MATERIALS AND METHODS

1. Plant Material

   Five different palm species such as *Borassus flabellifer* L., *Corypha umbraculifera* L., *Caryota urens* L., *Licuala peltata* Roxb. and *Livistona rotundifolia* Mart. were selected for the present study.

2. Collection of Materials

   Fruits of *Borassus flabellifer* were collected from a selected group of palms from Pattambi, Palghat district in June 2005 and 2006. Since it is of annual flowering type, the fruits are available only during May to July. *Corypha umbraculifera* is monocarpic. Fortunately, the fruits were available from a specific palm growing at Mankav, Kozhikkode district in June 2005 and also from another palm during May-June 2006. *Caryota urens*, being a regular flowering type, fruits are available throughout the year and was collected from Thalassery in January, 2005 and 2006. Like *Borassus flabellifer*, *Licuala peltata* and *Livistona rotundifolia* are also annual flowering types and the fruits of these two species were collected from trees cultivated in the Botanical Garden, Calicut University during the months of May and June, 2005 and 2006.

   Ripe fruits were collected manually and brought to the laboratory immediately. Fruits of each of the species were divided into two lots. One lot was dehusked/depulped by removing the mesocarp, followed by further cleaning. The other lot consisted of entire unhusked/pulpy fruits. The ripe fruits were soft and had a colour characteristic for each species. The entire fruits as well as dehusked/depulped seeds were used for the investigation.
Depending upon the availability, seeds were selected randomly from one or more bunches, pooled together and were used for storage and germination studies.

3. Storage Studies

Fresh fruits, immediately after collection, were used for storage studies under three different conditions. For this, both entire fruits and de-husked/depulped seeds were divided into three equal lots of 300-400 seeds in all the palms except *B. flabellifer* in which each lot consisted of 100 seeds only. Each seed lot was spread uniformly in open trays and kept at room temperature (designated as open RT). The second lot was stored in clean, air-filled polythene bags and kept at room temperature. The third lot was stored in air-filled polythene bags and kept in refrigerator at an average temperature of $4\pm2^\circ C$.

3.1. Sampling

Samples from the fruits/seeds stored under all different storage conditions were drawn at an interval (Table 1) of one or two weeks to evaluate storage behaviour. Fresh seeds were considered as controls.

3.2. Determination of Moisture Content

For the determination of moisture content of the seeds stored under different conditions, 10 seeds each in triplicate were drawn from the seed lots of all the storage conditions, at regular intervals as given in Table.1. The seeds were weighed accurately in a pre weighed container, using electronic balance and kept in a hot air oven set at 100°C for 1 hour. Then the temperature was adjusted to 60°C. After 24 hours, dry weight of the seeds was taken. The seeds were again kept in the oven at 60°C. The dry
weight determination was continued till the values became constant. Moisture content was calculated from the fresh weight and dry weight.

3.3. Germination Percentage

Ten seeds each in triplicate were drawn from the fruits/seeds stored under different storage conditions as described earlier. The samples collected were sown in garden pots filled with clean sand and kept for germination in the net house of the Department of Botany, University of Calicut.

In the case of *Borassus flabellifer*, heaps of soils of 1 m. high were made and the fruits as well as dehusked seeds were buried in it. The pots and soil heaps were watered regularly.

The number of seeds germinated on each day was noted. The percentage of germination was calculated as follows.

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\text{Percentage of germination} = \frac{\text{Number of seeds germinated}}{\text{Total number of seeds kept for germination}} \times 100
\]

4. Germination Studies

For the elucidation of biochemical aspects of germination /reserve mobilization, fresh seeds (control) of all palms were kept for germination under natural environmental conditions. Seeds were sown as described earlier.

The seedlings soon after the protrusion of the cotyledonary sheath were taken out of sand, washed and kept in trays lined with wetted germination paper for further development of the seedlings. For convenience of study, the seedlings were categorized into different stages based on morphological changes noticed during germination (Fong, 1978; Khudairi, 1958). The seeds
and seedlings at different stages of development were categorized as follows -
Stage 0, fresh seeds; stage 1, appearance of the cotyledonary sheath (1\textsuperscript{st} week); stage 2, extension of the sheath (2\textsuperscript{nd} week); stage 3, appearance of swollen apex (3\textsuperscript{rd} week); stage 4, the first plumular sheath develops and root shoot differentiation starts (4\textsuperscript{th}-5\textsuperscript{th} week); stage 5, primary leaf comes out of the sheath (6\textsuperscript{th}-7\textsuperscript{th} week); and stage 6, lateral roots developed (8\textsuperscript{th}-10\textsuperscript{th} week) (Table 2, Fig. 2-6). Approximate period taken for each stage of development varied among the different palms.

4.1. Biochemical Characterization of Seeds during Germination

The seeds/seedlings at different stages of germination were taken for biochemical analysis. Different parts of the seedling such as endosperm, haustorium, cotyledonary sheath and plumular sheath were separated from about 30 seedlings, pooled together and sampled during respective stages of germination and used for the studies. The biochemical components of the seed tissues, like, starch, total soluble sugars, reducing sugars, proteins and lipids were extracted and estimated according to standard procedures. HPLC for the analysis of galactomannan, assay of the enzyme amylase, PAGE studies for protein profile and also were carried out.

4.1.1. Determination of Dry Weight of Tissues

A known quantity of tissues of fresh seeds/seedling parts such as endosperm, haustorium, cotyledonary sheath and plumular sheath was taken, weighed and then kept in a hot air oven at 100\degree C for one hour and then at 60\degree C till the dry weight of the tissues became constant. Percentage of dry weight was calculated as suggested by International Seed Testing Association (1985).
4.1.2. HPLC Analysis of Galactomannan

4.1.2.1. Isolation of Galactomannan

Galactomannan was extracted from powered endosperms isolated from the seeds of palms under study, according to the procedure of Buckeridge and Dietrich (1996). The weighed samples were subjected to hot water extraction (80°C) for 6 hours. After filtration through cheese cloth the extract was centrifuged at 10,000 x g for 30 min at 5°C followed by precipitation of the supernatant with 3 volumes of ethanol and was left overnight at 5°C for completion of the precipitation. It was collected by centrifugation, dried and weighed. The polysaccharide precipitated contained typically more than 95% galactomannan.

The galactomannan thus isolated was hydrolysed with 5ml of 1N HCl by heating on a water bath for 14 hours. After cooling, 10 ml of ethanol was added and centrifuged to remove the precipitates if any. The supernatant was transferred to a Petridish and dried. The powder was dissolved in 2.0 ml of distilled water. The sample was then subjected to separation and identification of the components by High Performance Liquid Chromatography. HPLC system available at SCTIMST, Trivandrum, was used for the study.

Twenty μl of aliquot was injected into the HPLC system consisting of Waters u Bondapak –NH2 column, Waters 600 pump and Rheodyne 7725 injector. The mobile phase was acetonitrile: water (70:30) at a flow rate of 1.0 ml / minute. The sugars were detected by using Waters 2414 refractive index detector and quantified by comparison of the peak areas of the sample with those of standard solutions.
4.1.3. Starch

The starch content of different parts of the seed/seedling was determined at various stages of germination using the method of Pucher et al. (1948) as described by Whelan (1955).

4.1.3.1. Extraction

Tissues isolated from different parts of seeds/seedlings were cut into small pieces and pooled separately. From each of this, 200 mg of the tissue was weighed and homogenized in diethyl ether to remove the lipids that may interfere with the processes of extraction and purification of starch. Then the diethyl ether was decanted off and the residue was ground in 30% (v/v) perchloric acid for the extraction of starch. The homogenate was centrifuged at 4000 x g and the supernatant was collected. The residue was again homogenised in 30% (v/v) perchloric acid and centrifuged. The processes of homogenisation, centrifugation and extraction were repeated till it was ensured that the entire starch content of the tissue was extracted. Volume of the combined supernatant was noted. A known volume of the aliquot was taken from the combined supernatant and an equal volume of freshly prepared iodine–potassium iodide reagent was added to the tube and mixed well using a vortex shaker. The mixture was then kept undisturbed for 10-20 minutes and centrifuged for 10 minutes. The supernatant was decanted off. The excess iodine reagent present in the residue was removed by washing with alcoholic sodium chloride followed by centrifugation. After centrifugation, the coloured residue was treated with alcoholic sodium hydroxide till the blue colour was found disappeared. The residue was again washed with alcoholic sodium chloride. It was then dissolved in a known volume of 10% (v/v) sulphuric acid by heating in a water bath. After cooling, the supernatant was collected and used for the estimation of starch.
4.1.3.2. Estimation

Estimation of starch was done according to the protocol of Montgomery (1957). A known quantity of aliquot was made up to 1.0 ml and 0.1 ml of 80% (w/v) phenol was added to it and mixed well. Five ml of concentrated sulphuric acid was quickly added to the mixture from a burette and allowed to cool. The optical density of the resultant solution was measured at 540 nm using a Genesis-20 spectrophotometer. Soluble starch was used as the standard.

4.1.4. Amylase Assay

Activity of amylase was studied only in the seeds of *Borassus flabellifer* and *Corypha umbraculifera* during germination since tissues of different parts of these seedlings were available in sufficient quantities because of their larger size. Since the endosperm and cotyledonary sheath contained negligible amount of starch, these tissues were not chosen for the amylase assay. Tissues of seedling parts such as haustorium and plumular sheath were used for the study.

One gram tissue was homogenized in 10 ml, cold, .2 M sodium phosphate buffer (pH 7), using a pre-chilled mortar and pestle kept in ice bath. The homogenate was centrifuged at 16000 x g for 15 min in a Kubota (Model KR 20000 T) refrigerated centrifuge at 4°C. The supernatant was transferred to a clean test tube and kept in an ice bath. The extract was used for enzyme assay.

Amylase activity was estimated using the Dinitrosalycylic acid method as explained by Bernfeld (1995).
4.1.4.1. Preparation of Dinitrosalicylic acid (DNS)

Thirty grams of sodium potassium tartrate was dissolved in 50 ml of distilled water. The sodium potassium tartrate solution (50 ml) was mixed with 20 ml of 2 N NaOH.

The mixture of sodium potassium tartrate and NaOH was warmed in a water bath at a temperature of 45-60°C. One gram of DNS was added gradually with stirring till it was completely dissolved. The solution was cooled and made up to 100 ml. The reagent was kept protected from light and carbon dioxide.

4.1.4.2. Assay

Two hundred µl of 10% homogenate, 0.5 ml of 2.0 M sodium phosphate buffer of optimum pH and 0.2 ml of 4.0% substrate (soluble starch procured from Merck) were incubated for 30 minutes at optimum temperature. The activity of the enzyme was ceased by the addition of 1.0 ml of DNS at the 30th minute. The tubes were heated for 5 minutes in boiling water bath and then cooled. It was made up to 10 ml by adding double distilled water. The optical density of the solution containing the reduction product was measured using Shimadzu (UV-1601) UV-Visible spectrophotometer at 540 nm. Maltose was used as standard. Unit activity and specific activity were calculated.

4.1.4.3. pH Optimum

The optimum pH for enzyme activity was determined by incubating enzyme assay system for 30 minutes at 37°C with substrate, in buffers of a pH range 4.2-7.4 at intervals of 0.4 pH, in a water bath. The pH of the buffer in which the enzyme showed highest activity was taken as optimum pH.
4.1.4.4. Temperature optimum

The temperature optimum of amylase activity was determined by incubating the assay system for 30 minutes at a temperature ranging from 20\(^0\)C to 40\(^0\)C at an interval of 2\(^0\)C with substrate and buffer having optimum pH. The temperature at which the enzyme showed highest activity was considered as optimum temperature.

4.1.4.5. Enzyme proportionality

The enzyme proportionality range for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature with optimum pH, optimum substrate concentration and different volumes of 10\%(w/v) enzyme extract ranging from 50-400 \(\mu\)l.

4.1.4.6. Substrate saturation

The substrate saturation for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature with optimum pH 200 \(\mu\)l of 10\%(w/v) enzyme extract and different concentration of soluble starch 4\%(w/v) ranging from 100-260 \(\mu\)g/ml.

4.1.4.7. Unit activity

Unit activity was calculated as mg maltose/g tissue, formed during 30 minutes at 37\(^0\)C.

4.1.4.8. Specific activity

The amount of soluble protein in enzyme solution was determined by Lowry’s method (described later). The specific activity was calculated by dividing the unit activity by the amount of protein in mg present in the tissue.
4.1.5. Total Soluble Sugars

For the estimation of soluble sugars the method proposed by Montgomery (1957) was adopted.

4.1.5.1. Alcoholic Extraction

Tissues isolated from different parts of seeds/seedlings were cut into small pieces and pooled separately. Two hundred mg of tissue was homogenised in 80% (v/v) ethyl alcohol using a glass mortar and pestle. The homogenate was carefully transferred to a round-bottomed flask fitted with a water condenser and refluxed over a steam bath for 4 hours. The flask was cooled and the extract was transferred into centrifuge tubes and centrifuged at 4000 x g for 10 minutes. The supernatant was collected in a boiling tube. The residue was homogenised again in 80% (v/v) ethanol and refluxed for 1 hour. The extract was clarified by centrifugation; the supernatant was collected and combined with the original. The combined extract was dried in an evaporating china dish. The dry residue left in the china dish after evaporating the solvent was dissolved in a known volume of distilled water and aliquots were taken from this for estimation.

4.1.5.2. Estimation

The total soluble sugar was estimated using the method proposed by Montgomery (1957). From the sample, a known volume of aliquot was taken in a test tube and made up to 1.0 ml. To this 0.1ml of 80% (w/v) phenol was added and mixed well. Five ml of concentrated sulphuric acid was added to the tube quickly from a burette. After cooling, the optical density of the resultant solution was measured using green filter in a colorimeter. D-Glucose was used as the standard.
4.1.6. Total Reducing Sugars

For the estimation of total reducing sugar, an alcoholic extract of the seed/seedling tissues was prepared (described earlier).

4.1.6.1. Estimation of Reducing Sugars

An aliquot from the extract prepared for the estimation of total soluble sugar was used for the estimation of total reducing sugars according to the Nelson-Somogyi method (Nelson, 1944; Somogyi, 1952).

4.1.6.2. Preparation of Somogyi’s Copper Reagent

This reagent was prepared by dissolving 24 g of anhydrous sodium carbonate and 12 g of sodium potassium tartrate (Rochelle salt) in about 250 ml of distilled water. To this 4 g of copper sulphate as a 10% (w/v) solution was added and mixed followed by the addition of 16 g of sodium bicarbonate. Then 180 g of sodium sulphate was dissolved in about 500 ml of distilled water and boiled to expel air. After cooling, the two solutions were mixed and the volume was made up to 1000 ml (Somogyi, 1952).

4.1.6.3. Preparation of Nelson’s Arsenomolybdate Reagent

Nelson’s arsenomolybdate reagent was prepared by dissolving 25 g of Ammonium heptamolybdate in 450 ml of water. Then 21 ml of sulphuric acid was added and mixed well. To the mixture 3.0 g of disodium hydrogen arsenate dissolved in 25 ml of distilled water was added. The solution was mixed well and incubated for 24 hours at 37°C (Nelson, 1944).
4.1.6.4. Estimation of reducing sugars

From the sample, a known volume of aliquot was pipetted out and was made up to 1.0 ml using distilled water. To this 1.0 ml of Somogyi’s copper reagent was added. The mixture was then placed in a bath of boiling water and heated for 20 minutes. After cooling under tap water 1.0 ml of Nelson’s arsenomolybdate reagent was added with immediate mixing till the effervescence ceased. The intensity of colour was measured after proper dilution at 540 nm using a Photochem Digital Colorimeter. D-Glucose was used as the standard.

4.1.7. Analysis of Proteins

4.1.7.1. Total Proteins

Total protein content of the tissues of seed/seedling at various stages of germination was determined using the method of Lowry et al. (1951).

Two hundred mg of tissue was homogenised using a chilled glass mortar and pestle in a medium containing 50 mM phosphate buffer (pH 7.5) and 50 mM 2-mercaptoethanol. A known volume from the homogenate was pipetted out into a centrifuge tube and an equal volume of 10% (w/v) trichloroacetic acid was added and mixed well. This was kept in an ice bath for one hour for flocculation. The mixture was centrifuged for 10 minutes and the supernatant was decanted off. The precipitate was washed with 2.0% (w/v) trichloroacetic acid and centrifuged again. The washing and centrifugation of the precipitate was repeated twice with 15% (v/v) perchloric acid to remove starch and thrice with diethyl ether to remove lipid. The precipitate thus obtained was treated repeatedly with 80% (v/v) acetone and then with anhydrous acetone to remove the pigments.
The dry pellet obtained after centrifugation was digested in a known volume of 0.1N sodium hydroxide by heating in a bath of boiling water for 10 minutes. The digest was centrifuged and the supernatant was collected. From the supernatant aliquots of known volume were pipetted out in triplicate and made up to 1.0 ml with distilled water. To this, 5.0 ml of alkaline copper reagent was added and mixed well. After 10 minutes, 0.5 ml of 1N Folin-Ciocalteu’s phenol reagent was added and shaken well immediately. The tubes were kept for 30 minutes for colour development. The optical density was measured at 700 nm using Schimadzu UV visible spectrophotometer. Bovine serum albumin-fraction V powder was used as the standard.

4.1.7.2. Soluble Protein

The quantity of protein in the enzyme extract was determined according to the procedure of Lowry et al. (1951).

Two hundred mg of tissue was homogenised using a chilled glass mortar and pestle in a medium containing 50 mM phosphate buffer (pH 7.5) and 50 mM 2-mercaptoethanol. The homogenate was centrifuged at 4000 x g for 10 minutes. The supernatant was collected and the volume noted. From the supernatant, an aliquot of 2.0 ml was pipetted out into a centrifuge tube and an equal volume of 10% (w/v) trichloroacetic acid was added and mixed well. Further steps were done as described above for the estimation of total proteins.

4.1.7.3. Electrophoretic studies of protein profile

SDS Poly acrylamide gel electrophoresis was carried out according to the method of Gaal et al. (1980). Two hundred mg of the endosperm tissues of fresh palm seed /seedling was homogenised using a chilled mortar and pestle in 50 mM phosphate buffer, 50 mM 2-mercaptoethanol and 10 %
sodium dodecyl sulphate (SDS). The 10% homogenate was centrifuged at 16,000 x g for 20 minutes using a Kubota KR 20000 T refrigerated centrifuge at 4°C and the supernatant was collected.

4.1.7.3.1. Preparation of the Gels

The resolving gel was prepared by mixing 3.3 ml of acrylamide/bisacrylamide (30% T and 2.67% C), 5 ml of 1.0 M resolving gel buffer (pH 8.8), 50 µl of 10% ammonium persulphate, 50 µl of 10% SDS and 5.0 µl TEMED. The mixture was made up to 10 ml with deionised water.

The stacking gel was prepared by mixing 0.99 ml of acrylamide/bisacrylamide (30% T and 2.67% C), 3.0 ml of 0.5 M resolving gel buffer (pH 6.8), 30 µl of 10% ammonium persulphate, 30 µl of 10% SDS and 5.0 µl TEMED. The mixture was made up to 6 ml with deionised water.

4.1.7.3.2. Gel Casting

The gel was cast in a Genie mini vertical gel casting unit. The glass plates, the comb and the spacers of the casting unit were wiped clean with alcohol using tissue paper. Then the glass plates were wiped with acetone. The dried glass plates were clamped on the casting unit with the spacers placed in between them.

The resolving gel was poured into the casting unit and the top was layered with a small volume of deionized water to avoid contact with air. After the completion of the polymerization, the water was removed with strips of filter paper. Then the comb was placed and the stacking gel was poured carefully. The gel was topped with deionised water. After polymerization the comb was removed carefully and the wells were cleaned thoroughly. Forty µl of the extract containing 20% sucrose was added to each well. Bromophenol
blue was used as the tracking dye. Low molecular weight marker (Biorad) was loaded in one of the wells. Electrophoresis was carried out using the electrophoretic reservoir buffer, Tris-glycine, pH 8.4. Initially the gels were maintained at a voltage of 80 V. Once the stacking has taken place, the voltage was raised to 120 V and was maintained there till the electrophoretic run reached the bottom of the gel. At the end of the run the gel was carefully removed and was stained with 0.2% coomassie brilliant blue R 250 in methanol- acetic acid mixture. After 3 hours of staining the gels were destained in methanol-acetic acid and were stored in 7% (v/v) acetic acid.

The gels were analysed in a Biorad Geldoc and molecular weight of the bands was determined using the Quantity One software.

4.1.8. Total Lipids

Total lipid content in the endosperm tissues of seed and seedlings at different stages of germination was determined gravimetrically using the modified form of the Folch method (Folch et al., 1957; Slack et al., 1977; Christie, 1993). One gram of seed tissue was homogenized thoroughly in chilled diethyl ether using a clean glass mortar and pestle. The homogenate was centrifuged at 4000 x g for 10 minutes. The supernatant was collected in a pre-weighed china dish. The sediment was homogenized again with chilled diethyl ether and the process was repeated several times and the supernatants were added to the bulk in the china dish. The china dish containing the combined supernatant was kept in a hot air oven at 60°C for 24 hours. The china dish along with the contents left after evaporation was weighed again and the difference between the initial weight and the final weight was found out.
4.2. Statistical Analysis

All the analyses described above were carried out in 6-8 replicates and the values are expressed as mean ± standard deviation and standard error. The statistical significance was tested using Fisher’s t-test. Values of \( P < 0.02 \) were taken as statistically significant.