Materials and methods

Sheela S. “Physiological and biochemical studies on jackfruit seeds (Artocarpus heterophyllus Lam) during storage and germination”, Department of chemistry, University of Calicut, 2007
Materials and methods
Jackfruits (*Artocarpus heterophyllus* Lam.) for the present study were collected from a specific (marked) tree growing at Chathannur Village in Kollam District during November to February of 2004-'05 and 2005-'06. Such a particular tree was selected because this tree flowers earlier than the Jackfruit trees of Calicut University Campus and hence mature fruits become available during November to February as and when the rainy season erases. Since Jackfruit seeds are recalcitrant and desiccation is an important aspect of this investigation ‘seed moisture content’ should not be varied much during the collection of ripened fruits. The firm flesh variety (Anonymous, 2000) of Jackfruit was selected for the present investigation. Inflorescences were tagged on the day of anthesis to record the number of days taken for development and designated as days after anthesis (DAA). Fruits ripened on the mother plant (120 DAA) were collected manually and brought to the laboratory. Fruits were cut open and seeds were collected without damage. Seeds were depulped to remove perianth and aril.
Washing

Depulped seeds were soon washed thoroughly in running water followed by distilled water to remove any trace of perianth or aril. Washed seeds were wiped with clean towel.

Surface sterilization

Seeds were surface sterilised by wiping with a clean towel wetted with 80% ethyl alcohol and kept for surface drying for 15 minutes at room temperature.

DESICCATION STUDIES

Seeds of ripened fruits (120 DAA) only were used for desiccation, storage and germination studies. Surface sterilised seeds (approximately 500) collected from 3-4 of ripened fruits were kept immediately after sterilisation for desiccation in open trays at room temperature (30±3°C) and designated as Room-Open.

Sampling

Desiccated seeds were sampled for moisture content determination and viability studies at an interval of 4, 8, 12, 13, 14, 15 and 16 days after desiccation and for biochemical studies at an interval of 4, 8, 12, 14 and 16 days. Fresh seeds immediately after collection served as the control (0 sample).

STORAGE STUDIES

Surface sterilized seeds of ripened fruits (120 DAA) were kept for storage under different conditions. One part of the collected seeds (approximately 300 numbers) were kept in sealed polythene bags providing sufficient space for air, and kept at room temperature designated as (Room-
Polythene). Another lot consisting of about 300 seeds were kept in sealed polythene bags in the lowest tray of fridge (Refrigerator 4-8°C). Seeds of both types of storage were sampled for germination, moisture content determination and biochemical studies at an interval of 10 days each upto 130 days (until the seeds become non-viable).

Viability

Ten seeds each in duplicate of control, desiccated and stored seeds were sampled as described above and kept for germination in the Petri plates lined with moist Whatman No. 1 filter paper (between paper). The number of germinated seeds was noted daily and the germination percentage was calculated as given below

\[
\text{Germination percentage} = \frac{\text{No of seeds germinated}}{\text{Total number of seeds sown}} \times 100
\]

Seed Vigour Index

Ten seeds in duplicate of control, desiccated and stored seeds were kept for germination as described above and daily count of sprouted seeds were taken. The seed vigour index (SVI) was calculated from the daily count data (Copeland and McDonald, 1995) using the formula given below.

\[
\text{SVI} = \frac{\text{No of seeds germinated}}{\text{Days of first count}} + \frac{\text{No of seeds germinated}}{\text{Days of last count}}
\]

Moisture content determination

Ten seeds in duplicate of control, desiccated and stored seeds were taken and fresh weights were determined using Shimadsu Ax 120 Electronic balance. Then the weighed seeds were kept in hot air oven at 100°C for one hour and then at 60°C. Drying and weighing were repeated until concordant
values were obtained. Moisture content percentage was calculated as explained by ISTA (1985).

\[
MC\% = \frac{\text{Fresh weight of seeds} - \text{Dry weight of seeds}}{\text{Fresh weight of seeds}} \times 100
\]

**BIOCHEMICAL STUDIES**

Biochemical analysis of metabolites and amylase assay was carried out using control, desiccated and stored seeds. The seeds were sampled at different intervals as described earlier. Four seeds each in duplicate were decoated, separated into embryonic axis and cotyledons, chopped into small pieces and pooled. Samples for biochemical studies and dry weight determination were taken from this pooled tissue. Random sampling procedure was followed for each estimation. Separate samples were taken for dry weight determination, biochemical estimation of starch, sugars, total and soluble protein and amylase assay.

**Dry weight determination of tissues**

A known amount of fresh pooled tissue was taken and weighed tissue was kept in hot air oven at 100°C for one hour and then at 60°C until constant weight was obtained.

**Analysis of Starch Extraction**

The method of Pucher *et al.*, (1948) described by Whelan (1955) was used to determine the starch in seed samples. Two hundred milligram of tissue in duplicate was weighed and homogenized in a glass mortar and pestle by adding 30% perchloric acid. The homogenate was centrifuged for five minutes and the supernatant was collected. The residue was re-extracted six times with 30% perchloric acid to ensure the complete extraction of starch.
The supernatants after each centrifugation were pooled and the volume of the combined extract was noted. A known volume of the combined extract was pipetted and an equal volume of the freshly prepared iodine potassium iodide reagent was added and thoroughly mixed. After 10 minutes, it was centrifuged for 10 minutes and the supernatant was decanted. The precipitate was washed with alcoholic sodium chloride solution to remove the excess iodine potassium iodide reagent. After centrifugation, the blue precipitate obtained was treated with alcoholic sodium hydroxide solution till blue colour was completely discharged. It was then centrifuged and washed again with alcoholic sodium chloride solution to remove liberated iodine. The precipitate was dissolved in a known quantity of 10% (v/v) sulphuric acid by heating in a boiling water bath, cooled and centrifuged for 10 minutes.

Estimation

Estimation of starch was done according to Montgomery (1957). Suitable aliquot was taken and its volume was made up to 1 ml using double distilled water. To this 0.1ml 80% (w/v) phenol was added and shaken well. Five milliliter of concentrated sulphuric acid was added quickly from a burette and allowed to cool. The optical density of the solution was measured at 540 nm (No.4 green filter) using Systronics Colorimeter. Soluble starch procured from Merck Chemical Company was used as standard.

Analysis of Sugars

Extraction

Five hundred milligram tissue was homogenised in 80% ethyl alcohol in a glass mortar and pestle and refluxed for two hours. The homogenate was centrifuged and the supernatant was collected. The residue was again extracted with 80% ethyl alcohol and after each centrifugation the supernatants were combined. The combined extract was evaporated to dryness
over a boiling water bath and eluted in 4 ml double distilled water and cleared by centrifugation.

HPLC of Sugars

Samples extracted as described above were used for HPLC studies. It was conducted at the Laboratory for Polymer Analysis, Sree Chithra Institute for Medical Science and Technology, Thiruvananthapuram. HPLC system consisting of Waters u Bobdapak –NH₂ column, Waters 600 pump, 7725 Rheodyne, 7725 injector and Waters 2414 Refractive Index detector with sensitivity - 4 were used for the analysis. The mobile phase used was Acetonitrile/Water-70/30. The flow rate was 1ml per minute. The injection volume used was 20 μl for standards and samples.

Glucose, fructose, rhamnose, maltose, sucrose, galactose and raffinose procured from Merck Chemical Company were used as the standards. From the chromatogram of standards and samples, comparison was made and from the peaks and area of each sample, the amounts of individual sugars were calculated.

Amylase assay

Enzyme extraction

Eight hundred milligram tissue each of control, desiccated and stored seeds were used for the enzyme assay. Individual samples were homogenized in 8ml, chilled 0.2 M Phosphate buffer (pH-7.5), using a pre-chilled mortar and pestle kept in ice bath. A known aliquot of the homogenate was taken for the estimation of total protein. The homogenate was centrifuged in refrigerated centrifuge (Plastocraft model ROTA R4R V/FM) at 10,000 rpm for 10 minutes. The supernatant was used as enzyme source for amylase assay. A known aliquot of the supernatant was precipitated with 10% TCA for soluble protein estimation.
Enzyme Assay

Dinitrosalicylic acid method as explained by Bernfeld (1955) was followed to estimate amylase activity.

Preparation of Dinitrosalicylic acid

One gram of 3, 5-Dinitrosalicylic acid was dissolved in 20 ml of 2N NaOH and 50 ml of distilled water and stirred slowly to avoid capturing of carbon dioxide. Thirty grams of Rochelle salt (sodium potassium tartrate) was added, dissolved and made up to 100ml. The solution was kept in amber coloured bottle.

Assay: Following optimal conditions of the assay system were standardized.

pH optimum: The optimum pH for enzyme activity was determined by incubating enzyme assay system consisting of 0.5ml buffer of each pH, 0.2ml of enzyme and 0.3ml of substrate (soluble starch) for 30 minutes at 37°C in buffers of pH ranging from 4-8 at an interval of 0.4 pH. The enzyme action was ceased by the addition of 1ml of dinitrosalycilic acid reagent at the 30th minute. The tubes containing the reaction mixture were kept in boiling water bath for 5 minutes and then cooled. It was made up to 10ml by adding double distilled water. The optical density of solution containing the reduction product was measured using Genesis 20 (Bausch and Lomb) Spectrophotometer at 540nm. Maltose procured from Merck Chemical Company was used as the standard. The buffer pH in which the enzyme showed highest activity was taken as pH optimum.

Temperature optimum: The temperature optimum of enzyme activity was determined by incubating the assay system for 30 minutes at temperature ranging from 20°C to 40°C at an interval of 5°C with substrate and buffer
having optimum pH (5.3 and 8.0). The temperature at which the enzyme showed highest activity was considered as temperature optimum.

**Enzyme proportionality:** The enzyme proportionality range for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature (37°C) with optimum pH (5.3 and 8.0), 0.3ml of 2% (w/v) soluble starch and 10% (w/v) enzyme extract ranging from 0.05ml to 0.4ml at an interval of 0.05ml.

**Substrate optimum:** The substrate saturation for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature (37°C) with optimum pH (5.3 and 8.0), 0.1ml of 10% enzyme extract and different quantities of 2% (w/v) soluble starch ranging from 0.1ml to 0.4ml.

As per standardised optimal conditions, the assay system contained one hundred microlitre (0.1ml) of homogenate, 0.6ml of 0.1M sodium acetate buffer (pH-5.3) or 0.1M sodium phosphate buffer (pH-8.0) of optimum pH and 0.3ml of 2% substrate (soluble starch procured from Merck Chemical Company) was incubated for 30 minutes at optimum temperature (37°C) and the product formed was estimated according to Bernfeld (1955) using dinitrosalicylic acid. Unit activity and specific activity of the enzyme were calculated.

**Unit activity:** Unit activity, defined as mg maltose formed during 30 minutes at 37°C per g tissue (dry weight) was calculated.

**Specific activity:** Specific activity is units of enzyme activity per mg of protein. The amount of protein in enzyme solution was determined by Lowry's (1951) method. The specific activity of amylase was calculated by dividing unit activity by mg protein present in the tissue.
Confirmatory test for $\alpha$ and $\beta$ amylase:

$\alpha$-amylase: Confirmatory test for $\alpha$-amylase was carried out using the method of Kneen et al., (1943). The enzyme preparation was held at 70°C for 30 minutes in the presence of 0.2% (w/v) calcium acetate. The suspension was cooled, centrifuged in cold at 10,000 rpm for 10 minutes and the supernatant was used for the assay. $\alpha$-amylase withstand heat treatment in the presence of calcium acetate and $\beta$-amylase is destroyed.

$\beta$-amylase: To the enzyme preparation, cold 0.1N HCl was added slowly with stirring until pH lowers to 3.3. The suspension was held at 4-5°C for 18 hours and centrifuged in cold at 10,000 rpm for 10 minutes. The pH of the suspension was raised to 4.6 by cautious addition of 0.1N NaOH. The resulting suspension was again centrifuged in cold at 10,000 rpm for 10 minutes. The supernatant was used for the assay. $\beta$-amylase withstand the treatment.

Analysis of Total and Soluble Proteins

Extraction

The method of Lowry et al., (1951) was followed to estimate the total and soluble protein.

Aliquot of homogenate and supernatant taken during the enzyme assay was used for the estimation of total and soluble protein respectively. A known aliquot was pipetted and mixed with equal volume of 10% (w/v) trichloracetic acid and kept for flocculation for one hour in an ice bath. The protein precipitate was collected by centrifugation for 10 minutes and the supernatant was decanted off. The residue was washed with 2% (w/v) trichloracetic acid, followed by washing twice with 15% perchloric acid to remove the starch. The residue was washed with 80% acetone followed by anhydrous acetone and centrifuged. The precipitate obtained after centrifugation was digested in
known volume of 0.1N NaOH and kept in boiling water bath for 10 minutes and centrifuged.

**Estimation**

Known aliquots were taken from the supernatant and made up to 1ml. To this, 5ml of alkaline copper reagent was added and shaken well. After 10 minutes, 0.5 ml of 1N Folin-Ciocalteau reagent was added, immediately shaken well and kept for 30 minutes. The optical density was read at 700nm using Genesis 20 Spectrophotometer. Bovine Serum Albumin (BSA) fraction V powder procured from Merck Chemical Company was used as the standard.

**Electrophoretic Study of Protein Profile (SDS PAGE):**

Protein profile of the seed during desiccation was studied by SDS PAGE. One gram of the seed sample was homogenized in pre chilled glass mortar and pestle in Tris-HCl buffer of pH-7.2. The homogenate was centrifuged at 10,000 rpm for 10 minutes using Plastocraft - model ROTA R4R V/FM refrigerated centrifuge and the supernatant was collected. The protein sample was dissociated into its polypeptide subunits by adding equal volume of gel loading buffer containing 10% SDS, β-Mercaptoethanol, Glycerol, 0.1% Bromophenol blue and Tris HCl (pH-6.8). The mixture was heated for 2 minutes (Laemmli, 1970) in boiling water bath and immediately kept in freezer. The sub units were separated electrophoretically using GENEI electrophoresis unit in SDS-PAGE slab gel having 10% separating gel and 4% stacking gel. The gel after electrophoresis was stained with Coomassie brilliant blue and the bands were compared with known Bovine Serum Albumin (BSA) fraction V powder procured from Merck Chemical Company.
### Composition of the separating gel (10%)

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<th>Volume</th>
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</thead>
<tbody>
<tr>
<td>30% Acrylamide / 0.8% Bisacrylamide</td>
<td>3.33ml</td>
</tr>
<tr>
<td>4X Resolving Gel buffer</td>
<td>2.5ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 μl.</td>
</tr>
<tr>
<td>Double distilled water</td>
<td>4.00ml</td>
</tr>
<tr>
<td>% (w/v) Ammonium Per Sulphate</td>
<td>50 μl.</td>
</tr>
<tr>
<td>TEMED</td>
<td>5 μl.</td>
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</tbody>
</table>

### Composition of the stacking gel (4%)

<table>
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<td>4X Stacking Gel buffer</td>
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<tr>
<td>10% SDS</td>
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<tr>
<td>Double distilled water</td>
<td>3.00ml</td>
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<tr>
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<td>50 μl.</td>
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<tr>
<td>TEMED</td>
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</table>

The sample was electrophoresed using Tris-Glycine running buffer of pH 8.3.

**HISTOCHEMICAL STUDIES**

Axes and small cotyledons of control and desiccated seeds were fixed for histochemical studies.
Tissue preparation

Samples were fixed in FAA, dehydrated through alcohol-TBA series, infiltrated and embedded in paraffin wax (Johansen, 1940). Using a rotary microtome (LEICA, model RM 2125RT) the individual blocks were cut at 10μ thickness and the sections were mounted on glass slide using Haupt’s adhesive and used for histochemical staining. The sections were deparaffinised, hydrated and stained for localization of starch and proteins.

Localisation of Starch

Localisation of starch was done according to Berlyn and Miksche (1976) using periodic acid-Schiff’s reagent as well as Safranin-Iodine potassium iodide solution.

Periodic acid-Schiff’s reagent (PAS) staining

The hydrated sections were placed in 0.5% (w/v) periodic acid solution at 23°C for 15 minutes and the sections were washed using running tap water for 10 minutes. The sections were then placed in Schiff’s reagent for 10 minutes at 4°C and washed in tap water for 20 seconds. After washing in tap water, the sections were placed in 2% Sodium sulphite for 2 minutes and washed again in tap water for 5-10 minutes. The sections were dehydrated through alcohol series, cleared in xylene and were mounted in DPX.

Safranin - Iodine potassium iodide staining

The hydrated sections were dipped in dilute solution of safranin and washed immediately to remove excess stain. Iodine potassium iodide solution was added dropwise using filler followed by thorough washing in running water. Slides were immediately dried over the slide warming table and the sections were cleared in xylene and mounted in DPX.
Localisation of Total Protein

For the localisation of total protein, sections were stained with mercuric bromophenol blue according to Mazia et al., (1953) as explained by Berlyn and Miksche (1976). The hydrated sections were placed in bromophenol blue stain for 15 minutes and then in 0.5% acetic acid for 20 minutes. The sections were then placed in water. The sections were dehydrated through alcohol-TBA series, cleared in xylene and were mounted in DPX.

Scanning Electron Microscopic study of starch grains

Sample preparation

Control seeds were soaked in water, crushed and mixed well mechanically and filtered gently. Starch grains with water was kept undisturbed to settle down. The supernatant was drained off and the residue was washed three times with 0.2M Phosphate buffer (pH-7.2). Sufficient time was given to settle down and the residue was washed with distilled water repeatedly. The supernatant was completely drained and the residue dried in hot air oven at 40°C. Starch powder was collected after complete drying.

SEM study

Starch powder smeared after wetting with distilled water and covered with two sided cellophane tape adhesive. Aluminium stub with starch grain powder dried in hot air oven at 40°C slowly. After complete drying, gold ion sputtering was done and observed in the S–2400 Scanning Electron Microscope (Hitachi) available at Sree Chithra Institute of Medical Science and Technology, Thiruvananthapuram. Starch grain surface was scanned and exposed at 2000x, 3000x, 4000x and 5000x magnification.
Control (fresh) seeds only were used for the germination and reserve mobilization studies. Sixty fresh seeds in duplicate were kept for germination in Petri plates (between paper) in darkness. Samples were collected at the interval of 2, 5, 10, 20, 30, 40 and 50 days after germination / seedling growth. Four seeds / seedlings each collected on sampling days were decoated, separated the cotyledons and axis. For dry weight determination of seedling parts, the samples were kept in hot air oven at 100°C for one hour and then at 60°C till weight became constant.

During initial days, axis and cotyledons were sampled separately and used for dry weight estimation and biochemical studies. After the elongation of radicle i.e. 20th day onwards, the pooled cotyledon tissue alone was used for these studies. The biochemical estimation of starch, sugars, total protein, soluble protein and amylase assay were conducted as described earlier.

Histochemical localization of starch and proteins of cotyledons were done to localize these reserves during germination. Staining was done as per the methods described under desiccation studies. Observations of stained sections were done and photomicrographs were taken using Nikon microscope (ECLIPSE E 400) and Nikon Digital Camera (DXM 1200F) attached with digital image analyser.

Statistical analysis

All experiments starting from sampling were repeated minimum of 6 times using seeds of fruits collected 4-5 times each during two consecutive years and mean value was taken and the standard deviation / standard error was calculated. Test of significance was done following Fischer’s ‘t’ test.