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RESEARCH ARTICLE

Microscopical study and characterization of brachysclereid (stone cell) In *Manilkara zapota* (L.) P. Royen (Sapotaceae) fruit pulp

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Abstract

Microscopical study and characterization of brachysclereid in *Manilkara zapota* fruit pulp has been discussed here. Isodiametric shape of brachysclereid is present in sapodilla fruit pulp (Mesocarp). The amount of brachysclereid in fruit pulp is 3.2 gm. Chemical composition of brachysclereid (stone cell) is mainly composed of lignin. Mostly Guaiacyl lignin (G-lignin), and syringyl lignin (S-lignin) is present. This lignin has been identified by Fourier Transform Infrared Spectroscopy (FTIR) and UV-VIS spectroscopy method. The absorption peak at 288 nm. of UV-VIS spectrophotometer confirmed the G-lignin. The FTIR band of 1377cm^{-1} indicate the C-O stretching of syringyl ring and the band 1262 cm^{-1} indicate the C-O stretching of Guaiacyl ring. The Wiesner reagent and maule treatment also confirmed the presence of G-lignin. Under UV light the brachysclereid shows fluorescence signal.

Keywords: *Manilkara zapota*, G-lignin, S-lignin, FTIR, UV-VIS spectrophotometer, Brachysclereid, Fluorescence.

Introduction

Manilkara zapota (L.) P. Royen, is the most known fruit tree species of Sapotaceae. It is native to Mexico and Central America. Its cultivation is most expansive in coastal India (Maharashtra, Gujarat, Andhra Pradesh, Madras and Bengal States). The common English name of *Manilkara zapota* is sapodilla.

Manilkara zapota (L.) P. Royen fruit pulp which is grown in Purba Medinipur, Contai region. Sclereids are found in different plant organs in different form. Stone cells primarily known as brachysclereid. Stone cells are evolved by the deprivation of lignin on primary cell walls, subsequently secondary thickening of cell walls. It can occur singly or in groups in the flesh pulp of fruit.. Stone cells (brachysclereid) are abundant thick walled tissue cells in pear fruit and are principally compiled of lignin (Cai *et al*. 2010, Jin *et al*. 2013, Yan *et al*. 2014). Lignin is a hetero-polymer and comprise three types of unit, called as syringyl, guaiacyl, and *p*-hydroxyphenyl units. The content of lignin and ratio of these units in cell walls result from cell disintegration and contain the chemical or physical state of the cell wall.

Microscopic analysis gives necessary indication on the presence and ordination of cell wall ingredient. Numerous methods for microscopic examination have been displayed to find out lignin. Lignin typically absorbs ultraviolet (UV) lights. Thus, a UV microscope is a strong tool for its detection (Scott *et al.* 1969). The specific structure of lignin has been detected by other methods. O-4 linked coniferyl and sinapyl aldehydes in lignin (Pomar *et al*. 2002) can be detected by the Wiesner reaction (phloroglucinol-HCl reaction). The Maule colour test is one of the utmost efficient methods for detecting lignin. This test is rendered by sequential treatment with potassium permanganate, hydrochloride and aqueous ammonia.

Materials and Methods

Plant Sample

Living fruit of *Manilkara zapota* (L.) P. Royen is collected from Contai region, used as a sample.

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Fruit tissue preparation for Light Microscopy (LM) and Polarised Microscopy:

The collected fruit sample was fixed in FAA (formalin: glacial acetic acid: 90% ethanol= 5:5:90) solution for preservation. The sample was dehydrating at 30°C by 70, 85, 95 and 100 ethanol in percentage (30 min in each step) sequentially. Some pulp portion is taken from fruit and macerated with mortar and pestle. Macerated portion is treated with Wiesner reagent. 2-3 drops of 1% phloroglucinol-ethanol solution poured on the surface of sample, placed on a glass plate, and after 2 to 3 minutes, 1drop of 35% HCl poured again on the same sample. The sample covered by a glass cover slip for observation under LM (Light microscope) with model no. LEICA DM 3000 and brachysclereid images were taken. The remaining macerated pulp portion without Wiesner reagent is observed under polarised microscope.

Observation of lignin deposition in fruit pulp

With the help of bright field and UV light microscopy lignin depositions were observed. Lignin autofluorescence were observed by UV Light (Roussel and Clair 2015). 2-3 drops of 2% $C_6H_3(OH)_3$ solution were added to the thin cut prepared sample. Then the samples were incubated for 3 min. and then exuded in 3 mol/L HCl solution for 1 min.

Guaiacyl -lignin produced purple red (Engles and Jung 1988) by the use of Wiesner reagent. Finally, the samples were sealed with cover slip and performed under an optical microscope (LEICA-DM3000). Lignin was found significantly by the treatment of Maule stain. The appearance of G-lignin as yellow-brown in colour (Patten *et al.* 2005). The sample sections were exuded with 0.5% $KMnO_4$ solutions for 2 min, refined different times with distilled water, and then coloured in 3 mol /L HCl solution for 1 min. The MnO_2 fixed after the reaction was refined with distilled water, and ultimately colured with a 10% aqueous ammonia solution for 1 min, and then viewed under LEICA- DM3000).

Brachysclereid isolation from the sample

The method was carried out as described by (Nie *et al.* 2009) with some modifications. A warring blender is used to homogenize the ~ 5 g samples. 0.1 M NaCl is used to dilute the homogenize sample. The suspension was incubated for 30 min. at 22 °C. Again the sediment was incubated for 30 min. with 500 ml of 0.5 N NaOH and decanted. Ultimately the sediment was suspended in 500ml. of 0.5 N HCl for 30 min., decanted and washed with water. This washing cleansing process was repeatedly done for several times whilst the brachysclereid cells were free of extra cell debris. Brachysclereids were collected and then dried in oven. The dried brachysclereids were weighed for three times. 2.7% in wt. brachysclereid (*x*) presents in our sample and the used empirical Eq. (1) for calculation is

$$x(\%\ in\ wt.) = \frac{m}{M} \times 100 \quad (1)$$

Whereas M (in wt.) = used raw sample and m (in wt.) = collected dry brachysclereid.

Estimation of lignin content

Lignin content was standardization and modified as stated by (Zhang *et al.* 2017). 5mL of 95% ice-cold ethanol solution was used to homogenized frozen fruit (1g). The extract is centrifuged at 10,000*g for 10 min at 4^0C. The supernatant was taken and refined with ethanol/hexane=1:2 (v/v) for 3 times. The supernatant was dried at 60^0C for 24 h. The extracted with 1 mL of 25% C_2H_3BrO-CH_3COOH (1:4v/v) for 30 min at 70^0 in a water bath. Then, 1 mL of 2 mol/L NaOH, was added to stop the reaction. 0.1 mL of 7.5 mol/L NH_2OH-HCl and 2 mL CH_3COOH was added and centrifuge. Take 0.5mL of supernatant and dilute to 5mL with glacial acetic acid. The absorbance of the supernatant was measured at 280nm with a spectrophotometer.

Manilkara zapota fruit pulps were dried and grinding by mortar and pestle for powder form. FTIR data were taken for analyzing the presence of functional groups in the samples, using Fourier Transform Infrared Spectroscopy, model no. JASCO FTIR 420 at Jadavpur University, Kolkata, India. FTIR data were taken in the band of 4000-400 cm^{-1}.

Results

Light Microscopy, Polarised Microscopy and Fluorescence Microscopy analysis of Brachysclereid

Under bright-field microscope brachysclereid is seen in aggregates and also in single form. Its occur in clumps of 10-30 cells, are ovoid, spheroid, globose and clavate (Figure 1- E, F, G & H) in shape (R. Crang *et al.* 2018) and possess very thick lignified cell walls with numerous branched pits, narrow and broad lumen. This branch pit is called ramiform pit. With the treatment of Wiesner reaction the brachysclereid shows purple red colour. Under polarised microscope the brachysclereid shows birefringence (Figure 1- B & D). Birefringence is one of the significance features. It is applied in distinguished one component from another under polarized dark-field microscopy. Xylem, sclereids, calcium oxalate crystals, vessels, etc. are common birefringent plant components (Wang *et al.* 2016). With the maule treatment the G lignin shows yellow in colour (Figure 2- A-D). The autofluorescence (Franceschi *et al.* 1998, Lopez *et al.* 2004) consent an assessment of the substance localization of lignin in lignified tissues. It is suspected that autofluorescence is primarily due to lignin based on its normal emergence. From the fluorescence properties, blue signals (Figure 2-I) were present under UV light in brachysclereid and green signal (Figure 2- J) is present when blue light is excited.

Brachysclereid content in different Stage

Under light microscope the brachysclereid shows clump in nature. When the *Manilkara zapota* fruit pulp (mesocarp) is

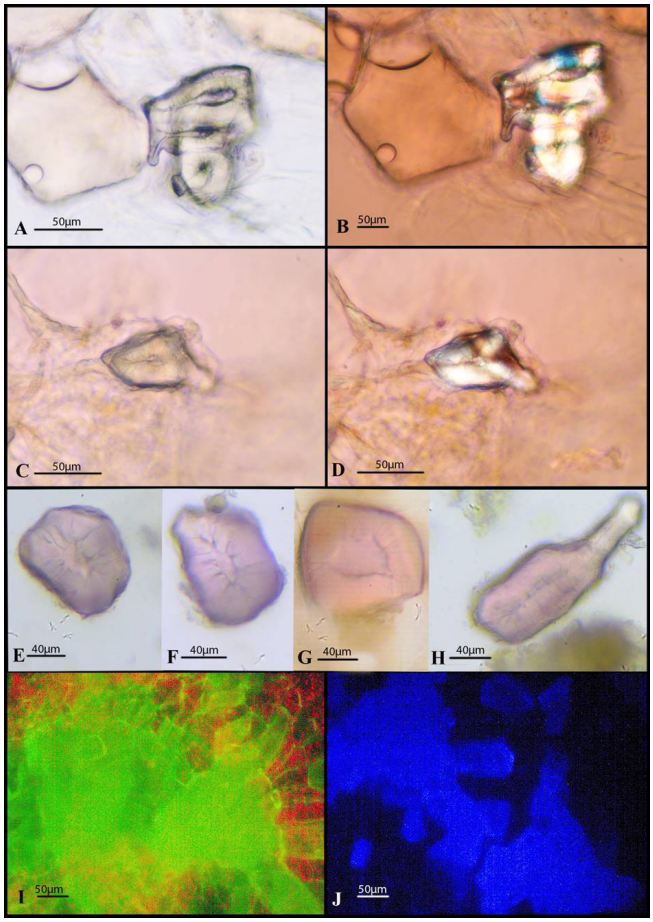
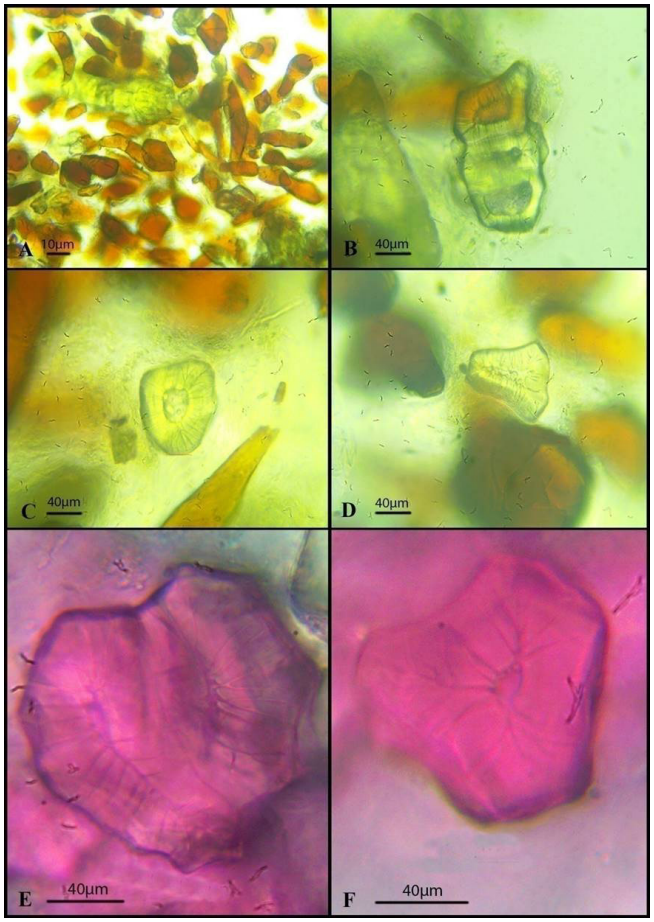


Figure 1: Detailed analysis of Brachysclereid of *Manilkara zapota* fruit pulp under LM (A, C, E, F, G, H), PM (B, D), FM (I-Blue Light, J-UV light)



Figue 2: Brachysclereid of *Manilkara zapota* fruit pulp with maule treatment shows yellow colour G-lignin containing sclereid, and brown colour parenchyma (A, B, C & D). Phloroglucinol- HCl stain shows purple red colour (E & F)

young in stage then the brachysclereid shows more density (Figure 3- C & D). In that time the fruit is very much hardy in nature and easily transported. But when the fruit is in fully ripened stage shows less density and decreases in number (Figure 3- G & H) (Li *et al*. 2017). At that time tissue is very much soft and cannot transport easily.

Analysis of lignin functional groups of *M. zapota* fruit pulp

UV Spectroscopy

Lignin determination is done by used of UV spectra analysis. The feature of UV absorption spectra of lignin have been

Table 1: FTIR absorption peaks and assignment of lignin extracted from the *Manilkara zapota* fruit pulp

Experimental Peak position (cm^{-1})	*Recommended Peak position (cm^{-1})*	*Assignments*	*Functional groups and structures in lignin/CaC_2O_4*	*References*
3340	3100-3400	O-H stretching	Associated –OH	You and Xu, 2016, Ahvazi *et al.* 2016, Ana *et al*. 2013
2937	2820-2960	C-H stretching	$-CH_2$, $-CH_3$	You and Xu, 2016, Ahvazi *et al.* 2016, Ana et al; 2013
1581,1514	1500-1600	Aromatic skeletal vibration	Benzene ring	You and Xu, 2016, Ahvazi *et al*; 2016, Ana *et al*; 2013
1458	1450-1470	C-H deformation	$-CH_2$, $-CH_3$	You and Xu, 2016, Ahvazi *et al*. 2016, Ana *et al*. 2013
1377	1375	C-O stretching	Syringil ring	Reyes-Rivera and Terrazas (2017)
1262	1270-1275	C-O stretching	Guaiacyl ring	You and Xu, 2016, Ahvazi *et al*. 2016, Ana *et al*. 2013
1135	1140	C-H stretching	Guaiacyl	You and Xu, 2016, Ahvazi *et al.* 2016, Ana *et al*. 2013
1037	1025-1035	C-O and C-H stretching	Aromatic ring and primary alcohol	You and Xu, 2016, Ahvazi *et al.* 2016, Ana *et al*. 2013
774	750=860	C-H stretching	Aromatic ring	You and Xu, 2016, Ahvazi *et al.* 2016, Ana *et al.* 2013

used for the qualitative and quantitative appraisement. To investigate the structural components of lignin, UV spectroscopy was used. In *M. zapota* UV spectrophotometer was measured in the 200-800 nm. range. The measurement

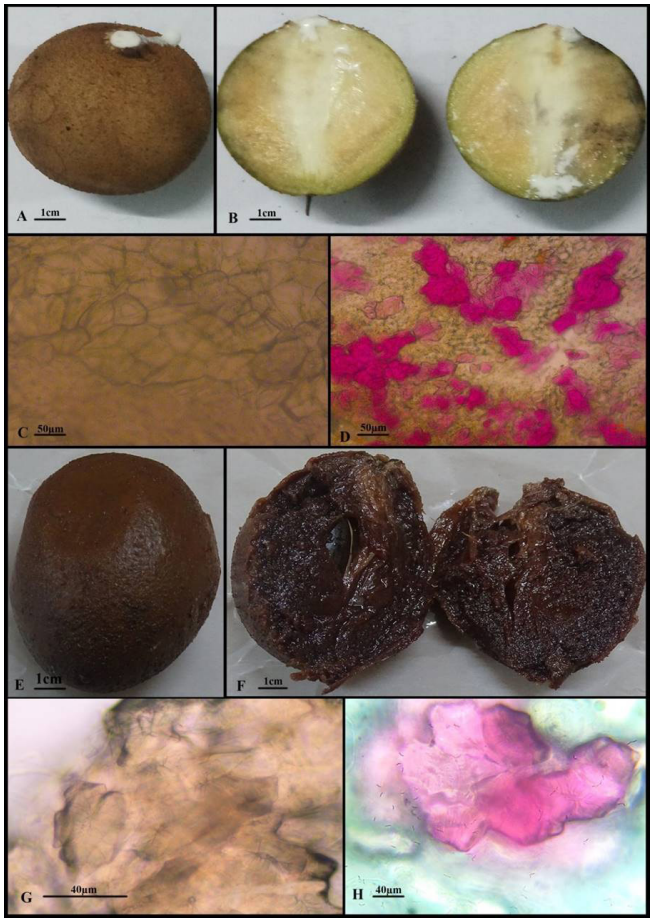


Figure 3: Young stage of *Manilkara zapota* fruit (A). Cross section shows hardy tissue portion (B). In mature stage the number of brachysclereid cluster is more in number and unstained (C) and stained with phloroglucinol-HCl (D). Full ripening of *Manilkara zapota* fruit (E). Cross section of fruit pulp shows soft pulp tissue portion (F). In ripening condition the number of brachysclereid cluster is less in number (G and H)

range of UV spectrophotometer of *M. zapota* is 200-800 nm. It showed an absorption peak maximum at 288 nm. (Figure 4). The characteristic absorption maximum peak of benzene ring is 288 nm, and it's may be assigned to the guaiacyl groups (Cai *et al.* 2010). And also shows typical benzene ring absorption peaked at 255 nm. (Wang *et al.* 2020).

Fourier Transform Infrared Spectroscopy (FTIR)

The utmost broadly used technique in the analysis of functional groups is FTIR spectroscopy. The richer brachysclereid cells of fruit pulp were selected for FTIR measurement. The applications of FTIR spectroscopy was very broad extent. According to the literature the peak assignments were conveyed. The main functional groups found in the fruit pulp were summarized in Table 1. In (Figure 5) and Table-1, the attributed to the contribution of lignin due to aromatic skeletal vibration (C=C) is around 1581cm^{-1} and 1514cm^{-1} (Liu *et al.* 2014). The band appearance at 1377cm^{-1} and 1135cm^{-1} can be assigneded to syringyl ring subsistent with C-O stretching and guaiacyl subsistent with C-H assigned, respectively (Reyes- Rivera and Terrazas 2017; Lu *et al.* 2017)

Discussion

The mature *M. zapota* fruit pulp mainly consisted of brachysclereid, parenchyma tissue (Wang *et al.* 2019). In this study we found the different microscopy structure of brachysclereid and its characterization. In young and mature stage the brachysclereid cell tissue increase in number and when it's fully ripening in stage the number of brachysclereid cell is decreased but parenchyma cells still present in a large number. From anatomical point of view it is revealed that stone cells were manifested from parenchyma cells (Whitchill *et al.* 2015). Likened to other cells, brachysclereid cells had much thicker cell wall, small, broad cell lumen and numerous ramiform pit. They had been easily distinguished by chemical reagents. With the treatment of Wiesner reagent brachysclereid shows violet red in colour. Brachysclereid of *M. zapota* fruit pulp were

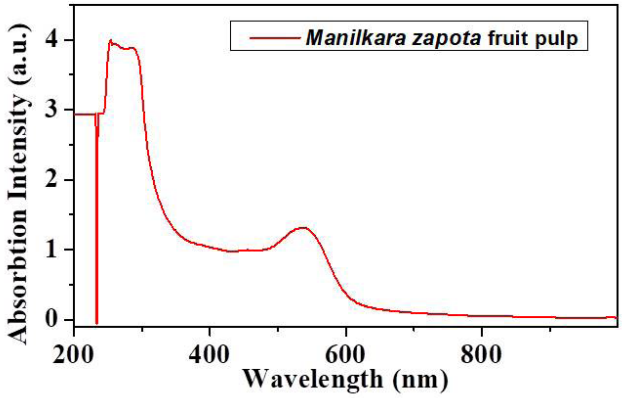


Figure 4: UV spectroscopy of lignin of ***M. zapota*** fruit pulp

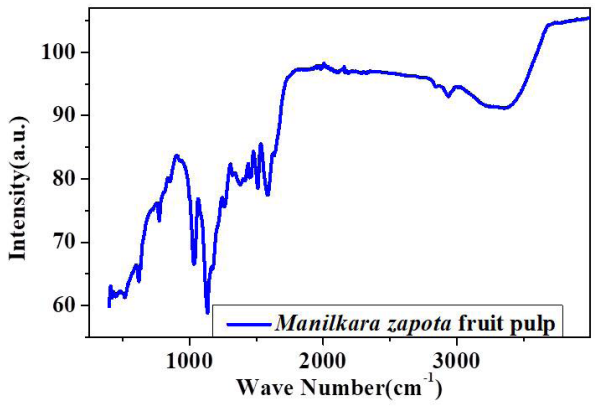


Figure 5: FTIR analysis of lignin of M. zapota fruit pulp

very lignified and comprise Guaiacyl-lignin and Syringyl-lignin (Menden *et al*. 2007). The Wiesner reagent, Maule test, UV spectrophotometric have revealed that G- lignin is present in *M. zapota* fruit pulp. FTIR spectroscopy technique revealed the functional group of G and S lignin. Lignin was a captious composition for the brachysclereid abrasion (Cheng *et al*. 2017), which also can be determined from robust fluorescent signal.

Conclusion

Experiments using phloroglucinal-HCL (Wiesner reagent), maule treatment and optical microscopy for the resolving of the brachysclereid and lignin extent in *Manilkara zapota* fruit pulp. The functional group of lignin is determined by FTIR technique. In Young stage the fruit pulp contain more density of sclereid cluster cell and it provides hardiness and rigidity to the fruit. But in ripening stage the fruit pulp contain less density of sclereid cluster cell and thus providing soft tissue to the fruit.

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Authors' Contribution Statement

Mamtaj Khatun and Amal Kumar Mondal planned the research. Mamtaj Khatun conducted the experiment, characterized and analysed the data. Amal Kumar Mondal edited the manuscript. All authors viewed and accomplished on the manuscript.

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