Physiology and Biochemistry of Seed Development and Germination in *Borassus flabellifer* L.

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DOCTOR OF PHILOSOPHY IN BOTANY

By

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DECLARATOION

I hereby declare that the thesis entitled "**Physiology and Biochemistry of Seed Development and Germination in *Borassus flabellifer* L.**" submitted by me in partial fulfilment of the requirements for the Degree of **Doctor of Philosophy in Botany**, **University of Calicut** is the bona fide work carried out by me and no part of the work has formed the basis for the award of any other degree or diploma.

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CERTIFICATE

This is to certify that the thesis entitled "**Physiology and Biochemistry of Seed Development and Germination in *Borassus flabellifer* L.**" Submitted by **Ratheesh chandra, P**. in partial fulfilment of the requirement for the Degree of **Doctor of Philosophy in Botany**, University of Calicut, is a bona fide record of the research work undertaken by him in this Department under my supervision during the period 2006-2010 and that no part thereof has been presented before, for the award of any degree or diploma.

Dr. Nabeesa Salim

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Ratheesh chandra, P.

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Dedicated to my beloved Parents….

INTRODUCTION

INTRODUCTION

The most recent estimate of palm family (Palmae or Arecaceae) reveals that the family contains 200 genera and 2,600 species (Dransfield *et al*., 2008). Many aspects such as systematics (Uhl and Dransfield, 1987; Tillich, 1995; Govaerts and Dransfield, 2005), anatomy and reproductive biology (Gatin, 1912; Tomlinson, 1960, 1961, 1990; Moore and Uhl, 1973; Iossi *et al*., 2006) of palms have been extensively studied.

Scientific study on palm seed germination began in the early nineteenth century with the contribution of Martius (1823-1850). Later Gatin (1912) surveyed morphology, anatomy and physiology of palm seedlings. According to Tomlinson (1960, 1990) and Uhl and Dransfield, (1987) seed germination is unique in palms and those authors described two types – admotive and remotive – germination. Germination behaviour and seedling characteristics of palm seeds have been elucidated by Moor and Uhl (1973) and Tillich (1995, 2000). Henderson (2006) narrated a historical survey of the studies on seedling morphology and anatomy and re-evaluated the seedling morphology in palm family.

Palm seeds, like many other monocotyledons typically possess a small embryo embedded in the copious amount of endosperm. During early phase of development, endosperm is liquid syncytium followed by cellularisation

and become hard later, by the deposition of polysaccharides stored in the form of thickened cell walls. The single cotyledon occupies a major portion of the embryo and has two main regions- the proximal portion that extends and protrudes during germination (cotyledonary sheath) to push the shoot-root axis of the seed to the ground and the distal portion which remains within the seed to develop into the haustorium during and following germination.

Studies on endosperm development have been focused mainly on the models of cereals (Parker, 1980; Kranz *et al*., 1998; Berger, 1999) and *Arabidopsis thaliana* (Mansfield and Briarty, 1993). However, scant information is available on the development of endosperm in palm seeds. Similarly, anatomical/ developmental studies of palm seed endosperm have lagged behind the biochemical studies such as analysis of biochemical constituents of the same. According to Bhatnagar and Sahwney (1981) the hard thick walled tissues of endosperm are found to difficult to process for microtomy and anatomical study. The endosperm structure of only one palm – the Date (*Phoenix dactylifera*) has been studied in detail (Keusch, 1968; DeMason *et al*., 1983).

Tomlinson (1960) described the Date palm embryo as small consisting of a single cotyledon with a proximal region which is involved in the initiation of germination and distal end is destined to develop into the haustorium. Although growth pattern of cotyledon has been investigated in

Phoenix dactylifera (DeMason, 1984) and *Washingtonia filifera* (DeMason, 1986) no systematic studies are carried out on the development of palm seed embryo and endosperm. Hence one of the objectives of the present study is anatomical and histochemical localisation of developing embryo in *Borassus flabellifer*, which is found to be embedded in the liquid endosperm during the early phase of seed formation. In addition to the developmental processes of the embryo, cell wall formation and thickening by the deposition of cell wall storage polysaccharides (CWSP) on the endosperm cell walls also was included in the present study.

Borassus flabellifer commonly known as Palmyra palm is a dioecious tree with a single cylindrical trunk with height of 10-25m. Each palm may bear 6-12 bunches of about 50 fruits per year. The fruit is round and dark-brown coloured on the bottom part and green on the top and changes the colour to dark-yellow when it ripens. The diameter of the palm fruit is 13-20 cm and commonly contains three pyrenes inside the fibrous mesocarp. The seeds are more or less hemispherical and flattened on cross section. The embryo is very small compared to the endosperm and is embedded in the endosperm which is hard and stony after the deposition of polysaccharides. At the micropylar end, the endocarp is thin thereby leaving an opening for the protrusion of the embryo during germination.

The first and foremost objective of the present study was the elucidation of embryo and endosperm development of *B. flabellifer* seeds. Histochemical and biochemical aspects of seed development were conducted on samples of developing seeds. Biochemical analysis of metabolites of embryo and endosperm also was another aspect. Anatomical/histochemical studies on mature embryo formed another important objective of the investigation.

Mannans/ galactomannan are cell wall storage polysaccharide commonly stored in the endosperm cell walls of many palm seeds (Meier and Reid, 1982; Daud and Jarvis, 1992). DeMason *et al*. (1989) investigated the endosperm development in Date palm following histochemical and ultra-structural techniques and stated that soluble sugars and proteins play important roles in the deposition of galactomannan on the cell walls. The endosperm of *Borassus flabellifer* is composed of galactomannan (Subrahmanyan *et al*., 1956; Mukherjee *et al*., 1961). According to Rao and Mukherjee (1962) galactomannan content of *B. flabellifer* endosperm contains galactose and mannose in the molar ratio 1:2.4 when extracted with alkali. But GLC study by Awal *et al*. (1995) showed that the endosperm consists of 97% mannose and 3% galactose in *B. flabellifer*.

Although the existence of galactomannan in the endosperm cells of *B. flabellifer* have already been reported, systematic studies to understand the

mechanisms of these cell wall storage polysaccharides and metabolites responsible for the synthesis of galactomannan reserves are lacking in palm seeds. So the work described in the present investigation was undertaken to gain insight into the overall importance of palm seed endosperm as a storage tissue during different stages of seed development. Distribution of galactomannan – constituent sugars such as galactose and mannose as well as other soluble sugars also were analysed to determine whether any change in the proportion of galactose to mannose residues occurs and to check the role of soluble carbohydrates in the synthesis of cell wall storage polysaccharides. Distribution of total and soluble proteins, protein profile, free amino acids and sugar content of endosperm are also formed important aspects of present study.

Palm seeds exhibit unique features during germination. Martius (1823) categorised palm seed germination into two types – germination admotive or admote germination in which the cotyledonary sheath develop adjacent to the seed and in the second type called germination remotive or remote germination which is characterized by marked elongation of the proximal part of the cotyledon to develop the cotyledonary sheath which carry the shoot and grow downward to the soil, get widens and the plumule is protruded from a distance away from the seed.

A preliminary study on *B. flabellifer* in our laboratory showed remotive type of germination (Radha, 2007). A perusal of literature showed that only scant information is available on the remotive type of germination in palms inclusive of *B. flabellifer* seeds (Subrahmanyan *et al*., 1956; Mukherjee *et al*., 1961; Awal *et al*., 1995).

As mentioned earlier, the distal portion of the cotyledon of palm embryo remains inside the seed and during germination, it is developed into the haustorium which grows tremendously and stores the metabolites of the degrading endosperm. Hence, complex temporal and spatial interactions do exist between the endosperm and haustorium during and following germination. Therefore, another important objective of the present study was the elucidation of comparative anatomical and physiological aspect of endosperm hydrolysis and haustorial development of *B. flabellifer* seeds during and following germination. The structure of embryo, endosperm as well as haustorium formation and endosperm degradation have already been elucidated in *Phoenix dactylifera* (Keusch, 1968; DeMason, 1985; DeMason and Thomson, 1981; DeMason *et al*., 1983, 1985) and in *Washingtonia filifera* (DeMason, 1986, 1988b; Chandra Sekhar and DeMason, 1988b). However, those authors neither did identify the source of endosperm breakdown at cellular level nor did elucidate the enzymes which might function on galactomannan degradation. Nevertheless, physiological state of

endosperm degradation during germination has been investigated in Date seeds (DeMason *et al*., 1985, 1992).

Previous studies on physiological and biochemical aspects of galactomannan-rich endosperm on Date seed suggested that hydrolysis of the cell wall could be accomplished by the activities of three enzymes– α-galactosidase, β-mannosidase and endo-β-mannanase because the storage galactomannans are used to feed the seedling until it reaches autotrophy (Reid 1971; Reid and Meier, 1973; DeMason *et al*., 1985, 1992). However, relative to the existence of voluminous literature on starch metabolism, most of the researches on the enzymes involved in the mobilization of endosperm reserves are on cereals in which aleurone layer is the digestive tissue that perform the function of enzyme synthesis. The metabolic reversal that takes place in the endospermous species that develop specialised digestive tissue is an intriguing aspect of seed development and germination. Very little is known about the physiological and biochemical aspects of galactomannan synthesis and accumulation during development and hydrolysis of the same during and following germination in palm seeds. However, the cell wall storage polysaccharides (CWSP) in palm seeds are mostly mannans/galactomannans and function as important storage compounds and their hydrolytic products are mobilised during germination.

Endo-β-mannanase is the principal enzyme involved in the mannan hydrolysis reported in Tomato (Bewley *et al.,* 1997; Mo and Bewley, 2003; Gong and Bewley, 2007) *Sesbania virgata* (Lisboa *et al*., 2006) Carob (Kontos and Spyropoulos, 1996) and Rice (Ren *et al*., 2008). Although palm family consists of more than 2600 species the members that have been analysed for endosperm development/ degradation represents just two or three species such as Coconut (Mujer *et al.*, 1984) Date (DeMason *et al.*, 1992) and Oil palm (Alang *et al*., 1988). Participation and control of at least three galactomannan hydrolysing enzymes such as α-galactosidase, endo-β-mannanase and β-mannosidase have been reported in Date seeds during germination and seedling growth (DeMason *et al*., 1992). In order to assess the participation of the enzymes in the galactomannan degradation the present study is directed also towards the elucidation of the mode of activity of the three enzymes- endo-β-mannanase, β-mannosidase and α-galactosidase and pattern of reserve mobilisation during and following germination of *B. flabellifer* seeds.

The mobilization of galactomannan–seed reserve of endosperm cells analysed biochemically by assaying enzymes such as α-galactosidase, endo-β-mannanase and β-mannosidase. In addition to the enzyme assay, rate of galactomannan degradation and mobilization of component sugars of galactomannan (galactose and mannose) to the developing haustorium also

was analysed. Similarly, distribution of starch, sugars, soluble proteins and free amino acids also were analysed in order to elucidate the metabolism of endosperm cell wall degradation.

As mentioned earlier, endosperm hydrolysis and development of haustorium are taken place simultaneously. Owing to the pivotal role of haustoria which is a transient storage organ developed during seed germination in palm seeds, developmental aspects of haustorium also was taken as an important objective. Accumulation of metabolites in the haustorium studied by histochemical localisation was aimed at to pinpoint the region and forms/structure of insoluble polysaccharides accumulated in the haustorium. Biochemical changes of starch degradation and involvement of amylase activity in the mobilisation of soluble carbohydrates to the developing seedlings also was studied.

A logical starting point for the study of *Borassus flabellifer* seed germination is the elucidation of embryo structure in the developing and ungerminated seed, mode of germination, seedling morphology and components such as cotyledonary sheath, plumular sheath/plumule and haustorium. The features considered in the present study include development and differentiation of embryo and endosperm on one hand and germination–associated morphology, metabolism of storage products and mobilization on the other.

REVIEW OF LITERATURE

REVIEW OF LITERATURE

Members of palm family (Arecaceae) show many characteristic features during seed development and germination. Palm seeds contain a relatively small embryo compared to the size of the seed and copious endosperm. The embryo appears embedded in the thick walled endosperm. At the time of seed dissemination, the endosperm may be solid, creamy, liquid or a combination of the three consistencies. According to Rao (1959), the endosperm of palm seed is of the nuclear type. The cell wall thickens during its formation and becomes hard due to the deposition of hemicellulose. Seed reserves are present in the massive endosperm and also in the single cotyledon of the small embryo.

Tomlinson (1960) investigated the mature Date (*Phoenix dactylifera*) embryo and described that it consists of three distinct regions- the tubular base, the petiole and the distal region which is destined to develop into the haustorium. According to him, during early stages of development, the cotyledon resembles a unifacial leaf and never turns green and certainly does not resemble the plicate photosynthetic leaves of palm seedlings. Biradar and Mahabale (1968) described the anatomy of palm species such as *Phoenix dactylifera*, *Caryota urens* etc. and suggested that seed anatomy of these species showed many reliable characters for identification.

Haccius and Philip (1979) described the initiation and early development of the cotyledon of Coconut (*Cocos nucifera*) and opined that the structure is comparable with other palms. According to those authors, the formation of cotyledon begins as a crescent shaped mound on one side of the stem tip, then broadest part of it over arches the apical meristem and finally, as a result of cell division the cotyledon rounds up.

Developmental aspects of palm seeds have been reported only in very few species. The embryo of palm seeds consists of single- massive cotyledon surrounding the epicotyl axis originating from a short root-hypocotyl axis (Foster and Gifford, 1974). According to DeMason and Thomson (1981), in Date seed, the cotyledon is having a distal portion which appears as elliptical in cross section with as an asymmetrical tip on one side. According to those authors, the cotyledon of Date palm is composed of three cell types with similar structure. These three layers are parenchyma, protoderm and procambium and the layers can be distinguished on the basis of position, size and shape within the embryo. The procambial strands in the cotyledon consists of a ring of sympodia which bifurcates two to three times to form several vascular strands. The major organelles in the *Phoenix dactylifera* cotyledonary cells are large protein bodies which are distributed throughout the cotyledon. Numerous small lipid bodies are also present along the membranes especially on the plasmalemma (DeMason, 1985).

According to DeMason *et al*. (1983) the Date endosperm consists of nucleated living cells with homogenous structure. In addition, numerous protein and lipid bodies, poorly developed plastids and mitochondria are also occurring in the endosperm cells. Comparative studies on the ultra structure and protein conformation of the embryo and endosperm of *Phoenix dactylifera* revealed that the cells of the cotyledon and endosperm function in reserve storage and the cells contain nuclei and cytoplasm – rich in lipid and protein bodies (Chandra Sekhar and DeMason, 1988a). Those authors suggested that there are several embryo – and endosperm – specific proteins which are presumed to play important roles in germination and seedling development.

In a series of papers on *Washingtonia filifera*, the structure and storage reserves of the endosperm have been described by DeMason (1986) and Chandra Sekhar and DeMason, (1988b). According to DeMason (1986) the endosperm of *Washingtonia filifera* consists of living cells which store insoluble carbohydrate in the form of thickened cell walls as well as proteins and lipids as protein and lipid bodies in the cytoplasm. The embryo of the *Washingtonia filifera* is located on the dorsal side of the seed near the raphe with root pole oriented towards the testa. The embryo consists of a proximal single cotyledon, an epicotyl, a small root pole which is blunt and flattened and a pointed root apex (DeMason, 1988b). The cotyledon is circular in at the base and tapers to a point and functions as a storage organ and composed of

parenchyma, protoderm and procambium. All cell types contain prominent nuclei, nucleoli, organelles as well as protein and lipid bodies.

During seed development in palms, reserve metabolites are accumulated in two areas; the massive – hard endosperm and single cotyledon of the small embryo (Panza *et al*., 2004). The reserves of endosperm cells are lipid, proteins and hemicelluloses. According to those authors, the reserves of cotyledon – lipids and proteins – are present in the form of lipid and protein bodies. Hemicelluloses of endosperm constitute mainly mannans or galactomannans in the form of thickened cell walls. Anatomical studies in the seeds of *Euterpe edulis* revealed that the endosperm consists of living cells with thickened cell walls. The cell wall is the site of mannan storage and is very hard resulting in difficulty to obtain sections for anatomical studies. The endosperm cells contain lipid and protein bodies also (Panza *et al*., 2004; Andrade, 2001).

Mechanisms of seed dormancy and germination and are poorly understood in palms. Many species show rapid germination, while others take several years to germinate (Wagner, 1982). Existence of wide variation in germination time among palm seeds was noticed by Koebernik (1971). Seed germination generally requires several weeks to over a year to take place (Basu and Mukherjee, 1972). Germination of Coco-der-mer (double coconut-*Lodoicea maldivica*) takes three years to germinate (Anonymous, 1976).

Germination in most palms was found to be highly erratic and several years might be required for all the seeds of a lot to germinate. Oil palm seeds take over a period of several years to germinate under natural conditions (Alang *et al.*, 1988).

Members of palm family show a unique feature during seed germination. The proximal part of the single cotyledon elongates to push one part of the cotyledon along with developing shoot-root axes, while the distal portion remains inside the seed, expand tremendously in size and functions as the haustorium which absorbs the metabolites of the endosperm and replaces the endosperm during germination/ seedling growth (Gatin, 1906; Tomlinson, 1960). According to those authors, the exact regions of the cotyledon which performs these functions and growth mechanisms of these portions are not fully known. Tomlinson (1960) reported that the palm seeds usually have small embryos and copious amount of endosperm. The cotyledon consists of two main regions: the distal portion or haustorium which remains within the seed during germination, and the proximal portion that extends to push the root and shoot axes of the seedling into the ground. In seedlings, the haustorium is developed as the endosperm disappears, until it nearly fills the seed cavity. In *Cocos nucifera*, the haustorium becomes so large that it fills the endosperm cavity (Tomlinson, 1961). The haustorium apparently absorbs degradation products from the endosperm. This may be eventually transported to the growing axis. Keusch (1968) suggested that the developing

haustorium is the source of endosperm – digesting enzymes. Tomlinson (1961) described that during germination, elongation of the cotyledonary petiole is more in *Borassus flabellifer* compared to *Hyphaene* and the embryo is carried some distance from the seed and buried deep in the soil thereby exhibiting remotive type of germination. In *Washingtonia filifera*, cotyledon is elongated slightly in conjunction with the root elongation and the emergence of ligule and plumule takes place later (DeMason, 1988 a, b).

Germination in palm seed is of hypogeal and cryptocotylar type (Tomlinson, 1960). Based on the mode of germination, Gatin (1906; 1912), following the earlier observations of Martius (1823), divided palms into two categories – germination admotive or admote germination and germination remotive or remote or remotive germination. In remotive germination, cotyledonary sheath develop adjacent to the seed and elongation of the cotyledon is slight. The hyperphyll is completely haustorial in function. In this type a short cotyledonary ligule is said to be present. In the 2nd type – remotive germination – the elongation of the cotyledon is marked resulting in a fast growth of cotyledonary sheath. Two sub types are recognized in remotive germination such as ligulate and eligulate. Uhl and Dransfield (1987) followed Martius's basic germination types with a variation in terminology such as adjacent and remote with the latter having two subtypes- remote ligular and remote tubular. Tomlinson (1990) also suggested the synonyms adjacent (admotive) and remote (remotive). Palms like *Cocos*

nucifera and *Areca catechu* are characterized by admotive type of germination and mode of germination in *Phoenix dactylifera* and *Corypha umbraculifera* are remotive.

In remotive germination, proximal part of the cotyledon after emerging out of the seed elongates along the connective, which is termed the cotyledonary sheath or apocole (Cook, 1939). Tomlinson (1961) introduced the term apocole to describe the elongating part of the cotyledon that buries the plumule in the case of remote germination. The lower part of the apocole grows downwards into the soil and widens like a sheath (cotyledonary sheath) some distance away from the seed/fruit. The embryo is carried into the bottom of the sheath. Davies (1978) described the remotive germination and according to the author the cotyledon after emerging out of the seed elongates along the connective (apocole) and its lower part grows downwards into the soil some distance away from the seed and the tube widens like a sheath. Simultaneously, the embryo moves away from the seed into the bottom of the sheath. Such a type of germination is met with *Borassus flabellifer* (Mukherjee *et al*., 1961) *Phoenix roebelenii* (Doughty *et al*., 1986) *Phoenix dactylifera* (DeMason, 1985) and *Washingtonia filifera* (DeMason, 1986).

According to Fong (1978) the first sign of germination in palm seeds is a small, positively gravitropic protrusion formed by the cotyledon bursting through the micropyle. This protrusion gets elongated in *Eugeissona tristis*

seeds and forms the extension or cotyledonary tube extricating the plantlet from inside the seed to well below the soil surface. As the cotyledonary tube grows, the apex enlarges slightly. The plumule is developed inside the swollen portion. In six-week-old seedlings, cotyledonary tube is oriented upright and 10 week-old seedlings show swollen apex. After about 12 weeks, plumular sheath or the cataphyll is found to be developed. Eophyll or the first plumular leaf appears in about 16 weeks after germination.

According to Davis (1978), the germination in *Elaeis guineensis* is neither remotive nor admotive. The absorbing organ attached to the spongy cotyledon is the radicle and the first root is an adventitious one. The author observed that, during germination, the endosperm above the embryo is ruptured and a disc consisting of a layer of endosperm, testa and the operculum are extruded from the germ-pore together with the fibre plug. More than one seedling is produced in many cases.

Germination and seedling development have been studied in palms like *Phoenix dactylifera* (DeMason, 1984, 1985; DeMason and Thomson, 1981) and *Washingtonia filifera* (DeMason, 1988a) in which germination occurs when the root pole of the embryo protrudes through the seed coat resulting from elongation in the lateral portion of the cotyledon. In these two species the proximal portion of the cotyledon extend to push the root-shoot axes of the seed into the ground and the distal part remains in the seed and develop

into the haustorium and hence seeds are characterised by remotive type of germination.

A historical survey of studies of seedling morphology and anatomy in the palm family is given by Henderson (2006). The traditional three germination types- adjacent ligular, remote ligular, and remote tubular—that have been commonly recognized are re-evaluated in this review. The study includes seedlings of 63 species, representing the six subfamilies of palms. According to the author, germination types determined by the length of the hyperphyll (cotyledonary petiole) are not completely valid. Instead, a combination of characters such as primary root orientation, coleoptile length, number of cataphylls, and eophyll plication correspond to the most recent classification of the family, and represent a better way of describing germination and seedling morphology of palm seeds.

DeMason (1988a) described the germination of *Washingtonia filifera*. The germination occurs when root pole of the embryo protrude through the seed coat by the elongation of the basal region of the cotyledon and one ligule is produced by the cotyledon followed by one second leaf. The distal part of the cotyledon remains within the seed and develops to the haustorium.

As described earlier, the single cotyledon functions initially as a storage organ. During germination, the proximal end of the cotyledon

elongates and protrudes as cotyledonary sheath and the distal portion expand as the haustorium which grows into the endosperm concomitant with the degradation of the endosperm (Keusch, 1968; DeMason and Thomson, 1981; DeMason *et al*., 1983, 1985). According to DeMason (1985) the endosperm digestion and haustorial expansion of *Phoenix dactylifera* seeds are completed with 10 weeks after germination. Haustorial anatomy and development also have been studied elaborately in the seeds of *Phoenix dactylifera* (DeMason, 1984) and in *Washingtonia filifera* (DeMason, 1988b). According to those authors, the proximal region of the cotyledon expands by intercalary meristematic activity at the base. Vascular tissue matures first in the junction between the two portions of the cotyledon and continues basipitally. The haustorium consists of isodiametric parenchyma cells that grow vertically forming randomly oriented elongated cells. Vascular bundles are developed simultaneously throughout the haustorium.

Germination in *Phoenix dactylifera* seeds occurs when the basal portion of the cotyledon elongates and protrudes through the seed coat (DeMason, 1985). The root then elongates along with the production and elongation of the ligule. The ligule is tubular, and a scale leaf appears through the tip. The first green simple plicate leaf appears from within the scale leaf. The seedling axis remains very close to the seed because of the very close elongation in the cotyledon base. Sequence of germination and early seedling development of *P. dactylifera* are divided into five morphological stages: the

resting and imbibing stage; radicle emergence; production of the ligule; production of the scale leaf; and production of the first foliar leaf (DeMason, 1985).

Iossi *et al*. (2006) investigated the morphology, anatomy and germination behaviour of Pigmy date palm (*Phoenix roebelenii*) seeds and observed that during germination, protrusion of seedling begins with an elongation of an operculum, through which the cotyledonary petiole is emitted with the embryonic axis at its tip. The plumule emerges out through a rift in the posterior part of the cotyledon. According to those authors, endosperm is hard and the embryo is not fully differentiated and occupies a lateral position in the seed. The undifferentiated embryonic axis is enveloped by the cotyledon and during germination the cotyledon become wider and pushes the radicle outside the seed.

Tillich (2007) described the overall process of morphology of palm seed germination in general and remotive type in particular. According to the author, in majority of palm seedlings the distance between the seed and plumule is brought about by the exclusive elongation of the unifacial proximal part of the cotyledonary hyperphyll. So the apocole (Tomlinson, 1961) is restricted to the part of the hyperphyll between the haustorium and cotyledonary sheath in remotive type of germination. In some cases, the cotyledonary sheath elongates above the soil surface and sharply bent

downward and keeps buried or close to the soil surface. By the meristematic activity of the marginal meristem of the cotyledonary sheath, a tubular structure, the ‘coleoptile’ is produced and by means of further growth, a distal opening is formed as a narrow slit and the first plumule is emerged through this slit or opening. The distal part of the cotyledonary hyperphyll becomes the haustorium which is completely hidden in the seed. Following germination, the haustorium grows considerably, replacing the endosperm and takeover the main storage function. According to Tillich (2007) the haustorium is the cotyledonary hyperphyll and is positioned in contact with the nutritive tissue-the endosperm. When the haustorium is embedded in the endosperm, its shape is cylindrical, globular or flattened depending upon the shape and size of the seed. The developing haustorium is always in contact with the degrading endosperm during and following germination.

Palm seeds, like other monocotyledons, typically possess a small embryo with abundant, non-living thick walled endosperm. The endosperm cell walls function as carbohydrate storage and are composed mainly of linear (1-4) α-D-mannan or galactomannan resembling cellulose in the conformation of the molecular chain (Meier, 1958; Meier and Reid, 1982; Aspinall, 1983).

The seeds of most members of the palm family are known to contain mannans and or galactomannans (Dea and Morrison, 1975). The reserve polysaccharides occur mainly as cell wall components of endosperm (Meier

and Reid, 1982; Aspinall, 1983; Bewley and Reid, 1985; Reid, 1985). Galactomannans have been reported as seed reserves in *Phoenix dactylifera* (Meier, 1958; DeMason *et al*., 1989) *Borassus flabellifer* (Mukherjee *et al*., 1961; Mulimani and Prasanth, 2002) and *Washingtonia filifera* (Meier and Reid, 1982; DeMason *et al*., 1989).

Subrahmanyan *et al*. (1956) first reported the isolation of galactomannan from the soft kernel of *Borassus flabellifer* by extracting with aqueous 10% sodium hydroxide and showed the presence of 78.3% mannose and 27.6% galactose. Subsequently Rao and Mukherjee (1962) extracted galactomannan from the Palmyra palm (*Borassus flabellifer*) with 4% alkali and found that galactose and mannose with molar ratio 1:2.4 consisting of a backbone of (1→4) β-D mannopyranose residues with galactose and mannose side chains. Conventional spectroscopic study on mature seed of *Borassus flabellifer* revealed that the kernel consists of linear chain of D-mannosyl residues linked (1→4) in the polysaccharide (Awal *et al*., 1995). Those authors conducted GLC analysis of the hydrolysed methylated polysaccharides and indicated that two mannosyl residues are the repeating units carried substituted at C-6, D-mannose in one case and D-galactose in the other and conformation of the (1-6) linkage was done by ^{13}C NMR spectrum. The backbone of structural pattern in the polysaccharide also appeared to be similar to the structure reported by Rao and Mukherjee (1962).

In plants, pure mannans are defined as essentially linear β (1-4) mannan pyranoside chain containing less than 5% of galactose. Mannans are the main reserve materials in the seed endosperm of Arecaceae family (Avigad and Dey, 1997; Percival, 1966). According to those authors, two mannans – A and B – having different molecular weights have been isolated from ivory nuts. Mannan A is alkali soluble and mannan B is insoluble in alkali and is extracted by precipitating from cuprammonium solution (Meier, 1958).

Samonte *et al*. (1989) studied the endosperm hydrolysis and galactomannan degrading enzymes in maturing normal and 'makapuno' coconut during germination. Makapuno is a mutant coconut characterised by soft fluffy solid endosperm and very viscous fluid. The endosperm consists of water soluble galactomannan with a ratio of 3:1 mannose:galactose. According to the authors some of the abnormal cellular properties are due to the altered ratio of the galactomannan. During maturation of the 'macapuno' coconut, an increased activity of α-D-galactosidase and resultant two fold reduction of galactomannan was occurred. At the same time β-mannanase and β-mannosidase enzymes also were very active. During maturation of normal coconut the solid endosperm content was reduced as a result of α-galactosidase, β-mannanase and β-mannosidase activity. The activity of all the enzymes were increased several fold both in the endosperm and haustorium. By this study it was concluded that the activity of enzymes and

lack of mobilization of nutrients to the embryo could be attributed to the non-germination of the ‘macapuno’ coconut.

Keusch (1968) carried out light microscopic and biochemical investigations on mannan mobilization in *Phoenix dactylifera* endosperm following seed germination and stated that mannan chains are depolymerised in the dissolution zone surrounding the haustorium and decomposition is brought about by hydrolytic enzymes. The end product of mannan hydrolysis was mannose and the role of an endo mannanase and β-mannosidase activity was reported. On absorption by the seedling, the mannose is converted to sucrose. On the basis of experimental finding, the author concluded that the haustorium secretes the enzymes necessary for mannan degradation. According to Keusch (1968) haustorium apparently absorbs the degradation products from the endosperm and these products undergo modifications and eventually transported to the seedling axis. This author hypothesized that the haustorium has two biological functions (1) secretion of hydrolytic exo enzymes into the endosperm and (2) absorption of hydrolytic products from the endosperm.

DeMason (1984) described the in Date distal part of the cotyledon is transformed into the haustorium during germination. During seedling growth of Date the exterior intercalary meristematic activity occurs in the base of the cotyledon and concomitantly the haustorium expands and increases in

volume. The haustorium of Date seeds consists of isodiametric cells with reserves such as lipids and proteins stored in lipid bodies and protein bodies respectively (DeMason, 1985).

Large mannan-rich endosperm of palm seeds scarcely imbibe during/ prior to germination and seedling development (Meier and Reid, 1982). According to those authors, some water is initially required only for the imbibition of the small embryo which at first grows only downward. Water is required in the endosperm only to maintain the very narrow 'dissolution zone' – which surrounds the haustorium in which the mannan reserves are broken down. The storage of pure mannan which are essentially hydrophobic, appear to be a part of the palm seed's xeromorphic germination strategy.

As mentioned earlier abundant endosperm is present in *Phoenix dactylifera* seed and constitute living thick walled cells which stores carbohydrate in the form- mainly of (1→4) β-D mannans (Chandasekhar and DeMason, 1988a). During germination one part of the cotyledon is transformed into the haustorium which absorbs the product of degradation of the endosperm reserves. In Date seed, β-mannanase and β-mannosidase alone were detected in the dissolution zone near the haustorium. Besides it has been proposed that the haustorium may not be responsible for the production of these enzymes. Instead enzymes would be activated in the endosperm cells which are living and metabolically active by an unknown signal coming from

the haustorium. However, the author did not rule out the hypothesis that inactive enzymes could have been secreted by the haustorium and activate in the endosperm.

According to DeMason *et al*. (1983) starch is less abundant in Date endosperm but abundant carbohydrate – mannans are stored as thickened cell walls. Aleurone layer is absent and all the endosperm cells are living. During germination, the thickened cell walls are digested after the hydrolysis of cytoplasmic contents and wall digestion progresses from the cytoplasmic side (DeMason *et al*., 1985).

DeMason *et al*. (1985) elucidated the presence of two cell wall hydrolases – endo-β-mannanase and β-mannosidase – in the endosperm of *Phoenix dactylifera*. According to those authors, these enzymes are present in the endosperm before germination and at germination, the major portion of activity is found in the softened endosperm adjacent to the haustorium. Those authors opined that the cell wall hydrolytic enzymes are synthesized by the haustorium and is secreted into the endosperm where it is activated or released by the regulating signal emanating from the haustorium. According to Chandra Sekhar and DeMason (1990) α-galactosidase activity increases 10 fold during cell wall digestion in the germinating seeds of *Phoenix dactylifera*. DeMason *et al*. (1992) assessed the role of α-galactosidase in the developing and germinating *Phoenix dactylifera* seeds and concluded that α-

galactosidase is synthesized during endosperm development and unique forms of these enzymes are associated with cell wall induction and cell wall mobilization in Date seeds.

According to Meier and Reid (1982) mannan group of cell wall reserves constitute mainly mannans formed by linear (1→4) β-linked chain of D-mannose residues and galactomannan in which the galactosyl residues are linked by (1→6) α-substitutes. 'Pure mannan' contains less than 10% non mannose. Meier (1958) reported that Date seeds are characterised by pure mannans (92%) and only 8% cellulose is present. In Ivory nut (*Phytelephas macrocarpa*) seeds, mannose is 97%, galactose 1.8% and glucose 0.8% (Aspinall *et al*., 1958). Alang *et al*. (1988) stated that in oil palm (*Elaeis guineensis)* seeds thick cell wall of endosperm constitute 47% of the dry weight and during germination the thick cell wall become markedly thinner, concurrent with a significant reduction in the insoluble carbohydrate and an increase in α-galactosidase and β-mannosidase activity in the degrading and residual endosperm. The insoluble polysaccharide of oil palm seed was found to be galactomannan. Eventhough Oil palm seeds contain 36% lipids, galactomannan is utilized more rapidly than lipid during the early stages of germination.

Excellent reviews on seed cell wall storage polysaccharide (CWSP) other than starch in general (Meier and Reid, 1982) and on mannans/

galactomannas in particular (Halmer and Bewley, 1982) cover an exhaustive description of extra cellular polysaccharides and their metabolic fate of the whole group of the compounds together. According to those authors, in addition to palm seeds, many legume seeds also have been reported to contain mannans and/or galactomannans as seed reserves in the endosperm tissues. Structure and metabolism of galactomannan have been well studied and documented in *Trigonella foenum-graecum*. (Reid and Meier, 1970; Reid, 1971; Reid and Meier, 1972; Reid and Meier, 1973; Meier and Reid, 1977; Reid and Davies, 1977; Reid and Bewley, 1979; Leung *et al*., 1981; Meier and Reid, 1982; Campbell and Reid, 1982; Spyropoulos and Reid, 1985; Dirk *et al*., 1999).

Gong *et al*. (2005) suggested that hard seeded species like *Trigonella foenum-graecum*, *Cassia tora*, *Ceratonia siliqua* and *Phoenix dactylifera* possess seeds with substantial endosperm with thickened cell walls composed of mannan and/galactomannan and as the endosperm completely surrounds the embryo it is possible that the endosperm restricts or prevents the germination owing to the nature of these cell walls. It has been suggested that the weakening of endosperm cell walls is required to allow the protrusion of radicle from the seeds (Bewley, 1997a). Light microscopic studies of these seeds revealed that there is localised modifications of endosperm cellular structure which offers less resistance to the radicle emergence, in all species except Date palm (Mayer and Poljakoff-Mayber, 1989). Although the

majority of endosperm cells have thick walls, those in the micropylar region of endosperm do not. In addition, there is a transition from thick walled to thin walled cells between lateral and micropylar endosperm that appears to be dependent on the cellular position relative to radicle tip, forming endosperm cap (micropylar endosperm) which disintegrates following penetration by radicle. In Asparagus and Coffee seeds, it has been reported that although the vast majority of the lateral endosperm have thickened cell walls those of micropylar endosperm adjacent to and overlying the radicle tip are having selectively thin walls (Williams *et al*., 2001; da Silva, *et al*., 2005).

Classification and evolutionary aspects of seed galactomannans of the family leguminosae revealed that significant variation exist in the yield of galactomannan at sub- familial and tribal levels (Buckeridge *et al*., 1995). Those authors further opined that the yield and composition of seed galactomannan from tropical legumes and their multifunctional polysaccharide content could be considered as an important factor for the adaptation of these species to the tropical humid, semi-arid and arid climates.

In some seeds, it is an established fact that dormancy is caused by the tissues that surrounds the embryo which acts as a mechanical barrier to the emergence of the radicle. It is proposed that germination is completed following the production of hydrolases within the surrounding tissues that secreted into the cell wall, causing weakening and so allowing the radicle to

breakout (Bewley, 1997 a, b). According to the author mannanase is a prominent component of endosperm cells of dormant seeds of several species and products of endo-β-mannanase activity has been suggested to be essential for germination of those seeds. According to Bewley (1997 a) in *Datura ferox* seeds there is a weakening of endo-β-mannanase activity hours prior to the completion of germination. However, the author opined that proof for the activity of this enzyme alone or in part is the cause of endosperm cell wall weakening remains to be established. In *Datura* seeds anatomical observation showed that erosion of the endosperm immediately in front of the radicle tip occurred prior to germination (Sanchez *et al*., 1990). Also, the content of cell wall polysaccharides – mainly galactomannan – markedly decreased in the micropylar portion of the endosperm before radicle protrusion in *Datura* seeds. Furthermore, the weakening of the endosperm is due to the breakdown of the endosperm cell wall which is mediated by the action of the cell wall degrading enzyme, endo-β-mannanase (Groot *et al*., 1988; Sanchez *et al*., 1990; Watkins *et al*., 1985).

Endosperm degradation and mobilisation of the products in seeds during germination depends on the synthesis and activity of many enzymes and the important well established enzymes are endo-β-mannanase, β-mannosidase, and α-galactosidase (Meier and Reid, 1982). Halmer and Bewley (1982) stated that hydrolysis of cell wall polysaccharides helps diffusion of enzymes and reserve degradation products from the endosperm to

the embryo (haustorium), thus to provide carbohydrates for seedling growth. According to those authors, in *Borassus flabellifer* endosperm cell walls provide hydrolysates which are translocated to the growing seedling as well as haustorium. In addition, cell wall degradation facilitates diffusion of enzymes and sources/ region of these enzymes is apparently the endosperm. The presence of endosperm hydrolysing enzymes, endo-β-mannanase and β-mannosidase in the endosperm and haustorium of germinating coconut seeds was reported by Samonte *et al*. (1989). Those authors suggested that β-mannanase and β-mannosidase activity is notably higher in the haustorium than in the endosperm indicating the greater rate of degradation of larger galactomannan polymers to smaller units occurs in the haustorium for the early uptake by growing seedling. Galactomannan is completely hydrolysed following germination to its monosaccharide constituents-mannose and galactose, which are then absorbed by the growing embryo (McCleary, 1983). In Lucerne (*Medicago sativa*) and Guar (*Cyamopsis tetragonoloba*) seeds, α-galactosidase action has been suggested to be required before the other two enzymes can hydrolyse cell wall galactomannans (McCleary and Matheson, 1975). According to Buckeridge and Dietrich (1996) in *Sesbania marginata* cell wall polysaccharides (galactomannan) is composed of main chain of D-mannose residues attached to each other through β (1-4) linkage. The main chain is branched in variable extends by units of D-galactose through α-(1-6) glycosidic linkage providing polymers with different mannose: galactose

ratio. Following germination, galactomannan is completely degraded by hydrolytic enzymes like α-galactosidase, β-mannanase or β-mannosidase resulting in the formation of monosaccharide constituents-mannose and galactose which are then absorbed by the growing seedling as the source of carbon.

Elucidation of galactomannan distribution and metabolism has been carried out elaborately in legume seeds (Anulov *et al.*, 1998; Buckeridge *et al*., 1995, 2000). According to Buckeridge *et al*. (2000) among the legumes the most studied species includes Guar (*Cyamopsis tetragonolobus*), Fenugreek (*Trigonella foenum-graecum*) and Carob (*Ceratonia siliqua*). In the seeds of fenugreek and guar, endosperm cells are nonliving and almost totally filled with galactomannan. Buckeridge *et al*. (2000) suggested that besides playing as a post germinative reserve, galactomannan can also serves as inhibiting substances in early stages of germination. According to those authors the mannanase may be considered as a bifunctional molecule which plays a role as a constraint for radicle emergence during germination and also as a storage polysaccharide after germination, the latter mainly in palm seeds where mannan yield is higher.

Mobilization of galactomannan and involvement of three hydrolysing enzymes α-galactosidase, endo-β-mannanase and β-mannosidase have been reported in legume seeds (Buckeridge *et al*. 2000). According to those authors

the galactomannan is disassembled to its monosaccharide constituents (free-mannose and galactose) and at the same time sucrose also is produced. Apparently sucrose is the sugar mobilized to the growing embryo as the carbon source; starch is transiently produced in the cotyledons. Dirk *et al*. (1999) proposed that storage cell wall degradation and starch synthesis are interrelated in *Trigonella foenum-graecum*.

According to Ganter *et al*. (2001) galactomannans are energy reserve polysaccharides found in the endosperm of seeds inclusive of palms. Hydrolysis of galactomannan in the endosperm of germinating seeds requires the presence of at least three enzymes, α-galactosidase (EC 3.2.1.22) for removal of the (1-6) α-D galactoside chains; β-mannanase (EC 3.2.1.78) for the hydrolysis of the (1-4) β-D mannan backbone into oligosaccharides; and β-mannoside mannohydrolase (β-mannosidase or exo-β-mannanase, EC 3.2.1.25) for complete hydrolysis of β-D-mannooligosaccharides to D-mannose. α-galactosidase and β-mannanase activities increase on germination concurrently with galactomannan depletion.

Unlike the palm seeds, endosperm capable of producing endo-β-mannanase has been reported in seeds like *Apium graveolens* (Jacobsen and Pressman, 1979) *Lactuca sativa* (Halmer and Bewley, 1979) *Lycopersicon esculentum* (Groot *et al*, 1988; Carrington *et al.*, 2002) *Sesbania virgata*

(Tonini *et al.,* 2006, 2010). All these seeds are having thin endosperm in which degradation is induced by gibberellic acid produced by the embryo.

Ren *et al*. (2007) investigated the relationships among α-galactosidase, endo-β-mannanase and β-mannosidase in degrading galactomannan in the cell wall of rice seeds during and following germination. The activities of α-galactosidase are present in the dry and pre-germinated seeds and increased during germination. However, the activity of endo-β-mannanase was detected only after germination. So those authors proposed that this enzyme plays a role in the mobilisation of galactomannan reserves and provides nutrients for seedlings. Studies on the hydrolysis of endosperm cell walls in Tomato seeds prior to radicle protrusion showed the activity of endo-β-mannanase is exclusively in the micropylar endosperm tissues prior to radicle emergence (Groot *et al*. 1988; Nonogaki and Morohashi, 1996). According to Toorop *et al*. (1996) endo-β-mannanase isoforms are present in the endosperm and the embryo of Tomato seeds but are not essentially linked to the completion of germination. Later, endo-β-mannanase activity has been reported in both endosperm and radicle tissues of Tomato seeds (Still *et al*. 1997). Galactomannan hydrolysis by the activity of β-mannanase, β-mannosidase and α-galactosidase have been investigated in *Lycopersicon esculentum* (Bewley *et al*., 1997; Mo and Bewley, 2002) *Trigonella foenum-graecum* (Gong *et al*., 2005) *Prosopsis juliflora* (Gallao *et al*., 2007) *Oryza sativa* (Ren *et al*., 2007, 2008) *Sesbania virgata* (Tonini *et al*., 2006, 2010)

Increased activity of endo-β-mannanase following germination have been reported in endospermic seeds in which the increase occurs after the emergence (Reid and Meier, 1973; Dirk *et al*., 1995, 1999; Wang *et al*., 2005). According to Wang *et al*. (2005) the role of endo mannanase in the breakdown of endosperm cell walls during mobilisation of major carbohydrates and protein reserves is presumed to be associated with degradation of cell walls in the starchy endosperm along with other hemicellulases thus facilitating the passage of α-amylases and proteases. A slight increase in the endo-β-mannanase activity during germination and greater increase following germination in rice seeds can be attributed to the earlier synthesis of this enzyme in the aleurone layer and subsequent accumulation in the starchy endosperm which constitute dead cells where enzymes does not occur. Those authors further opined that although β-mannosidase acts in concert with endo-β-mannanase to degrade polymeric mannans, the activity was very low during germination and post-germinative phases largely confined to the starchy endosperm and embryo.

Mo and Bewley (2003) reported the relationship between β-mannosidase and endo-β-mannanase activities in tomato endosperm during and following germination. Comparison of lettuce seed population and individual seed revealed that there exists correlation between the activities of both the enzymes in the micropylar endosperm. It is apparent that within the individual seed parts, there may be large differences in the amount of enzyme

activity which is an indication of lack of co-ordination of the activities of these two enzymes within the individuals of a population.

Enzyme activity and characterization of β-mannanase (1,4-β-D-mannan mannohydrolase) have been investigated in Tomato seeds (Nonogaki *et al*., 2000; Bourgault and Bewley, 2002 a, b; Bourgault *et al*., 2001, 2005). According to Bourgault and Bewley (2002 a) the activity of β-mannanase in Tomato (LeMAN4a) increases in the outer tissues during ripening and the enzyme is localised within the cell walls. While elucidating the crystalline structure of β-mannanase, Bourgault and Bewley (2002 b) have shown that the penultimate (L 398) residue is important for catalytic activity. According to those authors, variation in the C-terminal amino acids determines the activity of endo-β-mannanase of different cultivars of Tomato fruits during ripening. Bourgault *et al*. (2005) have elucidated the three dimensional crystalline structure of *Lycopersicon esculentum* β-mannannase and the structure had been compared with those of fungal β-mannanase and a model of substrate in LeMAN4a also has been proposed.

Recently, characterization and gene expression of enzyme endo-β-mannanase during and following germination was investigated in rice seeds (Ren *et al*., 2008). Enzyme assay and isoform analysis of whole seeds, scutellum, aleurone layer and starchy endosperm revealed that seeds begun to express endo-β-mannanase activity during germination and three isoforms of

endo-β-mannanase were detected. An endo-β- mannanase present in the dry seed was inactive. The amount of protein was decreased in the scutellum, but increased in the aleurone layer during and following germination. The author concluded that the increase in endo-β-mannanase activity in the rice grain may be due to the activation of extant protein and or the *denovo* synthesis of enzymes.

Scheller and Ulvskov (2010) reviewed and discussed the hemicelluloses and their biological roles and contribution to strengthening cell wall by interaction with cellulose and/ or lignins. According to those authors, these hemicelluloses including xyloglucans, xylans, mannans and glucomannans are synthesized by glycosyl transferases located in the golgi membranes and many glycosyl transferases are needed for the biosynthesis of xyloglucans and mannans. However, biosynthesis of xylans and β (1→3, 1→4) glucans remains elusive and recent studies have led to more questions than answers. In addition to the primary role of interaction with other polymers to ensure physical properties, hemicelluloses have been recruited to the function of seed storage carbohydrates.

MATERIALS AND METHODS

MATERIALS AND METHODS

Choice of Plant

Borassus flabellifer L. commonly known as palmyra palm belongs to the family Arecaceae was chosen for the present investigation. It is a dioecious robust palm reaching the height up to 10-25m., with a canopy of leaves which includes several dozens of fronds spreading 2-5 ft. across. The large trunk resembles that of the Coconut tree and is ringed with leaf scars. The fruits are spherical or hemispherical of about 8-15 cm in diameter with a smooth shiny epicarp – green when young and becoming deep violet or black when old. Usually three pyrenes are found in a fruit. The mesocarp is thick, fibrous and deep yellow in color with a fruity smell when ripe. The endocarp is stony, 3-5 mm thick and surrounds each pyrene. The seeds are more or less hemispherical, flattened on cross section with inner ridge both on ventral and dorsal side and with the testa adhering to the pyrene. The embryo is top shaped and is embedded in the endosperm near the micropylar end. The endocarp is stony and hard but remains as a soft shell adjacent to the region of the embryo thereby leaving an opening for the protrusion of the embryo during germination. The cotyledon is massive, solid and occupies a major portion of the embryo and is located towards the inner side of the seed.

Collection of Materials

Fruits of *Borassus flabellifer* were collected during January to June of 2007-2009 from a selected group of palms growing at Pattambi, Palghat District. Regular periodical collections were made to get fruits of all the developmental stages as well as mature ones for germination studies. Since the plant is very tall, collection of fruit-bunches was done manually using ropes and plastic nets to avoid damage during harvesting.

Developmental Aspects

Sampling

A preliminary survey revealed that about 6 months are required for the maturation of *B. flabellifer* fruits. So sample collection for seed developmental studies was done at specific intervals and approximate duration required for each developmental stage of fruit/seed is one month as given in Table 1. Collection of fruits was done during first week of every month.

Based on the stage of development, fruits were collected at six stages (Table 1) for various physiological/biochemical studies. A minimum of 20 fruits of each stage were collected at a time and were randomized. Each fruit containing three seeds (pyrenes) was cut to separate individual seeds.

Table 1. Approximate duration of sampling for the study of seed development of *Borassus flabellifer*

Stages	Sampling Intervals
1	November
2	December
3	January
4	February
5	March
6	April

Seeds consist of thick endocarp (which become very hard when mature), bulky endosperm and small embryo. In very young seeds embryo appeared very small and embedded in the gel-like (syncytium) endosperm. Later, the endosperm became solid and gradually got hardened. During sampling the embryo was removed carefully and the endosperm of 12-15 seeds was scooped and pooled together after breaking the endocarp. From the pooled tissues of sample endosperm and embryo were taken separately for various experiments.

Histochemical Studies

In young seeds embryo was embedded in the liquid endosperm, so sampling was done only after the endosperm became gel-like. The embryo

was scooped along with surrounding endosperm and the tissues were fixed in FAA (Johansen, 1940). When the endosperm became thick and hard, embryo and endosperm were sampled separately. Dehydration of fixed samples was done through alcohol-TBA series (Berlyn and Miksche, 1976), paraffin infiltrated and embedded. Using a Rotary Microtome (Leica Model RM 2125RT) the individual blocks were cut into 10 µm and the sections were used for anatomical/histochemical staining.

Staining

Deparaffinised sections were stained with Bromophenol blue to localize proteins (Maizia *et al.*, 1953) Hematoxylin (Berlyn and Miksche, 1976) and Toluidine blue (Khasim, 2002) for staining of cytoplasm and cell wall respectively.

PAS Staining

Insoluble polysaccharides were localized by staining with Periodic acid Schiff's (PAS) reagent (Berlyn and Miksche, 1976). The hydrated sections were placed in 0.5% (w/v) periodic acid solution at 23 ºC for 15 minutes and the sections were washed in running tap water for 10 minutes. The sections were then placed in Schiff's reagent for 10 minutes at 3±1 ºC and washed in tap water for 20 seconds. After washing, the sections were placed in 2% sodium bisulfite for 2 minutes and washed again in tap water

for 5-10 minutes. The sections were dehydrated through alcohol series and cleared in xylene.

Slides with stained sections were mounted in DPX and observed. Photomicrographs of the sections were taken using microscope (Nikon Model- ECLIPSE E 400) equipped with digital camera (Nikon Model- DXM 1200F) and image analyzer.

Biochemical Studies

Samples of embryo and endosperm were taken for biochemical studies also, at different intervals as described earlier. The following metabolites were analysed using standard methods.

Starch

The starch content of embryo and endosperm at various stages of development was determined by the method of Pucher *et al*. (1948) as described by Whelan (1955).

Extraction

Seeds (a minimum of 5-number) were cut and endosperm and haustorium were separated. Each tissue was separately cut into small pieces and from the pooled sample 1g tissues was ground using glass mortar and pestle in 30% (v/v) perchloric acid. The homogenate was centrifuged at 3000 x g and the supernatant was collected. The residue was again

homogenized in 30% (v/v) perchloric acid and centrifuged. The processes of homogenization and centrifugation were repeated till the entire starch content of the tissue was extracted. Volume of the combined supernatant was noted. A known volume of the extract was taken and an equal volume of freshly prepared iodine–potassium iodide reagent was added to the tube and mixed well using a vortex mixture. The mixture was then kept undisturbed for 20 minutes and centrifuged for 10 minutes. The supernatant was decanted off. The excess iodine reagent present in the residue was removed by washing the precipitate with alcoholic sodium chloride followed by alcoholic sodium hydroxide to remove bound iodine. The residue was again washed with alcoholic sodium chloride. It was then dissolved in a known volume of 10% (v/v) sulphuric acid and used for the estimation of starch.

Estimation

Estimation 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 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 was measured at 540 nm using a spectrophotometer (Genesys-20). Soluble starch was used as the standard.

Proteins

Total Proteins

Total protein in the endosperm/ haustorium was estimated according to 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). 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, kept in an ice bath for flocculation. The mixture was centrifuged at 3,000xg for 10 minutes and the supernatant was decanted off. The precipitate was washed with 2.0% (w/v) trichloroacetic acid and centrifuged. The 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 and centrifuged. 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 UV/visible spectrophotometer (Schimadzu Model-UV-1601). Bovine serum albumin-fraction V powder was used as the standard.

Soluble Protein

The quantity of soluble protein in the endosperm/haustorium 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). 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 earlier for the estimation of soluble proteins.

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 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 refrigerated centrifuge (Remi Model- C24-BL) at 4^{o}C and the supernatant was collected.

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.

Gel Casting

The gel was casted in a casting unit (Hoefer™ miniVE). The glass plates, the comb and the spacers of the casting unit were wiped clean with alcohol. Then the glass plates were wiped with acetone. The dried glass plates were clamped on to 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. After ensuring the polymerization of resolving gel,

stacking gel was poured and further comb was placed carefully so as to avoid the entrapping of air bubble. The gel was topped with deionized water. After polymerization the comb was removed carefully and the wells were cleaned thoroughly. Extract containing equalized quantity of proteins with 20% sucrose was added to each well. Bromophenol blue was used as the tracking dye. Low molecular weight marker (Biorad-PMWL) 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 the same voltage was maintained 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-water (4:1:5) and were further stored in 7% (v/v) acetic acid. The gels were analyzed in a Gel Doc (Bio-Rad) and molecular weight of the bands was determined using software (Quantity One).

Free Amino acids

Known weight of endosperm tissues at various stages of seed development was ground using mortar and pestle in 80% alcohol and refluxed for 5 hours in RB flask. Then the mixture was centrifuged at 3,000

x g for 10 minutes. Supernatant after centrifugation was collected in China dish and residue was re-extracted 3-4 times and the combined supernatant was dried at 55 °C in a hot air oven. The residue was eluted in known volume of distilled water, centrifuged and the supernatant was collected.

Ion Exchange Chromatography

One ml of the extract was passed through a column of freshly regenerated Dowex (50W-X8) according to Kliewer (1964) and eluted with distilled water. The fraction thus collected was used for the analysis of sugars. Ammonia solution (0.2 N) was used for the elution of bound amino acids from the column.

The sample obtained by eluting the amino acids from the Dowex Colum with 0.2 N ammonia solution was dried in a hot air oven at 60 °C and was used for amino acid separation and estimation by two dimensional descending paper chromatography.

Paper Chromatography

Known quantity of a sample was applied as one spot at the corner of the Whatman No.1 chromatography paper and separated by two dimensional descending chromatography. The solvent system for first run was butanol-acetic acid- water (100:20:50 v/v) and for the second run phenol-water (80:20 v/v).

Visualisation of the amino acids was done by spraying a mixture of 0.2% ninhydrin dissolved in acetone and heating in a hot air oven at 100 °C for 5 minutes. The individual amino acids were identified by comparing with simultaneously prepared chromatogram of standard amino acids. For estimation, individual spots of amino acids were cut and extracted in 80% acetone and the colour intensity was measured at 490 nm in spectrophotometer (Genesys 20).

Sugars

The fraction collected after ion exchange chromatography through the Dowex (50W-X8) column by eluting with distilled water was dried at 60 ºC in a hot air oven and dissolved in known quantity of distilled water and was used for the identification and estimation of sugars.

Paper Chromatography

The separation of sugars was done by one-dimensional descending paper chromatography using Whatman No.1 chromatography paper. The paper was marked off into two halves longitudinally so that at the end of development one half alone was sprayed with the reagent for the visualization of sugar spots. Known quantities of the samples were spotted on the paper in separate spots and allowed to run for 72 hours in a descending chromatography chamber using n-butanol, water and acetic acid (40:50:10) as the solvent system (Patridge, 1948). After running, the paper

was taken out, dried and cut into two halves. Sugar spots were visualized by spraying freshly prepared aniline-diphenylamine- phosphoric acid reagent according to Buchan and Savage (1952) and was developed in a hot air oven at 100 °C for 5 minutes. Sugar spots were identified by comparing it with simultaneously developed chromatogram of standard sugars. For quantitative analysis, sugars of unstained half of the paper was marked by superimposing the sprayed half of the paper according to Bacon and Edelman (1951). Quantitative determination of eluted sugars was done according to Montgomery (1957) as described earlier.

Insoluble Polysaccharides other than Starch (Galactomannans)

Extraction

Insoluble polysaccharide other than starch was extracted from the powdered dry endosperm tissue sampled at various intervals of seed development 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 homogenate 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. After centrifugation the pellet was dried and weighed. Known quantity of the powder was hydrolysed with 5 ml 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 Petri dish and dried at 55 °C in a hot air oven. It was then dissolved in known volume of distilled water.

HPLC

The sample was then subjected to separation and identification of the components by High Performance Liquid Chromatography (HPLC) system available at Sree Chitra Thirunal Institute for Medical Science & Technology (SCTIMST) Trivandrum. Twenty µl of aliquot was injected into the HPLC system consisting of Waters Spherisorb–NH2 column, Waters 600 series pump and Waters 717 plus auto sampler. The mobile phase was acetonitrile: water (75:25) which was set 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.

Germination Aspects

Mature fruits immediately after collection were separated into individual pyrenes, de-pulped and washed under tap water. Soil beds (4 x 4 m) were prepared by heaping soil at about 60 cm height and pyrenes were planted on the top of the soil heap, 10 numbers in a row and watered regularly. Most of the seeds started germinating during 35±5 days after

planting. Protrusion of cotyledonary sheath was considered as the sign of germination. Stages of seed germination were determined on the basis of morphological features and samples of seedlings were collected at regular intervals as given in Table 2.

Table 2. Approximate duration of sampling for the study of seed germination in *Borassus flabellifer*

Stages	Sampling Intervals
1	May
2	June
3	July
4	August
5	September
6	October

Seeds/seedlings were carefully taken out of the soil and washed thoroughly in tap water and separated into cotyledonary sheath, endosperm and haustorium. Since the endocarp and endosperm were very hard to break open, a marble cutter (Hi-Flex Model- HF-CM-4) was used. After cut-opening the pyrenes, endosperm and haustorium were sampled separately.

Histochemical Studies

Germinated seeds were separately sampled into haustorium and endosperm and the process of tissue- fixation, dehydration, paraffin infiltration, microtomy and staining were performed as described earlier.

Staining

Deparaffinised sections were stained with Bromophenol blue, Hematoxylin, Periodic acid Schiff's reagent and Toluidine blue as described earlier.

Biochemical Studies

As mentioned earlier, during germination, as the proximal end of the cotyledon grows outward carrying the embryo, the distal end of the cotyledon grows to a haustorium. The endosperm in contact with the haustorium appears as slimy due to the degradation of the hard endosperm tissues indicating that hydrolysis of the endosperm occurs in this region. So tissues for biochemical analyses and enzymes assay were collected by scooping this slimy endosperm tissue from the hard tissue which gradually becomes soft and gets reduced during and following germination associated reserve mobilization. Haustorium and hard endosperm tissue also were analyzed separately. A minimum of 5-10 seeds each were cut pooled and the tissues were taken for various analyses at each interval.

Starch, Proteins, Amino acids, Sugars, and Galactomannans

The tissues for metabolites such as Starch, Proteins, Amino acids, Sugars, and Galactomannans were sampled and analyzed during all the intervals by the procedure already described earlier.

Enzyme Assay

Assay of endosperm (galactomannan) degrading enzymes such as α-galactosidase, β-mannosidase and endo-β-mannanase were done.

α-Galactosidase

Assay was done based on the method proposed by Leung and Bewley (1981). One gram endosperm tissue of each stages of germination was scooped as described above and was ground using pre-chilled mortar and pestle in 10 ml low salt citrate-phosphate buffer (pH - 5.4) consisting of 8.3 ml of 70 mM NaCl and 1.7 ml citrate phosphate buffer with 50 mg polyvinyl poly pyrrolidone (PVPP) under chilled conditions. After centrifugation in a refrigerated centrifuge (REMI Model- C24-BL) at 4 °C and 12,000x g for 15 minutes, the supernatant was collected and α-galactosidase assay was done.

The assay system consisted of 0.1 ml 0.1 M citrate phosphate buffer (pH 5.4) and 0.2 ml para nitrophenyl α-D- galactopyranoside (10^{-3} M). Reaction was initiated by adding 25 µl enzyme extract (10% w/v) to the experimental tubes and incubated at 30 °C for 30 minutes. Reaction was

ceased by adding 1 ml cold Na_2CO_3 (10^{-1} M) and the resultant colour developed was measured at 440 nm using a Spectrophotometer (Genesys 20). Control tubes are supplemented with 25 µl enzyme extract after ceasing the reaction with 1 ml cold Na_2CO_3 (10^{-1} M).

pH Optimum

The optimum pH (5.4) for enzyme activity was determined by incubating enzyme assay system as described above for 30 minutes in buffers of a pH ranging from 4.0-7.0 at intervals of 0.2 pH. The pH of the buffer in which the enzyme showed highest activity was taken as optimum pH.

Temperature Optimum

The temperature optimum (30 °C) of α-galactosidase activity was determined by incubating the assay system for 30 minutes at a temperature ranging from 20 to 40 °C at an interval of 2 °C having optimum pH 5.4. The temperature at which the enzyme showed highest activity was considered as optimum temperature.

Enzyme Proportionality

The proportionality range (25 µl of 10% (w/v)) for enzyme activity was determined by incubating the assay system for 30 minutes at optimum

temperature (30 °C) with optimum pH (pH 5.4) and different volumes of 10% (w/v) enzyme extract ranging from 5-50 µl at an interval of 5 µl.

Substrate Saturation

The substrate saturation (200 µl-1mM para nitrophenyl α-D-galactopyranoside) for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature (30 °C) with optimum pH (5.4) and 25 µl of 10% (w/v) enzyme extract and different concentration of the substrate (para nitrophenyl α-D- galactopyranoside -10^{-3} M) ranging from 20-400 µl at an interval of 20 µl.

Unit Activity

One unit of α- galactosidase activity was defined as the amount of enzyme that catalyzes the hydrolysis of one mole of para nitrophenyl α-D-galactopyranoside per hour at 30 °C and at 5.4 pH.

β–Mannosidase

Method for β- mannosidase assay was adopted from Ouellette and Bewley (1986). One gram fresh endosperm tissue of each stage of germination of the seed was ground using pre-chilled mortar and pestle in 10 ml low salt citrate-phosphate buffer (pH - 5.4) consisting of 8.3 ml of 70 mM NaCl and 1.7 ml citrate phosphate buffer with 50 mg PVPP under chilled conditions. After centrifugation at 12,000x g in a refrigerated

centrifuge (REMI Model- C24-BL) at 4 °C for 15 minutes, the pellet was suspended in high salt buffer consisting of 1.3 ml 0.2 M citrate phosphate buffer (pH 6.0) and 8.7 ml of 1 M NaCl. The extract was allowed to stand on an ice bath for 30 minutes with occasional mixing and further it was centrifuged at 12, 000x g for 30 minutes. The supernatant was collected for the assay of β- mannanase.

The assay system consisted of 300 µl 0.2 M citrate phosphate buffer (pH 5.4), 1 ml BSA (0.25 mg ml^{-1}), and 150 µl para nitro phenyl β-D-mannopyranoside (10 mM). Reaction was initiated by adding 50 µl enzyme extract (10% w/v) in experimental tubes and incubated at 30 °C for 30 minutes. Reaction was stopped by adding 1ml cold Na_2CO_3 (200 mM). The control tubes were also added with 50 µl (10% w/v) enzyme extract. The intensity of the yellow color formed was measured at 400 nm using Spectrophotometer (Genesys 20).

pH Optimum

The optimum pH (5.4) for enzyme activity was determined by incubating enzyme assay system for 30 minutes at 30 °C with substrate, in buffers of a pH range 4.0-7.0 at intervals of 0.2 pH. The pH of the buffer in which the enzyme showed highest activity was taken as optimum pH.

Temperature Optimum

The temperature optimum (30 °C) of α- galactosidase activity was determined by incubating the assay system for 30 minutes at a temperature ranging from 20 °C to 40 °C at an interval of 2 °C with substrate and buffer having optimum pH (5.2). The temperature at which the enzyme showed highest activity was considered as optimum temperature.

Enzyme Proportionality

The enzyme proportionality range (50 µl) for activity was determined by incubating the assay system for 30 minutes at optimum temperature (30 °C) with optimum pH (5.4) optimum substrate concentration (para nitro phenyl β-D-mannopyranoside -10 mM, 150 µl) and different volumes of 10% (w/v) enzyme extract ranging from 10-100 µl at an interval of 5 µl.

Substrate Saturation

The substrate saturation (para nitro phenyl β-D-mannopyranoside-10 mM, 150 µl) for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature (30 °C) with optimum pH (5.2) and optimum enzyme concentration (50 µl of 10% (w/v) and different concentration of para nitrophenyl α-D- galactopyranoside (10^{-3} M) ranging from 10-200 µl at an interval of 20µl.

Unit Activity

One unit of β-mannosidase activity was defined as the amount of enzyme that catalyzes the hydrolysis of one mole of paranitrophenyl manno pyranoside per hour at 30 °C and at pH. 5.4

Endo-β-Mannanase

For the extraction of the enzyme, 1 g of slimy endosperm of each stage is ground in citrate phosphate buffer (pH 7.0) consisting of 3 M LiCl and 10% PVP in ice cold mortar and pestle. The extract was centrifuged at 12,000x g for 15 minutes and the supernatant is used for the endo-β-mannanase assay.

Preparation of Galactomannan substrate

Galactomannan substrate is prepared by dissolving 100 mg commercially available galactomannan (Locust bean gum from *Ceratonia siliqua* seeds purchased from Sigma) in 50 ml citrate phosphate buffer and stirred in an Orbitek environmental shaker while heating at 60 °C for 2 hours. The suspension was cooled and stirred for additional 12 hours. Later the suspension was centrifuged at 10000 x g for 10 minutes. The suspension collected after centrifugation was used as the substrate for the assay of endo-β-mannanase.

Assay of Endo-β-Mannanase

The assay system consisted of 200 µl citrate phosphate buffer (pH 4.0) and 50µl galactomannan substrate dissolved in the buffer (0.2%). Reaction was initiated by adding 200 µl enzyme extract (10% w/v) and the tubes were incubated at 32 °C. After 30 minutes reaction was terminated by adding 1ml 80% (v/v) alcohol. In the control tubes enzyme extract was added after terminating enzyme action. Sugar content of both experimental and control was estimated by HPLC and the difference between the control and experimental served as the activity of endo-β-mannanase.

Amylase

Biochemical analyses of tissues showed the presence of very high amounts of starch in the haustorium which is a temporary storage organ of seed reserves during germination of *Borassus flabellifer* seeds. So amylase assay was conducted in the tissues of haustorium sampled at different intervals of germination/ seedling growth.

Five hundred mg tissue of haustorium at each stage of germination was homogenized in 5ml phosphate buffer (pH- 7.0) using chilled glass mortar and pestle in an ice bath. The homogenate was centrifuged at 12,000x g using a refrigerated centrifuge (REMI Model- C24-BL) at 4 °C for 15 minutes and the supernatant was used as enzyme source of amylase assay.

Amylase was assayed according to Bernfeld (1955) using Dinitrosalicylic acid reagent using 2% soluble starch as the substrate.

Preparation of Dinitrosalicylic acid Reagent

Fifty ml of sodium potassium tartarate solution (60%) was mixed with 20 ml of 2N NaOH and the mixture was heated at 45-50 ºC. One gram of dinitrosalicylic acid was added gradually with constant stirring and made upto 100 ml.

Amylase Assay

The assay system consisted of 0.2 ml of 10% homogenate, 0.6 ml buffer (pH- 5.0) and 0.2 ml substrate (soluble starch) and incubated at 37 ºC for 30 minutes. The activity was ceased by adding 1.0 ml of Dinitrosalicylic acid. The tubes were heated in boiling water bath for 5 minutes, cooled and diluted to 10 ml using distilled water. The optical density was measured at 540 nm using UV-Visible Spectrophotometer (Shimadzu Model- UV-1601). Maltose was used as the standard.

pH Optimum

The optimum pH (5.0) for enzyme activity was determined by incubating enzyme assay system for 30 minutes at 30 °C with substrate, in phosphate buffer (0.2 M) of a pH range 4.0-6.5 at intervals of 0.5 pH. The

pH of the buffer in which the enzyme showed highest activity was taken as optimum pH.

Temperature Optimum

The temperature optimum (37 °C) of amylase assay was determined by incubating the assay system for 30 minutes at temperature ranging from 20 to 40 °C at an interval of 2 °C with substrate and buffer having optimum pH (5.0). The temperature at which the enzyme showed highest activity was considered as optimum temperature.

Enzyme Proportionality

The enzyme proportionality range (0.2 ml, 10% w/v) for activity was determined by incubating the assay system for 30 minutes at optimum temperature (37 °C) with optimum pH (5.0) optimum substrate concentration (soluble starch- 0.2 ml, 2%) and different volumes of 10% (w/v) enzyme extract ranging from 0.05-0.4 ml at an interval of 0.02 ml.

Substrate Saturation

The substrate saturation (soluble starch 0.2 ml, 2%) for enzyme activity was determined by incubating the assay system for 30 minutes at optimum temperature (37 °C) with optimum pH (5.0) and 0.2 ml of 10% (w/v) enzyme extract and different concentrations of substrate (soluble starch- 2%) ranging from 0.1-0.5 ml at an interval of 0.1 ml.

Unit Activity

One unit of amylase activity was defined as the amount of maltose formed per gram tissues at 37 ºC during a period of 1 hour.

Statistical Analysis

All experiments were repeated for a minimum of 6-8 times and the mean values ± standard error is given in Tables and Figures. Standard deviation and standard error were calculated. Test of significance was done by Fishers''t' test.

RESULTS

RESULTS

1. Seed Development

Seed development of *Borassus flabellifer* is a very slow process. The endosperm is liquid syncytium which gradually get solidified when the fruits attain about 3 month's growth. The embryo is found embedded in the endosperm (Fig. 1B). In the present study, embryo and endosperm was investigated at different intervals of seed development (Table 1).

1.1. Anatomical/Histochemical Studies

1.1.1. Embryo

As mentioned above, the developing embryo appeared as embedded in the liquid endosperm (Fig. 2A) hence anatomical/ histochemical studies of the embryo were possible only after 2nd stage of seed development (Table 1). It was very difficult to localise the embryo due to the gel-like texture of the endosperm. After 3 months of development, embryo was found located towards the periphery of the proximal part of endosperm near the testa. (Fig. 2B). In the 3rd stage, the embryo was enlarged in size and assumed globular shape (Fig. 2C). As growth advanced to 4th stage, embryo showed an enlarged single cotyledon and an underdeveloped root-shoot axis with the root pole pointing towards proximal side. Embryo development was almost completed

at this stage (Fig. 2D). In stage 5 the embryo appeared well developed consisting of a massive single cotyledon and the developed root pole pointing towards the proximal end (Fig. 3A). The development of plumule also was taken place at this stage consisting of a shoot apical region with developing lobes on either side. (Fig. 3A). In the 6th stage, the plumule was found to be surrounded by plumular sheath which appeared as two lobes on either side of the plumule in the longitudinal sections (Fig. 3B).

The cells of the embyo were comparatively smaller with conspicuous nucleus and densely stained compared to the endosperm cells (Fig. 2B). Proximal region of the embryo was with well formed root-apical dome and cellularisation was clearly seen in stage 6 (Fig. 3 B). This proximal part appeared as ‘root apex’ which protrudes and elongates as cotyledonary sheath during seed germination. The plumular region appeared as inverted towards the micropylar end in the opposite side of the root pole (Fig. 3 A&B).

Mature embryo is an oblong structure consisting of dome like ‘root apex’ at the proximal end and massive single cotyledon with a tapering end towards the bottom (Fig. 1 E). Both transverse and longitudinal sections of composite structure consisting of embryo and the surrounding endosperm showed cellular differentiation of both the structures (Fig. 1 G&H). The cotyledon of mature embryo consists of well-formed epithelial cells and cortex which are isodiametric and contains conspicuous nucleus and dense

cytoplasm (Fig. 5 A, B, C, D & E). Sections of peripheral layers of the embryo (cotyledon) stained with Periodic acid Schiff's reagent revealed very darkly stained comparatively thick walled cells (Fig. 6D). A number of differentiating vascular strands with feebly stained smaller cells were clearly seen in the sections of the cotyledon (Fig. 6 A&D).

1.1.2. Endosperm

In the samples of stage 1, cell wall formation of endosperm started from the periphery which is in contact with the testa (Fig. 4A) and during further stages in the centre the endosperm appeared semi-liquid and gel like, while the testa appeared well developed with cellular details (Fig. 4A&B). Endosperm cells with slightly stained and incomplete cell walls were visible and cellular details were not fully seen in this stage. The endosperm showed cellularisation in stage 3 and individual cells were fully formed but cell walls appeared thin and cell inclusions and nucleus were present (Fig. 4C&D). Cell wall thickening of endosperm started in the 4^{th} stage of seed development (Fig. 4D) and progressively increased during further developmental stages (Fig. 4E &F). Cell walls were found to be deeply stained with Periodic acid Schiff's reagent indicating the occurrence of insoluble polysaccharides. However, starch grains were totally absent in the endosperm cells in the samples of all developmental stages (Fig. 4A-F). The lumen of the cells appeared narrow and empty in the last stage. The staining of cell wall with

Periodic acid Schiff's reagent showed slight difference in the colour intensity and thickness of cell walls in the last stage (Fig. 4F).

Anatomy of the endosperm of mature seeds sampled from fully ripened fruits showed thick walled cells in transverse and longitudinal sections stained with hematoxylin (Fig. 5C&6C) Periodic acid Schiff's reagent (Fig. 5D&6D) and toluidine blue (Fig. 5E & 6E) whereas very feeble staining was imparted with bromophenol blue (Fig. 5B&6B). The lumen of the endosperm cells were devoid of any inclusions. Since the embryo is embedded in the proximal part of endosperm (Fig. 1B) the sections of intact embryo along with endosperm showed a layer of tissues (intermediate layer) in between the endosperm and embryo (Fig. 5&6). Eventhough this layer is a continuation of the endosperm cells, cell shape and structural details were not fully organised and cell wall thickness was uneven and comparatively less than that of endosperm cells. This layer consisted of undifferentiated cells and densely stained with hematoxylin, Periodic acid Schiff's reagent and toluidine blue (Fig. 5&6).

1.2. Biochemical Studies

1.2.1. Embryo

As mentioned earlier, embryo of *B. flabellifer* seeds is enclosed in the endosperm and in the developing seeds the embryo was inseparable from the endosperm. So biochemical studies were conducted only in the samples of

mature embryo which was having 0.7±0.2 cm length, 0.4±0.1 cm breadth and 0.28±0.1 g fresh weight (Table 8). Dry weight percentage was 26 and starch and protein content was comparatively low (Table 3).

Soluble sugar fractions of *B. flabellifer* mature embryo showed the presence of fructose, galactose and rhamnose. Rhamnose content was very high in comparison with other sugars (Table 3, Fig. 7).

Table 3. Dry weight, Starch, Proteins and Sugars in *Borassus flabellifer* Mature Embryo (mg g^{-1} dw)

Dry weight Percentage		26.03±1.41
Starch		12.62±1.12
Total Proteins		4.70±0.25
Soluble Proteins		1.23±0.09
Sugars	Rhamnose	11.75±0.92
	Fructose	0.52±0.04
	Galactose	1.58±0.11

1.2.2. Endosperm

1.2.2.1. Dry weight Percentage

During early stage of seed development, samples collected for dry weight content constituted mainly endosperm since the embryo was small and

inseparable from the endosperm. Dry weight of endosperm of the 1st stage was only 6.58% and slight increase was observed during the initial stages of development. After 3rd stage, significant increase ($P<0.01$) was observed (Table 4, Fig. 8A).

1.2.2.2. **Starch**

Starch contents of endosperm was very low (lesser than 1%) but significant decrease ($P<0.01$) was occurred in the 2nd stage followed by an insignificant increase in the third stage. Significant increase in starch content ($P<0.01$ each) was observed in stages 4 and 5 followed by a significant reduction in the final samples (Table 4, Fig. 8B).

1.2.2.3. Proteins

During seed development total protein content of endosperm was only 10.33 ± 0.8 mg g^{-1} dw and showed significant reduction ($P<0.01$) upto 4th stage. Rapid increase was observed in the samples of 5th stage and thereafter remained unchanged (Table 4, Fig. 8C).

Very low quantity of soluble proteins (1.17%) was present in the endosperm. But significant changes were noticed up to 5th stage of seed development. Only insignificant increase was occurred in the 6th stage (Table 4, Fig. 8C).

1.2.2.4. **Protein Profile**

Protein profile of *B. flabellifer* endosperm during seed development showed increasing number of protein bands in the samples of successive stages of development (Fig. 9). Only 9 bands, ranging in molecular weights 6kDa to 300 kDa are present in the early stages (1st and 2nd) of seed

development. Third stage onwards number of bands found to be increased. Bands were very prominent in stages 5 and 6.

Table 4. Changes in Dry weight, Starch and Proteins in Endosperm tissues of *Borassus flabellifer* Seed during Development (mg g^{-1} dw)

Parameter	Stages of Seed development					
	1	**2**	**3**	**4**	**5**	**6**
Dry weight Percentage	6.58±0.27	6.79±0.38	9.33±0.71	14.80±0.22	26.84±2.57	31.73±2.09
Starch	0.94±0.02	0.80±0.01	0.82±0.02	1.26±0.09	1.85±0.09	1.22±0.02
Total Protein	10.33±0.80	8.67±0.27	5.60±0.56	3.87±0.14	10.15±1.00	9.90±0.85
Soluble Protein	1.17±0.08	0.97±0.05	1.35±0.07	1.89±0.05	2.93±0.20	3.15±0.30

1.2.2.5. Free Amino acids

Endosperm tissues of *B. flabellifer* showed the presence of alanine, arginine, asparagine, aspartic acid, cystine(s), glutamic acid, glutamine, glycine, hydroxy proline, leucine, lysine, methionine, proline, serine, threonine, and valine during seed development (Table 5, Fig. 10).

Alanine was present in the endosperm only during early stages of seed development. A gradual but significant decrease was observed in the samples of 2nd and 3rd stages ($P<0.01$ each) and afterwards detectable amounts of alanine was not present.

Only trace amount of arginine was present in the endosperm during seed development. It was observed only in 3rd stage.

Asparagine was present in the samples of all the stages of seed development and showed only negligible changes during development except a sharp increase in the last stage.

In the 2nd stage of seed development aspartic acid showed no change compared to stage 1, but significant ($P<0.01$ each) reduction was observed in the samples of 3rd and 4th stages followed by an insignificant increase in the 6th stage.

Cysteine(s) was present in all stages of seed development. Significant increase was occurred in the 2nd stage followed by a marked increase the 3rd

stage. Insignificant change in cysteine content was occurred in the 4th stage. In the samples of 5th and 6th stages gradual but significant decrease was observed ($P<0.01$ each).

In the samples of all the stages of development, glutamic acid was present and gradual increase ($P<0.01$) was observed in the 3rd stage followed by significant reduction in the 4th and 5th stages ($P<0.01$ each). In the samples of last stage glutamic acid showed a significant increase ($P<0.01$).

Glutamine was present only in the initial stages of seed development and was reduced slightly in the samples of 2nd stage followed by a significant reduction in the 3rd stage. Only trace amounts of glutamine was present in the 4th stage and thereafter it was absent.

Glycine was present in all developmental stages of seed and showed significant changes ($P<0.01$) from stage to stage followed by an insignificant increase in the last stage.

Hydroxy proline was observed only in the initial stages of endosperm development exhibiting a significant increase in 2nd stage ($P<0.01$) followed a decrease ($P<0.01$) in the 3rd stage. Thereafter only trace amount was present and disappeared in the final stage.

Leucine showed gradual reduction during seed development and the reduction was significant ($P<0.01$) from stage to stage in all samples.

Lysine was not present in the initial stages of seed development. It appeared in the samples of 4th stage in trace amounts and considerable quantity was present in 5th and 6th stages (Table 5, Fig. 10).

Methionine was present throughout the developmental stages of endosperm in *B. flabellifer* seed and showed significant increase ($P<0.01$) in stage 2 followed by an insignificant reduction in the 3rd stage. Samples 4th stage showed marked reduction. In the 5th stage methionine was increased significantly ($P<0.01$) compared to stage 4. Only trace quantity of methionine was observed in the 6th stage.

Comparatively considerable quantity of proline was present in all samples of *B. flabellifer* seed development and showed gradual reduction upto 3rd stage. A marked increase was occurred in the samples of 4th and 5th stages. Only trace quantity of proline was present in the samples of final stage.

All stages of endosperm development showed serine and a gradual increase was occurred till 5th stage; change from stage 1 to 2 was significant ($P<0.01$). In the last stage a significant decrease ($P<0.01$) in serine content was observed compared to previous stage.

Threonine also was present in the samples of all stages of seed development. Samples of 2nd stage showed a significant decrease followed by a marked increase (4 times) in the stages 3. In the 4th and 5th stages notable decrease was observed followed by a significant increase in the final sample.

Valine was present in measurable quantity only in the samples of stage 3 and 4 of seed development and showed significant decrease ($P<0.01$) in 4^{th} stage. In the samples of 5^{th} and 6^{th} stages only trace amount of valine was present.

Table 5. Change in Free Amino acids in Endosperm tissues of *Borassus flabellifer* Seed during Development (mg g^{-1} dw)

Amino acids	Stages of Seed Development					
	1	**2**	**3**	**4**	**5**	**6**
Ala	0.59±0.04	0.41±0.03	0.29±0.01	T	T	ND
Arg	ND	ND	T	ND	ND	ND
Asn	0.56±0.05	0.58±0.04	0.60±0.05	0.61±0.04	0.63±0.05	3.48±0.02
Asp	0.38±0.02	0.37±0.02	0.31±0.01	0.21±0.01	0.21±0.01	0.26±0.02
Cys	0.22±0.01	0.24±0.01	0.61±0.03	0.66±0.04	0.44±0.03	0.34±0.02
Glu	0.91±0.07	0.93±0.08	1.12±0.09	0.53±0.04	0.25±0.01	0.71±0.05
Gln	1.43±0.09	1.26±0.08	0.45±0.03	T	ND	ND
Gly	0.22±0.01	0.19±0.01	0.22±0.01	0.33±0.01	0.55±0.03	0.60±0.03
Hyd Pro	0.49±0.03	0.96±0.07	0.74±0.06	T	T	ND
Leu	1.02±0.08	0.81±0.07	0.49±0.03	0.41±0.02	T	T
Lys	ND	ND	ND	T	0.56±0.04	0.53±0.04
Met	0.57±0.04	0.81±0.07	0.77±0.04	0.23±0.07	0.53±0.04	T
Pro	2.59±0.11	1.97±0.08	1.81±0.09	2.96±0.17	4.01±0.31	T
Ser	0.29±0.01	0.57±0.03	0.61±0.04	0.65±0.05	0.70±0.06	0.59±0.04
Thr	1.70±0.08	1.01±0.09	4.67±0.31	2.04±0.12	1.21±0.08	1.66±0.01
Val	ND	ND	0.16±0.01	0.11±0.01	T	T

(ND- Not detected, T-Trace quantity)

1.2.2.6. Sugars

During seed development, soluble sugar fraction of *B. flabellifer* endosperm showed the presence of raffinose, maltose, sucrose, glucose, fructose, galactose and mannose (Table 6, Fig. 11). Eventhough the relative position of the some spots in the chromatogram was adjacent to raffinose, these sugars may be some oligosaccharides coming under raffinose family.

Maltose was not present in the endosperm tissue initially but started appearing in the 2nd stage followed by a rapid increase (more than 3 fold) in the 3rd stage of development. Maltose content of the 4th stage samples was significantly reduced (more than 2 fold) and disappeared in the samples of 5th and 6th stages (Table 6, Fig. 11).

Sucrose was present in the endosperm tissues of all stages of seed development. Slight, but significant increase ($P<0.01$) was occurred in stage 2 and significant reduction ($P<0.01$ each) was observed upto 5th stage. In the samples of last stage of seed development sucrose content showed a sharp peak exhibiting five-fold increase compared to previous stage (Table 6, Fig. 11).

Comparatively vey high glucose content was present in the endosperm samples of all stages of seed development and there occurred significant increase from stage to stage up to 3rd stage ($P<0.01$ each). A sharp reduction was seen in the tissues of 4th stage compared to the previous stage. More than 18 fold decrease of glucose content was observed in the 5th stage and negligible quantity was present in the last sample (Table 6, Fig. 11).

Table 6. Change in Sugar content in Endosperm tissues of *Borassus flabellifer* Seed during Development (mg g^{-1} dw)

Sugars	Stages of Seed Development					
	1	**2**	**3**	**4**	**5**	**6**
Raffinose	ND	ND	20.12±2.11	25.96±2.51	ND	ND
Maltose	ND	12.64±1.12	43.26±4.12	19.53±1.81	ND	ND
Sucrose	18.41±1.61	22.63±1.93	18.84±1.81	14.09±1.31	7.79±0.61	38.62±3.41
Glucose	138.81±8.12	195.97±11.21	256.92±13.47	135.08±7.25	6.55±0.42	0.35±0.02
Galactose	ND	ND	ND	ND	1.76±0.05	1.14±0.09
Fructose	191.02±9.42	289.63±11.54	268.29±16.52	121.07±4.98	4.45±0.32	0.12±0.01
Mannose	ND	ND	ND	ND	ND	0.17± 0.01

(ND- Not detected)

Galactose was not present in the endosperm during the earlier four stages of *B. flabellifer* seed development. But considerable amounts of galactose was present in the 5th and 6th stages showing significant reduction ($P<0.01$) in the last stage. (Table 6, Fig. 11).

Fructose was present in the endosperm of all the stages of seed development and gradually increased up to 4th stage. A marked increase was observed in the samples of 2nd stage and remained unchanged in the samples of 3rd stage. Fructose content was reduced to less than one half in the stage 4. Samples of 5th and 6th stages showed reduction of fructose content which was more than 25 fold and 35 fold respectively, compared to the previous stages (Table 6, Fig. 11).

Mannose was present only in the final stage of endosperm in *B. flabellifer* seed development; that too only in negligible quantity (Table 6, Fig. 11).

1.2.2.7. Insoluble Polysaccharides other than Starch (Galactomannan)

HPLC analysis of endosperm tissue after acid hydrolysis and alcohol precipitation revealed the presence of galactomannans.

During seed development, the component sugar galactose showed gradual and significant increase from stage to stage ($P<0.01$ each). Mannose content was more in comparison with galactose in the samples of all

developmental stages and showed significant increase ($P<0.01$) in each stages (Table 7, Fig. 12).

Table. 7. Changes in Galactomannan in the Endosperm tissues of *Borassus flabellifer* Seed during Development (mg g^{-1} dw)

	Stages of Seed Development					
	1	**2**	**3**	**4**	**5**	**6**
Galactose	4.87	19.89	29.15	35.10	43.01	44.03
Mannose	13.74	53.67	84.45	102.31	159.05	196.91
Galactose: Mannose Ratio	1:3	1:3	1:3	1:3	1:4	1:5

The ratio of galactose:mannose also showed significant changes. During the initial stages of development, ratio was 1:3 and remained unchanged. Towards maturity-in the final stages- the ratio of galactose:mannose was increased from 1:3 to 1:4 followed by 1:5 in the final sample (Table 7, Fig. 12).

2. Seed Germination

2.1. Seed/ Seedling Morphology

Borassus flabellifer fruits contain usually three seeds (pyrenes). The hard endocarp of the seed encloses the horny endosperm and a small embryo (Fig. 1B). The embryo is located in a cavity of endosperm near the micropylar end. The endocarp of the seed is very hard, black in colour but adjacent to the embryo the endocarp is comparatively thin and somewhat soft and looks like testa. During germination the 'cotyledonary sheath' is protruded thorough this region (Fig. 1C).

As mentioned earlier, 35±5 days are required for seed germination in *B. flabellifer* and the mode of germination is remotive. The initiation of germination is shown by the protrusion of the 'radicle' like cotyledonary sheath which is very stout with a tapering growing tip (Figs. 13&14). Following germination the cotyledonary sheath show fast growth and the region above the tip become swollen (Figs. 13&14). After about 5 months of growth the cotyledonary sheath is elongated with a swollen region just above

the growing (tapering) tip. The swollen region carries the developing shoot consisting of stout plumular sheath and developing primary leaf (palmate) inside. The tip of the cotyledonary sheath become more tapering and develops into a 'tap root' like structure showing many secondary roots (Fig. 13 C&D). Next stage (5^{th}) shows longitudinal crack in the cotyledonary sheath above the swollen region. Through this slit, the robust, solid plumular sheath become visible and the primary leaf (palmate) located inside the plumular sheath protruded and later the primary leaf become green in colour (Figs. 13E&F, 14E).

2.1.1. **Total Seed Weight**

Total fresh weight of the *B. flabellifer* seed was 188-207 g fw inclusive of hard endocarp. During germination the total fresh weight of seed was calculated as the composite value of weight of endocarp, endosperm, developing haustorium and growing cotyledonary sheath carrying the miniature seedling. Significant increase ($P<0.01$) was noticed in the total weight of seed/seedling up to 3^{rd} stage. Thereafter it showed significant reduction from stage to stage. From the stage 4 to 5 the reduction of seed weight was very drastic (Table 8, Fig. 15).

2.1.2. **Weight of Endosperm**

Weight of endosperm of intact *B. flabellifer* seeds was 136.47±6.3 g fw. During germination it showed insignificant increase in the second and

third stages. Drastic reduction in endosperm weight was observed in 4th and 5th stages followed by an insignificant reduction in the final stage (Table 8, Fig. 15).

Haustorium showed total weight of 1.86±0.11g fw in the samples of stage 1 and gradual increase was occurred in all the stages of seed germination showing significant increase from stage to stage. In the 4th and 5th stages increase was very sharp compared to the previous stages and the final sample showed again significant ($P<0.01$) increase (Table 8, Fig. 15).

Table 8. Morphological features of Seed/Seedling components during and following Germination in *Borassus flabellifer*

Stages of Germination	Total *Seed weight (g fw)	Endocarp Weight (g fw)	Endosperm Weight (g fw)	Embryo			Cotyledonary sheath weight (g fw)	Haustorium Weight (g fw)
				m)Length	n)Breadth	w)Weight		
0	198.27±9.51	78.43±5.21	136.47±6.30	0.7±0.2	0.4±0.1	0.28±0.1	-	-
1	197.35±10.10	79.21±1.8	138.14±7.54				2.25±0.12	1.86±0.11
2	224.21±12.50	76.66±2.43	156.37±11.06				7.18±0.51	3.92±0.23
3	246.61±12.81	79.45±3.30	165.04±12.61				7.31±0.42	5.13±0.51
4	227.44±8.40	80.56±4.10	106.88±8.31				27.62±1.81	15.38±0.43
5	129.52±6.43	81.61±3.12	54.60±3.12				62.30±2.20	23.64±1.70
6	126.34±4.51	71.40±1.71	47.39±3.84				74.40±2.90	27.02±1.73

*Seed: One pyrene consisting of hard endocarp, endosperm and embryo and during germination it consists of endosperm, endocarp, haustorium and cotyledonary sheath.

2.2. Anatomical/ Histochemical Studies

2.2.1. Endosperm

Since the hydrolysis of the endosperm was occurred in the dissolution zone (slimy endosperm) cellular details of this region could not be localised by anatomical/ histochemical study and the hard endosperm which consisted of only thick walled cells did not show any detectable microscopic changes in the stained preparation.

As mentioned earlier, a dissolution zone was present around the endosperm near the embryo of mature-ungerminated seeds (Fig. 5A). But during and following germination localisation of this zone was not possible because during the tissue processing such as killing, fixing, dehydration, microtomy and staining the hard endosperm, haustorium and the dissolution zone got separated. So the inner region of the endosperm where the dissolution occurred showed stained materials without any cellular details (Fig. 16 A, B, C &D). However, haustorium was analysed histochemically during and following germination.

2.2.2. Haustorium

During germination**,** development of haustorium (distal portion of the embryo) starts simultaneous with the formation of cotyledonary sheath (proximal portion of embryo). Following germination the haustorium grows very fast and simultaneously the endosperm get hydrolysed and become thin. It takes about 5-6 months to hydrolyse the entire endosperm and some

remnants persist near the endocarp even after the formation of seedling (Fig. 17).

Sections of haustorial tissues of six developmental (seed germination) stages stained with bromophenol blue for proteins (Fig. 18) and hematoxylin for cytological observations (Fig. 19) showed that the cells were highly meristematic with conspicuous nucleus and cytoplasm and very high protein content in the first stage. Sections stained with Periodic acid Schiff's reagent (Fig. 20) showed clearly stained cell wall without any starch grains in the cells. During further development of haustorium (stages 2-3) cells became more vacuolated and protein content was reduced in the cortical cells. But the epithelial cells were with more proteins and more reduced vaculation. Developing starch grains were present (Fig. 20 B&C) in the haustorium in the samples of stage 2 and 3. Afterwards cell breakage and cell lysis were observed in the haustorial tissue and protein staining was confined to the distorted cell wall and cytoplasm. Even though cell wall breakage was occurred, starch grains size and number were increased in the tissues (Fig. 20 D&E). Haustorium showed fully damaged cells in the sample 6 and cellular details were completely lost. Starch grains appeared as scattered in the broken cell wall debris (Fig. 20E).

2.3. **Biochemical Studies**

2.3.1. Endosperm

Hard endosperm dry weight was 31.73% which maintained almost uniform during all stages of germination/ seedling growth (Table 9). No significant changes were occurred in the distribution of metabolites like starch, proteins, sugars and amino acids during seed germination. It showed similar values of metabolites distribution corresponding to that of the endosperm of control (ungerminated) seeds.

Slimy Endosperm (Dissolution zone)

2.3.1.1 **. Dry weight**

Dry weight percentage of slimy endosperm tissues of *B. flabellifer* seeds was 10.6% which showed only negligible changes upto 5^{th} stage. The samples of 5^{th} stage showed a sharp decrease and remained unchanged in last stage (Table 9, Fig. 21A).

2.3.1.2. Starch

Starch content of slimy endosperm was very low (5.4%) and showed only negligible changes during and following germination except stages 5 and 6 where significant decrease ($P<0.01$ each) was observed between stage 5 and 6 (Table 9, Fig. 21B).

2.3.1.3. Proteins

Protein content was very low (15 mg g^{-1} dw) in the endosperm tissues of *B. flabellifer* seeds (Table 9, Fig. 21C). During germination, total protein

content showed significant increase ($P<0.01$) in the 2nd stage, followed by a slight reduction in the 3rd stage. In the fourth stage significant increase ($P<0.01$) was occurred, thereafter gradual reduction ($P<0.01$) in protein content were occurred in the stages 5 and 6.

Table 9. Change in Dry weight, Starch, Soluble Protein and Total Proteins in *Borassus flabellifer* Slimy and Hard endosperm tissues during and following Germination (mg g^{-1} dw)

	Endosperm	Stages of Germination						
		0	1	2	3	4	5	6
Dry weight Percentage	Slimy	ND	10.60± 0.98	9.41±0.91	10.22±0.87	11.05±1.10	4.76±0.42	4.45±0.41
	Hard	31.73±2.10	28.17±2.02	31.20±2.17	29.80±1.98	30.10±2.71	30.87±2.64	29.63±2.22
Starch	Slimy	ND	5.43±0.45	5.12±0.33	5.54±0.52	5.39±0.51	4.67±0.31	3.14±0.24
	Hard	1.21±0.11	1.23±0.10	1.22±0.09	1.21±0.11	1.20±0.11	1.23±0.09	1.22±0.09
Total Protein	Slimy	ND	15.04±1.21	18.29±1.60	17.61±1.33	31.47±2.90	24.63±2.12	20.12±1.83
	Hard	9.88±0.81	9.87±0.83	9.85±0.84	9.89±0.85	9.87±0.86	9.88±0.88	9.89±0.86
Soluble Protein	Slimy	ND	0.18±0.01	0.87±0.08	0.98±0.07	1.18±0.11	1.20±0.09	0.68±0.05
	Hard	3.14±0.28	3.12±0.30	3.21±0.31	3.18±0.29	3.16±0.28	3.08±0.21	3.07±0.22

(ND- Not detected)

Soluble protein content of endosperm (0.18 mg g^{-1} dw) was very low in the slimy endosperm (Table 9, Fig. 21C) and showed slight increase ($P<0.01$) after 1st stage and thereafter only negligible increase was shown upto 5th stage, followed by a significant ($P<0.01$) decrease in the final stage.

2.3.1.4. Free Amino acids

Slimy Endopserm tissues of *B. flabellifer* seeds during germination showed the presence of alanine, arginine, glutamine, glutamic acid, glycine, hydroxy proline, proline, serine and threonine (Table 10, Fig. 22).

Alanine was absent in the samples of initial stages and started appearing in the 3rd stage onwards. The changes between the stages 5 and 6 were significant ($P<0.01$).

Arginine was found only in first two stages of seed germination and content was reduced significantly ($P<0.01$) in the samples of second stage compared to first stage.

Glutamine also was present only in the initial stages of seed germination and showed significant decrease $P<0.01$) in the 3rd stage and only trace amount was found in the samples of 4th stage.

Glutamic acid was present in all the stages of *B. flabellifer* seed germination and the quantity of distribution was uniform throughout germination.

Glycine was also found in all the stages of germination/seedling growth and only negligible changes were occurred during and following germination.

Table 10. Change in Free Amino acids in Endosperm tissues of *Borassus flabellifer* Seed during and following Germination (mg g^{-1} dw)

Aminoacids	Endosperm	Stages of Germination						
		0	1	2	3	4	5	6
Ala	Slimy	ND	ND	ND	0.42± 0.03	0.45± 0.02	0.37± 0.02	0.47± 0.03
		(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
Arg	Slimy	ND	0.23± 0.02	0.13± 0.01	ND	ND	ND	ND
		(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
Asn	Slimy	ND	ND	ND	ND	ND	ND	ND
		(3.48±0.15)	(3.47±0.12)	(3.47±0.13)	(3.45±0.22)	(3.46±0.23)	(3.48±0.18)	(3.47±0.22)
Asp	Slimy	ND	ND	ND	ND	ND	ND	ND
		(0.26±0.02)	(0.26±0.01)	(0.25±0.02)	(0.25±0.02)	(0.24±0.01)	(0.26±0.02)	(0.26±0.01)
Cys	Slimy	ND	ND	ND	ND	ND	ND	ND
		(0.34±0.02)	(0.34±0.03)	(0.31±0.02)	(0.32±0.03)	(0.35±0.03)	(0.35±0.03)	(0.34±0.03)
Glu	Slimy	ND	0.61± 0.05	0.64± 0.04	0.69± 0.04	0.65± 0.05	0.73± 0.06	0.66± 0.05
		(0.71±0.06)	(0.73±0.05)	(0.72±0.05)	(0.71±0.04)	(0.73±0.06)	(0.74±0.05)	(0.72±0.05)
Gln	Slimy	ND	0.76± 0.06	0.82± 0.07	0.53± 0.04	T	ND	ND
		(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
Gly	Slimy	ND	0.51± 0.04	0.62± 0.05	0.69± 0.06	0.73± 0.07	0.76± 0.07	0.73± 0.07
		(0.60± 0.05)	(0.62± 0.06)	(0.65± 0.05)	(0.59± 0.04)	(0.62± 0.04)	(0.60± 0.05)	(0.64± 0.03)
Hyd Pro	Slimy	ND	T	0.46± 0.03	0.52± 0.04	0.67± 0.05	0.62± 0.05	0.59± 0.04
		(ND)	(ND)	(ND)	(ND)	(ND)	(ND)	(ND)
Leu	Slimy	ND	ND	ND	ND	ND	ND	ND
		(T)	(T)	(T)	(T)	(T)	(T)	(T)
Lys	Slimy	ND	ND	ND	ND	ND	ND	ND
		(0.53±0.04)	(0.53±0.05)	(0.54±0.05)	(0.55±0.04)	(0.55±0.04)	(0.53±0.05)	(0.54±0.05)
Met	Slimy	ND	ND	ND	ND	ND	ND	ND
		(T)	(T)	(T)	(T)	(T)	(T)	(T)
Pro	Slimy	ND	ND	ND	ND	T	0.47± 0.03	0.78± 0.06
		(T)	(T)	(T)	(T)	(T)	(T)	(T)
Ser	Slimy	ND	0.76± 0.06	0.82± 0.08	0.89± 0.08	0.97± 0.07	0.79± 0.05	0.71± 0.06
		(0.59± 0.04)	(0.57± 0.04)	(0.59± 0.05)	(0.57± 0.04)	(0.58± 0.04)	(0.59± 0.05)	(0.60± 0.05)
Thr	Slimy	ND	0.83± 0.07	T	T	ND	ND	ND
		(1.66± 0.01)	(1.68± 0.01)	(1.63± 0.01)	(1.73± 0.01)	(1.71± 0.01)	(1.66± 0.01)	(1.68± 0.01)
Val	Slimy	ND	ND	ND	ND	ND	ND	ND
		T	T	T	T	T	T	T

(Values in parenthesis are amino acids in the hard endosperm; ND- Not detected, T-Trace quantity)

Hydroxy proline was present only in trace amount initially and all the stages showed considerable quantity with a significant increase ($P<0.01$ each) in stage 3 and 4 compared to that of final stages (Table 10, Fig. 22).

Proline was not present in the samples of initial stages of germination/ seedling growth but in the 4^{th} stage trace quantity was observed. Considerable quantity of proline was present in the 5^{th} stage and a significant increase ($P< 0.01$) was occurred in the final stage of germination/ seedling growth.

Serine was found to be present in all the stages of seed germination and showed no significant changes in quantity except a significant decrease ($P<0.01$) in stage 5.

Detectable amounts of threonine was present only in the first stage and only trace amounts were observed in the 2^{nd} and 3 stages (Table 10, Fig. 22).

2.3.1.5. Sugars

Paper chromatographic separation of sugar contents of *B. flabellifer* hard endosperm revealed sucrose, glucose, galactose, fructose, mannose and raffinose during germination while the slimy endosperm showed the presence of only maltose, sucrose and raffinose (Table 11, Fig. 23).

Maltose content of slimy endosperm was 16.7 mg g^{-1} dw in the 1^{st} stage. No significant changes were occurred upto 3^{rd} stage of germination. But a significant reduction ($P<0.01$) was occurred in stage 4. Samples of 5th stage

of germination showed significant increase ($P<0.01$) followed by significant ($P<0.01$) reduction in the final stage (Table 11, Fig. 23).

Table 11. Change in Sugar content in Endosperm tissues of *Borassus flabellifer* during and following Germination (mg g^{-1} dw)

Sugars	Endosperm	Stages of Germination						
		0	1	2	3	4	5	6
		Sugars (mg g^{-1} dw)						
Raffinose	Slimy	ND	63.04± 5.71	57.35±5.14	56.18±5.53	35.87±2.87	78.64±6.81	85.76±7.54
	Hard	ND	ND	ND	ND	ND	ND	ND
Maltose	Slimy	ND	16.71± 1.42	14.29±1.41	12.72±1.13	9.95±0.86	21.02±2.11	13.48±1.28
	Hard	(ND)	ND	ND	ND	ND	ND	ND
Sucrose	Slimy	ND	12.08± 0.87	23.54±1.91	36.63±2.47	68.52±3.72	97.14±6.64	117.1±10.35
	Hard	37.83±3.11	38.44±3.22	38.53±3.33	37.91±2.81	38.61±3.33	38.21±3.50	38.09±2.98
Glucose	Slimy	ND	ND	ND	ND	ND	ND	ND
	Hard	0.35±0.02	0.36±0.02	0.35±0.02	0.35±0.03	0.36±0.03	0.35±0.02	0.36±0.02
Fructose	Slimy	ND	ND	ND	ND	ND	ND	ND
	Hard	0.12±0.01	0.11±0.01	0.12±0.01	0.12±0.01	0.11±0.01	0.10±0.01	0.13±0.01
Galactose	Slimy	ND	ND	ND	ND	ND	ND	ND
	Hard	1.14±0.09	1.12±0.08	1.13±0.09	1.15±0.08	1.12±0.09	1.14±0.11	1.15±0.09
Mannose	Slimy	ND	ND	ND	ND	ND	ND	ND
	Hard	0.17±0.01	0.17±0.01	0.16±0.01	0.15±0.01	0.17±0.01	0.18±0.01	0.17±0.01

(ND- Not detected)

Sucrose content of slimy endosperm was comparatively lower than maltose content. During the entire period of germination continuous and significant increase was occurred from stage to stage showing almost 10 fold increase in the final stage compared to the initial stage.

Raffinose was not detected in the fresh (control) seeds; but found to be the major sugar which remained in very high quantity in all stages of germination. Reduction in raffinose was observed in the samples of all stages upto 5^{th} stage. Samples of 5^{th} stage showed a sharp increase and remained unchanged in the last stage of germination (Table 11, Fig. 23).

2.3.1.6. Insoluble Polysaccharides other than starch (Galactomannan)

Constituent monosaccharaides of galactomannan (galactose and mannose) of hard and slimy endosperm was analysed separately in the tissues during and following germination (Table 12 a&b). Hard endosperm showed both sugars in which mannose content was more compared to galactose. The content of galactose and mannose remained almost unchanged during all stages of germination/seedling growth. The ratio of galactose: mannose also was constant (Table 12a, Fig. 24A).

In the slimy endosperm galactose was present only in traces whereas abundant occurrence of mannose was observed. During seedling growth the mannose content was increased significantly up to 4^{th} stage and sharp reduction was occurred afterwards (Table 12b, Fig. 24B).

Table 12 a. Change in Galactomannan in Hard Endosperm tissues of *Borassus flabellifer* Seeds during Seed Germination (mg g^{-1} dw)

	Stages of Seed Germination					
	1	**2**	**3**	**4**	**5**	**6**
Galactose	39.12	41.82	39.62	39.72	38.76	43.97
Mannose	146.24	152.61	148.50	152.31	145.05	161.91
Galactose: Mannose Ratio	1:4	1:4	1:4	1:4	1:4	1:4

Table 12 b. Change in Galactomannan in Slimy Endosperm tissues of *Borassus flabellifer* Seeds during Seed Germination (mg g^{-1} dw)

	Stages of Seed Germination					
	1	**2**	**3**	**4**	**5**	**6**
Galactose	T	T	T	T	T	T
Mannose	238.42	205.67	197.68	138.42	106.86	29.95

(T-Trace quantity)

2.3.1.7. Enzymes involved in the hydrolysis of endosperm

α- Galactosidase (E.C. 3.2.1.22)

Standardization of optimal conditions for enzyme assay was done and the results showed that maximum activity was noticed at pH 5.4 at a temperature of 30 °C, with an enzyme proportionality range of 25 µl of 10% (w/v) extract and an optimal substrate (para nitrophenyl α-D-galactopyranoside) concentration of 200 µl- (1 mM) for a period of 30 minutes (Fig. 25 A, B, C &D).

α-Galactosidase was considerably active in the endosperm of ungerminated (control) seeds and during germination showed significant increase gradually upto 3rd stage ($P<0.01$ each). A sharp increase in the activity (about 3 fold) was observed in the samples of fourth stage followed by a sharp decline in stages 5 and 6 of germination/ seedling growth (Table 13, Fig. 26).

β- Mannosidase (E.C. 3.2.1.25)

Standardization of optimal conditions for β-mannanase showed maximum activity at pH 5.2 at a temperature of 30 °C, with an enzyme proportionality range of 50 µl of 10% (w/v) extract and an optimal substrate (para nitrophenyl β-D- mannopyranoside) concentration at 150 µl- (10 mM) for a period of 30 minutes (Fig. 27 A, B, C &D)

β-Mannosidase was not active in the ungerminated (control) seeds of *B. flabellifer*. During germination feeble activity was observed in the samples of stage 1 and increased significantly ($P<0.01$) in 2nd and 3rd stages of

germination followed a sharp peak in activity in the 4th stage. The activity was decreased significantly ($P<0.01$ each) in the samples of 5th and 6th stages of germination/ seedling growth (Table 14, Fig. 28).

Table 13. Activity of α-Galactosidase in the Endosperm tissues of *Borassus flabellifer* Seeds during and following Germination

Endosperm	Stages of Germination						
	0	**1**	**2**	**3**	**4**	**5**	**6**
Slimy	0.86±0.07	3.24±0.42	4.81±0.31	5.73±0.41	18.3±1.45	1.73±0.08	1.61±0.10
Hard	ND	ND	ND	ND	ND	ND	ND

(ND- Not detected)

Table 14. Activity of β-Mannosidase in the Endosperm tissues of *Borassus flabellifer* Seeds during and following Germination

Endosperm	Stages of Germination						
	0	**1**	**2**	**3**	**4**	**5**	**6**
Slimy	ND	0.04±0.01	5.71±0.51	8.44±0.65	32.94±2.50	16.33±1.21	5.55±0.05
Hard	ND	ND	ND	ND	ND	ND	ND

(ND- Not detected)

Endo-β-Mannanase (E.C. 3.2.1.78)

The product of the endo-β-mannanase activity analysed by HPLC showed the presence of only traces of mannose revealing incomplete degradation of large mannan chain of the endosperm reserve galactomannan.

2.3.2. Haustorium

2.3.2.1. Dry weight Percentage

Dry weight percentage of haustorium was maximum (18.4%) in the 1st stage of germination. Samples of 2nd stage showed a significant reduction ($P<0.01$) and remained unchanged till 4th stage. In the last two stages (5th and 6th) dry weight percentage of haustorium showed significant reduction ($P<.01$ each) (Table 15, Fig. 29A).

2.3.2.2. Starch

Haustorium of *B. flabellifer* seeds contained very low starch content initially and showed gradual increase in each stage. Increase of starch in the haustorium was significant ($P<0.01$ each) between each stage of germination/ seedling growth (Table 15, Fig. 29B).

2.3.2.3. Proteins

Total protein content of the haustorium in the first stage was 9.45mg/g dw and showed no change in the initial stages (upto 3rd stage) of germination/

seedling growth. But fourth stage onwards gradual decrease (insignificant) was observed in total protein content during and following germination (Table 15, Fig. 29C).

Table 15. Change in Dry weight, Starch, Soluble Proteins and Total Proteins in *Borassus flabellifer* Haustorium during and following Germination (mg g-^{1} dw)

	Stages of Germination					
	1	**2**	**3**	**4**	**5**	**6**
Dry weight Percentage	18.43±1.41	13.75±1.13	13.15± 0.82	12.83±1.10	10.17±0.74	8.89±0.43
Starch	4.30±0.32	11.52±0.92	32.12±3.10	37.86±3.54	56.07±4.85	95.87±8.54
Total Protein	9.45±0.83	9.13±0.91	8.21±0.71	7.32±0.62	7.11±0.53	6.56±0.44
Soluble Protein	0.67±0.04	0.84±0.07	0.91±0.08	1.11±0.09	0.65±0.05	0.61±0.06

Soluble protein content of haustorium was very low (less than 1%) in the initial sample and showed significant increase ($P<0.01$) only upto 4th stage of germination. In the last two stages the protein content was reduced significantly (Table 15, Fig. 29C).

2.3.2.4. Sugars

Haustorium of *B. flabellifer* seedlings showed the presence of sucrose, glucose, fructose, galactose, mannose and rhamnose (Table 16, Fig. 30). During haustorial development (seed germination) fructose content was decreased gradually up to 5th stage and disappeared in the final sample. Glucose was the abundant sugar, which showed gradual increase up to 5th stage in all samples and in the final sample also considerable quantity was retained (Fig. 30). Galactose was present in the samples of all stages except 6th stage whereas mannose was observed only in the early three stages. Similarly, sucrose also was present in these three stages. Rhamnose was distributed in all samples and content was decreased gradually (Table 16, Fig. 30).

2.3.2.5. **Amylase (E.C. 3.2.1.1)**

The optimal assay conditions for amylase activity showed that at pH 5.0, 0.2 ml enzyme (10% homogenate) in the presence of 0.2 ml (2% soluble starch) substrate at 37º C for a period of 30 minutes maximum activities was obtained (Fig. 31 A, B, C &D).

Table 16. Change in Sugar content in Haustorium of *Borassus flabellifer* during and following Germination (mg g^{-1} dw)

Sugars	Stages of Germination					
	1	**2**	**3**	**4**	**5**	**6**
Fructose	10.62±0.91	11.40±0.65	8.05±0.68	5.05±0.38	2.12±0.13	ND
Galactose	4.51±0.36	4.32±0.38	2.68±0.17	3.42±0.21	1.08±0.08	ND
Glucose	81.52±7.35	89.27±6.61	94.14±4.33	136.91±9.01	140.51±12.81	90.46±8.2
Mannose	12.51±1.11	11.98±0.83	9.49±0.42	ND	ND	ND
Rhamnose	12.05±0.83	12.08±0.91	13.02±1.21	9.80±0.78	9.71±0.78	8.74±0.54
Sucrose	6.82±0.47	6.73±0.58	7.72±0.68	ND	ND	ND

(ND- Not detected)

Table 17. Activity of Amylase in the Haustorium of *Borassus flabellifer* during and following Germination

Stages of Germination						
0	**1**	**2**	**3**	**4**	**5**	**6**
ND	34.28± 2.13	67.21± 4.58	141.08± 7.09	162.17± 10.26	104.92± 5.67	64.57± 4.26

(ND- Not detected)

Following seed germination, haustorium showed very high activity of amylase. The activity was increased gradually showing significant increase in each sample upto 5^{th} stage; maximum activity was observed at 4^{th} stage followedby a drastic decline during further stages of germination and seedling growth. (Table 17, Fig. 32).

DISCUSSION

DISCUSSION

The present study is primarily concerned with the developmental aspects of embryo and endosperm of *Borassus flabellifer* seeds. Seedling morphology, physiological and biochemical changes of reserve mobilisation during and following germination also is another aspect. Young fruits of *B. flabellifer* usually consist of three seeds (pyrenes) containing liquid endosperm and comparatively very small embryo which is embedded in the endosperm. Hence, the anatomical study of the endosperm and localisation of the embryo was found to be a task. Moreover, the thick fruit wall consisting of three layers of epicarp, fleshy/fibrous mesocarp and hard/ stony endocarp also caused practical difficulties in dissecting and processing the tissues for anatomical/ histochemical and biochemical studies. However, as mentioned in the materials and methods section, the entire processes of seed development and germination were investigated using samples of six comparable stages each (Tables 1&2).

In *Borassus flabellifer* seed, developing embryo is localised as an oblong structure embedded in the endosperm which occur as liquid syncytium initially and cell wall formation begins with stage 2. (Fig. 2B). During the development, proximal part of the embryo get slightly elongated towards the micropylar end of the ovule meanwhile, shoot apical organisation is started just below the micropylar region which consists of small meristematic cells

(Fig. 2D). Afterwards the embryo grows very fast and the proximal tip is developed into a differentiated dome which looks like root apical structure with well differentiated cellular organisation; positioned pointing towards the testa (Fig. 3A). Simultaneously the shoot apex gets differentiated below the proximal region (Fig. 3A). Shoot apex consists of developing plumular structure which is destined to develop thick fleshy plumular sheath later. In the longitudinal sections the plumular structure appears as two lobes on either side of the developing shoot apex. The position of the entire shoot is located as inverted in relation to the proximal tip of the cotyledon which is the structure destined to grow as cotyledonary sheath or 'root' during germination. According to Tomlinson (1960) in palm seeds the proximal tip of the cotyledon is root or 'hypocotyl' and during germination this region grows and protrude as cotyledonary sheath carrying the entire plumular (shoot pole) structure.

The mature embryo of *B. flabellifer* consists of a single cotyledon, root pole axis and differentiated shoot apical part (Fig. 1H). Similar structure of embryo in *Washingtonia filifera* as described by DeMason (1988b) consists of a single cotyledon, a root pole and an epicotyl. Development of the plumular sheath as well as its shape and size varies from seed to seed among palms and is a characteristic feature of 'remotive germination'. The details will be discussed under germination aspect.

The mature embryo of *B. flabellifer* is an elongated oblong shaped structure (Fig. 1E&H) having 0.7±0.2 cm length and 0.4±0.1 cm breadth and 0.28±0.1g fresh weight. As per the descriptions of mature embryo of *Phoenix dactylifera* and *Washingtonia filifera* (DeMason and Thomson, 1981; DeMason, 1988b) which are characterised by remotive germination, the size of both embryos are very small compared to that of *B. flabellifer*. Hence it seems that *B. flabellifer* seeds contain the largest embryo among palm seeds that are characterized by remotive germination. Nevertheless double coconut (*Lodoicea maldivica*) which produces the biggest palm seed shows remotive type of germination but the size and other details of its embryo are not known so far (Anonymous, 1976).

The cotyledon of *B. flabellifer* embryo consists of isodiametric parenchyma cells with conspicuous nucleus and dense cytoplasm (Figs. 5&6). More or less similar structural details have been reported in the developing cotyledon of *Phoenix dactylifera* (DeMason, 1984). But larger size and oblong shape of the *B. flabellifer* embryo is different from the size and shape of Date embryo. *B. flabellifer* cotyledon shows a well formed epithelial layer consisting of isodiamteric parenchymatous cells with conspicuous nucleus and dense cytoplasm. Cell walls of these cells are densely stained with Periodic acid Schiff's (PAS) reagent. Large numbers of vascular strands

consisting of undifferentiated cells are also present in the cotyledonary tissue (Fig. 6).

Analytical data of *Borassus* embryo shows 26% dry weight, starch and protein content are very low and lipid is not present. The cotyledon is not an important storage organ because the biomass as well as metabolites consists of negligible quantities compared to the endosperm which is the major storage organ comprised of insoluble polysaccharide – galactomannan which is considered as an important cell wall storage polysaccharide (CWSP). In *Phoenix dactylifera*, storage function has also been attributed to the cotyledon (Chandra Sekhar and DeMason, 1988a). According to those authors, presence of protein and lipids occur in cotyledonary cells of Date seeds.

Even though mature embryo of *B. flabellifer* contains only negligible starch, sugars such as rhamnose, fructose and galactose are also present (Table 3). Prior to germination, the embryo is in a resting stage and lack of significant amount of metabolisable carbohydrates is directly related to the 'resting' embryo which is located as a structure inserted to the dead endosperm cells (Fig. 1B). Occurrence of rhamnose in the embryo is found to be associated with structure and/ or function of plant cell walls because this deoxy sugar (1-6 deoxy mannose) is an important component of hemicellulose of plant cell walls. Absence of glucose in the embryo is presumably due to the utilization for the synthesis of rhamnose which is

formed from glucose-1-phosphate (Giraud and Naismith, 2000; Watt *et al*., 2004). According to those authors the biosynthetic pathways of rhamnose are not studied in plants. However, rhamnose is a common component of bacterial cell walls in general and capsules of many bacteria in particular and the rhamnose-rich cell wall capsule of pathogenic bacteria interact with host cell during infection and are vital for the bacterial survival. In *B. flabellifer* seeds the rhamnose sugar content and comparatively thick cell walls of peripheral layers of the cotyledonary cells which are differentially stained (Figs. 5&6) are seemed to be correlated with protection of embryo against pathogen since the embryo is situated in the middle of dead endosperm cells and hence vulnerable to infections.

Endosperm is a unique tissue of seeds in several aspects of development, physiology, cell biology and biochemistry. Due to its apparent morphological simplicity endosperm is viewed as a mass of storage cells and simple appendage to the embryo. As mentioned earlier, liquid endosperm (syncytium) is a characteristic of *B. flabellifer* seeds. Cell wall formation is initiated after one month as shown by hematoxylin staining and fully formed cell wall and nuclei are clearly seen in all cells (Figs. 4A,B,C&D). Secondary cell wall formation is started in the endosperm cells of stage 4 and simultaneously the nuclei of some cells are disappeared (Fig. 4D). During further developmental stages, cells are completely devoid of nuclei and

cytoplasmic inclusions get reduced. Cell wall thickening is increased resulting in the reduction of lumen of the cells and cell inclusions are totally absent (Figs. 4E&F). According to Berger (1999) endosperm development is characterised by four phases: syncytial, cellularisation, differentiation and death. All these four typical stages are obviously observed in the development of *B. flabellifer* seeds (Fig. 4). In cross sections as well as longitudinal sections the endosperm cells of mature seeds are devoid of any inclusions but are with very thick walls which are differentially stained with PAS (Fig 4.) indicating the occurrence of cell wall storage polysaccharides (CWSP) and non-meristematic or non-viable nature of these cells.

Even though detailed studies on endosperm development in palm seeds are meagre, some aspects have been investigated in *Cocos nucifera* (Bhatnagar and Sawhney, 1981). The development starts with free nuclei formation from the liquid syncytium. According to those authors, the multiplication of nuclei is started as the growth advances and the liquid syncytium is transformed into isodiametric meristematic cells. However, the pattern of endosperm development is dissimilar in palm species because cell wall thickness, hardness, chemical structure and cell wall storage polysaccharide distribution of endosperm cells exhibit distinct differences in these species.

Endosperm cells in the mature seeds of *B. flabellifer* are non-living as shown by tetrazolium chloride test (Fig. 1C&D). Embryo and endosperm development showed that differentiation of the embryo is almost complete before the deposition of thick cell wall materials on the endosperm cells. So it seems that before the endosperm cells become dead, the embryo gets fully differentiated. In Rice seeds, it has been reported that endosperm regulates embryo development (Hong *et al*., 1996). Similarly, characterisation of early expression of specific genes in the endosperm tissue which occupy the area surrounding the embryo is suggestive of the existence of interactions between the embryo and endosperm as reported in maize seeds (Opsahi-Ferstad *et al*., 1997).

Although some studies have been made on the structural and functional aspects of mature endosperm of dicots seeds such as *Trigonella foenum-graecum* (Meier and Reid, 1977; Reid and Bewley, 1979), *Lactuca sativa* (Jones, 1974), *Ceratonia siliqua* (Seiler, 1977) and monocots seeds like *Hordeum vulgare* (Bacic and Stone, 1981) these studies are found to be centred only on the cell wall thickening process of the endosperm and their carbohydrate components. In *Trigonella foenum-graecum* and *Ceratonia siliqua,* the thick cell wall of endosperm cells consists of galactomannan (Meier and Reid, 1982). Mature cell walls of *Phoenix dactylifera* endosperm are composed mainly of insoluble polysaccharides that are synthesized as

galactose rich galactomannan and later polymerized into relatively pure mannan (Meier and Reid, 1982; DeMason *et al*., 1992) Ivory nut (*Phytelephas macrocarpa*) has mannan (79% dw.) as storage polysaccharide (Bewley and Black, 1985).

Histochemical staining of endosperm cells with Periodic acid Schiff's reaction reveals the presence of PAS positive thick cell walls in *B. flabellifer*. But the cells are devoid of starch grains or any other cell inclusions (Fig. 4). Biochemical analysis of *B. flabellifer* endosperm showed gradual increase in dry weight (Table 4) and negligible starch content which is increased slightly during development (Table 4). This observation is in conformity with the view of Dey (1978) who opined that generally galactomannans do not exist together with starch. In *Washingtonia filifera* and *Phoenix dactylifera* seeds, endosperm cells are galactomannan-rich and starch content is negligible (DeMason, 1986, 1988b; DeMason *et al*., 1983).

Endosperm of mature *B. flabellifer* seeds are dead, devoid of nucleus and consist of thick walled cells with three layers clearly seen in PAS stained sections. Contradictory to this observation, mature endosperm cells of *Phoenix dactylifera* and *Washingtonia filifera* are nucleated and reported as living (DeMason *et al.,* 1983; Chandra Sekhar and DeMason, 1988 a, b). According to those authors the endosperm cells are thick walled which show (stained with PAS) three different layers and the cells are without starch.

According to Chandra Sekhar and DeMason (1988 a) the endosperm cells of Date are viable and the storage carbohydrate is deposited in the form of thickened cell wall materials. Lipids and protein bodies also have been reported in Date endosperm and isoelectric focussing of protein showed protein bands with heterogeneous molecular mass (Chandra Sekhar and DeMason, 1988 a). All the studies on Date seeds (DeMason, *et al*., 1983; Chandra Sekhar and DeMason, 1988 a) were conducted on dry seeds but the endosperm cells are reported as viable. According to Chandra Sekhar and DeMason (1988 b) endosperm cells of *Washingtonia filifera* also are thick walled and protein bodies and protein profiles are similar to those of *Phoenix dactylifera* endosperm. Contradictory to these features, mature seeds of *B. flabellifer* contain copious amount of endosperm and cells are non-living (Figs. 1B, C&D) and are characterised by the lack of cytoplasm and nucleus and/ or any other cell organelles (Figs. 4E&F). As mentioned earlier, in the present study, mature seeds of *B. flabellifer* treated with tetrazolium chloride confirmed the nonliving nature of the endosperm cells (Figs. 1C&D). Endosperm cells of mature seeds in general inclusive of almost all palm seeds are reported to be dead (Bhatnagar and Sawhney, 1981). According to Halmer and Bewley (1982) palm seeds are mannan/ galactomannan rich and the cells are dead. Although, endosperm tissue is viable in many germinating seeds of cereals and some palms, it is thought to be dead due to the massive accumulation of cell wall storage polysaccharides and the subsequent

desiccation may lead to total impairment of physiological activities (Larkins *et al*., 1992; Lopes and Larkins, 1993).

DeMason (1986) described that the endosperm cells of mature dry seeds of *Washingtonia filifera* are living and contain protein and lipid bodies within the cytoplasm. The cell wall is very thick and store insoluble carbohydrates-mannans. According to the author, slight differences exist in the microstructure of cell walls of *Phoenix* and *Washingtonia*. Similarly, protein bodies also differ structurally. A direct comparison between the endosperm cell structure of *Phoenix* and *Washingtonia* on one hand and *B. flabellifer* on the other is not feasible because the endosperm cells of the former species are living and that of the latter are non-living. Nevertheless, both types of endosperm cells are storage in function as suggested by DeMason (1986, 1988b) and thick cell wall constituents-mannan/galactomannans (hemicellulose) are the storage materials.

An interesting and unique observation in the mature seeds of *B. flabellifer* is the occurrence of a multicellular layer located in between the inner layers of endosperm and outer surface layer (epithelium) of embryo of mature seeds (Figs. 5&6). Although cellular details are not distinct under light microscope, this structure is stainable with Periodic acid Schiff's reagent for insoluble polysaccharides (Figs. 5D&6D), bromophenol blue for proteins (Figs. 5B&6B) and toluidine blue and hematoxylin for structural details (Figs.

5A&C, 6A&C). This intermediate layer present in between the endosperm and embryo is likely to play some vital role during germination of *B. flabellifer* seeds. Since the endosperm cells are dead, some sources or media of digestive enzymes/ hormones are essential for the degradation of endosperm cell walls during germination and subsequent reserve mobilisation. In cereal seeds, the endosperm is dead and the scutellum/ aleurone cells are differentiated as digestive tissues specialized for the secretion of enzymes that hydrolyse and mobilize endosperm reserves during germination (Jones and Jacobsen, 1991; Bewley and Black, 1994; Lopes and Larkins, 1993; Berger, 1999). Similarly, in the seeds of endospermous dicot like legumes, cotyledons are well differentiated and potentially function as the source of digestive enzymes as well as transient storage organ of metabolites translocated from the dead endosperm cells during germination (Reid and Meier, 1972). However, in most palm seeds, endosperm cells are dead and lack any viable tissues other than the cells of the embryo. The embryo consists of a large cotyledon, the distal end of which is transformed into the haustorium and proximal end grows as cotyledonary sheath carrying the plumule during germination. For the development of the haustorium and other seedling parts, storage metabolites are to be degraded and mobilized from the dead cells of the endosperm.

Structural and functional aspects of palm seeds during germination have received scant attention and the secretion of the digestive enzymes for endosperm hydrolysis is controversial (Mayer and Poljakoff-Mayber, 1989; Bewely and Black 1994; Larkins *et al.*, 1992; Lopes and Larkins, 1993). However, in the present study, anatomical or physiological role of the tissues present in between the endosperm and cotyledon (Figs. 5&6) are not investigated because sampling and dissection of the embryo embedded in the large and hard endosperm located inside a very hard stony endocarp is very tiresome and mechanical damage is very common in obtaining intact embryo along with the endosperm for anatomical/physiological experiments. However, steps are being taken to investigate the ultrastructure and functional aspects of this tissue during germination.

According to Lopes and Larkins (1993) endosperms of plant species that lack an aleurone layer must remain alive and be capable of producing hydrolytic enzymes to mobilize reserves during germination. Reid and Meier (1972) suggested that in endospermous legume like *Trigonella foenum-graecum* the seed reserve – galactomannan is broken down in a dissolution zone which begins at the aleurone layer of the endosperm and enlarge inwards towards the cotyledon. According to Spyropoulos and Reid (1985; 1988) during the early phase of germination, the embryo controls the galactomannan hydrolysis by growth hormones that are diffused from the axis tissues of

embryo to the endosperm. A more or less similar mechanism can be presumed to occur in *B. flabellifer* in which the synthesis of growth hormones rather enzymes is induced by the embryo and are diffused to the endosperm through the undifferentiated tissues present in between the endosperm and embryo when germination is started. This layer is found to be equivalent or similar to the dissolution zone of *Trigonella foenum-graecum* as suggested by Spyropoulos and Reid (1985, 1988). In Tomato seeds, it has been shown that for the production of full active hydrolysing enzymes such as β-mannosidase and endo-β-mannanase in the endosperm, the tissue must be in contact with the embryo at least during imbibition (Mo and Bewley, 2003). In *B. flabellifer* the undifferentiated cells of the intermediate layer consist of protein-rich and densely stained cell inclusions (Figs. 5&6) and hence are found to be physiologically active. So the vital role played by this tissue during germination cannot be ruled out. Similarly, in germinating seeds, the inner layers of the hard endosperm are getting hydrolysed, resulting in the formation of dissolution zone and their structural details are not distinct (Fig. 16). This zone is in contact with the developing haustorium in which the hydrolytic products are transiently accumulated.

De novo synthesis of galactomannan hydrolase has been reported in *Trigonella foenum-graecum* (Reid and Meier, 1972) and *Ceratonia siliqua* (Seiler, 1977). But in *Sesbania virgata the* enzyme necessary for

galactomannan degradation are already present in the protein bodies of the endosperm (Tonini *et al*., 2010). In *Borassus flabellifer* α-galactosidase is present in the mature seed and since the endosperm cells are dead and devoid of any cytoplasmic inclusions the enzyme is presumed to be localised in the intermediate layer layer- which is stained with bromophenol blue indicating the presence of proteins- present in between the endosperm and cotyledon.

As mentioned earlier, total protein content of endosperm during early stages of development is comparatively more and during 2-4 months it shows a reduction followed by significant increase whereas soluble protein is very low and exhibits only negligible increase. Protein metabolism of developing endosperm of palm seeds is not well investigated (Bhatnagar and Sawhney, 1981). However, according to DeMason *et al*. (1983) ultra structural studies of mature endosperm of *Phoenix dactylifera* revealed lack of machinery for *de novo* synthesis of enzyme proteins and hence total proteins is reported to be very low. Chandra Sekhar and DeMason (1988b) reported the presence of endosperm-specific proteins in *Washingtonia filifera* seeds. Maximum total and soluble protein content of *B. flabellifer* endosperm during the last stage of seed development is in consistent with the view of DeMason *et al*. (1985) who suggested that endosperm is a possible source of hydrolytic enzymes involved in autocatalysis. Since programmed cell death is characterized and reported in endosperm cells (Young *et al*., 1997), *de novo* synthesis of

enzymes specifically related to germination metabolism is not possible and hence precautious synthesis of soluble proteins at least polypeptides might have occurred in *B. flabellifer* seeds prior to germination. Mature endosperm of coconut seeds show the presence of hydrolytic enzymes like phosphatases (Balasubramaniam *et al*., 1973) and galactosidase (Mujer *et al*., 1984). Similarly, some endosperm specific proteins also have been reported in the mature endosperm of *Washingtonia filifera* (Chandra Sekhar and DeMason, 1988b) but their functions are not fully known. In spite of the absence of protein bodies in *B. flabellifer* endosperm, considerable amount of protein content observed (Table 4) may presumably include some hydrolytic enzymes which may be in the immobilised condition but are required for endosperm degradation during germination since *de novo* synthesis of enzyme proteins is not possible since the endosperm cells are non-living. In monocot endosperm, the protein bodies generally occur throughout the endosperm tissue (Berger, 1999). According to Lopes and Larkins (1993) accumulation of protein bodies in the endosperm cells is an adaptation that probably prevent their exposure to enzymes responsible for turnover of metabolic proteins. Other potential advantage of sequestering the proteins in membrane bound protein bodies which are often deposited in relatively non-hydrated condition is to facilitate seed desiccation during maturation.

Developing endosperm of *B. flabellifer* seeds consist of soluble sugar component such as sucrose, glucose and fructose in all developmental stages (Table 6). Sucrose occurs in low quantities which remain almost unchanged up to 4th stage with an upsurge in the last stage whereas both reducing sugars occur in abundance during the same period. Occurrence of sucrose, glucose and fructose in the tender kernel of *B. flabellifer* has already been reported (Subrahmanyan *et al*., 1956). According to those authors, even though free galactose and mannose are not present in the kernel, galactose and mannose are present as the components of insoluble polysaccharides. However, in the present study the distribution of sugars in the endosperm during different developmental stages reveal that sucrose translocated to the endosperm from the parent plant is incidentally converted into glucose/fructose resulting in their abundant occurrence as observed in the earlier stages of development (Table 6). These monosaccharides are presumed to be involved in the synthesis of galactomannan in the samples as deposits on the cell walls which constitutes about 24% of dry weight in the samples of 6th stage (Table 7). Nevertheless, free mannose and galactose are not present in the endosperm upto last stage, in which very low quantities of these sugars are present. Similar observations have been reported in legume seeds such as *Trigonella foenum-graecum* (Reid and Meier, 1970). According to Spyropoulos and Reid (1985, 1988) free mannose and galactose are not present in *T. foenum-*

graecum and these sugar-components are deposited simultaneously to form galactomannan.

As mentioned earlier, the endosperm in *B. flabellifer* is liquid syncytium in the early developmental stages and gradually get solidified and cell walls get hardened after stage 4. So it seems that since the synthesis and deposition of galactomannan is almost ceased, slight amount of galactose and mannose are retained coinciding with exorbitant reduction of glucose and fructose (Table 6). Simultaneously accumulation of sucrose is another interesting observation, presumably due to the lack of translocation and utilization for the synthesis of other metabolisable carbohydrates since metabolic processes are over and the endosperm cells of the mature seeds (stage 6) are dead. The occurrence of feeble amount of galactose and mannose in the final stage may be indicative of the cessation of galactomannan synthesis retaining these sugars as residual quantities.

Galactomannan synthesis and deposition in the endosperm cells are not investigated elaborately in palm seeds. However, galactomannan formation studied in *Trigonella foenum-graecum* endosperm using light and electron microscopy revealed that this polysaccharide is deposited in the form of thickening on the endosperm cells walls and deposition continues until there is no cytoplasm or only a remnant of it left in any of the endosperm cells except for the aleurone layer (Meier and Reid, 1977, 1982). Cell wall

thickening of *B. flabellifer* endosperm progressed continuously with the simultaneous disappearance of cell inclusions during seed development (Fig. 4). According to Meier and Reid (1982) galactomannan is deposited first on the walls of the innermost cells of endosperm next to the embryo and lasts in the cells bordering the aleurone layer. Contradictory to this view, in *B. flabellifer* seeds aleurone layer is absent and the cell wall thickening of endosperm cells starts from the periphery (testa) and spreads towards the centre (Figs. 2&4).

Analyses of galactomannan, estimated as component sugars – galactose and mannose revealed that galactose content increased from one stage to other during seed development (Table 7). Mannose content is more than galactose in all stages and pattern of distribution is similar to that of galactose, maintaining the galactose: mannose ratio as 1:3. But in the samples of 5^{th} and 6^{th} stages, the G:M ratio is 1:4 and 1:5 respectively, reflecting enhanced increase of mannose plausibly due to the hydrolysis by α-galactosidase activity in the endosperm of mature seeds. Involvement of α-galactosidase activity in *Phoenix dactylifera* (DeMason *et al*., 1992) *Sesbania virgata* (Buckeridge, 2010) *Senna occidentalis* (Bewley and Black, 1994) during the synthesis of galactomannan has been unequivocally reported. Galactose reduction during 5^{th} and 6^{th} stages, resulting in enhanced ratio of G:M=1:4 and 1:5 is found to related to the hardness of the endosperm in these

samples. In addition, significantly reduced moisture content (Table 3) also is contributing to the hardness of endosperm. This characteristic feature is directly related to the dry weight of the tissues which exhibits doubling of biomass compared to the earlier stages (Table 4). Involvement of a recently reported enzyme- mannan transglycosylase (Schroder *et al*., 2004) is presumed to occur in the synthesis and distribution of galactomannan during seed development in *B. flabellifer* because the ratio of galactose to mannose shows significant variation during seed development as a result of transglycosylation of galactomannan since the enzyme is able to transfer galactose to oligosaccharide derived from the hydrolysis of the same polysaccharide.

Galactomannan content of *B. flabellifer* tender kernel was first reported by Subrahmanyan *et al*. (1956). According to them galactose and mannose contents are 28% and 78% respectively. Subsequently, Rao and Mukherjee (1962) reported that alkali-soluble galactomannan of *B. flabellifer* kernel contain galactose and mannose in the molar ratio 1:2.4 consisting of a backbone of (1→4) β-D mannopyranosyl residues with galactose and mannose side chain. Awal *et al*. (1995) extracted *B. flabellifer* kernel in 10% aqueous alkali and after hydrolysis GLC estimation revealed the presence of mannose (97%) and galactose (3%). Discrepancy in the values of component monosaccharides of galactomannan in *B. flabellifer* in the present study may

be due to the difference in age of the seed and/or the variation in the solubility of this polysaccharide in different solvents and also dependant on the methods adopted. Awal *et al*. (1995) confirmed the structural units of galactomannan by GLC-MS spectroscopy. The present study reveals that galactomannan contents of *B. flabellifer* seeds show changes in the molar concentration of galactose and mannose which is found to be age dependant and directly related to the texture (hardness) of the endosperm of the mature seeds. However, Ganter *et al*. (2001) opined that the ratio of mannose to galactose depends on the plant source and the method of extraction and the ratio ranges in between 1 to 5.

The pathways of galactomannan synthesis during seed development are yet to be elucidated in plants in general and palms in particular. Earlier, Reid and Meier (1970) suggested that in *Trigonella foenum-graecum*, galactomannan begins to be formed at the early stages of seed development during which galactosyl and mannosyl residues are deposited on the cell walls and stachyose formation do occur during this process. However, the pathways of galactomannan synthesis in *B. flabellifer* are not comparable with *Trigonella foenum-graecum* due to the absence of stachyose in any stages of *B. flabellifer* seed development. According to Buchanan *et al*. (2000) in legumes, sucrose translocated to the developing endosperm from the parent plant is converted to mannose 1-phosphate and then to GDP mannose, in the

rough endoplasmic reticulum. Then a membrane-associated enzyme, GDP mannose-dependant mannosyl transferase transfers the mannose residues to the non-reducing end of a linear (1→4) β- linked mannose primer to form the growing backbone chain of galactomannan polymer. Simultaneously, UDP galactose formed as a result of epimerase activity on UDP glucose, is acted upon by another membrane-associated enzyme UDP galactose-dependant galactosyl transferase transfers a galactose residue to a mannose at or near the non-reducing end of the growing mannan chain forming (1→6) α linkage. The activities of these two transferase enzymes are increased in parallel during galactomannan synthesis and galactose residues cannot be transferred to preformed mannose chain. Buchanan *et al*. (2000) are of opinion that in plant cell walls where less abundant non-cellulosic wall materials such as galactomannans are present, this polysaccharide potentially interlock the microfibrils in the primary cell walls resulting in the firmness and very hard texture of cell walls. According to Schroder *et al*. (2004) galactomannan can make network to cross link with other polysaccharides in the cellulose.

Recent studies also have shown the presence of many glycosyl transferases which are required for the biosynthesis of galactomannan (Edwards *et al*., 1999; Gibeaut, 2000). Seed galactomannans of Tobacco and Coffee are synthesized by a galactosyl transferase similar to *Trigonella foenum-graecum* (Reid *et al.,* 2003). According to Reid *et al* (1995) the

relative concentration of UDP galactose and GDP mannose determine the substitution degree of galactomannans. Scheller and Ulvskov (2010) opined that for several reasons, mannans are likely to be synthesized as more highly substituted polymers that are subsequently trimmed by α-galactosidase and the mannans formed in some seeds such as Ivory nut are very low in galactose and hence essentially insoluble and therefore they are likely to be synthesized as more soluble precursors with much higher galactose substitutes. As mentioned earlier, during *B. flabellifer* seed development the endosperm show comparatively lower values of galactose: mannose ratio (1:3) in the earlier stages of development when the cell wall formation is just started and the galactomannans appear as liquid revealing comparatively more solubility. But, during last stages (5 and 6) the ratio of galactose:mannose is more (1:4 and 1:5) due to reduced galactose resulting in low solubility and formation of hard endosperm replacing the liquid endosperm. This observation is in conformity with the views of Scheller and Ulvskov (2010) and the consistency or texture of endosperm during seed development is directly linked with the distribution ratio of constituent sugars of galactomannan in the seeds of *B. flabellifer*.

Comparatively reduced content of galactose in the mature endosperm samples of *B. flabellifer* reveals the hydrolysis of galactose component due to α-galactosidase activity, which is an important event observed in the

endosperm of mature seeds assayed as the controls (ungerminated seeds) during the experiments on seed germination (Table 13, Fig. 26). More or less similar observation have been reported in *Senna occidentalis* in which an increase in α-galactosidase activity occurred during late stages of seed development resulting in an increase in M:G ratio from 2.3 to 3.3 (Bewley and Black, 1994). Occurrence of free galactose in the soluble sugar fraction is another evidence for the existence of α-galactosidase activity in the final stages of endosperm development in *B. flabellifer* (Table 6).

Endosperm of developing *B. flabellifer* seeds showed the presence of 11 amino acids and 2 amines in the first stage (Table 5). During the development, quantity of many amino acids is reduced and appears only in traces and many are disappeared. Quantitatively the changes of free amino acids are not directly related to the total protein content at any of the developmental stages (Tables 4&5) whereas qualitative differences do occur in the distribution of free amino acids and are found to be related to galactomannan biosynthesis. Generally, storage proteins are very low in palm seeds and the metabolism of proteins in the endosperm is yet to be investigated. However, scant descriptions of protein microstructure bodies in *Washingtonia filifera* and *Phoenix dactylifera* have been provided (DeMason, 1984, 1985).

Considerable quantity of asparagine and glutamine are present in the endosperm during all stages of development. In developing seeds, these two amines are the important translocating forms of nitrogen source from the parental plant to the developing seeds and synthesis of other amino acids occurs within the seeds (Mayer and Poljakoff-Mayber, 1989; Baskin and Baskin, 2001). Glutamine import has been reported to be declined with period of development in cereal seeds (Bewley and Black, 1985, 1994). Importance and metabolism of amino acids in cereals and palm seeds have received less attention but it is generally accepted that asparagine and glutamine are the important translocating forms and the amino groups of these amides provide nitrogenous component for newly synthesizing amino acids and carbon skeleton is furnished by the translocated carbohydrates (Bewely and Black, 1994). Distribution of glutamine and asparagine in *B. flabellifer* endosperm tissues is in consonance with the behaviour of cereal seeds during development (Mayer and Poljakoff-Mayber, 1989). After complete hardening of the endosperm cell walls the cells become dead and contain very high (maximum) amount of asparagine in *B. flabellifer* seeds presumably due to the cessation of metabolism and resultant loss of viability. According to Lopes and Larkins (1993) endosperm cells contain a variety of storage proteins and these proteins typically have high amide content and/or sulfur containing amino acids.

Although the role of free amino acids in the developing endosperm and its distribution are yet investigated in palm seeds, abundant occurrence of many free amino acids in *B. flabellifer* endosperm reveals the integral relationship of endosperm with the development of embryo because in *Arabidopsis thaliana* an amino acid transporter- AAP1 appears to be specifically expressed in the endosperm during early stages of embryo development (Hirner *et al.*, 1998). Presence of considerable quantities of free amino acids in the endosperm of *B. flabellifer* may be a prerequisite for the synthesis of enzyme proteins for endosperm hydrolysis during germination. However, since the endosperm cells are non-living, it is not certain whether the endosperm or the developing haustorium produce the galactomannan hydrolysing enzymes by incorporating the free amino acid retained in the endosperm.

Abundant occurrence of proline, hydroxy proline and threonine in the developing endosperm of *B. flabellifer* (Table 5) is found to be related to cell wall synthesis. As mentioned earlier, endosperm of developing *B. flabellifer* seeds is liquid when young and cell wall formation starts gradually during development and get completed with 4^{th} stage. The presence of hydroxy proline in the third stage reveals its involvement in cell wall formation. Various glycoproteins are involved in the final processing of cell wall substances containing a high proportion of hydroxy proline which is normally

absent or occurs in traces in other proteins (Anderson and Beardall, 1991) and glycoproteins are proline – or – hydroxy proline rich (Buchanan *et al*., 2000). According to those authors threonine rich glycoproteins are involved as an integral part during cell differentiation. Extensin is a glycoprotein component associated with cell wall synthesis and is the best studied hydroxy proline-rich glycoprotein (HRGPs) in angiosperm cell walls (Buchanan *et al*., 2000). Compared to all the free amino acids, hydroxy proline, proline and threonine are present in abundance and plausibly this observation is directly linked with cell wall synthesis in the endosperm cells of *B. flabellifer* during the conversion of liquid syncytium to cellular endosperm. Significant quantities of proline and threonine during later stages of endosperm development may be the residual amount since the cell wall formation is ceased during these stages. However, the metabolic role of other amino acids in the endosperm development is obscure.

Germination of palm seeds is erratic/ sporadic and *B. flabellifer* is not an exception. However, the present author succeeded in obtaining considerable number of germinated seeds/ seedlings during a period of about one month out of a large number of seeds planted for germination studies. Germination and seedling development of *B. flabellifer* seeds provide a unique system for investigating the embryo-endosperm interactions. As mentioned earlier, palm seeds possess small embryo and copious amount of

endosperm. A single seed of *B. flabellifer* consists of 136±6 g endosperm and 0.28±0.1 g embryo (fresh weight). About one month (35±5 days) is required for *B. flabellifer* seeds to germinate. The 'radicle' like proximal region of the cotyledon ('cotyledonary sheath') protrudes through the micropylar end of the pyrene (Figs. 13B&14B). This portion extends to push the shoot - root axis of the embryo out of the seed (Gatin, 1906; Tomlinson, 1960, Foster and Gifford, 1974). The elongating portion is termed as 'cotyledonary sheath' or apocole (Cook, 1939). As germination advances the cotyledonary sheath elongates and the tip looks like radicle (Figs. 14B&C). The region just above the tip which is hollow widens and thickens and carries the developing seedling (Fig. 14D). The growing cotyledonary sheath is positively gravitropic and grows into the soil. So the seedling is developed in the soil some distance away from the seed (remotive germination) (Fig. 13D). Then the extreme tip of the cotyledonary sheath differentiates into the true root which appears as tap root with many secondary roots. The broad/ bulky portion of the cotyledonary sheath grows further into a tubular structure inside of which a robust plumular sheath and plumule are developed (Fig. 13D). Later, a slit is formed on one side of the cotyledonary sheath and plumular sheath which carries the primary (palmate) leaf is protruded through the slit. According to Henderson (2006) morphology of seedling in Borasseae consists of straight symmetrical plumular-radicular axis, long cotyledonary sheath-which opens laterally, with swollen basal region and narrow growing tip and

single thick/ robust plumular sheath (cataphyll) which shows apical opening. In *B. flabellifer* seedling the plumular sheath is very thick, robust and 25-30 cm long, stout in the bottom and tapering toward the upper side with a groove through which the actual plumular (primary/palmate) leaf emerges (Fig. 14E). Simultaneously the distal part of the cotyledon that is retained inside the seed is developed into a haustorium, which grows fast replacing the endosperm and acts as a reservoir for the hydrolytic products of the endosperm during degradation (Fig. 17).

It is clearly seen that the proximal part of the cotyledon which protrudes as the cotyledonary sheath carries the developing root-shoot axis and the entire seedling is developed inside the cotyledonary sheath. The mode of germination is remotive in accordance with the view of Gatin (1912) and Uhl and Dransfield (1987). DeMason (1984, 1988a) reported remotive germination in *Phoenix dactylifera* and *Washingtonia filifera*. An elaborated morphological study on the germination behaviour of palm seeds in our laboratory confirmed remotive germination in *B. flabellifer*, *Corypha umbraculifera*, *Caryota urens*, *Licuala peltata* and *Livistona rotundifolia* (Radha, 2007). Morphological features of seeds/seedlings during germination have been investigated only in *Phoenix dactylifera* (DeMason, 1984, 1985; Chandra Sekhar and DeMason, 1988 a) and *Washingtonia filifera* (DeMason 1988a, b; Chandra Sekhar and DeMason, 1988b). According to those authors,

during seed germination of *Phoenix dactylifera* and *Washingtonia filifera*, the proximal part of the cotyledon elongates and protrudes out of the testa while the distal part of the cotyledon enlarges to form the haustorium which grows inside the endosperm by replacing it within the seed.

According to Bewley and Black (1994) germination is defined as the total metabolic processes which occur in a seed during and following water imbibition and results in radicle protrusion or some outward sign that the seed has germinated. The mobilisation of bulk reserves is therefore a post-germinative event associated with seedling development. Hence the usage 'during germination' pertaining to the seedling development and reserve mobilisation is contiguous. In the case of *B. flabellifer* the reserve mobilisation is a prolonged process (about 5-6 months) and the present author prefers the usage 'during and following' to cover the entire process of germination and seedling growth which is divided into 6 comparable stages. All the metabolites during these stages of seedling development and concomitant reserve mobilisation have been analysed. Five to six months are required for *B. flabellifer* seedling to emerge the green primary (palmate) leaf and then the seedling become autotroph. Keusch (1968) suggested that degradation of endosperm and expansion of haustorium in Date palm are completed by 10 weeks.

Studies on reserve mobilisation in palm seeds with remotive germination are very scanty, whereas seeds exhibiting admotive (adjacent) germination have been investigated during and following germination in *Cocos nucifera* (Balasubramaniam, 1976; Balasubramaniam *et al*., 1973; Samonte *et al.*, 1989) *Elaeis guineensis* (Alang *et al*., 1988; Opute, 1975; Oo and Stumpf, 1983). Preliminary aspects of endosperm degradation and haustorium development have been investigated in Date palm (*Phoenix dactylifera*) seeds (Meier, 1958; Keusch, 1968; DeMason, 1985; DeMason *et al*., 1985; DeMason *et al*., 1992) and in *Washingtonia filifera* (DeMason, 1988a; Chandra Sekhar and DeMason, 1988b).

As mentioned earlier, the morphology of *B. flabellifer* seedling is very heterogeneous and unique. So the estimation of metabolites are done only in the tissues of endosperm – which is the seed reserve – and the haustorium – which is intermediate or transient organ developed from the distal end of the cotyledon – to a large multi cellular organ. The hydrolytic products of the endosperm are temporarily stored in the haustorium and gradually translocated to the developing seedling until it becomes an autotroph. Reserve mobilisation studies of *B. flabellifer* seeds during and following germination are centred on the endosperm and haustorium because these two organs are integrally involved in the entire process. During germination of *B. flabellifer* seeds hard endosperm adjacent to the growing haustorium is degraded and

become soft and slimy. In comparison with the original hard endosperm tissue, the slimy portion shows wide variation in the distribution of dry matter and metabolites. Hence analyses of metabolites were carried out in both the hard and slimy endosperm tissues during and following germination.

Dry weight of hard endosperm is about 30% in the mature ungerminated seeds (Table 9) and following germination also it remains unchanged revealing more or less uniform water holding capacity due to lack of imbibition as suggested by Meier and Reid (1982), whereas in the slimy endosperm dry weight is reduced gradually proportional to the progress of seedling development (Table 9, Fig. 21A). According to Meier and Reid (1982) large mannan or galactomannan rich palm seeds scarcely imbibe during germination and only some water is essential for embryo growth. Water is required only to maintain the very narrow dissolution zone which surrounds the haustorium. Those authors opined that the storage of galactomannans which are essentially hydrophobic appears to be a part of the palm seed's 'xeromorph' germination strategy.

Starch content of *B. flabellifer* hard endosperm cells is very low and showed no changes during and following germination. But slimy endosperm contains comparatively more starch and showed slight changes during and following germination. So in the slimy endosperm a transient synthesis and/ or degradation of starch cannot be ruled out owing the occurrence maltose

(Table 11) which exhibit significant fluctuations in the distribution and resultant starch depletion (Table 9) during last stages of seedling growth.

Soluble sugars of hard endosperm consist of sucrose, glucose, galactose, fructose and mannose in the ungerminated (control) seeds (Table 11). In germinating seeds, hard endosperm showed the presence of all sugars as in the control seeds whereas only raffinose, maltose and sucrose are present in the slimy endosperm tissues. Sucrose content in the slimy endosperm tissues increases from stage to stage, accumulating in the final stage but maltose and raffinose exhibit only slight changes. Galactose and mannose are absent in the slimy endosperm which is the site of galactomannan degradation. Similar observations have been reported in *Trigonella foenum-graecum* which is a galactomannan-rich seed (Spyropoulos and Reid, 1985). As the galactomannan disappear in the endosperm, there is little accumulation of hydrolytic products- galactose and mannose which are rapidly absorbed by the haustorium, because the concentration of these sugars in the endosperm is close to zero in *Trigonella foenum-graecum* (Reid, 1971; Reid and Meier, 1972). However, according to Meier and Reid (1982) and Halmer and Bewley (1982) galactomannan mobilisation in the endosperm of seeds vary from species to species and in most cases the living aleurone layer is responsible for the synthesis and secretion of the polysaccharide – degrading enzymes.

However, living aleurone cells are absent in palm seeds and galactomannan degradation process is not well documented also.

Degradation of galactomannan which is the abundant seed reserve plays an important role in the germination and post germinative seedling development. Following germination, hydrolysis and mobilisation of galactomannans of endosperm cell wall provide source of metabolites for seedling growth (Bewley, 1997a, Wang *et al*., 2005). Complete breakdown of galactomannan requires a concerted activity of at least three enzymes – endo-β-mannanase (EC 3.2.1.78) β-mannosidase (EC 3.2.1.25) and α-galactosidase (EC 3.2.1.22). These enzymes have been investigated extensively in relation to seed germination in some legumes such as *Trigonella foenum-graecum* (Gong *et al*., 2005) *Prosopis juliflora* (Gallao *et al*., 2007) *Sesbania virgata* (Tonini *et al*., 2006, 2010), dicotyledonous species like *Lycopersicon esculentum* (Bewley *et al*., 1997; Bourgault *et al.*, 2005; Wang *et al.*, 2009) and monocotyledonous species like *Oryza sativa* (Wang *et al*., 2005; Ren *et al*., 2007, 2008).

Endosperm hydrolysis of seeds during germination has been studied in many cereals and albuminous legumes (Bewley and Black, 1985, 1994; Mayer and Poljakoff-Mayber, 1989; Baskin and Baskin 2001). Many legumes stores carbohydrates in the form of hemicellulose – galactomannans. Galactomannan hydrolysis during and following germination have been

exhaustively elucidated in *Trigonella foenum-graecum* (Reid 1971; Reid and Meier, 1973; Meier and Reid, 1977; Reid and Davies, 1977; Reid and Bewley, 1979; Leung *et al*., 1981; Campbell and Reid, 1982; Spyropoulos and Reid, 1985; Dirk *et al*., 1999). In these seeds synthesis of at least 3 enzymes occurs in the aleurone cells and is released to the dead cells of the endosperm where galactomannan deposits are present. These enzymes include α-galactosidase which cleaves 1 6 linkage between the galactose side chain and mannose backbone, endo-β-mannanase- that hydrolyse oligomers of mannose and β-mannosidase which cleaves mannose form the oligomer products of endo mannanase activity.

Considerable activity of α-galactosidase is shown by the ungerminated (control) seeds of *B. flabellifer* (Fig. 26) plausibly a prerequisite for attaining more solubility of the polysaccharides as suggested by Scheller and Ulvskov (2010). According to those authors trimming of galactose residue by α-galactosidase leading to high ratio of galactose: mannose and resultantly the liquefaction of stony endosperm to slimy texture is occurred during the early period of germination. According to Mo and Bewley (2003) and Wang *et al*. (2009), β-mannosidase, endo-β-mannanase and α-galactosidase are involved in the degradation of mannan containing cell walls of Tomato seed endosperm during germination. According to those authors, the synthesis of α-galactosidase occurs during seed development and is sequestered in protein

storage vacuoles and thus exhibit high activity in dry seed also. This view is in conformity with the behaviour of *B. flabellifer* endosperm in which α-galactosidase is found to be a constitutive enzyme sequestered in the endosperm. During early stages of germination, the activity increases 4 times exponentially and several fold increase is occurred upto 5th stage followed by an abrupt reduction (Fig. 26).

Following germination, α-galactosidase activity in the *B. flabellifer* endosperm showed a dramatic reduction in the final sample (Table 13) in which 4.5% dry weight and 30 mg/g galactomannan (Tables 9&12b) are retained revealing incomplete hydrolysis of the endosperm reserves. This observation is in agreement with the view of Minic and Jouanin (2006) according to whom complete degradation of galactomannan depends on the action of α-galactosidase and in addition, this enzyme is known to hydrolyse hydrolyses raffinose, stachyose and galactomannan oligosaccharides in Rice seeds. In *B. flabellifer*, raffinose content of endosperm is maximum in the samples of 6th stage (Table 11) which is coincided with the reduced activity of α-galactosidase indirectly revealing the role of this enzyme in the degradation of raffinose. Resultantly, the component sugars of raffinose namely galactose, glucose and fructose are not detected (Table 11).

Increased activity of α-galactosidase, endo-β-mannanase and β-mannosidase have been reported in *Cocos nucifera* during germination

whereas in ‘Makapuno’ coconut the activities of these enzymes are very low owing to the lack of germination in ‘Makapuno’ (Samonte *et al*., 1989). Similarly, Alang *et al*. (1988) opined that in *Elaeis guineensis* seeds during germination the thick endosperm cell wall become markedly thinner concurrent with increase in α-galactosidase and β-mannosidase activity in both degrading and residual endosperm.

Enhanced rate of α-galactosidase activity has been reported in dry (pre-germinated) seeds of Rice (Ren *et al.,* 2007) and in germinating seeds of Tomato (Feurtado *et al*., 2001). According to Lisboa *et al.* (2006) the enzyme α- galactosidase disbranch the galactose from the main chain of galactomannans and this step might be a necessary condition to grant access for β-mannanase to the main chain of galactomannan. The first enzyme involved in the mobilisation of mannan polymers is endo-β-mannanase which randomly cleaves the mannose backbone to release mannobiose and mannotriose and these oligosaccharides are in turn hydrolysed to mannose by β-mannosidase (Bewley and Reid, 1985; Reid, 1985; Bewley and Black, 1994; Bewley, 1997 a; Bewley *et al*., 1997). Contradictory to this view Dirk *et al*. (1995) opined that endo-β-mannanase become active most frequently following the completion of germination.

Assay for endo-β-mannanase (β-D-mannohydrolase) in the endosperm of *B. flabellifer* seeds during germination apparently shows no enzyme

activity since the reaction products estimated by HPLC did not show any component sugars. However, the activity of this enzyme is very crucial for the degradation of long mannan chain which is an important component of cell wall polysaccharide of *B. flabellifer* endosperm (Rao and Mukherjee, 1962; Awal *et al*., 1995). In *B. flabellifer* seeds, since the storage polysaccharide is galactomannan and α-galactosidase is very active even in ungerminated seeds, subsequent increase of this enzyme activity occurs during germination resulting in the formation of long mannan chain which is readily accessible to the activity of endo-β-mannanase is reported in many seeds such as Tomato (Still and Bradford, 1997; Still *et al.,* 1997; Nonogaki *et al*., 1992) Lettuce (Dutta *et al*., 1994) Fenugreek (Reid and Davies, 1977; Reid and Meier, 1972) and Carob (Kontos and Spyropoulos, 1996; Ouellette and Bewley, 1986).

Even though experimental results show no direct evidence for the activity of endo-β-mannanase, the activity of the enzyme in *B. flabellifer* cannot be ruled out because abundant occurrence of mannose was obtained in the assay of β-mannosidase. According to Wang *et al*. (2005) Bewley and Reid (1985) Mo and Bewley (2003) β-mannosidase is an enzyme that acts in concert with endo-β-mannanase to degrade the polymeric mannan. The products of endo-β-mannanase are mostly bioses and trioses of mannose as reported in many cereals such as Rice (Wang *et al*., 2005) Barley (Dirk *et al*., 1995) and dicots like Lettuce and Tomato (Mo and Bewley, 2003; Bourgault

et al., 2005). In Rice seeds, endo-β-mannanase activity is present in the aleurone layer and the enzyme presumably degrades cell wall to permit faster penetration of other galactomannan-and/or starch-degrading enzymes (Ren *et al*., 2008). More or less similar role of endo-β-mannanase is presumed to occur in *B. flabellifer* seeds also because thick and hard endosperm cell wall is not easily penetrable to other hydrolysing enzymes. Although comparable data are not available about the involvement of galactomannan-hydrolysing enzymes in palm seeds, significant amount of galactomannan is found to be mobilized during and following germination in *B. flabellifer* and for the activity of β-mannosidase random cleavage of long mannan back bone to bioses and trioses is essential. However, further investigation is essential for the elucidation of endo-β-mannanase activity in *B. flabellifer* seeds.

Even though the activity of β-mannosidase is not observed in ungerminated (control) seeds of *B. flabellifer*, several fold increase occurs during/ following germination revealing the availability of degradation products of endo-β-mannanase such as mannobiose, mannotriose etc. which are the substrates for β-mannosidase activity. This observation is corroborated with the endosperm degradation mechanism reported in Date palm seeds (DeMason *et al*., 1985, 1992). The overall increase in the activity of all the enzymes – endo-β-mannanase, β-mannosidase and α-galactosidase in the endosperm of *B. flabellifer* is almost similar to the more frequently reported

galactomannan hydrolysing enzymes in legume seeds (Meier and Reid, 1982; Leung *et al*., 1981) and Rice seeds (Wang *et al*., 2005; Ren *et al*., 2008).

Distribution of galactomannan analysed as constituent sugars – galactose and mannose in the hard endosperm of germinating seeds/ seedlings exhibited no changes (Table 12a) during the entire period of germination/ seedling growth similar to the distribution of biomass and other metabolites (Tables 10&11). On the other hand slimy endosperm which serves as the medium of hydrolytic enzyme activity show significant changes in the pattern of galactose-mannose distribution in the galactomannan (Table 12b). The quantity of galactose component in the galactomannan is found to be proportional to the activity of α-galactosidase as well as the parallel mobilization of this sugar and hence the product (galactose) appears only in traces (Table 12b). In the slimy endosperm the mannose content shows gradual increase with a peak at 4^{th} stage followed by significant reduction. This finding also is directly proportional to the activity of β-mannosidase during and following germination as mentioned earlier.

Reserve mobilisation in *B. flabellifer* seeds which is characterised by remotive germination involves the vital role of degrading endosperm tissues and developing haustorium because the haustorium is a transient reservoir where metabolites (products of endosperm hydrolysis) are accumulated and subsequently translocated to the developing seedling. As mentioned earlier,

haustorium is an organ developed from the distal portion of the cotyledon and its dry weight is more initially compared to the subsequent stages in which gradual reduction of biomass is observed because of profuse cell proliferation and dramatic increase in size as well as accumulation of soluble metabolites and resultant high moisture content as observed in the haustorium during seedling development.

Anatomy of the haustorium shows a distinct outermost epithelial layer consisting of isodiametric parenchymatous cells with conspicuous nucleus (Figs. 18, 19&20). Developing vascular bundles are also present. Dense staining with hematoxylin and bromophenol blue indicates the high meristematic activity and protein rich nature respectively (Figs. 18&19). In Date seeds, haustorial structure and function of vasculature in the translocation of metabolites have been described by DeMason (1984). According to the author, the tremendous increase in the size of the haustorium which keeps continual physical contact with the degrading endosperm and the vasculature near the epithelium are suggestive of the absorptive function. In *B. flabellifer* the haustorium is big and oblong shaped structure with plenty vascular bundles near the epithelial cells and the structural details are directly comparable to that of *Phoenix dactylifera* (DeMason, 1984). At the proximal end of the haustorium, a number of vascular strands are clustered which are in

contact with the growing cotyledonary sheath where the entire seedling is developing.

The haustorium development in terms of anatomical and ultra structural characteristic in *Washingtonia filifera* described by DeMason (1988 a, b) is almost similar to that of *B. flabellifer* despite its large size in the latter. Based on the observations in *Phoenix dactylifera* and *Washingtonia filifera*, DeMason (1984, 1988 a, b) suggested that the epithelial cells of haustorium are meant for absorption of the degradation products from the endosperm and also for the secretion of some enzymes to the endosperm. However, functional aspects of haustorium which is integrated with the endosperm in *Phoenix dactylifera* and *Washingtonia filifera* are not directly comparable with or not similar to *B. flabellifer* because the endosperm of the former species consist of living cells while that of the latter are non-living (DeMason, 1984, 1988b). Anatomy of *B. flabellifer* haustorium shows a well differentiated epithelial layer and cortical cells containing conspicuous nucleus and protein-rich and dense cytoplasm as observed in the sections stained with hematoxylin and bromophenol blue revealing their metabolic activity (Figs. 18, 19&20). As growth advances, cells become vacuolated and cell wall breakage and lysis occur (Fig. 18, 19&20). In the final stage, the haustorium becomes shrunken and cellular integrity is lost due to the mobilisation of metabolites to the developing seedling (Fig. 17).

According to Keusch (1968) mobilisation of mannan in Date seed following germination takes place by depolymerisation in the dissolution zone surrounding the haustorium. The haustorium secretes the enzyme β-mannosidase essential for the degradation and apparently absorbs the degraded products from the endosperm and these products eventually undergo metabolic changes and are transported to the developing seedling. The author opined that haustorium has two functions such as secretion of hydrolytic enzymes and absorption of hydrolysed products from the endosperm. In *Trigonella foenum-graecum* the endosperm cells are non-living and galactomannan rich, whereas the aleurone layer of the endosperm is composed of living cells which secretes the enzymes for degradation of galactomannan (Reid and Meier, 1972). According to Halmer and Bewley (1982) the dissolution zone formed as a result of cell wall hydrolysis also serves to aid the diffusion of enzymes and reserve degradation products from the endosperm to the embryo in addition to provide carbohydrate for mobilisation. In Lettuce, endosperm cell wall contains more than 60% mannan and is completely mobilized by β-mannosidase which is produced exclusively within the cotyledon (Leung *et al*., 1979; Halmer and Bewley, 1982).

According to Meier and Reid (1982) galactomannan mobilisation in *Ceratonia siliqua* takes place by the activity of α galactosidase and β-mannanase secreted by the endosperm cells which have living cytoplasm. In

palm seeds- *Phoenix dactylifera* and *Washingtonia filifera* the galactomannan hydrolysing enzymes- α-galactosidase and β-mannanase are reported to be secreted by the haustorium despite the occurrence of living endosperm (DeMason, 1984, 1988a, b; DeMason *et al*., 1985, 1992). However, in *B. flabellifer* seeds, neither the endosperm which consists of non-living cells nor the haustorium which is developed simultaneous to the hydrolysis of the endosperm can be considered as the sole source of enzymes for the degradation of galactomannan. In this context, as mentioned earlier, presence of the 'intermediate layer' consisting of cytoplasm and protein-rich inclusions is presumed to be involved in the secretion and/or diffusion of digestive enzymes because it raises an interesting question concerning the spatial and temporal role in the synthesis of digestive enzymes since the tissue occupies the region in between the viable embryo and nonviable endosperm and apparently it is a 'dissolution zone' similar to the seed structure of *Trigonella foenum-graecum* reported by Reid and Meier (1972). This layer is also similar to the dissolution zone formed in the endosperm during and following germination of *B. flabellifer* seeds.

Initially starch content of haustorium is very low but several fold increase is observed during the entire period of seedling growth (Table 15). Histochemical localisation of starch by Periodic acid Schiff's reaction confirms the occurrence of starch grains in the haustorial cells (Fig. 20). Starch grains are present in the 2nd stage of germination and the size and numbers are increased during later stages following germination (Figs. 20C,

D, E&F). Accumulation of starch in the haustorium of *B. flabellifer* seeds is evidently due to the synthesis and accumulation by utilizing the sugars translocated from the degrading endosperm cells. As mentioned earlier, dramatic increase of α-galactosidase and β-mannosidase activity on galactomannan of endosperm and resultant sugar translocation to the haustorium occurs in *B. flabellifer*. In addition to the translocation of sugars to the developing seedling, excess sugars are converted into insoluble starch transiently, in order to maintain the osmoticum. This process is found to be similar to the gluconeogenic origin of sugars and starch from lipids in the cotyledon of lipid rich seeds such as *Ricinus communis*, *Arachis hypogea* etc. (Mayer and Puljakoff- Mayber, 1989; Bewley and Black, 1985, 1994).

An interesting aspect of carbohydrate metabolism in the haustorial tissues of *B. flabellifer* is the synthesis and accumulation of starch and amylase activity during and following germination although both are not proportional to each other. Starch is continuously increasing in the haustorium until the seedling becomes autotroph, whereas feeble activity of amylase and accumulation of starch are proportional, revealing the lack of optimal conditions for the former and abundant occurrence of the latter presumably due to the cessation of metabolic events in the haustorial tissues during the final stages of seedling growth. So the metabolic scenario of haustorial tissue of *B. flabellifer* seedling is under the control of sink-source relationship in which the degrading galactomannan-rich endosperm is a powerful source which is well facilitated with essential specific enzymes whereas the

haustorium is a potential sink at least for a transient period. At the same time the haustorium functioning also as a source by supplying metabolites to the actively growing seedling revealing a balanced afflux of carbohydrates in the haustorium.

SUMMARY AND CONCLUSIONS

SUMMARY AND CONCLUSIONS

Development and germination of palm seeds provide a unique system for investigating the embryo-endosperm interaction. During early stages of development, palm seed is composed of liquid endosperm which become cellular and thick walled later. Palm seeds possess a small embryo compared to the abundant endosperm which function as organ for carbohydrate storage and are composed of mannans or galactomannans deposited on the cell walls as cell wall storage polysaccharides (CWSP). During germination, proximal portion of the cotyledon protrudes and elongates resulting in the formation of the cotyledonary sheath to push the root-shoot axis of the embryo. The distal portion of the cotyledon remains within the seed and develops in to the haustorium. During and following germination, the galactomannan-rich endosperm is hydrolysed into metabolisable carbohydrates and are transiently stored in the haustorium and eventually mobilised and utilized for seedling growth.

In the present study developmental and germination aspects of Palmyra palm (*Borassus flabellifer*) seed was investigated. The important objectives included are 1. Structure of embryo and endosperm analysed at different comparable stages of seed development, 2. Anatomical/ histochemical and biochemical analyses of metabolites in the developing embryo and

endosperm, 3. Morphology of germinating seed, 4. Histochemical localisation and biochemical analyses of seed reserves during and following germination.

Seed development was studied by collecting seeds at six different stages of development. Standard anatomical/ histochemical methods of tissue processing and staining techniques were followed for microscopic observations of the embryo and endosperm. Similarly, standard methods were followed to analyse protein profile, metabolites such as proteins, free amino acids, soluble and insoluble carbohydrates-sugars and starch. Cell wall storage polysaccharides – galactomannans were analysed by estimating its component sugars- galactose and mannose in the samples of all stages of seed development.

Seed germination aspects such as mode of germination, seedling morphology and distribution of metabolites during endosperm degradation and development of haustorium were conducted. Histochemical and biochemical aspects of germination and seedling developments were elucidated by analysing the distribution of metabolites in the endosperm and haustorium sampled at six comparable stages of germination/ seedling growth. Endosperm degradation was analysed as distribution of galactomannan content in the germinating seeds. Metabolites like proteins, sugars, starch and amino acids were also analysed in the tissues of endosperm and haustorium. Activity of galactomannan hydrolysing enzymes namely α-galactosidase, endo-β-mannanase, and β-mannosidase were also assayed at 6

different stages of germination/seedling growth. Accumulation and degradation of starch in the developing haustorium also was analysed by assaying amylase activity and estimation of starch and sugars during seedling growth

Developmental studies of *B. flabellifer* seeds revealed that endosperm was liquid syncytium in the early stages followed by cell wall formation which starts from the periphery and progress towards the centre. At maturity the endosperm cells became thick walled and non-viable due to the deposition of cell wall storage polysaccharides (CWSP). The embryo was very small constituting a massive cotyledon with a slightly pointed proximal end where the root-shoot axis was positioned. The mature seed showed a structurally well-formed cotyledon consisting of an epithelial layer of isodiametric cells and numerous provascular strands. In between the embryo and endosperm a microscopic layer of tissue consisting of undifferentiated cells (intermediate layer) was observed. This layer was positively stained for proteins and cytoplasm.

Distribution of dry weight, starch and protein content in the endosperm was increased gradually during seed development. Free amino acid and sugar contents showed significant variations depending on the developmental stages. Qualitative and quantitative differences in distribution of metabolisable carbohydrates was directly linked with the cell wall thickening due to the deposition of galactomannan which was estimated as the content of

component sugars – galactose and mannose. Similarly, distribution of sugars during the endosperm development was directly proportional to the galactomannan content in the respective developmental stages. During development, constituent sugars of galactomannan – galactose and mannose showed significant increase and the ratio of galactose: mannose was more in the mature endosperm and found to be related to the texture of the endosperm.

Mode of germination in *B. flabellifer* seed was remotive and duration for the completion of seedling development was about six months. Germination started with the elongation of proximal part of the single cotyledon as the growth of the cotyledonary sheath. The developing shoot – consisting of the plumular sheath and shoot apex – was carried along and came out of the seed. The growing tip of the cotyledonary sheath was positively gravitropic which anchored and grew deep into the soil and differentiated into the root system. The plumular sheath and developing plumular leaf (primary/ palmate) protruded through a slit on the lateral portion of the cotyledonary sheath and became green in colour and seedling attained autotrophy.

During and following germination, the endosperm hydrolysis was initiated in the central region which was in contact with the distal end of the cotyledon. So a dissolution zone was formed by hydrolysing the hard endosperm cells resultantly a slimy endosperm layer was developed. Distribution of metabolites in the hard endosperm remained unchanged during

germination, while slimy endosperm showed significant metabolic changes. Metabolite distribution of hydrolysing endosperm cells during and following germination was controlled by the hydrolytic enzymes such as α-galactosidase, endo-β-mannanase and β-mannosidase. Assay of these enzymes revealed that α-galactosidase was a constitutive enzyme and the other two were active during and following germination. Consequently the endosperm reserve-galactomannan content was reduced. The distal end of the cotyledon remained inside the seed and developed into the haustorium which grew fast replacing the degrading endosperm. Haustorial tissue was starch rich and the activity of amylase was very high during seedling growth.

The data were interpreted in the light of available literature and following conclusions were made.

1. *Borassus flabellifer* seed consists of comparatively small embryo embedded in the copious amount of endosperm.
2. Endosperm development is characterized by four phases such as syncytial, cellularisation, differentiation and death.
3. Seed storage material is galactomannan deposited as thickened cell wall of endosperm cells, which are non-living in mature seeds.
4. Distribution of proline, hydroxy proline and threonine in the developing endosperm is related to the cell wall synthesis during the conversion of liquid syncytium to cellular form.

5. In the mature seed, a specialised layer of undifferentiated endosperm cells (intermediate layer) is present in between the inner layer of the endosperm tissue and the outer surface of the embryo. This layer if presumed to play a metabolic role during germination by involving in the synthesis and/or diffusion of enzymes for endosperm hydrolysis.
6. Abundant occurrence of rhamnose in the mature embryo is related to its specific cell wall structure which is presumed to be essential for the protection of the embryo against pathogens since the embryo is embedded in the dead endosperm cells.
7. Cell wall storage polysaccharide is galactomannan and the enhanced ratio of galactose to mannose in the mature endosperm confers more hardness, less solubility in water and hence lack of imbibition during germination.
8. Mode of germination of *B. flabellifer* is remotive and about six months are required for the completion of germination and seedling development.
9. During and following germination hydrolysis of cell wall storage materials – galactomannans present as thickened cell walls (Cell wall storage polysaccharides) of endosperm – occurs to form galactose and mannose by the activity of α-galactosidase, β-mannosidase and endo-β-mannanase. But lack of accumulation of these sugars reveals subtle

metabolic changes of the soluble carbohydrates which are translocated to the developing haustorium.

10. Presence of α-galactosidase in the mature endosperm cells as a constitutive enzyme indicate the role of this enzyme in the synthesis of galactomannan by editing the structure of the cell wall storage polysaccharide.

11. The haustorium which is developed from the distal end of the cotyledon grows profusely by absorbing the hydrolytic products of endosperm and function as a transient organ to store the metabolisable carbohydrates mobilized from the endosperm which are simultaneously translocated to the growing seedling.

12. Localisation of starch by histochemical techniques revealed the synthesis/degradation of starch in the haustorium. Accumulation of starch and simultaneous amylase activity are indicative of the active-dual role of the haustorium as a transient ‘sink’ as well as ‘source’ during and following germination of *B. flabellifer* seed.

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