MATERIALS AND METHODS
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Biochemical and Nutritive Evaluation

Plant material: About 100 g seeds of *Iruguca utilis* Wall ex Wt. were procured originally from the Botany Field Laboratory at Maduravoyal, University of Madras, Madras in August 1978 from Dr. S. Janaki Ammal, Professor Emeritus, Department of Botany, Centre for Advanced Study in Botany, University of Madras, Madras. Subsequently, plants were raised every year in the plots of Botanical garden, Department of Botany, Bharathiar University, Coimbatore and the seeds were multiplied.

Chemicals

The chemicals used were from BDH (AR) or Sigma Chemical Company, St. Louis MO, USA throughout the study unless and otherwise specified.

Proximate analysis

Determination of moisture content:

The air-dry mature seeds (50 seeds at a time) were weighed, cut transversely with a sharp knife and incubated in an electric oven at 135°C for 2 hours. Then the samples were cooled in a desiccator and the weight was taken. The average loss in weight of the samples was calculated as moisture content and expressed on percentage basis.
Crude protein content

Preparation of seed flour:

About 50 g of air-dry and oven-dried seeds were powdered separately in a Wiley Mill Grinder to 60 mesh size. The fine seed powder, so obtained, will be referred hereafter as air-dry or oven-dried seed flour.

Digestion:

Nitrogen content of the seed flour was determined by microkjeldahl method (Humphries, 1955). One hundred mg of oven-dried seed flour was taken into a microkjeldahl digestion flask. To this 2 ml of 5% salicylic acid dissolved in conc. H₂SO₄ was added and mixed well. After 20 min, 0.3 g 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, 3 g potassium sulphate and 1 g selenium dioxide) followed by 1 ml of conc. H₂SO₄ 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 microkjeldahl 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 (33.3 mg of bromocresol green + 16.6 mg of methyl red dissolved in 10 ml of 95% ethanol).
The contents were titrated against N/50 sulphuric acid. 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 0.00020 g of N, which forms the basis for calculation of N content in the sample.

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

Ether extract (AOAC, 1970):

Two g of air-dried seed flour was extracted with ether in a soxhlet apparatus for 16 h according to the 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 on dry weight basis.

Crude fiber (AOAC, 1970):

After extraction with ether, the left-out residue was successively digested with 0.255 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 and ignited in an electric muffle furnace. After cooling, the contents were reweighed. The loss in weight was expressed as percentage of crude fiber on dry weight basis.
Ash content (AOAC, 1970):

Two g of oven-dried seed flour was weighed into a pre-weighed porcelain crucible. The crucible with the seed flour was placed in a 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 gave the ash content. The ash content was expressed as percentage on dry weight basis.

Nitrogen free extractives (NFE)

Percentage of nitrogen free extractives was calculated as given below:

\[ % \text{NFE} = 100 - (CP\% + EE\% + CF\% + Ash\%) \]

Where, CP = Crude protein
EE = Ether extract and
CF = Crude fiber

Total soluble carbohydrates
Extraction:

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

Estimation (Yorn and Willis, 1954):

From aliquots of the above extract, total soluble carbohydrates were estimated by the anthrone reagent method.
of Yemm and Willis (1954) using glucose as a standard at 620 nm in a Gilford spectrophotometer. The values were expressed as percentage on dry weight basis.

Extraction and estimation of total protein (true protein):

Extraction procedure (Basha et al., 1976):

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 (in a Sorval RC 5 B centrifuge). The procedure was repeated and the resulting TCA-washed pellet was resuspended twice in 80% ethanol (100 ml), centrifuging after each treatment, and finally air-dried. This material was suspended in 50 ml of 1 N 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 1 N NaOH solution and centrifuged. The supernatants were pooled.

Protein determination

The protein from 0.1 ml of the pooled supernatant was precipitated with cold 12.5% TCA for 30 min at 4°C. After centrifugation the protein pellet was redissolved in 0.1 N NaOH and from suitable aliquots, the protein was measured by the method of Lowry et al. (1951) using bovine serum albumin fraction V as a standard in a Gilford spectrophotometer.
The values of triplicate experiments were expressed as percentage of total (true) protein on dry weight basis.

Fractionation of different solubility classes of seed proteins and estimation

Extraction of albumin and globulin (Basha and Deevers, 1975):

Albumin and globulin fractions of seed proteins were extracted following the method of Basha and Deevers (1975), with slight modification. Two g of air-dried seed flour were extracted with 40 ml of 1 M NaCl, 20 mM 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 once more with the same buffer and centrifuged. The pellet after centrifugation was saved for the extraction of prolamin and glutelin in sequence. The above two supernatants were pooled and made to 70% saturation by adding solid (NH₄)₂ SO₄. 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 0.2 M NaCl, 5 mM phosphate buffer, pH 7.0 and dialysed against distilled water for 2 days with 5 changes of distilled water.

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

Extraction of Prolamin

The pellet saved from the above experiment was extracted with 75% ethanol 1:5 (V/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 0.1 N NaOH solution.

**Extraction of glutelin**

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

**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.

**Quantitative and qualitative analysis of free amino acids**

**Extraction**

Two g of defatted air-dried seed flour was extracted with 1:5 (W/V) 75% ethanol at 55°C for 1 hr. The content was filtered. The residue was extracted again with 1:3 (W/V) ethanol thrice as described above. The supernatants were pooled, cleared by centrifugation at 5,000 x g for 10 min. The clear supernatant was evaporated to 5 ml volume. The content was diluted to 100 ml with distilled water.

**Estimation** (Yemm and Cocking, 1955)

From suitable aliquots of the above extract, the free amino acid content was quantified by the ninhydrin method of Yemm and Cocking (1955), using leucine as a standard in a Gilford spectrophotometer at 570 nm. The values were presented as percentage of hot 75% EtOH extractable free amino acids on dry weight basis.
Qualitative analysis of free amino acids

The free amino acids extracted and clarified as in the above experiment in 75% EtOH, were evaporated to dryness in vacuo. The residue after dissolving in a known volume of KCl-HCl buffer, pH 2.2 was analysed using a Hitachi Perkin Elmer (Model KLA 3B) automated amino acid analyser. The value of individual amino acid is presented as μg/g of seed flour.

Analysis of total amino acids of seed flour

Twenty five mg of the defatted seed meal was hydrolysed by refluxing in 6 ml of 6 N HCl at 110°C for 24 h in vacuo. The contents were centrifuged. The clear supernatant was dried in a rotary evaporator to remove acid. After washing with glass distilled water and drying 3 times, the dried residue was dissolved in a known volume of KCl-HCl buffer, pH 2.2. Aliquots were analysed in a Hitachi Perkin Elmer (Model KLA 3 B) automated amino acid analyser.

Analysis of sulphur amino acids (methionine and cysteine) and glucosamine

Sulphur amino acids and glucosamine analysis of the acid hydrolysed seed powder was carried out by Dr. C. M. Roxburgh, according to procedures normally employed in the C.S.I.R.O. Division of Protein Chemistry, Parkville, Melbourne, Australia through the kind help of Dr. David R. Murray, Department of Biology, Wollongong university, Wollongong, Australia. Cysteine was estimated as S-sulpho-cysteine following reduction of the hydrolysate with dithiothreitol and oxidation
with O₂/sodium tetrathionate according to the procedure of Inglis and Liu (1970). The amino acids are presented as percentage of total recovered residues.

Mineral composition of the seed

Preparation of ash extract:

After ashing (described earlier, under proximate analysis), the ash material was carefully transferred to a 50 ml conical flask with three washings of glass distilled water. Two drops of conc. HCl were added to the conical flask and the contents were digested over a hot plate for 10 min. After digestion the contents were transferred to a 100 ml volumetric flask with two washings of glass distilled water. The volume was made up to the mark. This extract was used for the estimation of phosphorus, calcium and iron.

Phosphorus estimation (Dickman and Bray, 1940):

One ml of ash extract was pipetted into a 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 g. of ammonium molybdate was dissolved in 100 ml of warm 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 1 litre with glass distilled water). Blue colour was developed by adding 6 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 660 nm with 'ELICO' spectrophotometer. By referring to a calibration curve obtained as above with
different aliquots of known concentration of phosphorus, the phosphorus content of the sample was calculated and expressed as mg/100 g flour.

**Calcium estimation (Cheng and Bray, 1951):**

Calcium was estimated by versenate (EDTA) titration method of Cheng and Bray (1951). Five ml of ash extract was pipetted into a 50 ml conical flask to which 2 ml of 10% H<sub>2</sub>OH solution was added. A pinch of murexide indicator was added to the contents of the flask and titrated against 0.01 M versenate solution, until the end point (rose to violet). From the volume of 0.01 M versenate consumed, the content of Ca present in the sample was calculated with the known value that 1 ml of 0.01 M versenate (EDTA) corresponds to 0.4008 mg of Ca. The Ca content was expressed as mg/100 g of seed flour.

**Iron estimation**

The iron content of the ash extract was estimated by feeding the sample to an Atomic Absorption Spectrophotometer (Fechtron Model). The iron content was expressed as mg/100 g of seed flour.

**Antinutritional factors of the seed**

L-DOPA (3,4-dihydroxyphenylalanine) extraction and estimation (BRAIN, 1975):

One g of seed flour was extracted with 5 ml of 0.1 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 re-extracted 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 Gilford spectrophotometer) after correction for background absorption. Using L-DOPA (Sigma Chemical Co., USA) as a standard, the content of L-DOPA in the seed flour was calculated and expressed as percentage on dry weight basis.

Tannin extraction and estimation (ACAC, 1970)

One g of seed flour was weighed and transferred to each of four, 500 ml conical flasks. One hundred and fifty ml of distilled water was added to each flask. To the first 3 flasks 0.1% solution of standard tannic acid was added 10 ml, 15 ml and 20 ml respectively. The flasks were heated gently, and the contents boiled for 30 min. The contents of each flask were centrifuged at 3000 x g for 20 min. The supernatant from each flask was collected separately into 4, 250 ml volumetric flasks and the volume was made up to the mark. Ten ml aliquots from each extract was later transferred to 4 different 100 ml volumetric flasks and the following were added in sequence to each flask: 75 ml of distilled water, 2.5 ml of Molin-Denis reagent (To 750 ml of distilled water 100 g sodium tungstate, 20 g phosphomolybdic acid and 50 ml of phosphoric acid were added and mixed. The mixture was refluxed for 2 h and made up to 1 litre) and 5 ml of sodium
carbonate solution (350 g sodium carbonate was dissolved in 1 litre of distilled water at 75°C). After allowing it to stand overnight, it was filtered through glass wool and the volume was made up to the mark.

After 30 min the absorbance was measured at 740 nm. From the difference in absorbance of different samples, the tannin content of the seed flour was calculated and expressed as percentage on dry weight basis.

Assay for trypsin inhibitor activity (Chrispeels and Baumgartner, 1978)

The trypsin inhibitor assay was performed following the method of Chrispeels and Baumgartner (1978). About 500 mg of air-dried seed flour was extracted with 10 ml of ice-cold distilled water for 3 h 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.

Trypsin inhibitor activity was measured by adding increasing aliquots of extract (100-400 μl) to a standard amount of trypsin (2 μg) and 1.7 mg of benzoyl arginine-p-nitroanilide (benzoyl arginine-p-nitroanilide was a gift from Prof. I. Liener, Department of Biochemistry, College of Biological Sciences, Minnesota University, St. Paul, USA) in a total volume of 3 ml in 25 ml Tris-Cl buffer, pH 8.0 with 10 mM CaCl₂. The reaction was carried out for 30 min at 37°C and stopped by the addition of 1 ml 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. One unit of activity corresponds to that amount of trypsin inhibitor which gives a 50% inhibition of enzyme activity under the above conditions. The protein content of the extract was determined according to Lowry et al. (1951).

Assay for haemagglutinating activity (Sathe and Salunkhe, 1981)

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.

The method followed for assaying haemagglutinin activity was based on that described by Sathe and Salunkhe (1981). Human blood (blood groups A, B and O) were procured from Forensic Science Laboratory, Tamil Nadu Forensic House, Madras-4. Blood erythrocyte suspension (4%) was prepared by washing the blood samples separately 5 times with 0.9% NaCl (centrifuged at 5,000 x g for 15 min after each washing) and diluting the residue to approximate volume. Aliquots (100 µl) of albumin and globulin protein fractions were mixed separately with an equal volume of different groups of blood erythrocytes suspension (4%), and allowed to stand for 2 h at 21°C. The presence or absence of haemagglutination activity was reported as + = present, and - = absent. Analyses were performed independently twice.
Determination of fresh weight, dry weight, total-N, protein-N and trichloroacetic acid soluble-N contents of developing seeds and pericarps.

Plant material:
Seeds of Mucuna utilis were sown in the experimental plots of the Botanical garden of the department of Botany, Bharathiar University, Coimbatore. Flowers were tagged at anthesis. The tagged fruits were harvested first at 10 days after anthesis (DA) and then weekly between 14 and 56 DA. Each harvest at 10 and 14 DA consisted of 10-20 fruits from each of three replications and thereafter 5-10 fruits from each of three replications.

Determination of fresh and dry weights of developing seeds and pericarps:
Immediately after harvest, the seeds and hulls (pericarps) were separated, weighed, and were dried in an electric oven at 80°C for 24 h. Both fresh and dry weights of seeds and pericarps were recorded separately. The average values for three replicate samples were given.

Determination of total-N, protein-N and TCA soluble-N contents of developing seeds and pericarps:
The dried seed and pericarp samples were powdered separately to 60 mesh size in a Willey Mill. Care was taken to thoroughly clean the various components of the Willey Mill to avoid contamination of sample-powders. The powdered samples were stored in screw-cap bottles until further use.
Determination of total-N content

The total-N content of different stages of seed and pericarp samples were determined by microkjeldahl method of Humphries (1956) as described under proximate analysis.

Determination of protein-N content (Thimann and Loos, 1957)

Two hundred and fifty mg of sample powder was macerated with 20 ml of cold 10% TCA at 4°C and centrifuged at 12,000 x g for 15 min. The precipitate was washed with 10 ml of cold 10% TCA. The pellet was suspended in 10 ml of 5% TCA and was incubated at 80°C for 30 min to remove nucleic acids. The precipitate, thus obtained after centrifugation was analysed by microkjeldahl method as described for the estimation of total nitrogen content.

Determination of TCA soluble-N content

TCA soluble-N content was obtained by subtracting TCA insoluble nitrogen (protein nitrogen) from total nitrogen. Average values of triplicate samples were presented on dry weight basis.

Polyacrylamide gel electrophoretic (PAGE) pattern of buffer-soluble proteins, albumin and globulin of developing seed cotyledons

Plant material

Seeds were sown in the experimental plots of Botany Field Laboratory, Maduravoyal of Madras University, Madras. Flowers were tagged at anthesis. The tagged fruits were harvested at 10, 14, 21, 28, 35, 42, 49 and 56 DAA.
Harvested fruits were kept on shaved ice until they were dissected to obtain the developing seeds. Ten and 14-day seeds were not dehusked before extraction since, because of their small size and the tight bond between the integuments; seeds from subsequent harvests were dehusked. Therefore, extracts from 10 and 14-day-old samples were from whole seeds while the rest of the samples after 21 DAA were from embryonic tissue (for convenience called as cotyledon extracts).

Extraction of buffer-soluble proteins

The proteins from 10- and 14-day-old seeds were extracted according to Chen et al. (1976) in order to prevent the interaction of proteins with polyphenols. The whole seed samples were ground with a pre-chilled mortar and pestle with 1:1 (W/V) acid washed sand, and the paste was ground with 1:2-3 (W/V) of 0.05 M Tris-HCl buffer, pH 7.8 containing 0.2 M NaCl, 10 mM sodium metabisulphite, 1 mM KCl, 0.1% (W/V) bovine serum albumin and 20% (W/V) Dowex-1 (200 mesh) thoroughly, squeezed through 4 layers of cheese cloth and centrifuged at 16,000 x g for 30 min at 4°C.

The cotyledons of 21, 28, 35, 42, 49 and 56-day-old seeds were ground to a paste in a pre-chilled mortar with 1:1 (W/W) acid washed sand. The paste was ground with 1:5 (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/W) insoluble polyvinyl polypyrrolidone (PVPP, Sigma, USA)
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.

**Protein estimation:**

The proteins from 0.1 ml of the clear supernatant were precipitated with cold 12.5 TCA for 30 min at 4°C. The contents were centrifuged at 5,000 x g for 15 min and the pellet was dissolved in 0.1 M NaCl, and the protein was estimated according to Lowry et al. (1951).

**Extraction and separation of albumin and globulin from developing seed/cotyledons (Chassa and Beevers, 1975)**

The method followed for extraction and separation of albumin and globulin was essentially that of Chassa and Beevers (1975) with slight modification.

Samples of developing seed/cotyledons (excepting 10-day-old seeds) were ground to a paste in a pre-chilled mortar with 1:1 (V/V) acid washed sand. Then the paste was ground with 1:5-10 (V/V) 1 M NaCl, 20 mM sodium phosphate buffer, pH 7.0 and allowed to stand for 1 h at 4°C. The extracts were centrifuged at 20,000 x g for 15 min. The resulting pellet was again extracted with 1:2.5 (V/V) of the same buffer and centrifuged. The supernatants were pooled. A portion of the supernatant was used for protein estimation.

The proteins of the pooled supernatant were precipitated with solid (NH₄)₂SO₄, at 70% saturation. The rest of the procedure followed in separating albumin and globulin was the
same as has already been described under fractionation of different solubility classes of seed proteins.

**Protein estimation**

The protein content of water-soluble (albumin), salt-soluble (globulin) and the combined supernatants before (NH₄)₂SO₄ precipitation were estimated after TCA precipitation according to Lowry et al. (1951).

**Polyacrylamide gel electrophoresis (PAGE)**

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.**

1 N HCl - 48.0 ml

Tris (Hydroxymethyl aminomethane) - 36.6 g

N, N', N', N'-Tetramethyl ethylene diamine (TEMED) - 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

Crystallized, N-methylene bis acrylamide (Bis) - 0.3 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 per sulphate (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  \[ \text{--- 5.0 g} \]

Glycine  \[ \text{--- 25.8 g dissolved and the volume was made up to 1000 ml with glass distilled water. The pH was adjusted to 8.6, filtered and stored at 4°C. 1/10 strength of the above stock buffer was used.} \]

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

Solution A  \[ = 2.5 \text{ ml} \]
Solution B  \[ = 5.0 \text{ ml} \]
Solution C  \[ = 12.5 \text{ ml} \]

(All the 3 solutions were mixed thoroughly and the gel tubes were filled immediately, followed by water layering).

Polymerization of the gel:

Twelve open-ended glass tubes (5 mm x 80 mm) were stoppered with rubber caps at one end and placed with the open end up. The thoroughly mixed gel solution was pipetted into each tube to a height of 70-75 mm 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 a Pasteur pipette after polymerization.

**Electrophoresis**

Electrophoresis was performed in an apparatus containing 2 perspex containers or tanks (14/10 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 12 holes at its bottom at equal intervals along the circumference, 2 cm from the periphery. Each hole was fixed with a rubber grommet of 5 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, samples containing 240-280 \( \mu \)g of buffer-soluble protein were loaded per gel directly. Albumin and globulin protein samples 130-220 \( \mu \)g in 10% sucrose were loaded per gel tube for 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 the bases 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% (v/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 of the lower reservoir. Initially for 10 min the current supply was 2 mA per tube and later increased to 5 mA per tube for about 2 h or until the tracking dye had migrated to a distance of about 70 cm into the running gel. The entire process was carried out at 4°C.

Protein staining and destaining of the gels

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 on their removal.

The gels in which buffer-soluble proteins were electrophoresed were stained with 0.02% Coumassie Brilliant Blue G in a mixture of methanol, acetic acid and water in the ratio of 25:7:60 (V/V) (Jones and Lyttleton, 1972). Gels were left 24 h in the stain and destained later manually with the same solvent mixture without the stain until the bands were clear. The gels were stored in 7% acetic acid until zymograms were constructed.

The gels in which albumin and globulin protein were electrophoresed were stained with 0.5% Amido Black dissolved
in 7% acetic acid for 2 h. 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 scanned in Joyce Loeb Chromoscan.

Studies on seed germination

Plant material:

Seeds (more or less of the same weight; 1.14 ± 0.10 g) were soaked in running tap water overnight. The next day the seed coats were removed gently with the forceps, surface sterilised with 10% commercial bleach for 10 min, repeatedly washed 4-5 times with sterilised distilled water and germinated at room temperature (25 ± 2°C) in the dark in sterile vermiculite. The seedlings were irrigated with sterilised distilled water. The addition of water to the dry seeds was taken as zero (0) time germination. The cotyledons were collected at 2-day intervals during the germination course from day 0 to 18.

Determination of fresh and dry weights of cotyledons of germinating seeds:

Five to ten 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 24 h. The dried cotyledons were pooled and their weights were determined.
Extraction of soluble proteins (protease extract) from the cotyledons of germinating seeds (Baumgartner and Chrispeels, 1976 method with slight modification).

The cotyledons were harvested on alternate days from germinating seeds and they were homogenised to a paste in a precooled mortar with a pestle. The paste was extracted with 1 2-5 (v/v) 25 ml citrate-phosphate buffer, pH 5.4 containing 10 mM β-mercaptoethanol. The homogenate was centrifuged at 20,000 x g for 20 min at 4°C. The protein content of the supernatant was determined after cold 12% TCA precipitation according to Lowry et al. (1951). The supernatant, thus obtained, also served as the source of protease for determining the caseolytic activity. The buffer-soluble protein content was calculated and expressed in mg/pair of cotyledons.

Determination of buffer-insoluble proteins.

The pellet from the above experiment was suspended in 1.5-20 (v/v) 1 M NaOH and incubated at 45°C for 16 h. The contents were centrifuged at 20,000 x g for 20 min. The pellet was re-extracted with 2-10 ml of 1 M NaOH and centrifuged. The supernatants were pooled. After TCA precipitation, protein was measured by Lowry et al. (1951) method and expressed as mg/pair cotyledons.

The total protein content/pair of cotyledons was calculated by adding buffer-soluble protein content to buffer-insoluble protein.
Protease assay as caseolytic activity (Basha and Cherry, 1978 method with slight modification)

One ml of enzyme extract from cotyledons of 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 25 ml citrate-phosphate buffer, pH 5.4) in a water bath set at 37°C for 24 h. The reaction was terminated by adding 1.0 ml of 20% TCA. A reaction mixture in which 20% TCA was added immediately after the addition of enzyme extract served as a zero-time control. The samples were allowed to precipitate overnight at 4°C and were centrifuged at 5,000 x g for 10 min. Aliquots from the TCA-soluble supernatants were analysed for increase in ninhydrin positive material (proteolytic activity) by the method of Yemm and Cocking (1955). Proteolytic activity was presented on the basis of differences in absorbance (ΔA0.5) between the experimental samples and zero-time control/g protein/24 h.

PAGE pattern of buffer-soluble proteins from the extracts of the cotyledons of germinating seed

Extraction:

Cotyledons were harvested from germinating seeds on alternate days, washed thoroughly, blotted gently and ground to a paste in a chilled mortar with 1:1 (W/W) acid washed sand. The paste was extracted with 1:2.5 (W/V) extraction medium (Tris-Cl buffer 10 ml, pH 7.3, containing 0.1% (W/V) sodium ascorbate; 0.1% (W/V) cysteine-Cl and 17% (W/V) sucrose. The homogenate was incubated with 1:1 (W/V) PVPP 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.

Protein estimation:

Protein was measured by Lowry et al. (1951) method after TCA precipitation.

Electrophoresis:

Electrophoresis in 7.5% polyacrylamide gel was performed according to Davis (1964), elaborately described under seed development part.

Two hundred and forty to two hundred and eighty μg of protein was loaded per gel. The developed gels were stained with 0.02% Coumassie Brilliant Blue G (Sigma, USA) as has been described earlier.

Isoenzyme pattern

PAGE pattern of the isoenzymes of peroxidase, polyphenol oxidase, acetyl esterase, acid phosphatase and malate dehydrogenase from the extracts of developing seed/cotyledons and germinating seed cotyledons.

Extraction of enzymes:

The crude buffer-soluble protein, extracted (described earlier) for studying the PAGE profile of buffer-soluble proteins of developing seed/cotyledons and the germinating seed cotyledons formed the source of enzymes.
Electrophoresis

Gel electrophoresis in 7.5% polyacrylamide was performed according to Davis (1964), described earlier.

Two hundred to two hundred and thirty μg of protein was loaded per gel.

Staining of peroxidase isoenzymes (Pandey, 1967):

To 100 ml of 7% acetic acid was added 16.8 g of sodium acetate and the resultant solution was saturated with 1.5 EDTA. The solution was filtered, saturated with benzidine hydrochloride (Sigma, 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 photographed or examined for the construction of zymogram.

Staining of polyphenol (DOPA) oxidase isoenzymes (Van Loon, 1971):

Gels were equilibrated with 0.1 M phosphate buffer, pH 7.0 for 50 min and then incubated for 1 h in 0.01 M DOPA in the same buffer under vigorous aeration. Black bands indicate sites of polyphenol (DOPA) oxidase activity. Without aeration, it took longer time for the bands to appear; this resulted in less intense and more diffuse patterns. After staining the gels for polyphenol (DOPA) oxidase activity the gels were washed with glass distilled water and the zymograms were constructed immediately as the bands tended to diffuse on storage.
Staining of acetylcholinesterase isoenzymes (Reddy and Stahmann, 1972):
The gels were incubated for 30 min in 200 mM Tris-HCl buffer, pH 7.0 and then transferred to a staining solution containing 30 mg of \( \text{C}^- \text{naphthyl acetate} \) (dissolved in 2 ml of \( 90\% \) (v/v) acetone) added and mixed along with 100 mg of fast blue HR diazonium salt (Sigma, USA) in 90 ml of 10 mM Tris-HCl buffer, pH 7.0. After band development the gels were thoroughly washed with glass distilled water and stored in 7% acetic acid until the construction of zymograms.

Staining of acid phosphatase isoenzymes (Reddy and Stahmann, 1972)
The gels were incubated for 30 min in 200 mM acetate buffer, pH 5.0 and then transferred to a staining solution containing \( \text{C}^- \text{naphthyl phosphate} \) (1 mg/ml), fast blue RR diazonium salt (1 mg/ml) in 200 mM acetate buffer, pH 5.0. After band development the gels were thoroughly washed with glass distilled water and stored in 7% acetic acid until the construction of zymograms.

Staining of malate dehydrogenase isoenzymes (Fine and Costello, 1963)
The gels were incubated in a staining medium of the following composition.

\[
\begin{align*}
100 \text{ ml Tris-HCl, } \text{pH } 8.5 & \quad 23.3 \text{ ml} \\
2 \text{ ml malic acid (neutralized)} & \quad 1.5 \text{ ml} \\
NAD (30 \text{ mg/ml}) & \quad 0.6 \text{ ml} \\
Phenazine methosulphate, PMS (5 \text{ mg/ml}) & \quad 0.12 \text{ ml} \\
Nitro blue tetrazolium NBT (10 \text{ mg/ml}) & \quad 1.0 \text{ ml}
\end{align*}
\]
After the band development the gels were thoroughly washed with glass distilled water and stored in 7% acetic acid, until they were photographed.

The $E_f$ values of the bands were calculated as the ratio of the distance migrated by the bands in relation to the bromophenol blue dye front. Zymograms were constructed by direct examination of the gels.