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Mechanical Properties of Plant Cells, a Review, with Focus on Elastic Modulus of the Onion Epidermal Cell Wall based on Micro-Indentation with Atomic Force Microscopy and on Uni-axial Tensile Testing

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Abstract
This paper addresses the recently reported research findings showing that values of the commonly quantified compliant descriptor, elastic modulus (EM) of plant cells appear to differ drastically depending on the measurement method employed. When nano indentation methods are used the reported values tend to be in the 10 to 100 MPa range. When tensile testing methods are used the values tend to be in the 100 to 3000 MPa range. This paper reviews data from both methods on similar plant cells and attempts to determine the origins of these variations. Sample preparation, measurement method and cell structure could affect the interpretation of the elastic modulus (EM). Key details are reviewed of probe indentation and tensile stretching measurements and the differences are discussed. The conclusion is that a major reason for the differences in the results of the two methods lies in the degree of hydration of the plant cells during the measurements. The high elastic modulus values are those of very dry microfibrils stripped of any polysaccharide matrix material whereas the low elastic modulus values are obtained from microfibrils embedded in a fully hydrated polysaccharide-rich complex structure providing reinforcement material of the cell wall composite. A second reason points toward the extent of deformation in the two methods: the nano indentation takes place at a nano-sized area whereas the tensile test measures a deformation over an extensive length scale. Finally, the results suggest that the pectin network embedding the plant cell microfibrils plays a role in the elasticity of the cell wall. Two chemical tests are reported which shed light on the role of pectin in the elastic modulus data. Whereas the report focuses mainly on cell walls in skins of onions, they may be considered as surrogates for plant cells in general.

Key Word and Phrases
Plant Cell Walls, Nano Indentation, Tensile Testing, Microfibrils, Pectin, Elastic Modulus, Hydration, Polysaccharide Matrix, Atomic Force Microscopy, Force-Distance Curve, Onion Epidermis.

1. Background for Plant Cell Walls

Plant cell walls serve a variety of important biological functions for plants, such as providing mechanical support and determining their size and shape ranging from tall trees to garden vegetables. They have also been an interesting subject for engineering applications. Plant cell walls are essential in various commercial products such as paper, textile, plastic, etc. Also, the sophisticated composite structure, naturally occurring in cell walls, inspires innovative designs of engineering materials, such as carbon fiber plastic structures. All these applications require understanding of the composite structure of cell walls and their mechanical properties.

Primary plant cell walls are formed during the growing and dividing of plant cells. They provide mechanical support for the cells and can expand to allow cells to grow. Primary plant cell walls are polysaccharide-rich complex structures. The wall contains three main components: cellulose, hemicelluloses and pectin [1]. Cellulose microfibrils are embedded in a highly hydrated polysaccharide matrix which consists of hemicelluloses and pectin. Cellulose is comprised of many

parallel chains of 1,4-glucan which make cellulose crystalline, strong and indigestible. Cellulose microfibrils serve as reinforcement material of cell wall composite. Cellulose microfibrils are about 5 nm in thickness and have varied length. Hemicellulose is also a polysaccharide, but it is typically made up of chains of xylose interspersed with side chains containing arabinose, galactose, mannose, glucose, acetyl, and other sugar groups, depending on the plant type. [1]. Hemicelluloses separate microfibrils from each other but may tether them together into a cohesive network. Pectin is a gel phase that embeds the cellulose-hemicelluloses network. Pectin includes relatively simple polysaccharides and becomes soluble with mild treatments such as boiling water or mildly acidic solutions. Fig. 1 shows an example of a typical image of a cell wall obtained by Atomic Force Microscopy [1].

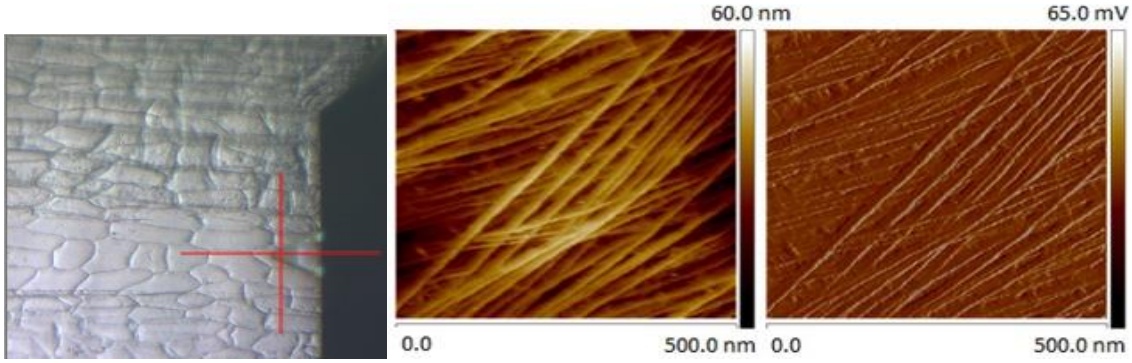


Fig.1 Left, optical image of a cell wall (celery epidermis); the red cross marks the location of the AFM imaging; middle and right images of a cell wall obtained by Atomic Force Microscopy in 500 nm scan of the celery fibrils; left image is Topography and right image is the AFM Peak Force Error Signal [1].

2. Indentation Methods of measuring Elastic Modulus

2.1 Introduction

Plant cell walls are complex composites which require delicate instruments and special techniques to study their structures and properties. The analysis of micro- or nano- mechanical properties of plant cell walls has become increasingly important in understanding cell wall structure and cell growth. Because of its micro-scale tip size, indentation technique can investigate the mechanical properties of thin, small and heterogeneous materials. Nanoindentation has been a new application tool on cellulose fibers and plant cell walls. hardness and Young's modulus of spruce cell-wall, bamboo cell walls, individual wood fibers and crop stalks cell walls have been examined [2]-[5]. These experiments were done with add-on force transducers or special indenter testing systems. However, in traditional nanoindentation experiments, samples were dried out and many were embedded in epoxy resin. Such sample preparations may cause undesired modifications and influence the mechanical properties of the cell walls.

Recent work has used an improved nanoindentation technique to measure the mechanical properties of single cells or tissue. The indentation depth is comparable to or larger than the cell wall thickness. The indent force is in a range of 1–100 μN [6] [7]. The nano-indentation with an AFM has been recently applied to living plant cells in conditions close to natural. Mechanical properties of suspended grapevine cells cultured in liquid medium [8], the primary cell wall of shoot apical meristems [9], rosette leaves [10], tomato cells [11] and epidermal cells of living roots of Arabidopsis thaliana [12] were measured using AFM based nanoindentation. The indentation depth is much lower (about 100 nm) in most of these studies. However, the experimental samples contain living cells with different turgor pressure, which has large influence on measured results. No conclusive results were reached on the cell mechanical properties with the effect of turgor pressure. Routier-Kierzkowska et al. measured the local stiffness of onion cells with different levels of turgor pressures. Their results showed that the local stiffness increases with larger turgor pressures [13]. Forouzesh et al. measured the moduli of plasmolyzed, normal and turgid

Arabidopsis cells. They found that the moduli are very similar for plasmolyzed and normal samples whereas they decrease in turgid sample which has the higher turgor pressure [14].

The aim of recent work, by Xi et al. [15] was to measure the elastic properties of cells walls isolated from onion scales using AFM based nano indentation in order to assess the feasibility of this technique for further study of plant cell walls. In this work, the freshly peeled single-layer onion abaxial epidermis wall was tested in liquid mode. The samples were independent of the turgor pressure influence and reflected only the properties of hydrated cell walls in their natural condition.

2.2 Force-distance curve

With its pico-newton force sensitivity and nanometer displacement accuracy, the AFM has been recognized as a promising tool to for studying materials properties. AFM for nanoindentation has emerged as a useful tool measuring the elastic moduli of biological samples. An AFM force-distance (F-D) curve is a plot of tip-to-sample forces versus the extension of the piezoelectric scanner measured with a position-sensitive photo detector. The F-D curve provides information of important mechanical properties such as adhesion, contaminants, viscosity, and local variations in the elastic properties of the surface.

In experiments, F-D curves are quite complex and vary especially with different samples. For the experiments by Xi et al., the onion epidermis sample and the tip were both negatively charged in water, so there was no adhesion in F-D curves. Figure 2 shows a representative F-D curve obtained by Xi et al on an onion epidermis cell wall in liquid [15].

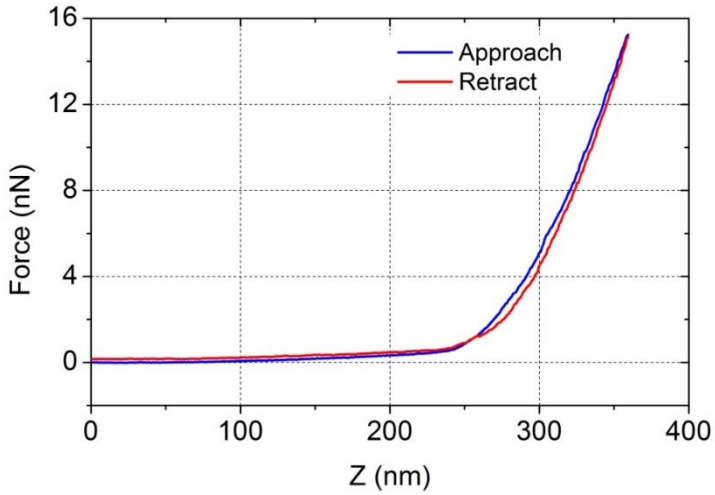


Fig. 2 A representative F-D curve (approach and retract) obtained in the experiment on onion epidermis cell wall in liquid. The y axis is the recorded force in nano-newtons and the x axis is the relative piezo displacement in nm by Xi et al [15].

2.3 The Hertz model

Xi et all used the Hertz model as the theoretical model for f-d curve analysis. The Hertz model assumes that the sample is isotropic, elastic and occupies infinite half space. It also assumes that the indenter is not deformable and there is no additional interaction between the tip and sample [16]. The elastic modulus E is obtained by a fit to the experimental F-D curve using the Hertz model. The F-D curve obtained by AFM nanoindentation is in fact a force- piezo displacement curve. To understand the force–sample deformation relationship, several specific parameters and equations were introduced.

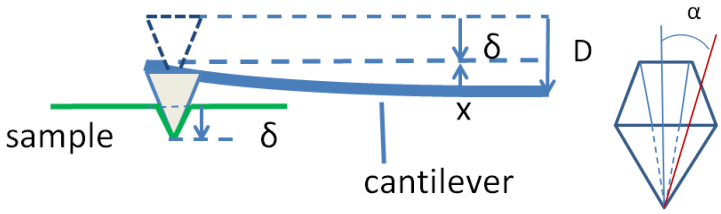


Fig. 3 Sketch of indentation distance and tip geometry by Xi et all [15].

As shown in Figure 3, when using the AFM tip to indent the sample, the recorded piezo displacement D is comprised of two parts: the tip cantilever bending x and the sample indentation δ. As shown in Figure 3, the cantilever is moved towards the sample by a carefully measured distance D.

$$D=x+\delta \tag{2.1}$$

According to Hertz model, the measured force based on a four-sided pyramid can be represented as [17]:

$$F = \frac{E}{1-\nu^2}\frac{\tan\alpha}{\sqrt{2}}\delta^2 \tag{2. 2}$$

where F is recorded force, E is sample's elastic modulus, ν is sample's Poisson's ratio, α is geometry angle of the indenter. In this analysis, ν takes the value 0.3 and α is estimated as 13^0 according to the parameters given by the manufacturer [15].

Knowing that x= F/k, where k is the cantilever spring constant, from the above two equations, one can obtain the relationship between recorded force F and recorded piezo displacement D:

$$F = \frac{E}{1-\nu^2}\frac{\tan\alpha}{\sqrt{2}}(D-\frac{F}{k})^2 \tag{2.3}$$

By fitting the experimental F-D retrace curves, one can get the sample's elastic modulus E. Figure 4. shows a sample of F-D curve fitting result by Xi et al [15] and it can be seen that the experimental data from the AFM retrace curve and the fitted data from the Hertz model are in good agreement.

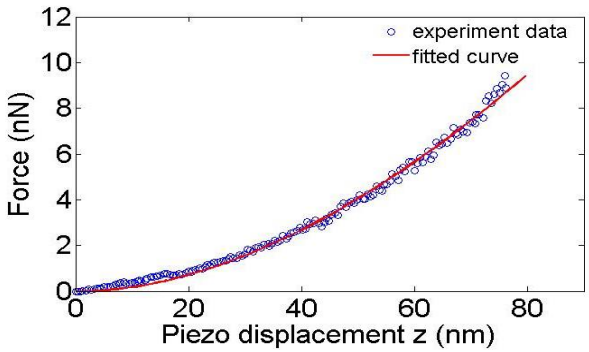


Fig. 4 A sample of F-D curve fitting. Experimental data in blue dots and fitted data in the red curve from the Hertz model showed good agreement; Xi et al [15].

2.4 Sample Preparations

The never-dried cell wall thickness of the abaxial epidermal cells was determined with optical microscopy by Xi et al [15]. The onion scale was hand-sectioned tangentially under a dissecting microscope (Olympus). Sections from each of the 4 scales were stained with 0.1 % Toludiene blue for 1 min at room temperature on a slide followed by rinsing with deionized water and imaged with an optical microscope (EVOS, AMG). The cell wall thickness was measured from each scale of three different bulbs. The thicknesses of different scales range from 2.4 μm to 11.5 μm. Substrate influence on the measured results is negligible when the sample deformation is less than 10% of sample thickness. In these experiments, the sample deformation was less than 150 nm so the substrate influence does not affect the accuracy.

Onion epidermis samples were prepared for AFM imaging by Xi et al [15] as described by Zhang et al [18]. Freshly peeled cell walls were fixed onto glass slides with edges glued. The epidermal strips were then immersed in a phosphate buffered saline solution (pH 7.4) containing 0.1% Tween-20 for 1 h. After that, the samples were rinsed with distilled water. Epidermal wall strips were probed by AFM (Veeco Dimension ICON from Bruker) in Peak Force QNM fluid mode. Nanoscope (v 8.10b44) was used to control AFM operation and Nanoscope Analysis (v 1.40) was employed for image processing. A cantilever holder from Bruker for liquid imaging was used to keep samples fully hydrated in water during the experiments. The tips used for images and indentation were ScanAsyst-Fluid+ probes from Bruker with spring constant given as 0.7 N/m. The spring constant of each tip was calibrated using a thermal tuning method before experiments [19]. All images were generated with tips scanning in the direction along the longitudinal direction of the cells. F-D curves were generated in ramp mode with controlled maximum force (15 nN) and constant tip velocity (800 nm/s).

2.5 Elastic moduli

The elastic moduli (EM) of the four scales (11^{th}, 8^{th}, 5^{th} and 2^{nd} scales) in different onions were measured by Xi et al [15] using AFM nanoindentation. 100 curves in 5 random cells in each scale were generated and analyzed. The results are shown in Figure 6. The error bar shows sample standard deviation. Changes in moduli are statistically significant. Previous research of onion epidermis scales indicate the orientation of cellulose microfibrils variation tendency along scales [1]. However, the elastic moduli do not always increase from young to old onion scales so there is no tendency along the scales. Fernandes et al. investigated the mechanical properties of living epidermal cells along the length of the Arabidopsis thaliana root. They found no clear differences in the mechanical properties along the length of the root [12]. The results of Xi et al [15] indicate similar conclusions with the elastic properties along the onion scales.

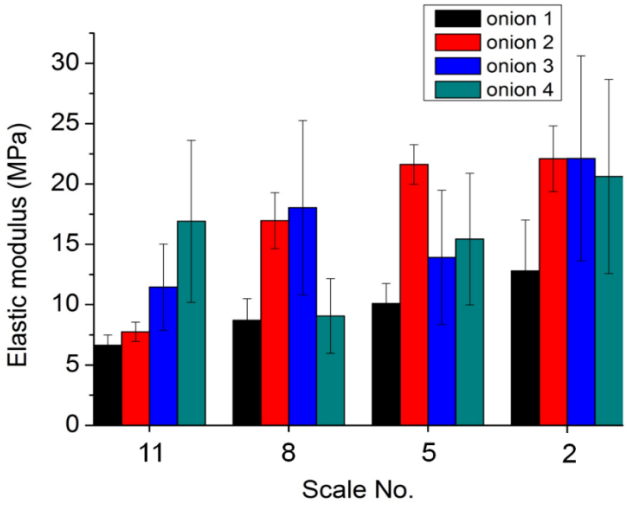


Fig. 6 Elastic moduli of different onion scales, scale 11, scale 8, scale 5 and scale 2 in four different onions. The error bar shows sample standard deviation. Changes in moduli are statistically significant by Xi et al [15].

2.6 Summary

Xi et al. [15] reported measurements on single layered, *fully hydrated* onion epidermis cell walls as test analogs. In the tests the AFM based nano-indentation method was used to observe changes in the elastic properties of onion epidermis cell walls which are made up of cellulose microfibrils , which are embedded in a highly hydrated polysaccharide matrix consisting of hemicelluloses and pectin. Cellulose is comprised of many parallel chains of 1,4-glucan which make cellulose crystalline, strong and indigestible. Cellulose microfibrils serve as reinforcement material of cell wall composite. Cellulose microfibrils are about 5 nm in thickness and have varied length. Hemicellulose is also a polysaccharide, but it is typically made up of chains of xylose interspersed with side chains containing arabinose, galactose, mannose, glucose, acetyl, and other sugar groups, depending on the plant type. Hemicelluloses separate microfibrils from each other but may tether them together into a cohesive network. Pectin is a gel phase that embeds the cellulose-hemicelluloses network. Pectin, especially homoglacturonan (HG) is negatively charged under cell wall physiological pH (around 5.0), Ca^{2+} serve as a bridge that cross-links the HG chains and increase the regional density of the cell wall surface, which adds rigidity to the cell wall [21]-[23]. Xi et al [15] took special care to use in their indentation measurements freshly peeled single-layer onion abaxial epidermis wall and tested in the liquid mode of atomic force microscopy, recently made available from Bruker, Inc.[15]. The samples were independent of the turgor pressure influence and reflected only the properties of hydrated cell walls in their natural condition. For the work reported here on onion cells the EM values ranged from 15 to about 40 MPa. Based on these findings the conclusion can be drawn that the AFM based nanoindentation method is a viable technique and thus opens the door to a systematic nondestructive examination and evaluation of complex cell wall structures in the future.

3. Tensile testing methods of measuring Elastic Modulus

3.1 Introduction

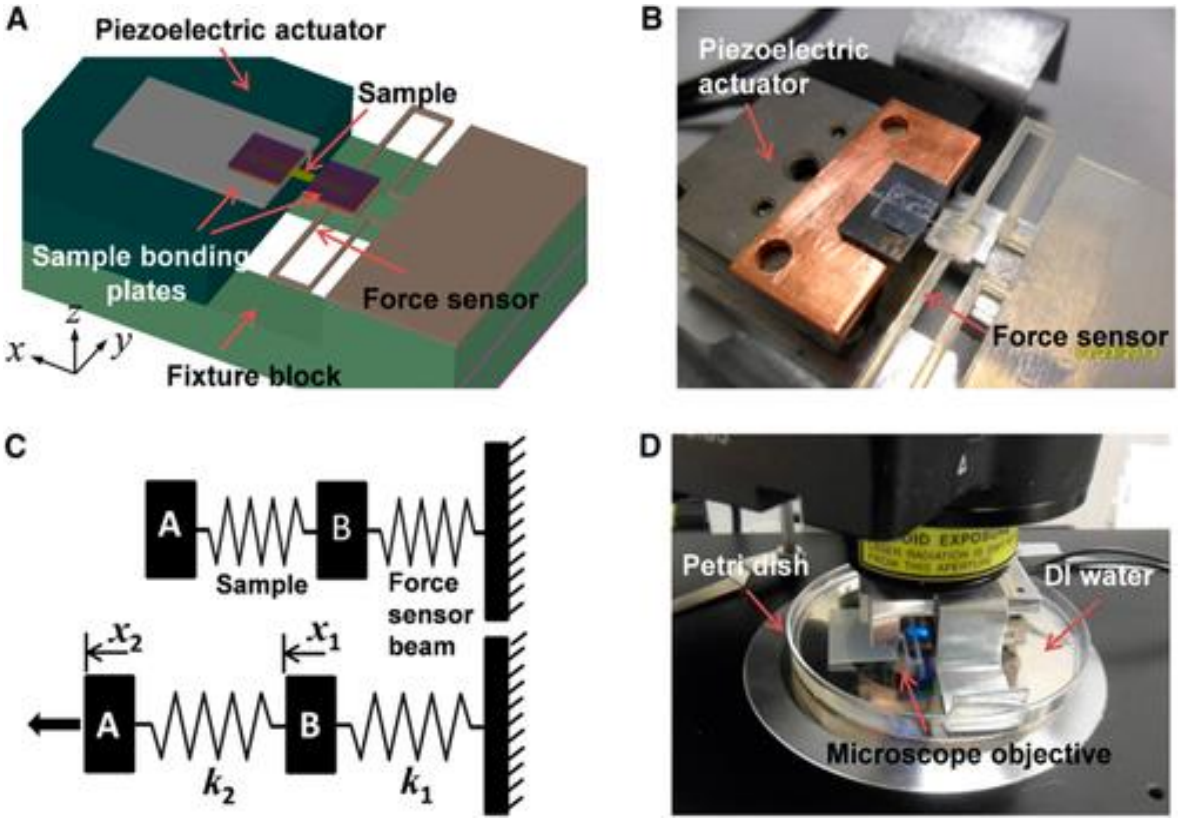
Zamil et al [24] reported a novel method used to characterize wall fragments under tensile loading. Cell wall fragments from onion outer epidermal peels were cut to the desired size (15 × 5 μm) using the focused ion beam milling technique, and these fragments were manipulated onto a microelectromechanical system (MEMS) tensile testing device. The stress-strain behavior of the wall fragments both in the major and minor growth directions were characterized in vacuo.

3.2 Methodology

The experimental system consisted of a force sensor and a piezoelectric linear actuator stage (AG-LS25, Newport Co., Irvine, California, USA) with a displacement resolution of 430 nm per step. The force sensor made of an acrylic-based polymer composite was fabricated by 3D prototyping. The sensor comprised a mechanical structure with serpentine beams and a sample gripping pad whose spring constant (force per unit displacement) was known. The piezoelectric actuator and force sensor were mounted on a fixture block to enable the expansion-type displacement of the piezoelectric actuator to be transferred to a sample-holding plate of the force sensor as shown in Fig. 7A and B. Figure 7C shows a schematic of the working principle of the experimental system. The force balance equation at the sample-holding plate of the force sensor is [24]

$$F = k_1 x_1 = k_2 (x_2 - x_1), \tag{3.1}$$

where F is the loading force, k_1 is the spring constant of the force sensor, which was known, x_1 is the displacement of the sample-holding plate, k_2 is the spring constant of the sample, and x_2 is the displacement of the linear actuator. Displacements x_1 and x_2 were obtained from the experiment and used to calculate the applied forces and the mechanical properties of the samples.



Fig, 7 Experimental system for tensile testing. (A) Three-dimensional schematic of the piezoelectric actuator and force sensor, which were placed on a fixture block. (B) Detail to show sample placement and bonding on sample-holding plates using epoxy glue. (C) Schematic of working principle of the actuator and force sensor during tensile testing. A is the actuator, B is force sensor. Known spring constant k_1 was calibrated before the experiment. Displacements x_1 and x_2 were obtained from the experiment. (D) A petri dish, containing the tensile testing device and deionized (DI) water, was placed on the stage of an inverted fluorescent confocal microscope. Zamil et al [24].

3.2 Sample preparation

Zamil et al [24] cut the outer onion epidermal tissues and bonded them to uniaxial displacement-controlled mechanical loading devices to apply and measure the force on the sample. Fluorescent polystyrene beads (500 nm in diameter) were dispersed on the sample surface under various levels of tensile load conditions to obtain displacement maps with a confocal fluorescent micro-scope. The resulting strain was measured using a digital image correlation technique by tracking individual bead displacements. The applied forces were obtained by measuring the displacement of the calibrated force-sensing device. Tissue- and cell-scale mechanical properties were quantified by calculating the applied stress and the corresponding global and local strains.

3.3 Elastic modulus measurements

Zamil e al [24] obtained elastic modulus values of individual cell walls of dehydrated and rehydrated samples were 3.0 ± 1.0 GPa and 0.4 ± 0.2 GPa, respectively. In contrast, the elastic modulus values of the global tissue-scale dehydrated and rehydrated samples were 1.9 ± 0.3 GPa and 0.08 ± 0.02 GPa, respectively. Poisson's ratio increased more than 3-fold due to hydration. The results on global, cell-to-cell, and point-to-point mechanical property variations suggest the importance of the mechanical contribution of extracellular features including the middle lamella, cell shape, and dimension. This study shows that a multiscale investigation is essential for fundamental insights into the hierarchical deformation of biological system.

3.4 Discussions and Conclusions

As Zamil et al [24] stated subcellular mechanical characterization of the cell wall can provide important insights into the cell wall's functional organization, especially if the characterization is not confounded by extracellular factors and intercellular boundaries. However, due to the technical challenges associated with the microscale mechanical characterization of soft biological materials, subcellular investigations of the plant cell wall under tensile loading have to be properly performed. Zamil et all report the mechanical characterization of primary onion epidermal cell wall profiles using a novel cryosection-based sample preparation method and a microelectromechanical system-based tensile testing protocol [24].

4.0 Comparison and Discussion

4.1 Comparison

In this review, the reported values of elastic modulus (EM) obtained from indentation and tensile deformations of soft biological tissues are compared. When the method of deformation is ignored, EM values for any given tissue typically span several orders of magnitude. If the method of deformation is considered, then a consistent and less ambiguous result emerges. On average, EM values for soft tissues are consistently lower when obtained by indentation deformations. Below the implications and potential impacts of these finding are discussed with the help of Table 1 summarizing the main results.

Table 1 Elastic Moduli as Measured by Different Methods and Under Various Conditions.

Measurement Method	Condition	E-Modulus(MPa)	Ref.
Nano Indentation	in air	110	[14]
Nano Indentation	in water	80	[14]
Nano Indentation	never dried (fully hydrated)	15 ± 8	[15]
Nano Indentation	Calcium Ion Effect	39 ± 3	[15]
Tensile Test (global Tissue)	dehydrated	1900 ± 300	[24]
Tensile Test (global Tissue)	re-hydrated	80 ± 20	[24]
Tensile Test(individual cells)	dehydrated	3000 ± 1000	[24]
Tensile Test (individual cells)	rehydrated	400 ± 200	[24]

Table 1 presents a summary of the elastic moduli as measured by the two different methods and under various conditions of hydration. The values obtained with nano indentation are typically lower than those obtained by the tensile testing method. The results from the two methods come closest when the plant cells are hydrated, i.e., EM= 80 MPa from tensile testing data and EM = 80 MPa from nano indentation data. The lowest value of EM, that is 15 MPa was obtained from never dried fully hydrated cells by nano indentation. One can conclude from the information and data presented that the values of EM for soft biological tissues depend on the method by which EM is obtained, on the sample preparation and the role of the network embedding the microfibrils.

4.2 Comparison

With regards to the testing method, as pointed out by McKee et al [25] tensile measurements consistently result in larger EM values compared with indentation measurements. The difference in YM between these two methods is an experimental confirmation that soft biological tissues are not homogeneous over the various length scales. Indentation measurements are localized to the region of indenter contact in the order of millimeters to nanometers depending on probe size/geometry. Tensile measurements induce macroscopic deformations that span the bulk of a tissue with the entire specimen stretched. Understanding the differences is straightforward with muscles or ligaments. These tissues are better suited to resist deformation from a given tensile stress in the direction of fiber orientation as compared to a localized indentation that might be perpendicular to the fiber orientation.

4.3 Hydration

The EM values in Table 1 indicate the importance of the state of hydration state. In earlier work [14] is was discovered that indentation measurements on Arabidopsis showed that EM values dropped from 110 to 80 in going from air to water. Xi et al [15] took special care to use in their indentation measurements freshly peeled single-layer onion abaxial epidermis wall and tested in the liquid mode of atomic force microscopy, recently made available from Bruker, Inc. [15]. The samples were independent of the turgor pressure influence and reflected only the properties of hydrated cell walls in their natural condition. For the work reported here on onion cells the EM values ranged from 15 to about 40 MPa. [15] When in the tensile measurements the tissue was thoroughly hydrated the EM values dropped significantly and reached values of 80 – 400 MPa. When the sample were first tested the cells were thoroughly dried and held in vacuum, in other words even residual H_2O was removed. Under these conditions the EM values reached 1900 MPa in the global issue experiments and as high 3000 MPa for individual cells. The conclusion is that a major reason for the differences in the results of the two methods lies in the state of hydration of the microfibrils.

4.4 Role of Pectin

Many researches have suggested that Pectin plays a major role in the cell wall elasticity. Pectin is a gel phase structure that embeds the cellulose-hemicelluloses network and becomes soluble with mild treatments. It serves as matrix material for the cell wall. Pectin accounts for a large proportion of cell wall in weight (about 42%) [26].

Xi et al [15] tested the effect of pectin network structure on modulus through control experiments. They chose to modify the pectin network with Ethylenediaminetetraacetic acid, (EDTA) for partial removal of pectin. In this treatment, 100 mM EDTA was added onto the cell wall sample taken from onion scale 5. This treatment is known to partially remove the EDTA-soluble pectin from the epidermis walls [27], [28]. Topography images were generated in the same area in water and after adding the EDTA solution. 80 F-D curves were probed in the sample area. The elastic moduli results are shown in Figure 8. The results show a decrease in the EM value from 22.8 MPA to 15.3 MPa a drop which shows the role of Pectin in determining the value of the elastic modulus.

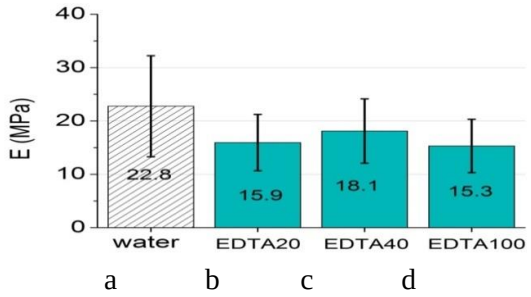


Fig. 8 Topography images of onion scale 5 in (from left to right) a) water, b) 20 minutes after dipping in 100 mM EDTA, c) 40 minutes after dipping in 100 mM EDTA, d) 100 minutes after dipping in 100 mM EDTA. e) Elastic moduli measured in water and after adding EDTA solution in the same area of cell wall shown in topography images. The error bar shows sample standard deviation.

In a chemical treatment the Calcium ion effect was explored by Xi et al [15]. 100 mM $CaCl_2$ solution was added onto the cell wall removed from onion scale 5. Topography images were generated in the same area in water and after adding $CaCl_2$ solution. 120 F-D curves were generated in the same area. Elastic moduli results are shown in Figure 9.

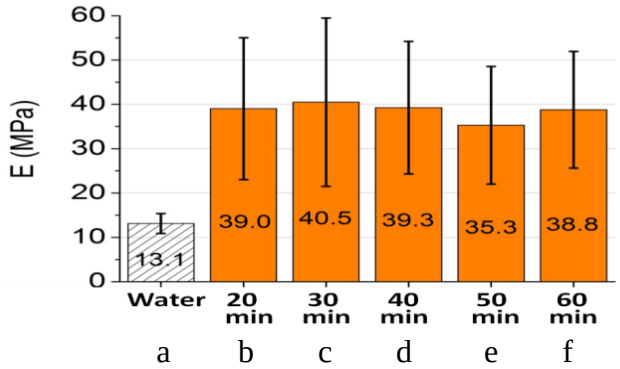


Fig. 9 Elastic moduli of onion cell wall from scale 5 in water and in $CaCl_2$ solution with time. The increase is statistically significant. Topography images of scale 5 in a) water, b) 20 minutes after adding 100 mM $CaCl_2$, c) 30 minutes dipping in $CaCl_2$ solution, d) 40 minutes in $CaCl_2$ solution, e) 50 minutes in $CaCl_2$ solution and f) 60 minutes in $CaCl_2$ solution; Xi et al [15].

As shown in Fig. 9 the values of the elastic modulus showed significant changes after adding 100 mM $CaCl_2$ solution [15]. Pectin, especially homoglacturonan (HG) is negatively charged under cell wall physiological pH (around 5.0), Ca^{2+} serve as a bridge that cross-links the HG chains and increase the regional density of the cell wall surface, which adds rigidity to the cell wall [21]-[23]. Due to the formation of gelation structures, elastic modulus increased dramatically after adding $CaCl_2$ solution and the standard deviation increased significantly on the calcium ion treated sample. The topography and elastic modulus remained relatively stable with time.

4.5 Discussion

The influence on cell wall elasticity when pectin was chemically modified is apparent. From the mechanical point of view, pectin serves as a matrix material in the cell wall biopolymer composite and has long been suspected of playing an important role in cell wall mechanics. Both the elastic modulus measurement results as well as the topographic images indicated a significant change as a result of the calcium ion treatment but a lesser change as a result of the EDTA (Ethylenediaminetetraacetic acid) treatment.

5. Conclusions

The research results reviewed here focus on cell walls in skins of onions as surrogates for plant cells in general and addresses the large difference in reported values for the elastic modulus of the onion epidermal cell wall. This review paper goes into some detail based on the publications of two groups each representing one of the two different methods but using the same type of plant cell, namely onion skin. The values of the elastic modulus are compared in a table and the differences are discussed. The conclusion is that a major reason for the differences in the results of the two methods lies in the degree of hydration of the microfibrils. A second reason deals with the extent of deformation in the two methods: the atomic force microscopy takes place at a nano-sized area whereas the tensile test is a deformation over an extensive length scale. Finally, Pectin which is the gel phase structure that embeds the cellulose-hemicelluloses network is seen to play a considerable role in the cell wall elastic modulus.

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