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Quantifying plant cell-wall failure *in vivo* using nanoindentation

Elham Forouzesh, Ashwani K. Goel, and **Joseph A. Turner,** Mechanical and Materials Engineering, University of Nebraska-Lincoln, W342 Nebraska Hall, Lincoln, Nebraska 68588-0526

Address all correspondence to Joseph A. Turner at **jaturner@unl.edu**

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Abstract

Nanoindentation experiments have been carried on *Arabidopsis thaliana* using spherical tungsten tips. Load–displacement plots obtained from experiments suggest that there is an optimum diameter of tip size which can be used to safely penetrate the tip through the cell wall. Based on the exact tip size used in the experiments and the measured load–displacement response, the failure stress was calculated using the experimental data in conjunction with a computational model. The value of failure stress was investigated in hypertonic (plasmolyzed), isotonic, and hypotonic (turgid) samples.

Introduction

The plant cell wall surrounds the cell and protects it against environmental stresses, fungi, pathogens, and insects such as aphids that have to penetrate through the cell wall for food.[1] Therefore, the effect of cell disease or genetic modifications on cell-wall composition and structure can be related to the consequential cell-wall failure properties.[2] In addition, studies of the failure properties of cell walls are important for bioprocessing applications in which it is necessary to break down the cell wall.[3] Furthermore, an understanding of the failure properties of the cell wall is crucial for successful delivery of materials inside the cells. Conventional drug delivery systems suffer from inefficiency or non-specific effects.[4,5] Thus nanosystems are being introduced for targeted drug delivery.[6] For this purpose, the carrier must be able to enter the cell wall without causing death or damage to the cell while at the same time penetrating through the wall. Therefore, it is important to understand the biomechanics of the cell wall to estimate the force needed to penetrate it while keeping the cell alive. Underestimation or overestimation of the force may lead to inadequate and unsuccessful delivery in the first case or damage to the cell in the second case.[7,8]

Failure stress represents the stress required for the cell wall to rupture. Different single-cell methods have been used to quantify these properties.[9] These methods include cell poking,[10] compression testing,[11,12] micro-indentation,[13,14] and nanomanipulation.[15,16] In these methods single cells are prepared by culturing. Application of these methods to very small cells and visualization of cell separation and breakage is very difficult with an optical microscope due to insufficient depth of focus and low resolution. The scanning electron microscope (SEM) has better resolution and depth of field. However, to study cells an environmental SEM (ESEM) should be used because this instrument permits the presence of a gas, such as water vapor. It has been shown that ESEM-based nanomanipulation[7,8,15] gives better information of cell shape during compression for very small cells. Nevertheless, in these kinds of measurements beam damage may be an issue. In addition, the values obtained from these experiments represent the stiffness of the whole cell rather than just the cell wall alone.

Previous studies mostly used compression tests and did not separate the effect of pressure from the wall properties.[12,16] The force required to burst the cell (which is called the cell-wall failure force) is a combination of parameters such as cell-wall material properties, turgor pressure, etc. The interpretation of measurements thus requires a careful consideration of these different effects.

Compared with the other methods, nanoindentation offers many advantages that make it a powerful technique for the measurement of mechanical properties of biomaterials.[17,18] The forces and displacements of commercial indentation instruments are typically in the μN and nm ranges, respectively. Thus, the sensitivity and measurement range is appropriate for measurements on plant cell walls that require higher indentation forces for penetration in comparison with mammalian cells that are often studied using the atomic force microscope (AFM).[19]

In this paper, a nanoindentation technique in conjunction with finite-element modeling is used to quantify the failure stress of plant cell walls. The nanoindentation measurements allow the onset of failure of the plant cell walls to be quantified very locally. Finite-element simulations are then used to interpret the measurements by allowing the mechanical response of the cell wall to be separated from effects due to turgor pressure. The response of the plant cell wall depends upon various geometrical and material parameters, which include the geometry of the cell wall, turgor pressure within the cell, the radius of

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the indenter and the mechanical properties of the cell wall. To investigate the effect of the turgor pressure on the failure stress and deformation at failure, experiments were conducted on normal, turgid, and plasmolyzed cells. To keep the cells in conditions as close to in vivo as possible, experiments were performed on single cells of leaves attached to the plant.

Experiments

Nanoindentations were performed on wild-type *Arabidopsis thaliana* (see Supplementary Materials). The size of the cells is such that it is impossible to monitor penetration of tips optically. However, the transducer of the instrument has a high resolution such that force and displacement can be measured during nanoindentation. Thus the system allows the onset of tip penetration into the cell wall to be quantified by examination of the load–displacement curves obtained from the measurements. Experiments performed by compression techniques have confirmed (using microscopes) that a drop in load–displacement curves can be associated with the onset of cell-wall failure.[7,8,14,20]

The first step in the experimental process was to determine the optimum tip size for nanoindentation, such that the tip can penetrate the cell wall with minimum cell disturbance. Tips with different sizes ranging from 25 to 1750 nm were prepared by etching tungsten. Studying the SEM images of the tips after nanoindentation revealed that tips with a radius smaller than 50 nm do not survive penetration and buckle during nanoindentation (Fig. S1). The load and displacement was measured with sufficient resolution such that the onset of failure was captured as a drop in force recorded by the high-resolution transducer of the nanoindenter. The drop in force is related to the onset of cell-wall failure (see online Supplementary Materials). Tips having a radius of ~50 nm showed the best results (Figs. S2–S3).

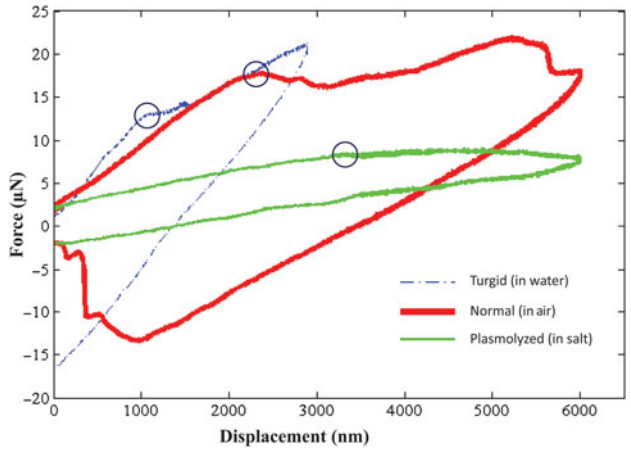


Figure 1. Example force–displacement curves obtained from nanoindentation measurements on plant cell walls with different environmental conditions; turgid (in water), normal (in air), and plasmolyzed (in salt solution). The circles indicate the discontinuities associated with wall failure. Tip radius = 50 nm.

Tips this size can penetrate through the cell wall without buckling while causing minimal disturbance to the cell when penetrating. Thus, the remaining experiments were performed using 50 nm tips. The method was then validated by changing one of the cell parameters; here cell turgor pressure, to determine its effect on the failure stress.

Results and discussion

From the force–displacement curves obtained from nanoindentation with the sharp tip, the force and displacement at the onset of the failure can be obtained. However, this force value cannot be related to the cell-wall failure stress directly. Parameters such as turgor pressure play an important role in cell-wall failure force.[21,22] Thus, to separate the effect of pressure from the cell-wall failure properties, the above experiments were simulated using finite-element modeling.

Interpretation of experiments with finite-element simulations

The experimental nanoindentation measurements were simulated so that the stress at different indentation depths could be quantified (see online Supplementary Materials). The model parameters such as viscoelastic properties, cell-wall modulus, and thickness, and turgor pressure were the same as reported previously[21,22] for a 22-day-old plant.

By measuring the displacement at which the step is observed in the experimental data and finding the stress from the simulations, the failure stress can be quantified. By pushing the indenter deeper into the material the stress value at the indentation point increases while the increase rate depends on the tip size. Smaller tip size leads to a higher stress value at similar indentation depth (higher stress concentration). The results of these simulations are shown in Fig. S4. Very blunt tips might need a very large deformation to initiate failure due to the fact that they mostly deflect the wall and cause the cell to lose water (volume change) via cell-wall permeability. Therefore, the failure step for a blunt tip either does not occur or it might occur at very large depths (out of the range of most experiments). These results are in agreement with the results obtained from the nanoindentation experiments (Fig. S3).

Effect of turgor pressure on cell-wall failure behavior

The turgor pressure affects the behavior of the cell in response to applied forces. However, the failure stress of the wall should be independent of turgor pressure, because it is an inherent property of the cell wall. Thus, measurements were performed using different environmental conditions: turgid (in water), normal (in air), and plasmolyzed (in salt solution). In each of these environmental conditions, the plant cell will have different turgor pressure values, highest in water, and smallest in salt solution.[21]

In Fig. 1, force–displacement curves from the nanoindentation experiments are shown for different environmental conditions. The failure point on each curve is shown with a circle that

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indicates the penetration point, which was determined when the slope of the curve changed by more than 60%. As expected, the displacement at penetration for the sample with a higher turgor pressure, the turgid sample in water, is smaller compared with the samples with lower turgor pressures (normal in air and plasmolyzed in salt solution). Contrary to the plasmolyzed cell, the turgid cell is expected to burst at a smaller deformation because the wall is already under a high stress from the turgor pressure. The results showed in Fig. 1 are in agreement with this hypothesis. Stenson et al.[11] also reported that a cell has to be deformed to a similar extent at failure regardless of the conditions to obtain adequate strains in the wall that leads to failure.

To quantify the failure stress and understand the experimental results, simulations of nanoindentation on cells with different turgor pressures (corresponding to the environmental condition) were created. These results are shown in Fig. 2. It can be observed that the sample in water (turgid) shows the highest increase in stress for a given wall deformation from the tip, whereas the sample treated with salt (plasmolyzed) shows the smallest increase in stress for a given deformation. Therefore, to reach to a specific stress for failure a turgid sample needs a smaller additional deformation in its cell wall compared with the other two samples.

The displacement at failure for each environmental condition was obtained from load–displacement curves generated during nanoindentation. These results are shown in Fig. 3. Implementing the failure displacement obtained from experiments into the simulation curves as shown in Fig. 2, it is possible to quantify the failure stress of the cell wall for the different environmental condition. According to these results, as shown in Fig. 4, the failure stress value for turgid and normal samples are almost the same (334 ± 8 MPa; $p > 0.05$). However, the plasmolyzed sample shows a lower failure stress (294 ± 7 MPa; $p < 0.05$ when compared with the other two samples). These results show that environmental conditions play a role in failure stress of a plant cell wall, but the precise mechanisms for such changes are beyond the scope of this work. Milani et al.[23] and Kierzkowska et al.[24] have studied the effect of plasmolyzation on the stiffness of a plant cell, both agreeing that the plasmolyzed cells have lower stiffness. These results also show that the failure stress may depend on the environmental state.

The predictive ability of this approach was tested using nanoindentation results in air with a larger tip ($R = 140$ nm). This measurement was not used in the stress analysis described above because of concerns associated with larger tips. The large deformations required to induce failure are expected to fall outside of the assumptions included in the analysis because of factors such as water migration out of the cell. Thus, the analysis was restricted to the smallest tips possible for which buckling did not occur. However, the outcome using the larger tip is illustrative. The measurement result for the larger tip is shown in Fig. S5 with a resulting penetration depth of 4600 nm. The simulation results from Fig. S4 when plotted using a log–log scale show a linear trend for large depths (see Fig. S6). From the slopes of these curves, we can extrapolate to the depths needed for the larger tip. The failure stress for this size tip was estimated as 310 MPa, ~90% of the value for the smaller tips (Fig. S6). Until further studies are completed, we recommend applying this approach for quantifying failure stress using the smallest tips possible.

These results can be used for understanding the failure mechanisms of the cell wall for other deformation cases and other plant cell types or possibly even subcellular compartments although additional measurement tools may need to be developed. The approach is also relevant for studying the effect of mutations on cell-wall mechanical properties. We anticipate that this method can also be used for understanding the behavior of insects known to penetrate cells for feeding. As shown here penetration through the epidermal cell wall of *Arabidopsis* requires a small tip size. Considering the large tip size of insect stylets (1–5 μm),[25,26] it is clear why these

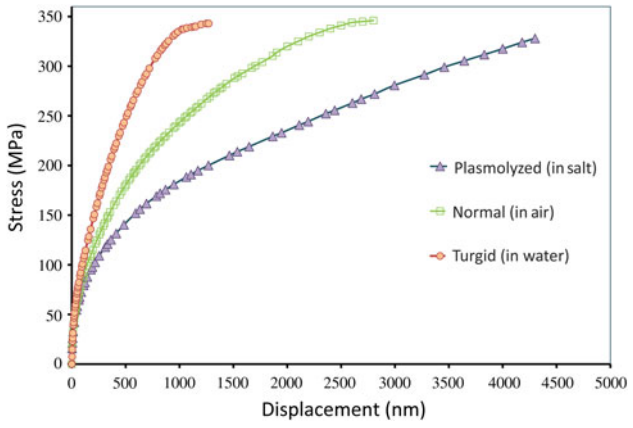


Figure 2. Stress–displacement results obtained from finite-element modeling with smallest, normal, and highest turgor pressures in plasmolyzed, normal, and turgid samples, respectively. Tip radius = 50 nm.

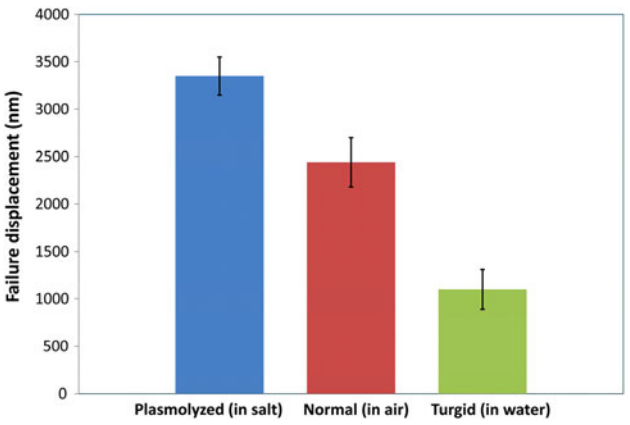


Figure 3. Displacement at failure values obtained from the experiments.

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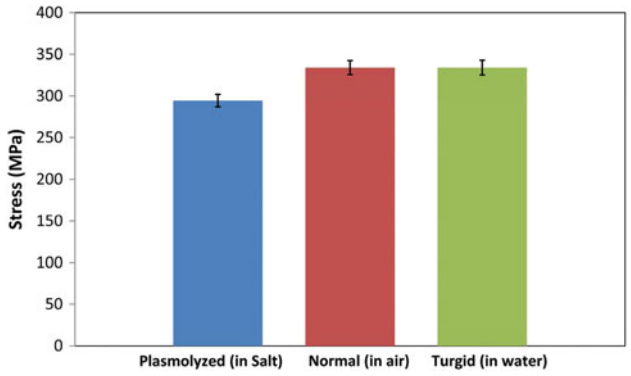


Figure 4. Failure stress of the cell wall at different environmental conditions obtained from the modeling and experimental results. The uncertainty shown is associated only with the measurements on the 15 samples of each type. The failure stress for the plasmolyzed state is statistically different ($p < 0.05$) than that of the other two states.

insects prefer piercing through the intercellular passes instead of the intracellular passes and use chemical secretions to lower the failure stress. The intercellular area is richer in pectin with a much lower expected failure stress because of the reduced amount of fiber-like reinforcement. Thus, the aphid can break into the cell without causing considerable damage to the cell. This conclusion is in agreement with the results obtained from EPG (electrical penetration graphs).[27]

Summary

Nanoindentation was combined with finite-element simulations to quantify the cell-wall failure stress. To validate the method, experiments were conducted in different environmental conditions including turgid (in water), normal (in air), and plasmolyzed (in salt solution). The displacement at failure for samples in water was the smallest due to the fact that the cell wall was already pretensioned by the high turgor pressure inside. The samples kept in salt solution showed the largest displacement at failure due to the low turgor pressure of the cells that does not exert as much stress on the cell wall. Failure stress for turgid and normal samples was found to be nearly identical, while the plasmolyzed sample showed a lower failure stress.

Acknowledgments

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Supplementary materials

For supplementary material for this article, please visit http://dx.doi.org/10.1557/mrc.2014.22

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