cold distilled water. They were stained immediately with 0.5% amido black (Sigma chemical) dissolved in 7% acetic acid for 2h. The gels were destained with 7% acetic acid, by shaking them
in a shaker until the bands were clear. The gels were stored in 7% acetic acid, until they were photographed/scanned. The destained gels were scanned in Schimadzu Gel Scanner Model, UV-240 in the range of 640-680 nm.

Automated Amino Acid Analysis of Purified Total Seed proteins

The total seed proteins from seed flour, were extracted by following the method of Basha et al (1976) as described earlier. After extraction the proteins were purified by cold 20% TCA precipitation and centrifuged as has been described already. Three hundred mg of seed proteins thus obtained were hydrolysed by refluxing in 6N HCl at 110°C for 24h in vacuo. The contents were centrifuged. The clear supernatant was dried in a rotary evaporator to remove the acid. After washing with glass distilled water and drying 3 times, the dried residue was dissolved in known volume of citrate buffer, pH 2.2. Known aliquots were analysed in LKB-Biochrome Automated Amino Acid Analyser, Model-4151 Alpha plus. The different amino acid contents are presented as g/100g protein.

Extraction of Total Lipids and HPLC Analysis of Free Fatty acid methyl esters (Schuster, 1985)

Total lipids were extracted from 3 g of seed flour with a mixture of chloroform and methanol in the ratio of 2:1, respectively following the method of Folch et al (1957). To the total lipids extracted from the seed flour, 10 ml of ethanol, 3 ml of 28% ammonium hydroxide, 25 ml of petroleum ether and 25 ml
of diethyl ether were added in a separating funnel, shaken for 5 min. and allowed to stand for 20 min. Later, the bottom phase was drained off and the ether phase was dried to which 3 ml of 0.5N NaOH in methanol was added and heated in a steam bath for 15 min. To this, 5 ml of water followed by 2 N HCl were added slowly until the pH was approximately 2. The fatty acid methyl esters were then extracted with 5 ml of petroleum ether and 5 ml diethyl ether from the acidified methylated lipid extract. Fatty acids were analysed using a Hewlett Packard HPLC system using the following parameters.

Column : Hypersil O.D.S. µm column

Mobile phase :

Solvent A - Water
Solvent B - Acetonitrile
Flow rate - 0.45ml/min
Stop time - 25 min

Solvent gradient :

At 5 min : % B30
At 15 min : % B70
At 17 min : % B80
At 25 min : %B100

Detection wave length : 230 nm

Retention time and area percentages of fatty acid methyl esters were recognized. Identifications were tentatively made based on the retention time.
Analysis of Mineral Profiles of the Seed

The minerals like zinc, copper, manganese, iron, and lead were analysed by employing Atomic Absorption Spectrophotometer. The other five minerals (calcium, magnesium, sodium, potassium, and phosphorus) were estimated by following different methods.

Mineral Analysis by Atomic Absorption Spectrophotometer

Sample Digestion

Five hundred mg of air-dried seed flour was mixed with 10 ml of conc. HNO₃ and 4 ml of 60% perchloric acid and 1 ml of conc. H₂SO₄ and the contents were kept for overnight. After that it was heated on a hot plate containing conc. H₂SO₄ in a beaker until the brown fumes ceased coming out and then allowed for cooling. After cooling, it was filtered through Whatman NO-42 filter paper. After filtration the filtrate was made up to 100 ml with glass distilled water.

Estimation (Issac and Johnson, 1975)

By feeding the sample to an Atomic Absorption Spectrophotometer (PERKIN-ELMER, Model-5000), the following elements were estimated with appropriate wave lengths.

<table>
<thead>
<tr>
<th>Name of the mineral</th>
<th>Wavelength used for estimation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Copper</td>
<td>324.6 nm</td>
</tr>
<tr>
<td>Zinc</td>
<td>213.7 nm</td>
</tr>
<tr>
<td>Manganese</td>
<td>279.4 nm</td>
</tr>
<tr>
<td>Iron</td>
<td>246.8 nm</td>
</tr>
<tr>
<td>Lead</td>
<td>283.1 nm</td>
</tr>
</tbody>
</table>
The mineral contents were expressed as mg/100g of seed flour on dry weight basis.

Estimation of Calcium and Magnesium

Calcium

Five ml of triple acid digested extract was taken in a China dish, to which 10 ml of 10% NaOH and 0.1g of murexide indicator powder (40 g of potassium sulphate or potassium chloride was ground with 10g ammonium purpurate) were added and titrated against 0.02N versenate. (19g of EDTA was dissolved in 5 litres of distilled water and standardized against 0.2N sodium carbonate solution) and adjusted until the colour changes from red to violet.

Calcium and Magnesium (Jackson, 1967)

Five ml of triple acid digested extract was taken in a China dish. To this 10 ml of ammonium chloride - ammonium hydroxide buffer, pH 10 and few drops of Eriochrome Black T indicator (0.1g Eriochrome Black T was dissolved in 25 ml of methanol containing 1g of hydroxylamide hydrochloride) were added and titrated against 0.02 versenate solution until the colour changes from red to blue.

Calculation

Percentage of calcium in the sample = Titre value of calcium x \( \frac{100}{5} \) x \( \frac{100}{0.5} \) x 0.0004

Percentage of magnesium in the sample = Titre value of calcium + magnesium - titre value of calcium

or
Titre value of calcium + magnesium x 0.96. Calcium and magnesium contents were expressed as mg/100g of seed flour.

**Estimation of Sodium and Potassium**

Sodium and potassium were estimated by using Flame Photometer, Model-EEL. The sodium and potassium contents were calculated by referring to the calibration curves of sodium and potassium, respectively, and expressed as mg/100g of seed flour.

**Phosphorus Estimation** *(Dickman and Bray, 1940)*

One ml of triple acid digested extract was pipetted into 100 ml volumetric flask. To this 50 ml glass distilled water was added followed by 5 ml of ammonium molybdate - sulphuric acid reagent (Solution A : 25 mg of ammonium molybdate was dissolved in 100 ml of distilled water. Solution B : 280 ml of conc. H₂SO₄ was diluted to 800 ml. Solution A was added slowly with constant stirring to solution B and the volume was made up to 1000 ml with glass distilled water). Blue colour was developed by adding six drops of 2.5% stannous chloride solution. The total volume was made up to 100 ml. The intensity of the blue colour was measured at 650 nm in a spectrophotometer. The phosphorus content present in the sample was calculated by referring to a standard curve of phosphorus and expressed as mg/100g of seed flour.

**Vitamin Analysis of the Seed Flour**

**Extraction and Estimation of Ascorbic Acid** *(Hawks, 1954)*

Ascorbic acid is oxidised to dehydroascorbic acid by bromine water. The excess bromine is removed by aeration. The dehydro-
ascorbic acid is treated with thiourea and then coupled with 2,4-dinitrophenylhydrazine and finally aerated with 85% H_2SO_4 to produce a red colour which is measured spectrophotometrically at 540 nm.

**Extraction**

One g of air-dried seed flour was taken and ground with small amounts of 4% oxalic acid, centrifuged and the supernatant was collected and made up to 25 ml. From the above supernatant 10 ml of the solution was pipetted out into a clean dry conical flask, a drop of bromine water was added and the excess of bromine was removed by aeration and was made up to 50 ml with 4% oxalic acid. Similarly 100 mg of ascorbic acid was weighed and made up to 100 ml with 4% oxalic acid. 10 ml of the stock was pipetted out into a 100 ml standard flask and made up to the mark with 4% oxalic acid. From this 10 ml was taken and transferred to a clean dry conical flask and few drops of bromine water was added till the solution became yellow. The excess of bromine was aerated and the solution was made up to 50 ml with 4% oxalic acid. Five ml of this stock solution contains 100 μg/ml of ascorbic acid.

**Estimation**

A suitable aliquot obtained from the above extract was taken into a test tube. To all the tubes a drop of 10% thiourea solution and 1.0 ml of 2,4-dinitrophenylhydrazine (2,4-DNPH) were added and made up to 6 ml with 4% oxalic acid. After incubating
for 3 hrs. at 37°C, the tubes were removed and cooled in ice. After adding 4 ml of 85% H\textsubscript{2}SO\textsubscript{4}, the colour was read at 540 nm in a Spectronic 20D spectrophotometer using ascorbic acid as a standard. The values were expressed as percentage on dry weight basis.

**Extraction and Estimation of Niacin (NIN/ICMR, 1983)**

**Extraction**

Niacin reacts with cyanogen bromide to give a pyridinium compound which reacts with aromatic amine like aniline to give a yellow colour. This colour is read at 420 nm in a spectrophotometer.

One g of seed flour was ground with 30 ml of 4N H\textsubscript{2}SO\textsubscript{4}, the mixture was steamed for 30 min. After transferring to a 50 ml flask with distilled water, the solution was filtered and 5 ml of 60% lead acetate solution was added. The pH was adjusted to 9.5 using thymol blue, till a light blue colour developed. After centrifugation, 2.0 ml of conc. H\textsubscript{2}SO\textsubscript{4} was added to the supernatant and left for an hour. After that, any lead sulphate that was precipitated, was centrifuged off and 5 ml of 40% zinc sulphate was added. The pH was adjusted to 8.4 with 10N NaOH, phenolphthaleine gives a slight pink colour at the end point. Once again, the supernatant was collected and the pH was adjusted to 7.0 using bromothymol blue as indicator. The solution turned just green.
Estimation

One ml of the above extract was taken in a test tube and was made up to 6 ml with distilled water. Cyanogen bromide solution was added shaken well and after 10 min. 1ml of 4% aniline was added to each of tubes. The yellow colour developed was read after 5 min. in a Spectronic 20D spectrophotometer at 420 nm, using niacin as a standard. The values were expressed as percentage on dry weight basis.

Antinutritional Factors of the Seed

Extraction and Estimation of L-DOPA (3,4-dihydroxyphenylalanine) (Brain, 1976)

One g seed flour was extracted with 5 ml of 0.1N HCl over a boiling water bath for 5 min. After cooling, an equal volume of ethanol was added. The mixture was shaken mechanically for 10 min. The contents were centrifuged at 5,000 x g for 10 min. The supernatant was retained. The pellet was reextracted with an equal volume of ethanol and the extract was clarified by centrifugation. Both the supernatants were combined and made up to a known volume with ethanol.

L-DOPA content in the extract was quantified by measuring the ultraviolet absorption at 283 nm (in a Wibro-Beckman Spectrophotometer, Model-26) after correction for background absorption. Using L-DOPA (Sigma chemical) as a standard, the content of L-DOPA in the samples was calculated and expressed as percentage on dry weight basis.
Extraction and Estimation of Tannins, Total Free Phenols from Seed Kernel and Coats

Extraction of Tannins and Total Free Phenols (Maxon and Rooney, 1972)

One g of air-dried powders of seed kernel and seed coats were taken in 100 ml flasks, separately to which was added 50 ml of 1% HCl in methanol. The samples were shaken on reciprocating shaker for 24h at room temperature. The contents were centrifuged at 10,000 xg for 5 min. The supernatants were collected, separately.

Estimation of Tannins (Burns, 1971)

From suitable aliquots of the above extract, tannin contents were quantified by Vanillin - HCl method using phloroglucinol as a standard at 500 nm in a Spectronic 20D spectrophotometer. The average values of triplicate estimations of all samples were expressed as g/100g seed kernel or g/100g of seed coats on dry weight basis.

Estimation of Total Free Phenols (Bray and Thorne, 1954)

One ml of the above extract was pipetted out into different test tubes to which 1 ml of folin-ciocalteu reagent followed by 2 ml of sodium carbonate solution were added and the tubes were shaken and placed in a boiling water bath for exactly 1 min. The test tubes were cooled under running tap water. The resulting blue solution was diluted to 25 ml with distilled water and the absorbance was measured at 650 nm in a Spectronic 20D spectrophotometer. Whenever precipitation occurred, it was removed by centrifugation at 5000 x g for 10 min. before measuring the absor-
The amount of phenols present in the samples was determined from a standard curve prepared with catechol. A blank containing all the reagents minus extract was used to adjust the absorbance to zero. Average values of triplicate estimations were expressed as g/100g of the seed kernel or g/100g of seed coats on dry weight basis.

**Assay for Trypsin Inhibitor Activity** (Chrispeels and Baumgartner, 1978)

Five hundred mg of air-dried seed flour was extracted with 10 ml of ice-cold distilled water for 3h. in the cold with occasional shaking. The contents were centrifuged at 20,000 x g for 15 min. The supernatant was used as the source of trypsin inhibitor activity.

Trypsin inhibitor activity was measured by adding increasing aliquots of extract (100-400 µl) to a standard amount of trypsin (2µg) and 1.7 mg of benzoyl arginine - P-nitroanilide (trypsin and benzoyl arginine-P-nitroanilide were received as gift chemicals from Prof. Irvin E. Liener, Minnesota University, U.S.A.) in a total volume of 3 ml in 25mM tris-HCl buffer, pH 8.0 for 30 min. at 37°C and stopped by the addition of 1N acetic acid. The absorbance at 410 nm was measured and the data were plotted to determine the aliquot size necessary to give a 50% inhibition of enzyme activity under the above conditions. The protein content of the extract was determined according to the method of Lowry et al (1951).
Assay for Haemagglutinating Activity (Liener, 1976c)

Albumin and globulin protein fractions (as obtained under fractionation of different solubility classes of seed proteins) were employed as protein samples for determining haemagglutinating activity. Human blood (blood groups A, B and 0) were procured from blood bank of Ray Vijay Clinical Laboratory, Coimbatore.

Preparation of Red Blood Cells

Blood erythrocyte suspension was prepared by washing the blood samples (A, B and 0) separately, with phosphate-buffered-saline and centrifuged for 3 min. at low speed 1000 x g supernatants were removed with Pasteur pipette. The washing procedure was repeated for three times. The washed cells were diluted by mixing 12 drops of cells with 24 drops of phosphate-buffered-saline.

Extracts (5 drops) of albumin and globulin protein fractions were mixed, separately, with an equal volume of different groups of blood erythrocyte suspension and allowed to stand for 20 min. at room temperature. Then spinned for 3 min. gently at 1000 x g. After centrifugation the tubes were gently shaken, the presence or absence of haemagglutinating activity was reported as given below.

- : No clumping, pellet disperses easily
+ : Clumping, pellet partially disperses
++ : Clumping, no dispersion of pellet

Analyses were performed independently twice.
Physiology of Seed Germination

Treatments Employed to Overcome Seed Dormancy

The seeds of *M. monosperma* are large-sized and dark brown in colour. They have hard and thick seed coats. The collected seeds stored in plastic containers at room temperature for more than 4 weeks, when sown in the experimental plots of botanic garden, did not germinate. Hence in the present study an attempt was made to overcome seed dormancy in both the collected germplasms.

After storing the collected germplasm seed materials for more than four weeks, several lots of healthy seeds more or less of uniform weight (7.0 ± 0.5g of Kerala germplasm and 6.0 ± 0.5 g of Tamilnadu germplasm) were separated. One lot of seeds for each germplasm was maintained as control by sowing in different experimental plots. The other lots of seeds from both the collected germplasms were subjected to the following physical and chemical treatments.

Physical Treatments

Each lot from both the collected germplasm seed materials at a time were,

1. soaked separately in running tap water for 24h, 48h and 72h and later sown in the experimental plots,
2. roasted on a hot plate for 30 min. and later sown in experimental plots,
3. boiled in water separately for 30 min. and later sown in separate experimental plots.
4. punctured with a sharp nail and later sown in separate experimental plots.

Chemical Treatments

Separate lots of seeds from both the collected germplasms were subjected to the below mentioned chemical treatments. After each treatment the treated seeds were washed thoroughly and sown in separate experimental plots.

1. Soaking in conc. H$_2$SO$_4$ for 4h
2. Soaking in 70% H$_2$SO$_4$ for 4h
3. Soaking in conc. HCl for 4h
4. Soaking in conc. HNO$_3$ for 4h
5. Soaking in 20% NaOH for 4h

Thirty days after sowing in different experimental plots in the botanical garden, the percentage of seed germination for control samples as well as the various other samples subjected to physical and chemical treatments were calculated and the results were presented.

Studies on Seed Germination

Plant Material

Seeds of both the germplasms, more or less of the same weight as mentioned earlier were punctured with a sharp nail and were soaked in running tap water overnight. The next day, the imbibed seeds were washed thoroughly with distilled water and germinated at room temperature in sterile vermiculite. The seedlings were irrigated with distilled water. The addition of water to
the dry seeds were taken as zero (0) time germination. The cotyledons were collected at 3 day intervals during the course of germination from day 0 to 21. The collected cotyledons and the embryonal axes were kept on shaved ice until they were used.

**Determination of Fresh and Dry Weights of Cotyledons of Germinating Seeds**

Three to five pairs of cotyledons in 3 replications were harvested, washed thoroughly in tap water, followed by rinsing with distilled water, blotted gently and their fresh weights were determined. Immediately, the cotyledons were dried in an electric oven at 80°C for 24h. The dried cotyledons were cooled and their weights were determined. The results on fresh weight and dry weight of a pair of cotyledons were expressed in g/pair of cotyledons.

**Determination of Fresh and Dry Weights of the Developing Embryonal Axes**

The plumule emerges out on the 12th day after imbibition. The growing embryonal axes were harvested at 3-day intervals during the course of germination from day 12 to 21. Three to five axes in 3 replications were harvested, washed thoroughly in tap water, followed by rinsing with distilled water, blotted gently and their fresh weights were determined. Immediately, the axes were dried in an electric oven at 80°C for 24h. The dried axes were cooled and their weights were determined. The results were expressed in g/embryonal axis.
Extraction and Estimation of Total Soluble Carbohydrates and Starch in the Germinating Cotyledons/Embryonal Axes

One g of cotyledons/embryonal axes was homogenised to a paste in a mortar with a pestle. The paste was extracted with hot 80% ethanol for the estimation of total soluble carbohydrates and the residue was extracted with 52% perchloric acid for the estimation of starch respectively, as has been mentioned earlier.

Estimation

Estimation of total soluble carbohydrates and starch was carried out as has been described earlier. The total soluble carbohydrates and starch contents were calculated and expressed in mg/pair of cotyledons or mg/embryonal axis on dry weight basis.

Extraction and Determination of the Activities of the Enzymes, α-amylase and β-amylases, from the Germinating Cotyledons/Embryonal Axes (Bernfeld, 1955; Kruger, 1972; Niku-Paavola et al., 1972)

Extraction of α-amylase

One g of the cotyledons/embryonal axes was macerated to a paste in a pre-cooled mortar with a pestle and extracted with 5-10 ml (W/V) of ice cold 10 mM calcium chloride solution overnight at 4°C. The contents were centrifuged at 54,000 x g at 4°C for 20 min. by using an ultracentrifuge (Beckman, Model-1260). The supernatant was used as the enzyme source.

Extraction of β-amylase (free form)

The free β-amylase was extracted from acetone defatted samples (one g of cotyledons/embryonal axes), with 66 mM phosphate
buffer, pH 7.0, containing 0.5 M sodium chloride. The extract was centrifuged at 20,000 x g for 15 min. in a Super Speed Refrigerated Centrifuge, Model MB-20. The supernatant was used as the source of free form of α-amylase.

Extraction of β-amylase (bound form)

The residue obtained from the above step was extracted with 66 mM phosphate buffer, pH 7.0, containing 0.5% 2-mercaptoethanol and centrifuged at 20,000 x g for 15 min. The clear extract was used as the source of bound form of β-amylase. All steps were carried out at 4°C.

Determination of Activities of the Enzymes, α-amylase and β-amylases, from the Extracts of the Germinating Cotyledons/embryonal Axes

The reducing sugars produced by the action of α- and/or β-amylase react with dinitrosalicylic acid and reduced to a brown-coloured product, nitroamino-salicylic acid.

Materials Required

Sodium acetate buffer, 0.1M, pH 4.7

1% Starch Solution

It was prepared by dissolving one g of starch in 100 ml of 0.1M sodium acetate buffer, pH 4.7.

Dinitrosalicylic Acid Reagent

One g of dinitrosalicylic acid was dissolved in 20 ml NaOH and 50 ml water. Then 30g of sodium potassium tartarate was added and made up to 100 ml with distilled water.
Maltose Solution

50 mg of maltose was dissolved in 50 ml distilled water in a standard flask and stored in a refrigerator.

Assay

0.1 ml of all the three enzyme extracts were diluted to 1 ml uniformly with distilled water. To this 1.0 ml of 1% starch solution was added, and then incubated at 27°C for 15 min. The reaction was stopped by adding 2 ml of dinitrosalicylic acid reagent. Then, the solution was heated in a boiling water bath for 5 min. and cooled under running tap water. The volume was made up to 10 ml by adding 6 ml of distilled water. Later the absorbance of the diluted reaction mixtures were read at 560 nm. A standard graph was prepared with 0-100 μg maltose.

Calculation

A unit of α- or β- amylase was expressed as the number of mg of maltose produced during 15 min. incubation with 1% starch solution. The activities of all the three enzymes assayed were expressed as mg of maltose released during 15 min. of incubation of diluted enzyme extracts with 1% starch solution.

Extraction and Estimation of Different Solubility Classes of Seed Proteins from Different Stages of the Germinating Seed Cotyledons/Embryonal Axes

One g of cotyledons/embryonal axes was homogenised to a paste in a pre-cooled mortar with a pestle. The albumin and globulin fractions of proteins were extracted following the method of Basha.
and Beevers (1975) which has been described earlier. The pellet saved from the above experiment was extracted with 0.4N NaOH 1:10 (W/V) overnight and centrifuged at 20,000 x g for 20 min. The supernatant contains both the prolamin and glutelin fractions.

**Protein Determination**

The protein content of different solubility classes of proteins separated were estimated following the method of Lowry et al (1951) after TCA precipitation, as has been described already. The values were expressed as mg/pair of cotyledons or mg/embryonal axis on dry weight basis. The total protein content was calculated by adding the values of albumin, globulin, prolamin and glutelin contents.

**PAGE profiles of the Albumins and Globulins Extracted from the Germinating Seed Cotyledons**

The profiles of albumins and globulins extracted from different stages of germinating seed cotyledons were obtained by electrophoresis in 7.5% polyacrylamide gel following the method of Davis (1962), elaborately described elsewhere. One hundred and eighty to two hundred µg of albumin and globulin proteins were loaded per gel.

**Protease Assay (Caseolytic Activity)** (Basha and Cherry, 1978; method with slight modification)

**Extraction**

One g of the cotyledons/embryonal axes was homogenised to a paste in a pre-cooled mortar with a pestle. The paste was
extracted with 1:2-5 (W/V) 25mM citrate-phosphate buffer, pH 5.4, containing 10mM 2-mercaptoethanol. The homogenate was centrifuged at 20,000 x g for 20 min. at 4°C. The supernatant thus obtained served as the source of protease for determining the caseolytic activity. The protein content of the supernatant was determined after cold 20% TCA precipitation according to the method of Lowry et al (1951).

Assay

One ml of enzyme extract from cotyledons/embryonal axes of the germinating seeds was added to 1.0 ml of 0.5% (W/V) casein solution (0.5 g of casein dissolved in 100 ml of 25mM citrate-phosphate buffer, pH 5.4) in a water bath set at 37°C for 2 h. The reaction was terminated by adding 1.0 ml of 20% TCA. A blank was kept without adding the enzyme extract. The samples were allowed to precipitate overnight at 4°C and were centrifuged at 5000 x g for 10 min. Aliquots from the TCA-soluble supernatants were analysed for increase in ninhydrin positive materials (proteolytic activity) by the method of Rosen (1957). Proteolytic activity was presented on the basis of mg protein/2 h/mg amino acids released.

Extraction and Estimation of RNA and DNA in the Germinating Seed Cotyledons/Embryonal Axes (Jayaraman, 1981)

The procedure for the extraction of nucleic acids (RNA and DNA) is given in the form of a flow chart.
The material was homogenised in a mortar with pestle with 5ml of ice cold 10% TCA (W/V).

- Centrifuged at 3000 x g for 10 min
- Supernatant was discarded
- The precipitate was suspended in 5 ml of ice cold 10% TCA
  - Centrifuged at 3000 x g for 10 min
- Supernatant was discarded
- The precipitate was suspended in 5ml of ethanol : ether mixture (1 : 1)
  - Centrifuged at 3000 x g for 10 min
- Supernatant was discarded
- The precipitate was suspended in 5ml of 0.5 N NaOH, mixed well and kept at 37°C for 16h
  - Centrifuged at 3000 x g for 10 min
- Supernatant contains RNA in hydrolysed form as well as proteins
  - Centrifuged at 3000 x g for 10 min
- Precipitate contains some proteins and most of DNA. One ml of perchloric acid was added and heated in a boiling water bath for 10 min. and cooled.

- Precipitate was discarded
- Supernatant contains nucleotides released from RNA. The volume was made up to a known value
- Supernatant contains nucleotides released from DNA. The volume was made up to a known value.
Estimation of RNA (Jayaraman, 1981)

A suitable aliquot of RNA extract obtained from the above mentioned procedure presented in the form of a flow chart was taken into a test tube and the volume was made up to 2 ml with distilled water. To this, 3 ml of orcinol reagent was added and the contents of the test tube were boiled in a water bath for 15 min. After cooling, the concentration of RNA in the samples was quantified at 665 nm in a Spectronic 20D spectrophotometer, using commercial yeast RNA (Sigma chemical) as a standard. The values were expressed in mg/pair cotyledons or mg/embryonal axis.

Estimation of DNA (Jayaraman, 1981)

A suitable aliquot of DNA extract obtained from the above mentioned procedure given in the form of a flow chart was taken into a test tube and the volume was made up to 3 ml with distilled water. To this 5 ml of diphenylamine reagent was added and heated in a boiling water bath for 15 min. After cooling, the DNA content of the samples were quantified in a Spectronic 20D spectrophotometer at 595 nm using calf-thymus DNA (Sigma chemical) as a standard and the values were expressed in mg/pair of cotyledons or mg/embryonal axis on dry weight basis.

Extraction and Determination of the Activity of Peroxidase in the Germinating Seed Cotyledons/Embryonal Axes

Extraction

One g sample material was ground to a paste in a chilled mortar with a pestle using one of the acid washed sand. The paste was extracted with 1:2.5 (W/V) extraction medium (tris-HCl buffer
10 mM, pH 7.3, containing 0.1% (W/V) sodium ascorbate; 0.1% (W/V) cysteine -HCl and 17% (W/V) sucrose). The homogenate was incubated with 1:1 (W/V) polyvinylpyrrolidone (PVPP, Sigma chemical) for 15 min. The homogenate was squeezed through 4 layers of cheese cloth and centrifuged at 16,000 x g for 30 min. at 4°C. The supernatant was used as the enzyme source.

**Measurement of Peroxidase Activity** (Perur, 1962)

One ml aliquots of enzyme source as obtained above were added to tubes containing 20 ml of sodium acetate buffer, pH 4.5, to which 0.5 ml of 0.05N H₃O₂ and 0.5 ml of 1% aqueous solution of pyrogallol were also added at 'O' time (i.e. time of adding of the reagents H₂O₂ and pyrogallol). A blank was maintained by adding all the above mentioned reagents without adding enzyme source. Ten min. after mixing all the reagents (i.e. 'O' time) in both the blank and various samples, the absorbance was read at 425 nm using a Spectronic 20D spectrophotometer. The peroxidase activity was expressed as OD of 'O' time and after 10 min. of incubation for all the samples assayed.

**PAGE Pattern of the Isoenzymes, α-amylose and β-amyloses (free and bound forms), from the Extracts of Germinating Cotyledons**

**Electrophoresis**

The isoenzyme pattern of α-amylose and β-amyloses (free and bound forms) extracted from the cotyledons of different stages of germinating seeds was studied employing polyacrylamide gel electrophoresis. Disc gel electrophoresis in 7.5% polyacrylamide
with 0.5% starch was performed according to the method of Davis (1964) as has been described earlier. The protein estimation of the enzymes was carried out according to the method of Lowry et al (1951) after cold 20% TCA precipitation as has been described already. One hundred and eighty to two hundred μg of protein was loaded per gel.

**Staining Method for α-amylase and β-amylases (free and bound forms)** (Tanksley and Orton, 1983)

**α-amylase**

Endohydrolysis of 1,4-α-D-glycosidic linkages in polysaccharides containing three or more 1,4 α-linked D-glucose units.

**β-amylases (free and bound forms)**

Hydrolysis of 1,4-α-D-glycosidic linkages from the non-reducing end of polysaccharide chains with the liberation of successive maltose units.

Stain: Starch-iodine system

**Staining Solutions**

A. Sodium acetate buffer 50mM pH 5.6 - 100 ml
   Calcium chloride 1 M - 2 ml

B. Iodine 10 mM; Potassium iodide
   14 mM 1:1 (V/V) - 100 ml

**Procedure**

The gels were incubated in solution A at 30-50°C for 1h. Solution A was discarded, and the gels were rinsed thoroughly with distilled water. The gels were stained with solution B for 3-5 min.
zones of enzyme activities appeared as transparent bands against a dark-blue background. Iodine solution was discarded. The gels were rinsed with water and the zymograms were constructed immediately since the zones of colour developed tend to fade out quickly at the end of the reaction.

**Staining of Peroxidase isoenzymes (Pandey, 1967)**

Gel electrophoresis in 7.5% polyacrylamide was performed according to Davis (1964), described earlier. One hundred and eighty to two hundred μg of protein (enzyme) was loaded per gel.

To 100 ml of 7% acetic acid was added 16.8 g of sodium acetate and the resultant solution was saturated with 1% EDTA. The solution was filtered, saturated with benzidine hydrochloride (Sigma chemical, USA) and filtered again. 0.1 ml of 3% H₂O₂ was added to the above solution before incubating the gels. After incubation, the gels were washed thoroughly and stored in 7% acetic acid until they were examined for the construction of zymograms.