International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 01-04-2020; Accepted: 17-04-2020: Published: 01-05-2020
www.botanyjournals.com
Volume 5; Issue 3; 2020; Page No. 01-05

Morpho-anatomy, histochemistry and physico-chemical profile of storage scale leaf of *Borassus flabellifer* L

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Abstract
The storage scale leaf of *Borassus flabellifer* L. is analyzed to understand its structural and chemical properties. It is elongated, club-shaped, with a light brown fibrous sheath enclosing the light yellow coloured smooth scale leaf. The anatomical features include an outer fibrous layer, small inner epidermis, large parenchymatous ground tissue which has closed, collateral vascular bundles found to be scattered. Histochemical analysis is helpful in localizing the biochemical storage components and the major storage component was starch, minimal quantities of proteins and lipids are distributed in the scale leaf. Total ash (1.39%), acid insoluble ash (0.02%), water soluble extractive (13.53%), alcohol soluble extractive (0.82%) and moisture content (6.77%) are the physico-chemical properties evaluated. The results show low moisture content which indicates less microbial growth, low ash values indicate less inorganic content or foreign matter and high purity of sample. High extractive values indicate the good chemical constituents present in the storage scale leaf.

Keywords: anatomy, histochemistry, scale leaf, physico-chemical analysis

1. Introduction
Borassus flabellifer L. is commonly called as the Palmyra palm. It belongs to the family Arecaceae. *Borassus* in Greek means “leathery covering of fruit”; *flabellifer* means “fan-bearer” [1]. The Palmyra, probably native to India, is a grand feature in the landscape of the tropical regions. The other local names of the palm includes Toddy palm, wine palm, Cambodian Palm, Panai maram, Pana, Taalimara, Tal and Tad [2]. It is the national fruit of Cambodia and is also cultivated in the Indian subcontinent and South East Asian countries because the dry, tropical weather supports its growth [3]. The Palmyra palm grows slowly and the sex of the tree cannot be identified until it starts to produce flowers. It usually flowers only after 12-15 years of maturity [4]. Seed germination is of remotive tubular type. The cotyledonary sheath encompasses the plumule and the radicle at its tip growing downwards into the soil. The cotyledonary sheath develops into a solid cylindrical structure, often marked externally by pneumathodes. The seedling produces a single scale leaf or cataphyll which contains abundant storage starch. The cotyledon and scale leaf become firm and hard and the latter grows to a thickness of about 1 cm by the growth and enlargement of its cells. The fully grown scale leaf has enlarged to a size of about 30-40 cm in length and about 3-4 cm in breadth at its broadest part. This scale leaf is the edible part of the seedling, locally known as "Panam kizhangu” [5].
Histochemistry is a microscopic technique that involves various microscopic procedures and bright field dyes and fluorochromes for the characterization and visualization of the different biochemical components present in the cells and tissues [6, 7]. Many research works on Borassus flabellifer L. were carried out on root, seed and fruit. Phytochemical and GC-MS analysis were carried out and 28 compounds were identified on root of Borassus flabellifer L. which can be used for various medical condition [8]. In another phytochemical analysis on the extracts and fresh palm sugar of *Borassus flabellifer* L. showed the presence of reducing sugar, terpenoids, tannins, flavonoids, and coumarin in all extracts, but only reducing sugar was present in palm sugar [9]. The ungerminated seed embryos of palmryah was evaluated to understand the nutritional quality with respect to minerals and fiber components, total phenols, and antioxidant properties [10]. In our recent study, the storage scale leaf was evaluated for its nutritional properties and found this edible part serves as a good source of food both in boiled and raw condition containing carbohydrates, fiber, fat and protein [11].
Although nutritional, phytochemical and pharmacological studies are essential to determine food source and the medicinal potential of a plant part to be treated as drug source, morpho-anatomical and chemical investigations will continue to be the first step in determining options for substitution to ensure safety and efficacy [12]. The aim of the present investigation on the storage scale leaf is to provide comprehensive information for standardization as food or drug source using anatomical, histochemical and physico-chemical studies.

2. Materials and methods
2.1. Source of the material
Healthy and fresh storage scale leaves of *Borassus flabellifer* L. were procured from Tambaram Market, Chennai, India and used for the present study. The sample was identified and authenticated at the Centre for Floristic Research and Herbarium, Department of Botany, Madras Christian College. The sample was subjected to morpho-anatomical, histochemical and physico-chemical analysis.

2.2. Specimen Preparation for Macroscopic and Microscopic Analysis
Fresh storage scale leaf was macroscopically studied to examine for its organo-leptic characters according to standard procedures and reported in the results [13]. For

anatomical and histochemical studies, free-hand sections were taken and staining was performed with various histochemical stains, dyes and reagent to understand the structure and localize biochemical substances. A wide-range of bright- field dyes and reagents were used to identify the storage components such as starch, lipids, proteins and secondary metabolites present in the storage scale leaf of Palmyra [14].

2.2.1. Safranin O [15]
Safranin O stain was prepared by dissolving 1g of the dye in 100ml of distilled water. Free-hand sections were stained with 1% Safranin stain for 2-5minutes; excess stain in the sample was washed with distilled water.

2.2.2. Toluidine Blue O [16]
0.5% of Toluidine Blue stain was prepared by dissolving in 100 ml of distilled water. Sections were stained for 1-5 minutes. Excess stain was removed by washing in distilled water. Proteins stained blue. Lignified material stained purple to blue in colour. This well-known metachromatic stain also stained several other components of the ell and cell wall.

2.2.3. Iodine Potassium Iodide Reagent (I_2KI) [14]
I_2KI reagent was prepared by dissolving 2g of Potassium Iodide and 0.2g of Iodine in 100 ml of distilled water. Free-hand sections were stained with I_2KI reagent for 1 minute, mounted in the same solution or diluted glycerine. Matured starch grains appeared bluish black in colour.

2.2.4. Coomassie Brilliant Blue R 250 [17]
0.02% Coomassie Brilliant Blue was prepared in Clarke's solution at pH 2. Clarke's solution was prepared by adding 1 part of glacial acetic acid to 3 parts of absolute ethanol. Free-hand sections were placed in 50% ethanol, stained with Coomassie Brilliant Blue for 10 minutes, thoroughly washed with Clarke's solution and mounted in dilute glycerine and observed under light microscope. Mature protein bodies appeared blue in colour.

2.2.5. Sudan IV [18]
Sudan reagent was prepared by dissolving 0.7 g of Sudan IV in 95% ethanol. Free-hand sections were placed in 50% ethanol for a few minutes, and then the sections were stained with filtered Sudan IV for 5-20 minutes and mounted in dilute glycerine. Lipids stained red in colour.

2.2.6. Congo Red [19]
Dissolve 2g of Sodium Chloride in 32ml of deionized water. Dissolve 0.5g of Congo Red powder in 68 ml of 100% absolute alcohol. Then combine the two solutions. Free-hand sections were taken and stained using Congo Red for 1minute then washed with water and mounted using glycerine.

2.2.7. Calcofluor White M2R [20]
10 mg of Calcofluor White M2R was dissolved in10 ml of distilled water. Free-hand and semi-thin plastic sections were stained for 20-30 minutes. Specimens were thoroughly washed with water, mounted in dilute glycerine and observed under UV excitation microscope. Cell walls fluoresced intensely in blue. This fluorochrome also reveals the presence of nuclei and protein bodies.

2.2.8. Acridine Orange [21]
Acridine Orange was used for the detection of nucleic acids and proteins. Acridine Orange stock solution was prepared by dissolving 0.1g of Acridine Orange in 100 ml of distilled water. The working solution was prepared just prior to use. 1 ml of Acridine Orange stock solution was diluted with 9 ml of phosphate buffer at pH 6.0. Free-hand sections were placed in the buffer for 2 minutes, stained with Acridine orange working solution for 15 minutes, thoroughly washed 2-3 times in buffer solution and mounted in buffer or in very dilute glycerine and observed immediately with the fluorescent microscope. This fluorochrome is used for localization of nucleic acid and also as general fluorochrome for observing the structural details.

2.2.9. Auto-Fluorescence
Free-hand sections were mounted in distilled water and were observed under fluorescent microscope to observe natural fluorescence of the cells and its components.

2.3. Microscopy and Photography
Leica fluorescence microscope was used for observation and colour photographs were taken using the same microscope.

2.4. Physico-chemical Analysis
Physico-chemical properties such as moisture content, ash values viz., total ash, acid insoluble ash and extractive values viz., water soluble extractive, alcohol soluble extractive were determined using suitable procedures [22].

3. Results and Discussion
The present study provides a detailed insight on the structural aspects of the scale leaf of *Borassus flabellifer* L. Histochemical studies were performed in order to reveal the presence of storage components such as starch, protein, lipid, alkaloids, etc.

3.1. Morphology of Storage scale leaf
The storage scale leaf is elongated, club-shaped, with a light brown fibrous sheath enclosing the light yellow coloured in appearance. The length varies from 23.20 cm to 28.80 cm and the weight varies from 45.30g to 51.03g. The base is broad and it has a tapering end from which the new shoot originates. It is odourless, with a starchy taste. The organo-leptic characters of storage scale leaf is presented in Table 1.

Table 1: Morphological characters of *B. flabellifer* storage scale leaf

Organo-leptic Characters	Storage scale leaf
Shape	Club shaped, broad at base, tapering towards end
Weight	45.30-51.03g
Length	23.20-28.8cm
Colour	Light brown fibrous sheath and light-yellow scale leaf
Odour	No specific odour
Texture	Seed coat fibrous, storage scale leaf smooth
Taste	Starchy

3.2. Anatomical studies on storage scale leaf
The structural detail of the storage scale leaf is analyzed using Safranin O. The transverse section of mature scale leaf shows single-layered epidermis followed by the homogenous parenchymatous ground tissue, the

parenchymatous cells near to the epidermis is small and it becomes enlarged in the central portion (Fig. 1). Vascular bundles are found to be scattered in the ground tissue. Vascular bundle is closed collateral, and a fibrous bundle sheath is present surrounding the vascular bundle. Safranin O stains xylem and bundle cap red in colour indicating the presence of lignin (Fig. 2). The apical region of the storage scale leaf where new leaf shoots are originating is also sectioned and stained with Toluidine Blue. Transverse section shows the epidermis and the vascular bundle stained blue in colour and the green-coloured chlorenchymatous photosynthetic tissue (Fig. 3). In a previous study on the Palmyra root, the anatomical details of the root are well presented [23].

3.3. Histochemical studies on storage scale leaf

In this study, the storage scale leaf is subjected to histochemical analysis for the localization and identification of storage biochemical and phytochemical constituents. Starch is the major storage component of the storage scale leaf. Iodine Potassium Iodide (I_2KI) reagent readily reveals the presence of starch by showing bluish black colouration as shown in fig. 4. Starch is found abundantly in the storage parenchymatous cells which forms the fundamental ground tissue and it is absent in the epidermis and vascular bundle region. The size of the starch bodies is smaller in the peripheral region and they are larger in the central region of the ground tissue and also the starch grains are densely packed. I_2KI reagent is one the best method for the qualitative localization of starch [24].

Proteins are found to be distributed in the storage parenchyma cells surrounding the vascular bundle. Coomassie Brilliant Blue staining reveals the presence of protein by showing blue coluration (Fig. 5). Lipids are localized using Sudan IV stain. Lipids are found in the peripheral regions of the scale leaf mostly in the epidermis and sub-epidermal parenchymatous ground tissue as red colouration (Fig 6). The parenchymatous tissue surrounding the vascular bundles located near the peripheral region also show positive red colouration. No significant colour change is seen in the middle region of the parenchymatous ground tissue indicating the absence of lipids.

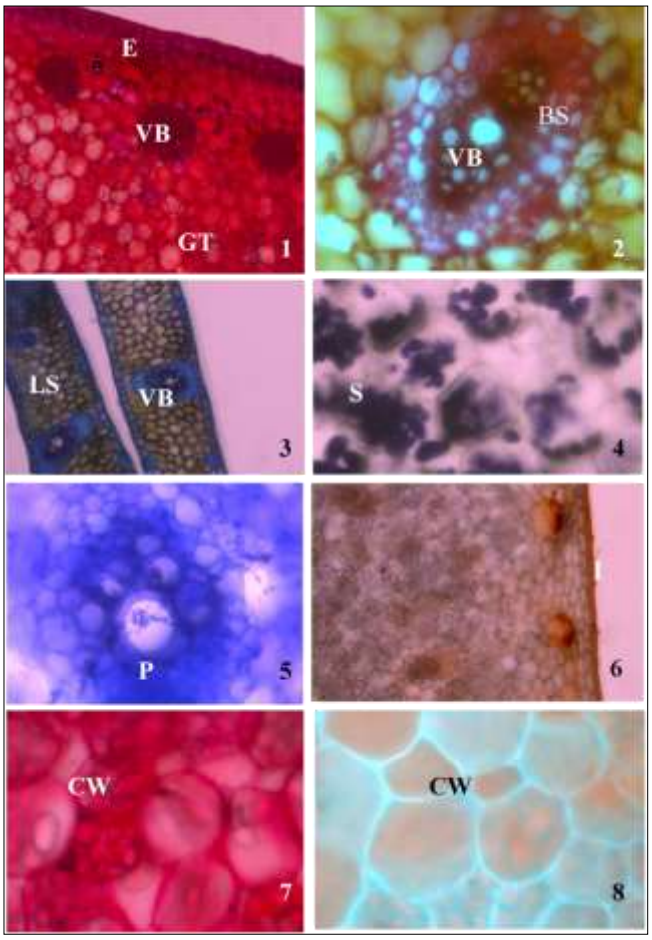
Congo Red and Calcoflour White fluorochromes were used for cell wall staining. The bright red fluorescence of the cell wall is revealed by the Congo Red staining as shown in Fig. 7. An enlarged view of cell wall stained by the fluorochrome Calcoflour is shown in Fig. 8 and Fig. 9 shows an enlarged view of a vascular bundle and fibrous bundle sheath stained by Calcoflour White exhibiting bluish fluorescence of the walls of the vascular tissues.

Both fluorochromes helps us to understand the cell wall pattern and the distribution of the tissues. Figure. 11 shows the enlarged view of the Acridine Orange staining of vascular bundle showing yellow fluorescence and the fibrous bundle sheath cells showing yellow fluorescence of the cells. In Fig. 12 an enlarged view of a vascular bundle is seen fluorescing in red colour especially the xylem tissue due to auto fluorescence. No fluorochrome is used for staining and the section is mounted with water to detect the natural fluorescence. Table 2 summarizes the Histochemical localization of the biochemical components in epidermis, ground tissue and vascular bundles.

Table 2: Histochemical localization of storage biochemical components in *B. flabellifer* storage scale leaf

Biochemical components	Epidermis	Ground tissue	Vascular Bundle
Starch	-	+	-
Protein	-	+	-
Lipid	+	-	-

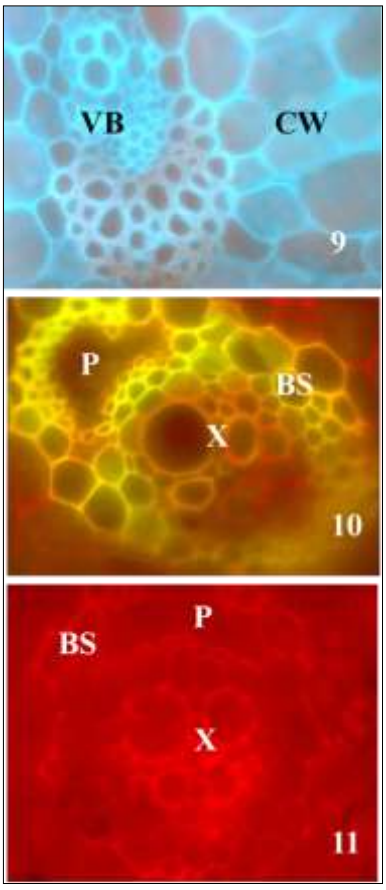
+ indicates the presence - indicates the absence



Keywords: Fig. 1 - stained with Safranin O 40x**,** Fig. 2 - Magnified view stained with Safranin O 400x, Fig. 3 - storage scale leaf tip showing young leaf shoots stained with Toluidine Blue O. 100x, Fig.4 - Magnified view of starch grains in the ground tissue stained with I_2KI. 400x, Fig. 5 - stained with Coomassie Brilliant Blue. 100x, Fig. 6 - stained with Sudan IV showing lipids in the peripheral region. 100x, Fig. 7 - Magnified view of cell wall stained with Congo Red. 400x, Fig. 8 - Magnified view of cell wall of parenchymatous ground tissue stained with the fluorochrome Calcoflour White M2R. 400x.

(E: Epidermis, VB: Vascular Bundle, GT: Ground Tissue, BS: Bundle Sheath, LS: Leaf Sheath, S: Starch, P: Protein, L: Lipid, CW: Cell Wall)

Fig. 1-8. Structural and histochemical localization of free-hand transverse sections of storage scale leaf.



Keywords: Fig. 9 - Magnified view of vascular bundle stained with Calcoflour White M2R showing xylem 400x, Fig. 10 - Magnified view of vascular bundle stained by Acridine Orange showing xylem and phloem 400x, Fig. 11 - Magnified view of auto fluorescence of xylem and fibrous bundle sheath cells excited with blue light 400x.

(VB: Vascular Bundle, CW: Cell Wall, BS: Bundle Sheath, X: Xylem, P: Phloem)

Fig. 9-11. Histochemical localization of free-hand transverse sections of storage scale leaf

3.4. Physico-chemical analysis on storage scale leaf

The physico-chemical evaluation is important in checking purity and adulteration of the sample [25]. Physico-chemical parameters such as moisture content, total ash, acid insoluble ash, water soluble & alcohol soluble extractives were determined and presented in table 3. The total ash content of the sample is 1.39% and acid insoluble ash is 0.02%. Ash values indicate less inorganic content or foreign matter and high purity of the sample. The water-soluble extractive value 13.53% and the alcohol soluble extractive value is 0.82%. High extractive values indicate the good chemical constituents present in the powder. The value of loss on drying (moisture content) at 105°C is 6.77% and the low moisture content indicates less microbial growth.

Table 3: Physico-chemical analysis on *Borassus flabellifer* L. storage scale leaf sample

Parameters	Results
Total ash	1.39%
Acid insoluble ash	0.02%
Water soluble extractive	13.53%
Alcohol soluble extractive	0.82%
Loss on drying 105˚C (moisture content)	6.77%

4. Conclusion

Morpho-anatomical study shows that, the scale leaf is a storage organ and it helps in germination as it has the plumule and radical at both the ends. Histochemical analysis results conclude that the major storage biochemical substance is starch. Proteins and lipid are also present. Physico-chemical parameters indicate the purity and chemical constituents of scale leaf. This study concludes that the storage scale leaf forms a good and inexpensive source of nutrition for humans.

5. Acknowledgments

The authors are grateful to the Principal and Secretary and the Head of the Department, Department of Botany, Madras Christian College (Autonomous) for their support and providing necessary facilities in carrying out this study.

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