Advanced Nanoscale Characterization of Plants and Plant-derived Materials for Sustainable Agriculture and Renewable Energy

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ADVANCED NANOSCALE CHARACTERIZATION OF PLANTS AND PLANT-DERIVED MATERIALS FOR SUSTAINABLE AGRICULTURE AND RENEWABLE ENERGY

by

MIKHAEL SOLIMAN
B.S. The German University in Cairo, 2010

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Materials Science and Engineering in the College of Engineering and Computer Science at the University of Central Florida Orlando, Florida

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Major Professor: Laurene Tetard
ABSTRACT

The need for nanoscale, non-invasive functional characterization has become more significant with advances in nano-biotechnology and related fields. Exploring the ultrastructure of plant cell walls and plant-derived materials is necessary to access a more profound understanding of the molecular interactions in the systems, in view of a rational design for sustainable applications. This, in turn, relates to the pressing requirements for food, energy and water sustainability experienced worldwide.

Here we will present our advanced characterization approach to study the effects of external stresses on plants, and resulting opportunities for biomass valorization with an impact on the food-energy-water nexus.

First, the adaption of plants to the pressure imposed by gravity in poplar reaction wood will be discussed. We will show that a multiscale characterization approach is necessary to reach a better understanding of the chemical and physical properties of cell walls across a transverse section of poplar stem. Our Raman spectroscopy and statistical analysis reveals intricate variations in the cellulose and lignin properties. Further, we will present evidence that advanced atomic force microscopy can reveal nanoscale variations within the individual cell wall layers, not attainable with common analytical tools.

Next, chemical stresses, in particular the effect of Zinc-based pesticides on citrus plants, will be considered. We will show how multiscale characterization can support the development of new disease management methods for systemic bacterial diseases, such as citrus greening, of great importance for sustainable agriculture. In particular, we will focus on the study of new
formulations, their uptake and translocation in the plants following different application methods. Lastly, we will consider how plant reactions to mechanical and chemical stresses can be controlled to engineer biomass for valorization applications. We will present our characterization of two examples: the production of carbon films derived from woody lignocellulosic biomass and the development of nanoscale growth promoters for food crop. A perspective of the work and discussion of the broader impact will conclude the presentation.
To my beloved Araks, my parents, and my siblings Mark and Kuki
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1 INTRODUCTION

The emergence of reports from all major scientific agencies worldwide\(^1-^6\) stresses the urgency of designing efficient strategies and policies that address the food, water and energy components of the nexus synergistically to keep up with the pressures imposed by growing populations on our planet\(^7\) (Figure 1.1). Integrated management approaches and cross-functionality will need to be introduced into policy-making in order to adequately and efficiently solve the challenges faced in each sector while ensuring the other sectors are not adversely affected.

![Diagram of the food-water-energy nexus](image)

Figure 1.1. The food-water-energy nexus illustrating the interdependence of the components

An example of the unintended consequences of segmented policy-making is dedicating significant water use for agriculture in the hopes of protecting food security, without addressing the needs of populations living in the same regions with scarce water resources\(^8-^10\). Another example is subsidizing biofuels to supplement energy security, which can create more competition for land and water resources, inadvertently adding stress to food and water security. An increase in food prices has been attributed to a shift in land use from food crops to crops
dedicated to biofuel production\textsuperscript{11}. As a result, nexus thinking is focused on addressing system efficiency rather than enhancing productivity in each sector alone\textsuperscript{12}. Moreover, a nexus perspective would improve the understanding of intricate connections between the three sectors.

Three main pressures for food, water, and energy have been identified: world population growth, growth of the middle class in developing nations, and climate change\textsuperscript{12,13}. The increasing world population and demands for resources to sustain or improve quality of life for the expanding middle class worldwide, pose significant threats to the accessibility to finite resources in food, energy, and water\textsuperscript{3,5-7}. Climate change adds to the strain on the nexus by lowering the quality of arable land and threatening clean water resources\textsuperscript{6}. In addition to more efficient technologies, it is also imperative to utilize the available resources more efficiently than before.

Interdisciplinary scientific research has a crucial role to play in creating innovative solutions to meet the needs formulated in the scope of this nexus\textsuperscript{14}. Recent advances in nanotechnology, for instance, may hold a key to ensuring the availability of food to the growing world population\textsuperscript{15-18}. Nanotechnology also holds promise in the energy\textsuperscript{19,20} and water\textsuperscript{21,22} sectors. For instance, multi-walled carbon nanotubes were previously shown to have 3-4 times the capacity to absorb contaminant metallic ions in water compared to traditional sorbents used in water purification\textsuperscript{23}. In terms of energy security, nanotechnology proves useful in a wide range of applications. Nanostructured composites, for example, are being considered as anode materials to improve capacity and recharging rate in lithium ion batteries\textsuperscript{24,25}. In food applications, nanotechnology is promising improvements not just in enhancing agricultural production, but in packaging and food safety as well. The use of carbon nanotubes for pathogen detection in food packaging has been studied previously\textsuperscript{26}. 
The work presented in this dissertation more directly pertains to the energy and food security sectors. The motivation is to boost our fundamental understanding of plant responses to external mechanical and chemical stresses using cutting edge characterization with high spatial resolution and sensitivity, to eventually reach rational design of plant properties and processing for new solutions applicable to food, energy, and water.

1.1 Studying the Structure and Composition of Plant Cell Walls for more Efficient Bioenergy Production

In any given country, energy security could be defined as the reliable continuous supply of energy at an affordable price\textsuperscript{27,28}. Energy security may also refer to energy production and consumption in a sustainable manner with minimal effects on the environment\textsuperscript{28}. It is estimated that fossil fuels accounted for 85.5\% of total world energy consumption in 2016\textsuperscript{29}. Given the limited supply and the accelerating rate of depletion of the supply, this statistic suggests how fragile the world’s energy security is for the foreseeable future\textsuperscript{1}. In addition, fossil fuels release a significant amount of greenhouse gases exacerbating the effects of climate change\textsuperscript{30}. Based on the definitions above, continued reliance on fossil fuels as the world’s primary source of energy goes directly against the interest of safeguarding energy security and sustainability.

There are several approaches to overcome the issue of limited energy supply\textsuperscript{27}. First, more efficient energy consumption can be achieved through measures such as manufacturing cars that have better fuel economy\textsuperscript{31}, installing building insulation to reduce heat loss\textsuperscript{32,33}, and replacing incandescent light bulbs with fluorescent lamps or light-emitting diodes\textsuperscript{34}. However, this does not solve the underlying issue of depleting resources to alarmingly low levels. Therefore, alternative energy sources that are renewable and clean should be seriously considered. A
number of alternative sources are being studied\textsuperscript{35,36} including: solar power\textsuperscript{37,38}, wind energy\textsuperscript{39}, tidal energy\textsuperscript{40}, biofuels\textsuperscript{41-43}, geothermal energy\textsuperscript{44} and hydroelectric power\textsuperscript{45}. Each of these has its advantages and limitations with regards to how they influence the other sectors of the food-energy-water nexus. For example, dedicating more land and water use for the production of biofuels can affect food production as well as clean water supplies. However, biofuels offer a renewable source of carbon, and if the right practices are implemented, can significantly help offset the coming rise in demand for energy with minimal effect on the other sectors\textsuperscript{43}. A number of approaches to minimize the impact of bioenergy production on food and water supply include: using land that is not dedicated for food production, implementing double land use in which biofuel crops are grown in between seasons of growing food crops\textsuperscript{46}, and using crop residues and residues from forestry operations instead of discarding them as waste\textsuperscript{47,48}.

Currently, most of the biofuels produced in the US are obtained from cornstarch\textsuperscript{49,50}. This threatens crop yields available for consumption and food prices. As a result, the US Energy Independence and Security Act of 2007 specified that at least 16 of the 36 billion gallon target of biofuel production by 2022, should come from non-starch sources such as cellulosic biomass\textsuperscript{41,51}. Cellulosic biomass sources such as switchgrass would also require less irrigation than starch sources, thereby further reducing potential stresses on water security\textsuperscript{35}.

There is, however, a major obstacle in using cellulosic biomass; namely cellulose extraction due to the evolved resistance mechanisms to cell wall deconstruction, also known as recalcitrance\textsuperscript{52,53}. The plant cell walls consist of cellulose fibrils in a matrix of lignin and hemicellulose\textsuperscript{54}. This complex system is notoriously difficult to break down\textsuperscript{52,53,55,56}. As a result, the process of cellulose extraction is energy intensive, involving pretreatments and producing
significant waste. Therefore, studying in finer details the intricate structure and composition of the cell wall, which was not previously possible due to the lack of functional analytical tools for nanoscale characterization, could provide potential solutions to design more efficient processes to extract cellulose. Moreover, genetic modifications of plant systems informed from this new understanding for higher cellulose content compared to lignin would also be highly valuable.

1.2 Studying the Uptake and Effects of Chemical Treatments in Plants for Better Plant Disease Control

The term “food security” refers to the continued access to food and nutrition in terms of affordability and safety. Any disruption in food supply at a sufficient scale is considered a significant threat to any nation’s security. Nowadays, the food supply of the world is under constant threat due to factors such as climate change, which is predicted to cause an average 8% decline in food production in Africa and South Asia by the 2050s, and plant diseases which threaten eradicating important crops such as citrus, potatoes, and tomatoes.

Plant diseases pose the most significant threat in the short term. At least 10% of global food production is lost to plant diseases, at a time when hundreds of millions of people have inadequate access to food. In general, plant pathogens are difficult to control due to numerous factors including variation in time of infection and geographical spreading, as well as the large number of dangerous species capable of infecting all of the major crops. It is therefore imperative to find innovative measures to curb the negative effects plant diseases have on the food security of the globe. Additionally, these solutions must not affect the other two components of the nexus adversely. For instance, the increased use of pesticides threatens to contaminate water resources and levy larger energy costs in terms of storage and frequent
application using fuel-operated machinery. Therefore, solutions that could more efficiently alleviate the effects of plant diseases are sorely needed. One such approach is using nanoparticles and novel treatments designed from materials which have good antimicrobial properties thus killing the bacteria rapidly and which can also act as growth promoters to improve crop yields.

1.3 Considering the Importance of Tailoring Characterization Methods and Protocols to the Problem

To tackle the aforementioned challenges, it is imperative to develop approaches capable of capturing the variability of natural systems while accessing the interaction-level information at the subcellular level. Characterization, especially at the nanoscale, constitutes a pillar of the materials science and engineering field. As more breakthroughs are achieved in nanotechnology and design of materials, it becomes more necessary to have the ability to probe materials properties adequately at the nanoscale. This is true for the wide areas of research in which the potential of nanotechnology is being realized.

In the previous sections, the relation between the work that is presented in this dissertation and the food and energy components of the food-energy-water nexus was brought to light. At this point, it is important to highlight the crucial role played by using the appropriate characterization methods (Figure 1.2) in furthering our knowledge of plant structures and properties at the nanoscale, as well as in studying the novel treatments designed to protect food crops from plant diseases and enhance crop yields in view of rising global demands. In terms of plant structure and properties, it is vital to utilize characterization tools covering multiple scales from the nanoscale level to study cellulose microfibrils with diameters ranging from a few nanometers to tens of nanometers, to several micrometers to probe plant cell walls and their layers for instance.
Tracking the uptake of treatments in the vascular tissue of plants will also require the ability to access the phloem, which is normally on the order of several tens of micrometers.

Figure 1.2. Characterization methods at the corresponding length scales probed. The recent access of advanced atomic force microscopy (AFM), scanning electron microscopy (SEM), and transmission electron microscopy (TEM) enables the study of several key areas such as the nanoscale structure and composition of the plant cell walls, the interactions of the main components, and the uptake of nanoparticle treatments in plants. Nanoscale infrared spectroscopy (nanoIR) and energy dispersive x-ray spectroscopy (EDS) enable chemical characterization with high resolution.
The mechanisms behind the plant response to mechanical and chemical stresses still need to be studied in order to make informed decisions on how to utilize these phenomena to our advantage in securing the world’s natural resources. Within the scope of this work, the phrase “mechanical stress” is considered in its more general meaning since the effects of different stress components usually considered in materials science (such as torsion or shear) have not been studied extensively in plant cells. Nanoscale characterization tools such as advanced modes of atomic force microscopy are still not employed to their full potential to extract the wealth of knowledge contained in complex systems like the plant cell walls. Moreover, these same tools could also be used to study the uptake and effects of promising nanoparticle treatments inside plants and better appreciate the mode of action of these treatments and how they can eliminate dangerous plant pathogens.

1.4 Outline of the Present Work

In some plant systems, exposure to mechanical stresses induces a change in composition of the plant cell walls. In chapter 2, we illustrate this by presenting the example of Poplar tension wood which forms an extra cellulose-rich layer in the cell wall dubbed the gelatinous (G) layer. The structure and composition of Poplar tension wood are studied using a number of characterization techniques including confocal Raman microscopy and advanced atomic force microscopy (AFM) based methods. Subtle changes in the Raman signature obtained from tension wood, as compared to opposite and normal wood, are identified using statistical analysis methods. Potential chemical and mechanical properties variations within the individual cell wall layers are also explored with advanced AFM techniques.
Chapter 3 focuses on the effects of chemical stresses in plants. In particular, we study novel nanoparticle-based treatments to combat plant diseases. The experimental work performed to answer key scientific questions about the uptake and effects of treatments designed for use in citrus plants to combat citrus greening disease, a plant bacterial infection which is currently decimating citrus crops in Florida and other regions worldwide, is discussed. A protocol combining multiscale characterization techniques is successfully developed which can potentially aid growers in designing future strategies for applying these treatments in the field efficiently to move towards precision agriculture.

Chapter 4 deals with two examples illustrating how the external stresses plants experience can be utilized to develop added value products in agriculture. In the first example, the properties of biomass films are studied, as well as the effects of varying processing parameters on them. Apart from energy production, the financial efficiency of biorefineries may be improved by realizing the added-value potential of the waste produced from processing biomass into biofuels. Obtaining new organic materials from biomass as opposed to petroleum would constitute a step in the direction of sustainability. In the second example, efficient delivery of growth promoters using nanoparticles to enhance the yield and physiological traits of tomato plants is explored. The ability of the plants to uptake the growth promoters is also studied. The example underlines the potential of nanoparticle-based efficient delivery of growth promoters in minimizing the negative impact of overuse of fertilizers and pesticides on the environment.

Chapter 5 concludes with a discussion of the implications of the results described in chapters 2, 3, and 4 and relates them to the broader perspective of the energy-water-food nexus. Future directions and recommendations in each topic are also considered.
2 EFFECTS OF MECHANICAL STRESS IN PLANTS – THE EXAMPLE OF GRAVITY IN POPLAR WOOD

2.1 Background

Mechanical stress can affect the growth and development of all plant species. Plants can experience mechanical stress from natural (wind, gravity), or artificial (touch from foreign objects) sources. Changes in the plants structure and composition at the cellular and subcellular levels, such as altering the cellulose orientation inside the cell walls have been observed. These variations manifest themselves at the macroscale in the form of variations in root density, stem size, the number of leaves, the surface area of leaves, the concentration of chlorophyll, and even resistance to disease and drought. These phenomena occur as a result of plant response at the cellular and subcellular levels, but have not been fully resolved with conventional characterization tools. The ability to study these changes at the nanoscale using advanced characterization techniques would significantly aid in understanding how these stresses at the macroscale can induce alterations in the arrangement of the building blocks of plant cell walls, as well as changes in composition.

Here we consider reaction wood as an illustration of the potential effect mechanical stresses such as gravity (gravitropism) can have on wood tissues. In reaction wood, tension and compression wood are formed as a manifestation of a plant’s natural mechanism to prevent cracking or breaking. Tension wood forms in hardwoods, whereas compression wood forms in softwoods. In hardwoods, tension wood forms on the side that is experiencing tensile stress as the branch is bending. As shown in the Scanning Electron Microscope (SEM) image (Figure 2.1), the transition from opposite to tension wood along a stem cross section appears to be abrupt. It is
now understood that cellulose content in tension wood is high compared to normal wood\textsuperscript{80} and is associated with the formation of an extra layer, called the gelatinous (G) layer, in the cell walls\textsuperscript{81,82}. It was previously reported that G layers contain more cellulose with higher crystallinity than the other layers of the cell wall\textsuperscript{81}. Moreover, it has been claimed that the content of S lignin in tension wood is higher than in normal wood, while the content of G lignin is lower\textsuperscript{80,83}. However, in terms of crystallinity, quantified values for the crystallinity index of cellulose in tension wood were obtained when measuring the tissue as a whole, and not the individual cell wall layers\textsuperscript{84-86}. In most cases, the crystallinity index of cellulose is measured using X-ray Diffraction (XRD) following extensive treatment, such as grinding into powder or extraction using solvents and thermal treatments, which might influence results. For the S/G ratio, chemical characterization techniques which require breaking down the sample, such as gas chromatography, are often used\textsuperscript{87}. However, certain chemometric methods have been used to quantify the S/G ratio previously based on characterization methods such as nuclear magnetic resonance (NMR)\textsuperscript{83} and Raman spectroscopy\textsuperscript{88}.

Figure 2.1. Schematic representing the arrangement in a section of reaction wood, with tension wood area represented in red (left). SEM images capture a transition between tension and opposite wood. The red line indicates the transition in cell wall structure between tension wood and opposite wood. Zoom on the tension wood cells (right) showing the G layer in the walls.
The capacity of the plant to naturally select cellulose content in the G layer suggests that there are important key parameters to understand regarding the formation of plant cells. In turn, the molecular understanding would unlock engineering processes beneficial to producing biomass that is suitable for biofuel production or for other applications aiming at valorizing lignocellulosic biomass\textsuperscript{80,89}. Currently biomass deconstruction of the lignin-hemicellulose-cellulose complex matrix for further processing is challenging and mainly driven by trial and error choices of solvents and pretreatment conditions. In order to extract cellulose from the cell walls, lignin must first be removed using a combination of chemical and thermal processes\textsuperscript{90,91}, which are energy intensive\textsuperscript{80}.

In this study, reaction wood represents a useful model system to develop approaches capable of quantitative probing of nanoscale properties that can be related to larger scale measurements. The development of metrology tools capable of differentiating cellulose and lignin and their interactions in plant cell walls, at the mesoscale and nanoscale, is of prime interest in this work. Such model system is relevant according to the surge of isolated studies using nanoscale metrology tools to explore the complex properties of plant cell walls. Despite the production of data depicting the localized variations in plant cell walls\textsuperscript{92-94}, a staggering lack of understanding of the molecular interactions between the wall’s biopolymers constitutes a critical barrier to realizing the full potential of biomass in energy and material design applications. Isolated and qualitative analyses of nanoscale images are insufficient to impact material design related to plant-derived materials. Multiscale and quantitative considerations must be considered to connect observations made with imaging and spectroscopy to the results obtained with more conventional characterization for plant science.
Confocal Raman microscopy has been used to analyze various components of plant cell wall layers in their natural state, including the ratio of cellulose to lignin content or the orientation of cellulose fibers in the different layers of the cell wall. Agarwal et al. showed further data analysis can reveal traits such as cellulose crystallinity. Others have used Raman spectroscopy to investigate the polymorphic modifications of cellulose. These measurements were conducted on the plant tissue as a whole, however, and not on individual cell walls or cell wall layers. Due to the natural variations from one cell wall to another, statistical tools such as Principal Component Analysis (PCA) and k-means clustering have also been considered to identify significant variations in the properties of the systems. Despite the rich information contained in Raman spectra, optical diffraction limits the lateral resolution to a few hundred nanometers. This considerably impedes the exploration of structures and molecular interactions within the cell wall, which would require resolving nanoscale features. To overcome this bottleneck, SEM and AFM-based techniques have been considered to explore nanoscale structure and properties in plants. For instance, several groups used AFM and force distance curve analysis to measure the local stiffness of cell walls and discovered variations in local stiffness between different cell wall layers.

As discussed in the preceding paragraphs, advanced characterization methods have already unveiled a significant amount of information about the structures and properties of plants and plant cell walls. Nevertheless, there are several questions that still remain, and the full potential of these characterization methods and data analysis techniques is yet to be realized. In this chapter, the goal is to expand on the current level of advanced plant characterization by seeking to extract the maximum possible information from techniques that are already being used and by
utilizing novel advanced AFM-based methods to probe potential variations at the nanoscale and within individual cell wall layers.

We aim to answer the following questions:

1) *Can spatially resolved spectroscopy unveil variations in the composition and structure of the cell walls of plants that experienced mechanical stress?*

2) *Can we establish new correlations between small scale and layer-scale properties?*

3) *What are the limitations of existing characterization tools?*

As previously mentioned, here we use reaction wood as a model system to demonstrate the rich information that can be obtained with confocal Raman microscopy and nanoscale imaging techniques and discuss the connections that require more in-depth studies. We analyze the cell wall morphology and composition of the outer rings, inner rings, and pith regions on both sides (tension and opposite) of a stem cross section of Poplar wood. The results are compared to those obtained in normal (unstressed) wood from the same origin. More specifically, we use statistical methods to track changes in peak width and position for cellulose and lignin characteristic Raman bands. Lignin S/G ratio and cellulose crystallinity are among the properties calculated from our datasets. To explore finer variations in the cell wall, we use Atomic Force Acoustic Microscopy (AFAM), a technique combining acoustic actuation and AFM, to study potential variations in the local elastic properties of the cell walls\textsuperscript{110,111}. In order to complement the results of AFAM, nanoscale infrared spectroscopy (nanoIR)\textsuperscript{112} is used to analyze variations in chemical signature at the nanoscale within the individual cell wall layers.
2.2 Methods

Sample Preparation:

Twenty five micrometer thick cross sections of fresh Poplar wood were prepared using a cryomicrotome (LEICA CM 3050S cryostat) following the work described by Jung et al. Cryo-sectioning was performed at -8 °C, and cutting speed was manually controlled. A piece of poplar stem with less than 2 cm diameter was fixed on the metal plate using glue instead of embedding to avoid any contamination by the embedding material. The cross sections were placed on a microscope glass slide before imaging. For electron microscopy, the sections were sputter coated with a thin layer (~2 nm) of gold-palladium (Au/Pd). SEM imaging was performed with a working distance of ~ 7 mm, a beam energy of 5 kV, and an electromagnetic lens aperture of 20 μm.

Confocal Raman Microscopy:

A detailed description of confocal Raman microscopy is provided in Appendix A. Confocal Raman imaging and spectroscopy was performed using WITec Alpha 300 Raman and AFM (RA) equipped with a 532 nm laser with a maximum power output of 50 mW. The laser power used for sample excitation in this study varied from 5 to 10 mW. A grating of 600 grooves per mm was used, and integration time was 1 s. The spectral resolution for the grating used is less than 3 cm⁻¹. Spectra were acquired every 500 nm using a 20X objective (Carl Zeiss EC Epiplan NA 0.4). For finer details, a 100X objective (Carl Zeiss EC Epiplan NA 0.9 DIC) was used with spectra acquired at 100 nm steps. It is important to note that due to the diffraction limit, the resolution of the measurement using the 100X objective was around 300 nm, which means that an average of three ‘identical’ spectra was acquired every 300 nm throughout oversampling.
Data Analysis:

WITec Project FOUR+ software was used for data analysis including cosmic ray removal (CRR), baseline correction, noise filtering, peak fitting, and cluster analysis. For CRR, a filter size of 4 and a dynamic factor of 8 were applied. This pretreatment was followed by baseline correction using the shape method, with shape size of 100, noise factor of 1 and smoothing using Savitzky-Golay algorithm with an order of 3. The traits were studied by monitoring the intensity of respective peaks for cellulose and lignin. In addition, Lorentz fitting was used to determine the position of the maximum and the full width at half maximum (FWHM) of the peaks. Values collected were recorded to form maps and histograms. The draw field feature was used to limit the data analysis to selected regions in a given image.

K-means clustering\textsuperscript{115,116} with three clusters for normal wood and four clusters for tension wood was performed on large data sets using WITec Project FOUR+ software. In addition, Principal Component Analysis\textsuperscript{117,118} (PCA) was carried out using Unscrambler X software. For PCA, 20 spectra were selected in each cell wall layer identified by the k-means clustering analysis. Prior to PCA, the spectra were mean normalized using Unscrambler X.

Crystallinity of Cellulose:

The crystallinity $X_c$ of cellulose was determined based on Agarwal’s work\textsuperscript{99,100} using the following equation:

$$X_c = \left(\frac{I_{380}}{I_{1100}}\right)^{-0.286} \times 0.0065 \times 100 \text{\%}$$

(1)

where $I_{380}$ and $I_{1100}$ are the respective intensity of the peaks at 380 cm$^{-1}$ and 1100 cm$^{-1}$, which correspond to bending of ring C-C-C groups and asymmetric stretching of the $\beta$-(1-4)-glycosidic
bond in cellulose, respectively. This equation was obtained by a calibration method which involved obtaining Raman spectra from different cellulose samples with known crystallinities and estimating the peak intensity ratio of the aforementioned peaks for each sample. The equation is then obtained using regression analysis and can be used to estimate cellulose crystallinity under any conditions; however, several considerations have to be met. The measurements must be conducted at constant humidity, since it has been shown that humidity variations influence the crystallinity of cellulose. Additionally, the effects of other components of the plant cell wall should be considered, such as the increased background fluorescence of lignin. Background removal is, therefore, essential prior to calculation of the peak intensities to ensure an accurate result.

*Atomic Force Microscopy (AFM), Atomic Force Acoustic Microscopy (AFAM), and Nanoscale Infrared Spectroscopy (nanoIR):*

A detailed explanation of AFM is provided in Appendix C. AFM and AFAM were performed using WITec Alpha 300 RA. A cantilever with spring constant \(k \approx 2.7 \text{ N/m}\) was used (Tip B, Mikromasch 11-series). For AFAM, the sample was placed on a piezoelectric transducer vibrated at the cantilever contact resonance (68 kHz) with amplitude of 10 V. The amplitude and phase of the cantilever were measured at each point on the image by a lock-in amplifier (Stanford Instrument SRS SR844) using the actuator driving frequency as reference. The setup for AFAM measurement is shown in Figure 2.2. Time constant was set at 10 ms and sensitivity was optimized for the signal measured. Amplitude and phase signals were rerouted to the WITec.
controller (external channel 1 for amplitude, and external channel 2 for phase) for image formation.

![Diagram of AFAM setup](image)

**Figure 2.2**: Setup used for Atomic Force Acoustic Microscopy (AFAM). A sinusoidal waveform controls the actuation of the piezo element placed underneath the sample. The cantilever signal $S(t)$ is analyzed by lockin amplifier detection to isolate the amplitude and phase of the component at the reference frequency (actuation frequency). Amplitude and phase are recorded at each point of a predefined map to form the AFAM image.

Nanoscale infrared spectroscopy (nanoIR) measurements (Figure 2.3) were performed using the nanoIR2 platform (Anasys Instruments). In nanoIR, a pulsed tunable laser is used to excite the sample locally directly under the AFM tip. As the sample absorbs the IR light, it expands and contracts based on the frequency of the pulsed laser. The cantilever oscillates with an amplitude that is directly related to the sample absorption at each excitation wavelength. By recording the amplitude of the cantilever response at each wavelength, an IR spectrum is easily obtained for the position probed with the AFM tip. Another measurement involves sweeping the pulsed laser frequency ($f$) to detect shifts in the contact resonance which can be correlated to changes in the nanomechanical properties. For this measurement, a gold-coated cantilever (Anasys Instruments Model PR-EX-nIR2-10, $k \sim 0.07$-$0.4$ N/m) was mounted to scan the sample in contact mode. A
pulsed Quantum Cascade Laser (QCL) (Daylight Solutions MIRcat-1100, Serial No. 6380) was used to excite the sample. Local IR spectra were acquired between 1530 and 1810 cm\(^{-1}\) while fixing the laser pulse rate at the contact resonance of the cantilever (~ 173 kHz). IR amplitude and frequency maps were obtained by scanning specific regions of the sample while recording the cantilever response amplitude and contact resonance at each data point.

![Figure 2.3. Setup for nanoIR measurement. A pulsed tunable QCL laser is used to excite the sample locally underneath a gold-coated AFM tip which is in contact with the sample. The amplitude of the cantilever response to photothermal expansion of the sample is used to monitor sample absorption at various wavelengths. The contact resonance of the tip-sample system can also be monitored by sweeping the frequency of the pulsed laser.](image)

2.3 Characterization of the Cell Walls of Normal Wood

2.3.1 Chemical Characterization

Pure cellulose, lignin, and hemicellulose samples were initially tested to identify their characteristic Raman peaks (Figure 2.4). Cellulose (Figure 2.4 (a)) and lignin (Figure 2.4 (c)), the two main biopolymers present in the cell wall, exhibit characteristic peaks, the main ones being at around 1100 cm\(^{-1}\) and 1600 cm\(^{-1}\), respectively. A pure hemicellulose sample was also probed (Figure 2.4 (b)), and exhibits similar Raman bands to cellulose, but shifted towards lower
frequencies (~ 1090 cm\(^{-1}\) and 1120 cm\(^{-1}\) compared to ~ 1100 cm\(^{-1}\) and 1125 cm\(^{-1}\) in cellulose, respectively). An average Raman spectrum of a typical Poplar plant cell wall is also shown (Figure 2.4 (d)). Full assignments for the characteristic peaks are listed in Table 2.1.

![Raman Spectra](image)

Figure 2.4. Average Raman spectra obtained from (a) pure cellulose, (b) pure hemicellulose, (c) pure lignin, and (d) Poplar plant cell wall
Table 2.1. Assignments for characteristic Raman peaks observed in Poplar wood plant cell walls and pure biopolymer components.

<table>
<thead>
<tr>
<th>Peak Position (cm(^{-1}))</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>2940</td>
<td>C-H str. in hemicellulose(^{120,121})</td>
</tr>
<tr>
<td>2890</td>
<td>C-H str. in cellulose(^{92,95,98})</td>
</tr>
<tr>
<td>1650</td>
<td>Ring C=C str. In lignin(^{92,97,98})</td>
</tr>
<tr>
<td>1600</td>
<td>Aryl ring sym. str. in lignin(^{92,97,98,121})</td>
</tr>
<tr>
<td>1380</td>
<td>HCC, HCO, and HOC bending in cellulose and hemicellulose(^{98,120})</td>
</tr>
<tr>
<td>1335</td>
<td>Aryl-OH and Aryl-OCH(_3) in S lignin(^{88,122,123})</td>
</tr>
<tr>
<td>1280</td>
<td>Aryl-OCH(_3) in G lignin(^{122,124})</td>
</tr>
<tr>
<td>1140</td>
<td>Aromatic C-H in plane def. in lignin(^{98})</td>
</tr>
<tr>
<td>1120-1125</td>
<td>COC sym. str. in cellulose and hemicellulose(^{92,95,97})</td>
</tr>
<tr>
<td>1090-1100</td>
<td>COC asym. str. in cellulose and hemicellulose(^{92,97,98})</td>
</tr>
<tr>
<td>380</td>
<td>Ring CCC bending in cellulose(^{98})</td>
</tr>
</tbody>
</table>

Figure 2.5 shows maps of the intensity, in addition to distributions of the position and width, of each peak acquired in the outer region of the cross section. As can be seen in Figure 2.5 (b), the regions where the intensity of the lignin peak at 1600 cm\(^{-1}\) is highest are concentrated in the cell corners. On the other hand, the cellulose peak at 1100 cm\(^{-1}\) exhibits more uniform intensity.
indicating the presence of cellulose in the walls. Figures 2.5 (c) and (d) show the distributions of the peak positions for the peaks around 1100 cm\(^{-1}\) and 1600 cm\(^{-1}\), respectively. The peak corresponding to cellulose has an average position of around 1094 cm\(^{-1}\), whereas the peak corresponding to lignin has an average position of around 1598 cm\(^{-1}\). Similar analysis was performed in the inner region and the pith of the same normal wood cross section and shows that the peak positions of cellulose and lignin varied compared to the values obtained in the outer region, with cellulose having an average peak position at ~1097 cm\(^{-1}\) and lignin at ~1602 cm\(^{-1}\) in the inner region, whereas in the pith cellulose has an average peak position at ~1099 cm\(^{-1}\) and lignin at ~1605 cm\(^{-1}\). This shows that the different rings present within a single cross section can have some variation in their properties.
Figure 2.5: Raman intensity maps for cellulose (a) and lignin (b) obtained in the outer regions of a normal wood cross section. The map for cellulose was constructed using the intensity of the peak at 1100 cm$^{-1}$ corresponding to C-O-C stretching, and the map for lignin was constructed using the intensity of the peak at 1600 cm$^{-1}$ corresponding to aryl ring stretching. Distributions of peak position for the peak at 1100 cm$^{-1}$ (c) and 1600 cm$^{-1}$ (d). Distributions of peak width for the peaks at 1100 cm$^{-1}$ (e) and 1600 cm$^{-1}$ (f).
Higher resolution Raman mapping was also acquired (with a 100X objective) on normal wood cell walls to study variations between the individual cell wall layers (Figure 2.6). Chemical maps of cellulose (Figure 2.6 (b)) and lignin (Figure 2.6 (c)) distribution in the cell wall were constructed using the intensities of the bands at 1100 cm$^{-1}$ and 1600 cm$^{-1}$, respectively. The chemical maps show that lignin is more concentrated in the middle lamella and the cell corners. Cellulose is more uniformly distributed across the cell walls. K-means clustering (Figure 2.6 (d)) is capable of differentiating between the layers, with three clusters separating the middle lamella, the S layer, and the lumen. The average spectra calculated from each cluster show that the middle lamella has higher intensity for peaks representative of lignin at around 1335 cm$^{-1}$ and 1600 cm$^{-1}$ compared to the S layer. On the other hand, the peak representative of cellulose (around 1100 cm$^{-1}$) has about the same intensity in the S layer and the middle lamella, which suggests significant presence of cellulose in the middle lamella.
Figure 2.6: Higher resolution confocal Raman mapping of a normal wood plant cell wall. (a) Optical image of selected cells in the epidermis of the normal wood cross section. (b, c) Maps representative of cellulose (b) and lignin (c) constructed by calculating the intensity of their respective Raman peak at 1100 cm\(^{-1}\) and 1600 cm\(^{-1}\). (d) Average spectra of the regions identified by k-means clustering (three clusters). Map (inset) illustrating the overlay of the 3 clusters corresponding to the lumen (red), secondary (S) layer (green) and middle lamella (blue).

Based on the results of k-means clustering, 20 individual spectra were randomly selected from the S layer and the middle lamella for principal component analysis (PCA) (Figure 2.7). PCA further helps identify the main vibrational bands responsible for variation between the S layer
and the middle lamella. The scores plot (Figure 2.7 (b)) for the first and second principal components (PC1 and PC2) show that there is separation between the spectra obtained from the middle lamella and those obtained from the S layer along the axis representing PC1, which is responsible for 75% of variation in the data. Separation along PC2, which is responsible for 4% of variation, is not observed. The loadings of PC1 (Figure 2.7 (c)) show that the major sources of variation occur at 1335 cm\(^{-1}\) and 1600 cm\(^{-1}\), corresponding to aryl ring vibrations in lignin, and at 2890 cm\(^{-1}\) corresponding to C-H stretching in cellulose. It is interesting to note that the results showed less variation in the cellulose band around 1100 cm\(^{-1}\) based on the small PC 1 loading observed for this band, which agrees with the k-means clustering result.

Figure 2.7: Principal component analysis of the Raman spectra obtained from the middle lamella and S layer in normal wood. (a) Average of the 20 selected spectra selected from the middle lamella and the S layer. (b) Principal component scores representative of the middle lamella (blue) and the S layer (red). (c) Loadings of the first principal component.
2.4 Characterization of the Cell Walls of Tension and Opposite Wood

2.4.1 Chemical Characterization

The effect of gravity on the plant cell wall was studied using reaction wood as a model system. To test this effect, we studied the plant cell wall in both the tension side and the opposite side of a Poplar tension wood cross section (Figure 2.1). The aim here is to highlight any alteration in the biopolymer constituents (cellulose and lignin) in the cell walls. We compare the composition of the tissues in different areas of the cross section, including inner and outer rings, and the pith (as labeled in Figure 2.1). Here we first explore the structure and composition of the cell layers in the outer region of the section.

![Raman mapping and cellulose and lignin distribution](image)

Figure 2.8. Raman mapping and cellulose and lignin distribution in the outer region of a Poplar cross section. (a,b) Maps of the peak intensity (\(I_{1100}\)) at 1100 cm\(^{-1}\) representative of cellulose in the tension (a) and opposite (b) side of the section. (c,d) Distribution of peak position (c) and peak width (d) for the cellulose band in the range 1095-1108 cm\(^{-1}\). (e,f) Maps of the peak intensity (\(I_{1600}\)) at 1600 cm\(^{-1}\) representative of lignin in the tension (e) and opposite (f) side of the section. (g,h) Distribution of peak position (g) and peak width (h) for the lignin band in the range 1600-1608 cm\(^{-1}\).
In Figure 2.8, the distributions of cellulose and lignin in the cell walls of the outer region of the tension and opposite sides of the cross sections were calculated based on the Raman spectra obtained in both sides of the stem cross section. All measurements were carried out with the same configuration and settings. The maps presented in Figure 2.8 (a, b) were constructed by calculating the intensity at the peak maximum (~1100 cm\(^{-1}\)) corresponding to the vibration of β-(1-4)-glycosidic bond in cellulose. A shift in the position of this peak is found from 1102 cm\(^{-1}\) in the tension side to 1099 cm\(^{-1}\) in the opposite side, and peak width from 42 cm\(^{-1}\) in the tension side to 40 cm\(^{-1}\) in the opposite side. The shift is above the spectral resolution of the instrument. This highlights variations in the cell wall cellulose structure between the tension and opposite sides. Similar shifts are observed in the lignin peak position (1602 cm\(^{-1}\) in the opposite side to 1605 cm\(^{-1}\) in the tension side) and width (from 33 cm\(^{-1}\) in the opposite side to 31 cm\(^{-1}\) in the tension side) of the band characteristic of lignin around 1600 cm\(^{-1}\) (C=C in the aromatic ring). These changes could indicate that the lignin structure or environment at the molecular level is different between the tension and opposite sides. Additionally, the maps constructed by calculating the maximum peak intensity of the Raman band at 1600 cm\(^{-1}\) (Figure 2.8 (e,f)) indicate that lignin is more concentrated in the middle lamella and cell corners.
Figure 2.9. Higher resolution confocal Raman mapping of a tension wood cell wall. (a) Optical image of selected cells in the tension wood. (b, c) Maps representative of cellulose (b) and lignin (c) constructed by calculating the intensity of their respective Raman peak at 1100 cm\(^{-1}\) and 1600 cm\(^{-1}\). (d) Average spectra of the regions identified by k-means clustering (four clusters). Map (inset) illustrating the overlay of the 4 clusters corresponding to G layer (red), secondary (S1, S2) layer (green, brown) and middle lamella (blue).

To better understand the shifts in cellulose and lignin characteristic peak position and width observed in Figure 2.8, we acquired a higher resolution map of the plant cell wall composition on the tension side using a 100x objective (Figure 2.9). The distribution of cellulose in Figure 2.9
(b) and lignin in Figure 2.9 (c) is in good agreement with the maps in Figure 2.8 (e,f) in that lignin concentration is the highest in the lamella and cell corner while cellulose is more prominent in the S and G layers.

The average spectra calculated from each cluster reveal variations in cellulose content and cellulose to lignin ratio between the layers. The results confirm that the G layer (red in Figure 2.9 (d)) is very rich in cellulose as seen from the large peak in the 1050-1150 range cm\(^{-1}\), which can also be assigned to hemicellulose\(^{120,125}\), and rather low lignin signal around 1600 cm\(^{-1}\). On the other hand, the middle lamella presents a large peak around 1600 cm\(^{-1}\), indicative of higher lignin content. The bands at 1335 cm\(^{-1}\) and 1380 cm\(^{-1}\) are indicative of bending vibrations resulting from cellulose\(^{98}\) but the peak at 1335 cm\(^{-1}\) may also be attributed to aryl-OH or aryl-OCH\(_3\) vibrations in lignin\(^{88,98,122}\). The cellulose band intensity at ~ 1100 cm\(^{-1}\) in the lamella is the lowest of all layers. The secondary wall reveals two different signatures with the region closest to the lamella showing strong peaks for lignin and cellulose, in addition to a strong peak at 1335 cm\(^{-1}\). The region closest to the G layer shows a much lower peak at 1335 cm\(^{-1}\) and 1600 cm\(^{-1}\), which suggests a larger contribution from lignin to the 1335 cm\(^{-1}\) band.

The properties of tension wood were further investigated by performing PCA on the spectra extracted from the Raman image (Figure 2.10). In contrast to k-means clustering, PCA differentiates three layers: the middle lamella, the S layer, and the G layer. The advantage of k-means clustering in this study is the ability to overlay the clusters identified with the plant cell wall image. The average spectra corresponding to each layer are presented in Figure 2.10 (a). Two predominant PCA components are identified: PC1 accounts for 58% of the variations, while PC2 accounts for 31%. Figure 2.10 (b) shows that the data is grouped into three main clusters.
corresponding to three regions of the cell wall. The G layer is clearly separated from the S and middle lamella by PC1, confirming large variation in the Raman spectra with the biggest variations found at 2900 cm\(^{-1}\), 1600 cm\(^{-1}\), and 1335 cm\(^{-1}\) (Figure 2.10(c)). The loadings and the scores for PC1 confirm the presence of larger amounts of lignin in the S and ML layers and larger concentration of cellulose in G.

Figure 2.10. Principal component analysis (PCA) of the Raman spectra obtained on the G, S and ML layers of the tension wood. (a) Average Raman spectra, (b) Principal component scores of PC1 and PC2 representing cluster for G (black), S (red), and ML (blue). (c) PC1 loadings with respect to relative Raman shift.

Next, the distinct signature of the S and G layers is explored by selecting spectra from the two regions marked by the masks (light blue) in Figure 2.11 (a,b). The distribution of peak positions and widths for cellulose at 1100 cm\(^{-1}\) (Figure 2.11(c,d)) and 1380 cm\(^{-1}\) (Figure 2.11(e,f)) are plotted for the G layer and the S layer. Significant shifts in the position and width of both characteristic Raman bands are observed. The peak around 1380 cm\(^{-1}\) corresponds to HCC, HCO, and HOC bending in cellulose and hemicellulose\(^{92}\). The increase in the position of the center of the distribution from 1379 cm\(^{-1}\) in the S layer to 1382 cm\(^{-1}\) in the G layer is significant since the spectral resolution is less than 3 cm\(^{-1}\). This shift may be explained by a change in the
environment (such as residual stresses) surrounding these molecules, or the orientation of the cellulose microfibrils\textsuperscript{96}. For instance, lignin is present in a greater amount in the S layer than in the G layer\textsuperscript{54}. The changes observed in the 1100 cm\textsuperscript{-1} peak width with a much narrower peak in the G layer than in the S layer, suggest different properties of the cellulose microfibrils in the S and G layers. Similar variations have previously been attributed to changes in crystallinity\textsuperscript{99,126}. Additionally, changes in cellulose characteristic peak positions have been attributed to changes in stress and strain in the microfibrils\textsuperscript{127,128}, as well as orientation\textsuperscript{97}.

Figure 2.11. Comparison of the cellulose Raman bands in S and G cell wall layers in tension wood. Areas marked in blue in the G layer (a) and S layer (b) represent the positions from which the spectra are extracted to construct the histograms in (c-f). (c,d) Distribution of the 1100 cm\textsuperscript{-1} peak position (c) and width (d). (e, f) Distribution of the 1380 cm\textsuperscript{-1} peak position (e) and width (f).
To shed more light on the potential variations in cellulose structure, we calculated crystallinity based on the univariate model developed by Agarwal\textsuperscript{99} (see section 2.2 and Table 2.2). The method was first tested on two controls: bacterial cellulose and microcrystalline cellulose (Avicel PH 101, 50 μm particle size, moisture between 3-5%). The respective calculated values, 72.4% for bacterial cellulose and 59.1% for Avicel, are found to be slightly lower than values previously reported by Agarwal\textsuperscript{99} but in the same order of magnitude. In addition, the values are in agreement with Park et al. who used a combination of XRD peak deconvolution and nuclear magnetic resonance (NMR) to calculate cellulose crystallinity\textsuperscript{119}. Our calculations reveal that cellulose crystallinity varies between the S and G layers in the tension side. The G layer exhibits the highest crystallinity with ~ 86% compared to ~ 68% in the S layer and the ML. Overall cellulose crystallinity of the G layer is higher than Avicel PH101 and bacterial cellulose. In opposite and normal wood, it is observed to be slightly lower on average. However, the variation within each layer is significant, which is natural due to the localized nature of the measurement. A qualitative comparison of the values measured in the G, S and ML layers in tension wood tissues is possible by plotting the peak intensity ratio between the cellulose peaks at 380 cm\textsuperscript{-1} and 1100 cm\textsuperscript{-1} (I\textsubscript{380}/I\textsubscript{1100}) as shown in Figure 2.12. The plot illustrates that a higher intensity ratio on average is indicative of more crystalline cellulose, as in the G layer.
Table 2.2. Cellulose crystallinity in plant cell walls of tension wood: G layer, S layer, and ML.

Control samples include bacterial cellulose and Avicel PH101

<table>
<thead>
<tr>
<th></th>
<th>$I_{300}/I_{1100}$</th>
<th>% Crystallinity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Avicel PH 101</td>
<td>0.413 ±0.09</td>
<td>59.1 ±12.9</td>
</tr>
<tr>
<td>Bacterial Cellulose</td>
<td>0.499 ±0.05</td>
<td>72.4 ±7.2</td>
</tr>
<tr>
<td>G layer (tension wood)</td>
<td>0.59 ±0.1</td>
<td>86.1 ±13</td>
</tr>
<tr>
<td>S layer (tension wood)</td>
<td>0.47 ±0.07</td>
<td>67.9 ±10</td>
</tr>
<tr>
<td>Middle Lamella (tension wood)</td>
<td>0.47 ±0.07</td>
<td>67.9 ±10</td>
</tr>
<tr>
<td>S layer (opposite wood)</td>
<td>0.46 ±0.07</td>
<td>66.4 ±10</td>
</tr>
<tr>
<td>Middle Lamella (opposite wood)</td>
<td>0.46 ±0.07</td>
<td>66.4 ±10</td>
</tr>
<tr>
<td>S layer (normal wood)</td>
<td>0.449 ±0.04</td>
<td>64.7 ±5.8</td>
</tr>
<tr>
<td>Middle lamella (normal wood)</td>
<td>0.451 ±0.05</td>
<td>65 ±7.2</td>
</tr>
</tbody>
</table>
Figure 2.12. Distribution of the intensity ratio between the cellulose peaks at 380 cm$^{-1}$ and 1100 cm$^{-1}$ ($I_{380}/I_{1100}$) in the G layer (red), S layer (blue), and ML (black) of tension wood. Cellulose crystallinity of the mean intensity ratio is indicated for each layer.

Next, we explored the differences between the cellulose and lignin signatures in the tension and opposite sides. The ratio $I_{1600}/I_{1100}$, with $I_{1600}$ the peak intensity of the lignin band at 1600 cm$^{-1}$ and $I_{1100}$ the peak intensity of the cellulose band at 1100 cm$^{-1}$, is calculated in the two regions. The results are presented in Figure 2.13. The center of the distribution varies from 1.6 for the opposite side to 0.5 for the tension side, which agrees with the presence of a thick G layer mainly composed of cellulose in the tension wood cell walls. This result could also suggest that the cellulose or lignin properties vary in the S layer and/or ML between the tension side and the opposite side.
Figure 2.13. Distribution of the ratio of $I_{1600}/I_{1100}$ calculated for the tension side (red) and opposite side (black) with all cell wall layers combined, in the xylem outer layer.

In order to more closely examine these potential differences, we analyzed the cellulose and lignin peaks in spectra acquired from the S layer, and compare the results between the tension and opposite sides. In doing so, we find a peak shift from ~1099 cm$^{-1}$ in the opposite to ~1103 cm$^{-1}$ in the tension side (Figure 2.14(a)), which is larger than between the S (~1102 cm$^{-1}$) and G (~1103 cm$^{-1}$) layers in the tension side. For lignin, the center position shifts from ~1599 cm$^{-1}$ in the S layers of the opposite side to ~1605 cm$^{-1}$ in tension side, as shown in Figure 2.14(b). This indicates variations in the lignin structure and interactions with the surrounding. The changes observed may contribute to different S/G ratios or different arrangements of the cellulose fibers. Lastly, for the ML, noticeable shifts in lignin peak position are observed as shown in Figure 2.14(d). Overall, these results show that the differences between the tension and opposite sides observed in Figure 2.8 not only arise from the existence of the G layer in the tension side but also from structural and compositional changes in the S and ML layers. To the best of our knowledge, this aspect of studying variations between the tension and opposite sides has not been reported.
previously. Table 2.3 summarizes the positions and widths of the characteristic cellulose and lignin peaks measured in the wood experiencing mechanical stress during growth.

Figure 2.14. Variations in cellulose and lignin in the S (a,b) and ML (c,d) layers of the cell wall in tension (red) and opposite (black) wood. Peak position distributions for characteristic cellulose band around 1100 cm$^{-1}$ (a,c) and lignin around 1600 cm$^{-1}$ (b,d).
Table 2.3. Cellulose and lignin peak positions and widths for the different regions of the stem described in this study.

<table>
<thead>
<tr>
<th>Material</th>
<th>Cross Section Side</th>
<th>Peak Position (cm⁻¹)</th>
<th>Peak Width (cm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All Layers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cellulose</td>
<td>Opposite</td>
<td>1099</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1102</td>
<td>42</td>
</tr>
<tr>
<td>Lignin</td>
<td>Opposite</td>
<td>1602</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1605</td>
<td>31</td>
</tr>
<tr>
<td>G Layer</td>
<td>Cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1103</td>
<td>41</td>
</tr>
<tr>
<td>S Layer</td>
<td>Cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Opposite</td>
<td>1100</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1103</td>
<td>48</td>
</tr>
<tr>
<td>Lignin</td>
<td>Opposite</td>
<td>1600</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1605</td>
<td>38</td>
</tr>
<tr>
<td>Middle Lamella</td>
<td>Cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Opposite</td>
<td>1099</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1103</td>
<td>55</td>
</tr>
<tr>
<td>Lignin</td>
<td>Opposite</td>
<td>1600</td>
<td>32</td>
</tr>
<tr>
<td></td>
<td>Tension</td>
<td>1605</td>
<td>41</td>
</tr>
<tr>
<td>Normal Wood: All Layers</td>
<td>Cellulose</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1094</td>
<td>43</td>
</tr>
<tr>
<td>Lignin</td>
<td>N/A</td>
<td>1598</td>
<td>34</td>
</tr>
</tbody>
</table>

We further studied lignin by calculating the ratio of S to G lignin (or S/G ratio) in the cell walls based on assignments of Aryl-OH and Aryl-OCH₃ around 1335 cm⁻¹ in S lignin and Aryl-OCH₃ around 1280 cm⁻¹ in G lignin. Next, we compare the intensity ratio I₁₃₃₅/I₁₂₈₀ after background subtraction. We note that although the ratio calculated is not a direct measurement of the actual S/G ratio, it has been previously shown that the two quantities are linearly correlated. The results, presented in Figure 2.15, unveil significant differences between the cell walls of the tension, opposite and normal wood.
In normal wood, the S/G ratio is lower than in tension wood. However, when comparing the S/G ratio between the tension and the opposite side of the reaction wood section, we find that in the inner region, the opposite side has a higher S/G ratio whereas in the outer region, the tension side has a higher S/G ratio. This could be correlated to previous results, showing a shift in peak positions in opposite direction for cellulose and lignin in the outer region (Figure 2.16).

All aforementioned comparisons were also carried out in the outer region closer to the edge of the cross section. Similar results are observed with shifts in peak position and width for both lignin and cellulose in the inner region, while smaller variations are observed in peak position for both cellulose and lignin in the pith. However, a significant change in peak width is observed between the tension and opposite sides in the pith layer, suggesting that the biopolymers exhibit varying properties between the tension and opposite sides even in the pith (Fig. 2.17).

Figure 2.15. S/G ratio between the tension (red) and opposite (black) sides compared with normal wood (blue). (a) The distribution of S/G ratio in the xylem inner layer of tension and normal wood sections. (b) The distribution of S/G ratio in the xylem outer layer of tension and normal wood sections.
Figure 2.16: Comparison of cellulose and lignin peaks position and width distributions in the outer (a-d) and inner regions (e-h). (a,b) Cellulose peak position and width distribution in the tension and opposite sides in the outer region. (c,d) Lignin peak position and width distributions in the tension and opposite sides in the outer region. (e,f) Cellulose peak position and width distributions in the tension and opposite sides in the inner region. (g,h) Lignin peak position and width distributions in the tension and opposite sides in the inner region. [adapted from Soliman et al.129].
Figure 2.17. Cellulose and lignin distribution in cell walls in the pith. (a,b) Maps of the peak intensity (I_{1100}) at 1100 cm\(^{-1}\) representative of cellulose in the tension (a) and opposite (b) sides of the section. (c,d) Distribution of peak position (c) and peak width (d) for the cellulose peak in the range 1095-1105 cm\(^{-1}\). (e,f) Maps of the peak intensity (I_{1600}) at 1600 cm\(^{-1}\) representative of lignin in the tension (e) and opposite (f) sides of the section. (g,h) Distribution of peak position (g) and peak width (h) for lignin in the range 1596-1608 cm\(^{-1}\).

2.4.2 Nanoscale Properties of Tension Wood

Thus far Raman spectroscopy has revealed chemical and structural changes, such as crystallinity of cellulose, in the cell walls across the stem; however, a tool that can help visualize these changes at the nanoscale remains highly desirable. Confocal Raman microscopy suffers from the optical diffraction limit, restricting its lateral resolution. To overcome this challenge, we consider
a platform capable of measuring nanoscale variations in mechanical or chemical properties observed with functional AFM (AFAM and nanoIR, see section 2.2).

AFAM maps of the local variations in mechanical properties or subsurface features has previously been reported. Figure 2.18 (e), representing the AFAM phase signal, highlights the details unveiled with nanoscale imaging, well beyond the resolution achieved with confocal Raman spectroscopy or standard AFM (Figure 2.18 (b,c)).

Figure 2.18. Nanoscale investigation of mechanical properties with AFM and AFAM. (a) Topography image of the tension Poplar wood cross section. (b and c) High resolution topography images. (d and e) Corresponding AFAM phase images obtained for the higher resolution topography maps in (b) and (c), respectively.
Figure 2.19. (a) Distribution of the phase value extracted from the S (red) and G (blue) layer in the AFAM phase map in Figure 2.18 (e). (b) Comparison in phase variation across nanoscale features in the cell walls of the S layer (red) and G layer (blue) along the lines in Figure 2.18 (e).

Interestingly, a comparison of the phase measurements recorded across the S and the G layer suggests that the acoustic wave propagation is affected by the nature of the material. Figure 2.19 (a) shows the distribution of the phase measured using AFAM in the S and G layers in Figure 2.18 (e). The plot reveals a variation of around 0.4° in the center average of the phase measured, with both distributions exhibiting roughly the same width. Figure 2.19 (b) shows profiles extracted from the S and G layers along the section markers, red and blue lines indicated in Figure 2.18 (e). The curves show a variation in phase on the order of a few degrees within a 200 nm distance in both the S and G layers. This finding suggests that the phase signal in AFAM measurements is sensitive to local variations in structure and/or properties in each layer of the cell wall. The phase signal is particularly important in this measurement as it can relate to the contact stiffness and damping in the tip-sample system\textsuperscript{133}. In general, the phase increases with increasing contact stiffness, and decreases with increasing damping\textsuperscript{133}. This suggests that AFAM
is a promising tool to monitor the viscoelastic properties of individual plant cell wall layers at the nanoscale.

Figure 2.20: Nanoscale infrared spectroscopy measurement using nanoIR. (a) Topography image of a cell wall on the tension side. (b) Image of the amplitude of cantilever response at the contact resonance. The image is acquired at the region marked by the white square in (a). (c) Image of the contact resonance frequency acquired at the same region. Both images in (b) and (c) were acquired at wavenumber (ν) = 1640 cm⁻¹. (d) nanoIR spectra acquired at points 1 and 2 marked in (b). (e) First derivative of the nanoIR spectra shown in (d). (f) Contact resonance spectra acquired at points 1 and 2 marked in (c).

Lastly, while AFM can give valuable structural information and AFAM has promising potential in investigating the physical properties of soft materials at the nanoscale, they cannot give direct chemical information at this resolution. However, it is highly desirable to obtain chemical
information at the nanoscale. To address this shortcoming, the chemical characterization capability of infrared spectroscopy with the nanoscale spatial resolution of the AFM has been developed in the form of nanoIR\textsuperscript{112}. Here, we used nanoIR to investigate the chemical properties within a S and G layer of the tension side. The maps of the cantilever response amplitude and contact resonance frequency at a fixed laser wavenumber (1640 cm\textsuperscript{-1}, which corresponds to OH bending vibrations adsorbed on cellulose)\textsuperscript{134,135} are presented in Figure 2.20. Furthermore, IR single spectra were acquired at locations exhibiting low and high amplitudes indicated by points 1 and 2 in Figure 2.20 (b). The amplitude of the resonance was measured at fixed pulsed laser frequency of 173 kHz (Figure 2.20 (d)). The variations in the IR map indicate chemical inhomogeneity within the S layer; however, the IR spectra shown in Figure 2.20 (d) acquired from points 1 and 2 do not exhibit significant variation in the IR signature. To confirm this, we obtained first derivatives of the IR spectra (Figure 2.20 (e)) which again do not show variation in the chemical signature between points 1 and 2. On the other hand, the contact resonance measured at points 1 and 2 (Figure 2.20 (f)) showed a clear shift from 175 kHz in point 1 to 173 kHz in point 2, which is indicative of a higher local stiffness in the plant cell wall at point 1 than at point 2 since a higher contact resonance frequency corresponds to greater local stiffness. One possible explanation for this result is that the nanochemical measurement includes photothermal expansion of a larger volume underneath the AFM tip, which means that the measurement is actually detecting an average contribution of highly heterogeneous materials in the volume, whereas the nanomechanical measurement involves just the local interaction between the tip and the sample at the point being measured without displacement of the point measured. These findings suggest that AFAM may be influenced by chemistry and/or stiffness, but the two cannot
be readily decorrelated at this point. Nonetheless, this approach paves the way to a deeper understanding of individual cell wall layers composition and properties in plant tissues.

2.5 Summary

Confocal Raman microscopy and statistical analysis reveal variations in cellulose and lignin properties and their distributions in the tension, opposite and normal wood. These features could not be resolved without sub-micrometer microscopy capabilities. Using k-means clustering, the different layers of the cell wall on the tension side can be identified, and variations in the peak position and width of two of the characteristic cellulose Raman bands are observed in the different layers. The S/G ratio for lignin is estimated to be higher in tension wood than in normal wood which is in agreement with previous studies. The crystallinity of cellulose within the cell wall layers, estimated from the Raman spectra, reveals that G layers exhibit the highest crystallinity while cellulose in the S layer and the ML of tension wood is found to be slightly more crystalline than in opposite and normal wood.

These results show that we can monitor variations in the composition and structure of the cell walls of plants that experienced mechanical stress using spatially resolved spectroscopy techniques. We also show that it is possible to monitor nanoscale mechanical properties using advanced AFM-based methods such as AFAM and nanoIR. This enables characterization of properties within the individual cell wall layers. One limitation encountered in using nanoIR is the fact that the QCL pulsed laser excites a larger volume in the sample, which hinders the measurement of nanoscale chemical composition, especially in a complex system as the plant cell wall. The nanomechanical measurement, on the other hand, involves effects that are
localized to where the tip is in contact with the sample, and is therefore more sensitive to nanoscale variations within the cell wall layers.

One challenge that remains is to link the nanoscale variations to the changes observed at the layer-scale (~ few micrometers). An interesting future study is to use AFAM to monitor nanoscale mechanical property changes within the S layer in tension wood, and to determine if there is any correlation between these changes and the shifts in cellulose or lignin Raman peak position and width observed in Figure 2.14.

In the long term, these studies can significantly aid in materials design applications for bioenergy and added value products manufacturing from lignocellulosic biomass. The ability to control the properties of plant cell walls at the nanoscale can enable the design of more favorable structure and composition to enhance the efficiency of biomass deconstruction. This path holds promise in terms of sustainability and protecting the food-energy-water nexus.
3 EFFECTS OF CHEMICAL STRESS IN PLANTS – THE EXAMPLE OF PESTICIDES IN CITRUS

3.1 Background

Plants can experience chemical stresses from the multitude of sources of artificial chemicals and pesticides applied on an industrial scale to crops around the world. The effects of chemicals used to treat plants, prevent or eradicate plant diseases and promote crop yields is an important topic of research due to its implications on the environment and safety of consumers. The use of nanoparticles is especially significant. For instance, carbon-based fullerol nanoparticles have been shown to increase bitter melon fruit yields by up to 59%, and the use of ceria nanoparticles in wheat showed a 12.7% improvement in shoot biomass and a 36.6% improvement in grain yield. As indicated in the examples, nanoparticles can induce stresses which can potentially cause fundamental changes in the plants properties. In this chapter, we address the issue of chemical stress in plants. We focus on an application relevant to sustainable agriculture. More specifically, we address the need to combat plant diseases that are severely reducing citrus crop yields.

To address the basic necessity of providing food for the world’s growing population, the importance of increasing the efficient use of arable land will continue to grow in coming years. The goal is to double the production of food worldwide by 2050. To achieve this, significant obstacles shall be overcome, such as eradicating epidemics of plant diseases and pest infestations, limiting overuse of pesticides with harmful side effects on the environment, managing soil erosion, and addressing declining biodiversity and desertification due to climate change and other threats.
Over the years different methods have been used to cope with these challenges such as improving soil quality\textsuperscript{141} and efficient water use\textsuperscript{142}, as well as enhancing crop yields with fertilizers\textsuperscript{143} and pesticides\textsuperscript{144}. Nutrients have also been used to improve agricultural production either by soil application to improve soil fertility\textsuperscript{141,145} or by foliar application to the plant directly\textsuperscript{146-149}. In fact, nitrogen supply alone is responsible for 30-60\% of yield in crops such as grain, corn, and cotton. Researchers also claim that this figure would be even higher if other nutrient inputs such as phosphorous or potassium were factored in\textsuperscript{143}.

As beneficial as nutritious fertilizers are for agricultural production, they provide limited support for plant disease treatments. A number of pathogens continuously threaten crops, putting food production in jeopardy around the world\textsuperscript{61}. Bacterial infections such as \textit{pseudomonas syringae} pathovars and \textit{Ralstonia Solanacearum}, both of which infect a variety of plant species including tomatoes\textsuperscript{150,151}, potatoes\textsuperscript{152}, and tobacco\textsuperscript{153,154}, have caused significant economic losses to the agricultural industry\textsuperscript{61}. \textit{Ralstonia Solanacearum} has caused losses of \textasciitilde\textcurrency{}1 billion per year in potato crops alone. This particular pathogen is challenging due to its adaptability, variation in strains, and the wide range of plants it can infect, making potatoes unusable for potato-based products. \textit{Ralstonia Solanacearum} is a soil-borne pathogen and it infects the plant by entering through wounds or cracks in the root, then translocating systemically to the shoot system of the plant where it colonizes the xylem tissue\textsuperscript{61}. Xylem and phloem-restricted pathogens constitute an important threat to food production\textsuperscript{155-157}, due to the difficulty associated with detecting the early onset of the disease, as well as the lack of efficient methods to directly target (control or eradicate) the bacteria inside the plants using existing pesticides. Another systemic disease, called Citrus Greening and also known as Huanglongbing (HLB)\textsuperscript{158}, constitutes our case study here. HLB, which infects the phloem of citrus plants, has been decimating citrus crops in Florida,
and other regions of the world such as Brazil and Asia\textsuperscript{159}. There are also alarming signs the disease is spreading to California and Texas. In California, the first detection of HLB occurred in 2013, and until now the disease does not seem to have spread to all citrus groves\textsuperscript{160}. In Texas, the disease was first detected in 2012\textsuperscript{161}, and is already spreading at an alarming rate. In Florida, HLB is caused by the bacterial species \textit{Candidatus Liberibacter asiaticus} (\textit{CLas}). The bacteria are transferred to the phloem of the citrus plants through a vector, the Asian Citrus Psyllid (\textit{ACP})\textsuperscript{162}, an insect that feeds on the sugar transported through the phloem by extending a stylet which can penetrate into the vascular system of the leaves. During the feeding process, \textit{ACP}s can inject the bacteria into the phloem, as shown in Figure 3.1. The pathogens can rapidly transfer from the leaves phloem to the roots\textsuperscript{163}. As the bacterial colonies grow, the transport of important nutrients and water is hindered, slowly causing lower fruit yield with a sugar to acid content unsuitable for consumption. Overall, HLB has caused a US$7.8 billion loss in Florida’s citrus industry alone between 2007 and 2016\textsuperscript{164}.
Figure 3.1. (a) Representation of a psyllid feeding on sugar in the phloem\textsuperscript{165}. (b) Fluorescence image of psyllid stylet sheath left behind which shows the path the stylet takes to reach the phloem\textsuperscript{166}. Scale bar in (b) represents 50 μm.

A number of methods have been and continue to be considered to combat HLB to varying degrees of success. Thermal treatment (or thermotherapy) has initially shown some promise\textsuperscript{167}. Best management practices and quarantining of infected regions have helped in sustaining some citrus production; however, as is the case with the former methods, none of these solutions directly target and eliminate the disease inside the plants. Breeding and genetic engineering have also showed some improvement in the resistance of the plants to the disease but will likely require several years before the technology becomes fully matured\textsuperscript{168,169}. All these methods are very costly to the growers.

In the meantime, growers are using a variety of insecticide products to control ACP\textsuperscript{170}, as well as antibiotics to control HLB\textsuperscript{171,172}. Conventional insecticides targeting ACP have showed some
promise\textsuperscript{162,170}. For instance, field trials by researchers at the University of Florida’s Institute of Food and Agricultural Sciences (UF-IFAS) yielded a guide for growers to apply several commercial insecticides such as Imidacloprid and Fenpropathrin\textsuperscript{173}. However, it has been recently shown that ACP already developed resistance to Imidacloprid, Fenpropathrin, as well as other key insecticides in Florida\textsuperscript{174}. Moreover, these insecticides can control the vector, but do nothing to the bacteria colonizing the vascular system of the plants\textsuperscript{170}. Antibiotics have been considered to act systemically by directly targeting the bacteria through direct injection\textsuperscript{172}. However, it was shown that the antibiotics do not reach the target vascular tissue at a concentration high enough to kill the bacterial\textsuperscript{171}.

With these considerations in mind, a new nanoparticle-based treatment was designed by Dr. Swadeshmukul Santra at the University of Central Florida\textsuperscript{175}. The vision for this treatment was to overcome the limitation of standard pesticides by allowing systemic movement of bactericidal actives in the plant. To achieve this, the core particles should maintain a diameter below 5 nm to cross the natural barriers inside the plant, such as the casparian strip which blocks the apoplastic route of transport\textsuperscript{176}. In addition, to circumvent resistance developing for copper (Cu) in bacterial colony, the treatment was developed with ZnO, which has also been shown to have antibacterial properties\textsuperscript{177-179}, but is used less commonly than Cu. Pathogens are still not resistant to Zn. The ZnO-based nanoparticle treatment was dubbed Zinkicide\textsuperscript{TM}\textsuperscript{175}. Figure 3.2 shows some of the favorable attributes of Zinkicide\textsuperscript{TM}. The formulation developed by Santra’s lab\textsuperscript{175} has already shown some promise in combatting another plant disease, Citrus Canker\textsuperscript{180}. Three modes of application have been considered for this treatment to date: foliar, root uptake, and trunk injection (Figure 3.3).
Figure 3.2. Attributes of Zinkicide™ that have been demonstrated to date.
In addition to its antibacterial activity, Zn is considered as an important element with several significant roles in plants\textsuperscript{181,182}. In wheat plants, it was found that the uptake of Zn is a carrier-mediated active process, and that Zn reaches the shoot part of the plant within 3 hours of root uptake\textsuperscript{183}. The uptake and effects of Zn and ZnO nanoparticles are also significant due to their potential use to tackle issues in agricultural production\textsuperscript{177,184-186}. In cucumber, corn, lettuce, and radish, it was found that \textasciitilde35 nm Zn and \textasciitilde20 nm ZnO nanoparticles reduce growth of roots and shoots when applied at a concentration of 2000 mg/L\textsuperscript{177,187}. Conversely, \textasciitilde1.2 \textasciitilde6.8 nm oblate spherical and hexagonal ZnO nanoparticles, applied at 10 mg/L concentration, increased the growth of roots and shoots in clusterbean\textsuperscript{188}.

Due to the nanoparticulate form of the active ingredient of Zinkicide\textsuperscript{TM}, an extensive process for regulation and approval by the Environmental Protection Agency (EPA) before a product can be

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**Figure 3.3.** Representation of the different modes of application of the treatments
commercialized for agricultural use is required. A second Zn-based treatment was designed by Dr. Santra’s team to address the time sensitive issue of citrus greening without nanoparticles. This second treatment is called Multi-functional Surface Subsurface Systemic Therapeutic (MS3T). MS3T is made of three components that are either regulated by the EPA, recognized as safe by the Food and Drug Administration (FDA), or naturally occurring. These components are: Kaolin clay, Quaternary ammonium compound (Fixed-quat), and a Ternary Solution (TSOL) that consists of a Zn-chelate compound formed by a complex of Zn ions, urea, and hydrogen peroxide. Clay is meant to protect the surface of the leaves by providing a repellant to relieve the pressure from insects such as ACPs. The quaternary compound is meant to act as a locally systemic active, by topical bacterial treatment in the stomatal regions. Fixed-quat is non-phytotoxic and the aim is to immobilize it onto a solid support (leaf) to maintain its antibacterial efficacy. TSOL is designed to be the systemic component of MS3T that shall enter the leaves and translocate through the vascular system to target the bacteria inside the phloem. Based on this design, MS3T should be sprayed on the leaves. Similar approaches aiming at improvements in agricultural production have been reported with foliar application to combat phytopathogens. Grain yield in cereals could be improved with urea, this was partly attributed to the good source of nitrogen brought to the plant. Disease symptoms were also significantly reduced in melon using foliar spray treatments in the pre-flowering stage. Foliar application of silicon was shown to improve plant resistance to powdery mildew in various plant species, while foliar application of phosphates improved innate resistance to pathogens in plants such as cucumber, pepper, rice, maize, and grapevine.

In this chapter, the uptake of Zinkicide™ through the roots and the uptake of MS3T through the leaves will be discussed. Different aspects of uptake will be covered including where the
treatment goes inside the plants following increasing durations of exposure to the treatments. Additionally, the effects of the treatment on the plant tissue at the cellular and subcellular levels will be discussed. The aim is to answer the following questions:

1) Can the plants uptake the treatments through various routes of application (foliar and root uptake)?

2) Can the treatments reach the phloem where the target pathogens reside?

3) Can we track the uptake and translocation of the treatments inside the plant?

4) How fast does uptake occur?

5) What form do the treatments take inside the plant?

6) What are the effects of TSOL and Zinkicide™ on the plant tissue at the cellular and subcellular levels?

First, the chemical composition of both treatments is studied using infrared spectroscopy (Raman, FTIR). This also helps in determining the molecular structure of the active ingredients. The morphology of both treatments is also examined using electron microscopy at the nanoscale. For Zinkicide™ the stability is studied considering the requirement for long-term storage in warehouses. Two formulations of Zinkicide™ are considered: the initial research grade synthesized in Dr. Santra’s lab, and the agricultural grade developed in partnership with Trademark Nitrogen (TMN).

Next, the uptake and translocation of the active ingredients of Zinkicide™ and MS3T treatments in young citrus seedlings are examined. In order to study the different aspects concerning the uptake and internalization of the treatments, a sequence of characterization methods are used. To
test whether there are any variations in the chemical signature of the plant materials following uptake of Zinkicide™, infrared spectroscopy (Raman spectroscopy and Fourier Transform Infrared Spectroscopy (FTIR)) is performed. The distribution of the treatment is also examined to track its translocation throughout different segments of the seedling over time. X-ray fluorescence spectroscopy (XRF) is chosen for this measurement as it can test a large number of samples in a relatively short amount of time. XRF has already been used previously to detect Zn concentrations in planta201,202. It is also necessary to determine whether the treatments can reach the phloem where the bacteria reside. To achieve this, EDS combined with SEM imaging are used. Finally, the future implications of the study will be explored, and what still needs to be studied in order to obtain a more comprehensive knowledge about these treatments and their utility in handling HLB as well as their potential use in other plant systems and diseases.

3.2 Methods

3.2.1 Analytical methods

**Infrared Spectroscopy:** Raman spectroscopy measurements were conducted using a WITec alpha 300 AR confocal Raman microscope. The integration time was set to 1 s per spectrum, with the laser power set to ~ 5 mW. Spectra were acquired in an array of 100 spectra across a region of 10 μm by 10 μm on each sample.

FTIR measurements (see Appendix B) were acquired on a Perkin Elmer Spectrum 100 FT-IR Spectrometer. The instrument is set to perform Attenuated Total Reflectance (ATR) measurements, and a diamond/ZnSe crystal is used. Spectral resolution was set to 4 cm⁻¹, and an iris aperture with a diameter of 8.9 mm was used. The force applied on solid samples to keep
them in contact with crystal was kept constant for all measurements. Three FTIR spectra per sample were acquired and averaged for data analysis.

Data was plotted and analyzed in OriginPro 8.5 software.

**SEM:** SEM imaging was performed on a Zeiss Ultra-55 FEG SEM. SEM images were acquired with beam energy of 10 kV, a working distance of 13 mm, and an electromagnetic lens aperture of 20 μm. Three different regions of the samples were imaged when examining the morphology and structure at various scales.

**EDS:** EDS measurements were also performed on the same Zeiss Ultra-55 FEG SEM system using a silicon drift X-ray detector (Noran 7 system). The beam energy used was 10 kV, and a working distance of 13 mm and aperture of 60 μm were used. The elemental analysis was captured between 0.6 kV and 10 kV.

**X-ray Fluorescence Spectroscopy (XRF):** XRF measurements were performed on a PANalytical Epsilon 1 Range instrument with software (Epsilon3), used to calculate the Zn percentage inside the sample as indicated in the standard protocols discussed by Norrish and Hutton. In brief, the concentration is calculated based on direct measurements of peak and background intensities for each element and correlating the result to the elements mass absorption coefficient. The instrument’s built-in software (PANalytical Omnian) is calibrated by the manufacturer to accurately estimate the background intensities for each element. This calibration model also accounts for natural variations in other elements present in the plants (such as Manganese or Iron) and the inter-elemental effects. However, the content of elements such as carbon, oxygen, and hydrogen is not accounted for. For accurate measurement, it is important to include the matrix material of the sample (in this case, it is cellulose since the samples are made mostly from
plant cell walls). This step is crucial as it allows the software to identify any radiation scattered at low energies as coming from cellulose. Therefore, a model was setup in the software which accounted for the matrix material by setting it to cellulose with the chemical formula: C6H10O5.

For each sample, three measurements were performed, and the average Zinc content was calculated.

3.2.2 Plant treatment and preparation methods

**Single leaf assay:** To test the movement of the treatments (Zinkicide™ and the systemic component of MS3T, TSOL) in the vascular system of the leaves, a simple leaf assay (Figure 3.4) was developed in which a single leaf was placed in the treatment solution in a small vial and left for the duration of uptake. Following uptake, the leaf tissue was sectioned for Raman and SEM/EDS studies on the midrib cross section to detect characteristic signatures of the treatments.

![Figure 3.4. Single leaf assay in which a leaf is cut from a seedling and placed in a vial filled with the treatment solution to test the active ingredient’s movement in the vascular system.](image)
**Whole seedling assay to test root uptake of Zinkicide\textsuperscript{TM}:** Seedlings were removed from the soil and the roots were cleaned, before being immersed in the treatment for a selected duration of uptake. At timepoints 6 h, 12 h, 18 h, and 24 h, the seedlings were sectioned at different locations for characterization. Figure 3.5 (a) shows the whole seedling assay setup. During treatment, the seedlings were placed inside a growth chamber in order to simulate the field conditions in Florida. The temperature of the growth chamber was set to cycle between 25 °C and 31 °C, and the humidity was set to cycle between 60% and 80%. Day/night cycles were also simulated.

**Setup to test foliar uptake of MS3T and systemic component TSOL:** The foliar uptake of MS3T as well as the systemic Zn active TSOL alone was tested to compare the uptake with and without the clay and Fixed quat components. Both treatments had a concentration of 1600 ppm metallic Zn. The treatments were sprayed at a rate of two sprays per leaf (one spray at the top and one at the bottom side of the leaf blade). After spraying, the seedlings were placed inside the growth chamber to match the conditions in the field. The growth chamber settings were as outlined in the previous section concerning root uptake of Zinkicide\textsuperscript{TM}. The seedlings were left inside the growth chamber for varying uptake durations: 2 h, 4 h, 6 h, 12 h, and 24 h. For each duration, three seedlings were treated, in addition to three control untreated seedlings. Following uptake, the plant leaves, stem, and roots from treated and untreated seedlings were washed in order to remove any residues from the surface of the plant materials. After washing, the plant material was left to dry in an oven at ~ 60 °C for 24 h. After drying, the plant material was ground into fine powder.
**Washing procedure:** The washing protocol involved extensive cleaning of the leaves, stem, and roots, to ensure removal of any residues remaining on the surface after foliar spray treatment since our aim is to quantify the uptake of the treatments. First, the plant material is rinsed gently in a mixture of 40 ml of 0.1% alconox in 2L of DI water. Next, the plant material is rinsed three times in pure RO water, while ensuring the water is discarded in-between each step. The plant tissue is then placed in 0.75M of HCL for 30 s prior to final rinsing in DI water. Following washing, the plants were left to dry overnight in oven at 60 °C.

**Powder preparation for spectroscopy:** Plant material was grinded into powder using a spice and nut grinder. Each sample was grinded for an equal duration to homogenize the size of the pieces in the powder (due to the lack of a Wiley mill).

**FTIR measurement on plant tissue:** FTIR measurement was performed on the powder samples. For each sample, at least five spectra were acquired. PCA was performed on the data using a specialized software (The Unscrambler X) following treatment of the data as follows: The FTIR spectra were first converted from transmittance to absorbance spectra. Baseline adjustment and mean normalization were then performed before PCA was run on the dataset.

**Tissue sectioning for imaging:** When uptake duration and washing (if necessary) were completed, each seedling was cut into four segments (Figure 3.5 (b)).

**SEM and EDS measurement on plant tissue:** To perform the measurement, first the leaf blade of four leaves obtained from the four segments was cut after flash freezing in liquid nitrogen to preserve the conditions inside the leaf. The sections were then coated with gold-palladium using a sputter-coater (Emitech K550) at 30 mA for 1 minute and 30 seconds. SEM imaging was done
with a 10 keV electron beam, a 60 μm electromagnetic lens aperture, and a 13 mm working distance.

![Image of seedling setup](image)

Figure 3.5. Setup for the uptake experiment. (a) Whole seedling assay setup. (b) The shoot part of each seedling is divided into four segments. (c) Sectioning the leaf blade to obtain leaf extract.

3.3 Characterization of Zinkicide™ and MS3T

3.3.1 Characterization of Zinkicide™

The initial formulation of Zinkicide™ investigated was pure research grade Zinkicide™ chemicals, and henceforth is referred to as Zinkicide™ SG6. During the initial stages of the project, Zinkicide™ SG6 was characterized using infrared spectroscopy (Raman spectroscopy, FTIR) in order to identify the chemical signature of its main components. The individual components and reagents used in synthesis were also tested to facilitate the assignment of the characteristic Raman and IR bands observed.
The Raman spectra of Zinkicide™ SG6 (Figure 3.6 (a)) show characteristic bands at 810 cm\(^{-1}\), 870 cm\(^{-1}\), 1050 cm\(^{-1}\), 1260 cm\(^{-1}\), 1390 cm\(^{-1}\), and 1630 cm\(^{-1}\). The band at 810 cm\(^{-1}\) is attributed to out of plane C-H deformations in sodium salicylate, which is used as a coating agent for the nanoparticles in this formulation. Bands from sodium salicylate are also observed at 1260 cm\(^{-1}\) corresponding to benzene ring vibrations, 1390 cm\(^{-1}\) corresponding to symmetric carboxylic group stretching, and 1630 cm\(^{-1}\) corresponding to asymmetric carboxylic group stretching\(^{205}\). The band observed at 870 cm\(^{-1}\) is attributed to O-O stretching in hydrogen peroxide, while the band at 1050 cm\(^{-1}\) is attributed to N-O stretching vibrations in nitrate\(^{206}\).

More recently, a modified formulation suitable for industrial scale production was adopted and dubbed Zinkicide™ TMN 110. This formulation was synthesized using agricultural grade reagents; however, the composition is protected by proprietary information. Two versions of this formulation were synthesized: TMN 110 and TMN 113. TMN 110 is the final agricultural grade formulation for Zinkicide™ including all ingredients and reagents used in synthesis. TMN 113 is excluding hydrogen peroxide. Zinkicide™ TMN 110 was characterized to identify its chemical signature with a view to future studies of its uptake and effects inside citrus plants, in addition to aging studies. The Raman bands corresponding to sodium salicylate(810 cm\(^{-1}\), 1020 cm\(^{-1}\), 1260 cm\(^{-1}\), 1390 cm\(^{-1}\), 1460 cm\(^{-1}\), 1630 cm\(^{-1}\), and 3070 cm\(^{-1}\)), hydrogen peroxide (870 cm\(^{-1}\)), and nitrate (1050 cm\(^{-1}\)) observed in Zinkicide™ SG6 are also observed in Zinkicide™ TMN 110 (Figure 3.6 (b)). The ratio of the peak intensity of hydrogen peroxide (870 cm\(^{-1}\)) to the peak intensity of nitrate (1050 cm\(^{-1}\)) is significantly lower in Zinkicide™ TMN 110 than in Zinkicide™ SG6. This suggests that a lower amount of hydrogen peroxide relative to nitrate is used in the synthesis of Zinkicide™ TMN 110 compared to Zinkicide™ SG6.
FTIR spectra obtained from both Zinkicide™ SG6 and TMN 110 also show some differences between the two formulations (Figure 3.7). Peaks assigned to sodium salicylate are observed at 740 cm⁻¹, 810 cm⁻¹, 860 cm⁻¹, 980 cm⁻¹, 1030 cm⁻¹, 1480 cm⁻¹, and 1580 cm⁻¹ for Zinkicide™ SG6 (Figure 3.7 (a)). Additionally, a strong broad band in the range of 1310 – 1380 cm⁻¹ is observed which corresponds to nitrate. Similar bands are observed in Zinkicide™ TMN 110 (Figure 3.7 (b)), but with shifts in peak positions. For example, the band at 740 cm⁻¹ corresponding to out of plane C-H deformation in salicylate shifts to ~ 760 cm⁻¹, and the band at 1580 cm⁻¹ corresponding to asymmetric carboxyl group stretching in salicylate shifts to ~ 1550 cm⁻¹. The band at 1480 cm⁻¹ observed in Zinkicide™ SG6 is not present in Zinkicide™ TMN 110. The reason behind the shifts in peak positions as well as the disappearance of the band at 1480 cm⁻¹ can be due to the fact that Zinkicide™ TMN 110 contains additional chemicals that could be interacting with the salicylate groups. The composition of Zinkicide™ TMN 110 is protected by proprietary information; however, the changes observed in FTIR and Raman spectra can shed some light on how the ingredients interact with one another, and possibly on the structure or defects present in the nanoparticles, which in turn can aid in optimizing the treatment for best performance in the future. Full Raman and IR peak assignments are summarized in Table 3.1.
Table 3.1. Raman and IR Peak Assignments for Zinkicide™ Components.

<table>
<thead>
<tr>
<th>Material</th>
<th>Peak Position (cm⁻¹)</th>
<th>Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Raman</td>
<td>IR</td>
</tr>
<tr>
<td>Sodium Salicylate</td>
<td>3070</td>
<td>2900 - 3070</td>
</tr>
<tr>
<td></td>
<td>1630</td>
<td>1550-1580</td>
</tr>
<tr>
<td></td>
<td>1460</td>
<td>1460 - 1480</td>
</tr>
<tr>
<td></td>
<td>1390</td>
<td>1370</td>
</tr>
<tr>
<td></td>
<td>1260</td>
<td>1280</td>
</tr>
<tr>
<td></td>
<td>1020</td>
<td>1030</td>
</tr>
<tr>
<td></td>
<td>810</td>
<td>810 - 900</td>
</tr>
<tr>
<td></td>
<td>Not Observed</td>
<td>700 - 760</td>
</tr>
<tr>
<td>Nitrate</td>
<td>1320</td>
<td>1310 - 1380</td>
</tr>
<tr>
<td></td>
<td>1050</td>
<td>1040 - 1050</td>
</tr>
<tr>
<td>Hydrogen Peroxide</td>
<td>870</td>
<td>830 - 840</td>
</tr>
<tr>
<td>Zinc Peroxide</td>
<td>840</td>
<td>400 - 450</td>
</tr>
</tbody>
</table>
Figure 3.6. Raman spectra of (a) Zinkicide™ SG6 and (b) Zinkicide™ TMN 110. Characteristic bands of hydrogen peroxide (870 cm\(^{-1}\)) and nitrate (1050 cm\(^{-1}\)) are present in both formulations. The ratio of peak intensity of hydrogen peroxide to nitrate is significantly lower in Zinkicide™ TMN 110.
Figure 3.7: FTIR spectra obtained from (a) Zinkicide™ SG6 and (b) Zinkicide™ TMN 110.

Significant variations in the IR spectra are observed between both formulations, for example the band at 740 cm\(^{-1}\) in Zinkicide™ SG6 shifts to 760 cm\(^{-1}\) with a much lower intensity in Zinkicide™ TMN 110.
Zinkicide™ TMN 110 and 113 were also studied using SEM imaging to observe their structures at the nanoscale. (Figure 3.8). The SEM images highlight how one ingredient, hydrogen peroxide, can affect the morphology of the aggregates at the nanoscale, with Zinkicide™ TMN 110 showing a round shape and Zinkicide™ TMN 113 showing a plate-like structure.

![SEM images of Zinkicide™ TMN 110 and TMN 113 aggregates](image)

Figure 3.8. SEM images of (a) Zinkicide™ TMN 110 and (b) Zinkicide™ TMN 113 aggregates. TMN 110 exhibited a round structure, whereas TMN 113 showed plate-like structures.

**Aging study for Zinkicide™ SG6**

Given that the formulations are meant to be stored for several months, we studied the stability and potency of the formulations – nanoparticles aging in reagents or in water- over the course of 12 weeks. This was achieved by acquiring Raman spectra of the solutions on a weekly basis for a period of 12 weeks. Figure 3.9 explains the set of solutions that were tested in the aging study and are discussed here.
Figure 3.9. The solutions tested in the aging study for Zinkicide™ SG6. The solutions were tested on a weekly basis including the original Zinkicide™ SG6 including the excess reagents, a control with all ingredients except Zinc nitrate, a washed solution left to age in water from the initial time point, and a washed solution that is obtained at each time point after aging in the reagents. Each solution was shaken prior to obtaining 10 μL droplets for measurement.

The aging of Zinkicide™ SG6 was studied by monitoring the ratio of the intensities of hydrogen peroxide (870 cm\(^{-1}\)) and water (3400 cm\(^{-1}\)) Raman bands in Raman spectra obtained from Zinkicide™ SG6 aging in excess ingredients conditions. This ratio was chosen because of a reduction in the hydrogen peroxide peak intensity (870 cm\(^{-1}\)) observed in initial aging studies. Raman spectra (Figure 3.8) of the solutions at the initial time point show the characteristic bands of Zinkicide™ SG6 such as the hydrogen peroxide peak (870 cm\(^{-1}\)) and the nitrate peak (1050 cm\(^{-1}\)). The spectrum of washed Zinkicide™ SG6 shows a peak at 840 cm\(^{-1}\) which is attributed to the O-O stretching vibration in Zinc Peroxide (ZnO\(_2\))\(^{210}\). The presence of this band suggests that
the nanoparticles not only consist of ZnO but also have a ZnO$_2$ component which might have a key role in the efficacy of the nanoparticles against HLB.

First we consider the aging of the Zinkicide™ SG6 formulation. Following the first 12 weeks, the ratio of the peroxide peak (870 cm$^{-1}$) to the water peak (~3400 cm$^{-1}$) decreases from ~ 0.7 to ~ 0.55 in the Zinkicide™ SG6 formulation aging in the reagents (Figure 3.9). On the other hand, the ratio of these peaks remains around 0.9 for the control solution. The measurement was repeated after 18 weeks and shows further decay in the same ratio to ~ 0.4 in Zinkicide™ SG6, and ~ 0.7 in the control solution. In terms of the washed solutions (Figure 3.12), the peak at 840 cm$^{-1}$ corresponding to ZnO$_2$ is present even at week 12 of the study for both the washed solution aging in water (Figure 3.12 (a)) and the washed solution obtained after aging in the reagents (Figure 3.12 (b)). A decay in the ratio of the ZnO$_2$ peak (840 cm$^{-1}$) to the water peak (3400 cm$^{-1}$) is observed from ~ 0.7 to ~ 0.4 by week 12 for the washed aging in water solution; nevertheless, the measurement was repeated after 18 weeks and the ratio is found to be around 0.55 which suggests that ZnO$_2$ is fairly stable.

Overall, this type of study can help understand which ingredients of Zinkicide™ SG6 will be stable over long storage periods. These results are also correlated to efficacy measurements conducted on bacterial species such as *E.Coli* where the efficacy of the Zinkicide™ SG6 was noted to decrease with time$^{211}$. It is possible that the decay observed in the hydrogen peroxide peak (870 cm$^{-1}$) intensity is correlated with the reduction in efficacy; however, further measurements are needed to confirm.
Zinkicide™ SG6 (a), Washed Zinkicide™ SG6 (b), and the control solution (c) at the initial time point of the aging study. Zinkicide™ SG6 exhibits hydrogen peroxide (870 cm\(^{-1}\)) and nitrate (1050 cm\(^{-1}\)) peaks, whereas the washed solution exhibits a Zn peroxide peak (840 cm\(^{-1}\)).
Figure 3.11. Evolution of hydrogen peroxide and Zinkicide\textsuperscript{TM} SG6 with respect to water over time. During the first 12 weeks, the ratio of the intensities of peroxide at $\sim$870 cm\textsuperscript{-1} to water at $\sim$3400 cm\textsuperscript{-1} decays over time in Zinkicide\textsuperscript{TM} SG6, but stays fairly constant for the control solution.
Figure 3.12. Average Raman spectra obtained at different time points during the aging of the washed Zinkicide™ SG6 after aging in water (a) and aging in reagents (b). The peak representing ZnO$_2$ is present after 12 weeks.

**Aging study for Zinkicide™ TMN 110**

The stability of initial formulations of Zinkicide™ synthesized using agriculture grade was also studied to aid in the material development. An aging study similar to that for Zinkicide™ SG6 was performed by monitoring the relative intensities of the hydrogen peroxide and nitrate Raman bands at 870 cm$^{-1}$ and 1050 cm$^{-1}$, respectively (Figure 3.13). In this case, nitrate was chosen since it was assumed to remain constant being one of the reagents for the reaction to form the nanoparticles. Five different initial formulations were tested, including the research grade
Zinkicide™ SG6 (which is referred to as Zinkicide™ 1 in this study). Table 3.2 summarizes the differences between each of the five formulations.

Table 3.2. Characteristics of the five formulations tested during the development of agricultural grade Zinkicide™.

<table>
<thead>
<tr>
<th>Formulation</th>
<th>pH</th>
<th>Concentration Zn (ppm)</th>
<th>Peroxide Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zinkicide™ 1</td>
<td>6</td>
<td>50,000</td>
<td>1</td>
</tr>
<tr>
<td>Zinkicide™ 2</td>
<td>6</td>
<td>50,000</td>
<td>0.5</td>
</tr>
<tr>
<td>Zinkicide™ 3</td>
<td>6</td>
<td>20,000</td>
<td>1</td>
</tr>
<tr>
<td>Zinkicide™ 4</td>
<td>6</td>
<td>20,000</td>
<td>0.5</td>
</tr>
<tr>
<td>Zinkicide™ 5</td>
<td>7</td>
<td>50,000</td>
<td>1</td>
</tr>
</tbody>
</table>
Figure 3.13. Aging of several initial formulations of agricultural grade Zinkicide\textsuperscript{TM}. The ratio of the intensities of peroxide (~870 cm\textsuperscript{-1}) and nitrate (1050 cm\textsuperscript{-1}) is presented.

In this study, the intensity ratio between the peaks representing hydrogen peroxide (~870 cm\textsuperscript{-1}) and nitrate (~1050 cm\textsuperscript{-1}) was used to monitor any potential changes in the formulations over time. In all formulations the ratio was found to decrease with time suggesting decay of hydrogen peroxide. As discussed in the aging study for Zinkicide\textsuperscript{TM} SG6, this might indicate a loss of efficacy as an antibacterial agent over time. This limitation was circumvented by the time Zinkicide\textsuperscript{TM} TMN 110 was developed.
3.3.2 Characterization of MS3T and its Components

MS3T was designed with a vision to rapid deployment in the field due to the imminent threat of wiping out citrus groves in Florida. The main components of MS3T (Clay, quaternary ammonium compound (fixed quat), and TSOL (Zn-chelate active)) are already being used separately in agriculture. Based on these considerations, MS3T should not require the time-consuming process of EPA approval that is being enforced for Zinkicide™. One of the goals of the project is to study uptake of the active components of MS3T, and to investigate the effects on citrus plants. Before starting the uptake studies, given that the structure of TSOL is not fully understood, we monitored changes in the characteristic IR and Raman bands of the components of TSOL (Zn nitrate, urea, and peroxide) to help in determining how these chemicals interact.

First, the individual components of MS3T were examined (Figure 3.14), and the FTIR spectrum of clay exhibits distinct characteristic bands (Figure 3.14 (b)). For instance, the peaks at 3620-3690 cm\(^{-1}\) are attributed to stretching vibrations of the inner hydroxyl (OH) groups lying between the tetrahedral and octahedral layers\(^{212}\). Moreover, peaks observed at 1000 cm\(^{-1}\) and 1110 cm\(^{-1}\) are attributed to stretching vibrations of Si-O bonds\(^{206}\). Finally, the bands observed between 750 cm\(^{-1}\) and 990 cm\(^{-1}\) are attributed to metal OH bending vibrations, for example Al\(_2\)OH bending\(^{212}\).

In the quaternary compound, the bands observed in the region of 2850 to 2920 cm\(^{-1}\) are attributed to C-H stretching vibrations\(^{206}\), whereas the band observed at 1620 cm\(^{-1}\) is attributed to OH bending\(^{206}\). The band observed at 1460 cm\(^{-1}\) is attributed to C-H bending and CH\(_2\) and CH\(_3\) deformation. In TSOL (Figure 3.14 (a)), the bands observed between 3260 cm\(^{-1}\) and 3440 cm\(^{-1}\) are attributed to N-H stretching vibrations as well as NH\(_2\) rocking\(^{206,213}\). These likely correspond to urea in TSOL. Moreover, the band at 1620 cm\(^{-1}\) is attributed to OH bending, while the bands
observed between 1340 and 1380 cm\(^{-1}\) are attributed to the nitrate group\(^{206}\). Table 3.3 summarizes the peak assignments for MS3T and its individual components.

**Table 3.3. Characteristic IR peak assignments for MS3T and its individual components.**

<table>
<thead>
<tr>
<th>FTIR Peak (cm(^{-1}))</th>
<th>Peak Assignment</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSOL</td>
<td></td>
</tr>
<tr>
<td>1630</td>
<td>OH Bending(^{206}), NH vibration in Urea(^{213})</td>
</tr>
<tr>
<td>1460</td>
<td>C-N Stretching in Urea(^{213})</td>
</tr>
<tr>
<td>1310-1380</td>
<td>(\text{NO}_3^-) Stretching(^{208,209})</td>
</tr>
<tr>
<td>1160</td>
<td>C-N Stretching in Urea(^{213})</td>
</tr>
<tr>
<td>1050</td>
<td>(\text{NO}_3^-) Stretching(^{208,209})</td>
</tr>
<tr>
<td>Quat</td>
<td></td>
</tr>
<tr>
<td>2850-2950</td>
<td>C-H stretching and CH(_3) or CH(_2) deformations in methyl and decyl groups(^{206})</td>
</tr>
<tr>
<td>1460</td>
<td>CH(_2) Scissor Vibration(^{206})</td>
</tr>
<tr>
<td>1380</td>
<td>Symmetric CH stretching in CH(_3) group (C-CH(_3))(^{206})</td>
</tr>
<tr>
<td>1290</td>
<td>CH(_2) Twisting Vibration(^{206})</td>
</tr>
<tr>
<td>950</td>
<td>CH(_3) Rocking Vibration(^{206})</td>
</tr>
<tr>
<td>750-810</td>
<td>CH(_2) Rocking Vibration(^{206})</td>
</tr>
<tr>
<td>Clay</td>
<td></td>
</tr>
<tr>
<td>3620-3690</td>
<td>Inner hydroxyl (OH) groups, lying between the tetrahedral and octahedral sheets(^{212})</td>
</tr>
<tr>
<td>1000-1110</td>
<td>Si-O Stretching(^{209})</td>
</tr>
<tr>
<td>900-990</td>
<td>Al(_2)OH bending(^{212})</td>
</tr>
<tr>
<td>MS3T</td>
<td></td>
</tr>
<tr>
<td>3620-3690</td>
<td>Inner hydroxyl (OH) groups, lying between the tetrahedral and octahedral sheets in clay(^{212})</td>
</tr>
<tr>
<td>1340-1380</td>
<td>(\text{NO}_3^-) Stretching in TSOL(^{208})</td>
</tr>
<tr>
<td>1000-1110</td>
<td>Si-O bond in clay(^{209})</td>
</tr>
<tr>
<td>900-990</td>
<td>Al(_2)OH bending in clay(^{212})</td>
</tr>
<tr>
<td>750-810</td>
<td>CH(_2) rocking in Quat(^{206})</td>
</tr>
</tbody>
</table>
Figure 3.14. FTIR spectra obtained from (a) TSOL, (b) clay, (c) quaternary compound, and (d) MS3T. TSOL exhibits a strong broad peak at 1340-1380 cm\(^{-1}\) indicative of nitrate, as well as a peak at ~1620 cm\(^{-1}\) corresponding to N-H vibrations in urea. MS3T exhibits peaks that can be traced back to the individual components clay, quaternary compound, and TSOL.
**Characterization of TSOL**

TSOL is the active component of MS3T. In order to determine the structure of TSOL at the molecular level, a titration experiment was designed to better understand the interaction of peroxide, urea, and Zn nitrate to form TSOL. To understand the interactions, the following binary compounds were tested: urea and hydrogen peroxide, urea and Zn nitrate, and Zn nitrate and hydrogen peroxide, in addition to the TSOL formulation including all three components. Figure 3.15 shows the fingerprint region of the FTIR spectra of TSOL, urea, urea and hydrogen peroxide, and urea and Zn nitrate. The first observation is that the FTIR spectrum of TSOL closely matches that of urea and Zn nitrate, which shows that urea and Zn form a complex inside TSOL. Additionally, we observed that the C-N asymmetric stretch band in urea (~1460 cm\(^{-1}\)) shifts to higher frequencies in TSOL, while the C=O group in urea (~1680 cm\(^{-1}\)) shifts to lower frequencies in TSOL. This confirms that the Zn metal is binding to the oxygen atom in the carbonyl group in the Zn-urea complex formed in TSOL\(^{214}\). The similarity between the spectra of pure urea and urea-peroxide suggests that little or no interaction takes place between the two components. In order to further explore the structure of TSOL and extract the most information from the IR spectra, modeling and simulation of the molecular structure of TSOL was performed by Dr. Loukas Petridis’ group at University of Tennessee and the Oak Ridge National Laboratory\(^{215}\).
Figure 3.15. FTIR spectra of urea (U), urea and Zn nitrate (U+ZN), urea and hydrogen peroxide (U+P), and TSOL. The IR spectra for TSOL and urea and Zn nitrate show shifts in the bands corresponding to C=O and N-C-N vibrations in urea compared to the IR spectrum of urea.

To corroborate the results of FTIR, a titration experiment was designed in which the concentration of one of TSOL’s constituents was varied (Figure 3.16). Five different solutions of TSOL were prepared with different concentrations of Zn nitrate. The concentrations (urea : hydrogen peroxide : Zn nitrate) were as follows 1:1:0.1, 1:1:0.2, 1:1:0.5, 1:1:0.7, and 1:1:1. Raman spectra were acquired for each solution as described previously.
Figure 3.16. Raman spectra of the different solutions of Zn nitrate and urea used in the titration experiment. As the concentration of Zn nitrate increases, the peak at 1010 cm\(^{-1}\) corresponding to N-C-N stretching in urea shifts to higher frequencies.

Previous studies\(^{213,214,216}\) of metal-urea complexes have shown that when the C-N band shifts to higher frequencies, the metal binds to the oxygen of the carbonyl group in urea. As can be seen in the Raman spectra shown in Figure 3.16, with increasing Zn nitrate content, the peak for symmetric stretching of N-C-N stretching resulting from urea shifts from \(\sim 1010\) cm\(^{-1}\) at a Zn nitrate to urea ratio of 0.1 to \(\sim 1025\) cm\(^{-1}\) at a ratio of 1. This shift to a higher frequency indicates that Zn forms a complex with urea in TSOL where it binds to the oxygen atom in the carbonyl group\(^{216}\).
Characterization of fixed-quat

The quaternary compound (fixed quat) in MS3T was also characterized using confocal Raman spectroscopy and FTIR in an experiment to better understand the interaction between the quaternary ammonium compound and silica particles present in the clay. In order to characterize fixed-quat in MS3T, we tested three materials: fixed quat particles, fixed quat gel, and silica nanoparticles (as control). This study was done to understand how quat and clay interact in MS3T, as Si is abundant in clay.

Figure 3.17. FTIR spectra of Silica nanoparticles, fixed quat particles, and fixed quat gel (left). The peak attributed to Si-O-Si in the silica nanoparticles (1070 cm\(^{-1}\)) shifts to lower frequencies in the fixed quat samples (right).

In both fixed quat particles and fixed quat gel, the peak attributed to Si-O-Si (1020–1070 cm\(^{-1}\)) is shifted to lower frequencies suggesting that binding between the quat molecules and the silica nanoparticles occurs (Figure 3.17). Raman spectroscopy further confirms this result in which the peak attributed to Si-O-Si (1080–1090 cm\(^{-1}\)) is shifted slightly towards lower frequency (Figure 3.18). These shifts in the Raman and IR bands corresponding to Si-O-Si vibrations
suggest that the quaternary ammonium compound has strong interactions with the Si-O-Si group when immobilized by the silica nanoparticles. This result suggests that, when mixed with clay in the MS3T formulation, the quat molecules will interact with the silica particles present in the clay. This might help in the localized delivery of quat to the stomata of the leaves.

Figure 3.18. Raman spectra of Silica nanoparticles, fixed quat particles, and fixed quat gel. The peak for Si-O-Si stretching (1090 cm\(^{-1}\)) shifts to 1080 cm\(^{-1}\) in the fixed quat samples.

3.4 The Uptake and Translocation of Zinkicide\textsuperscript{TM} and MS3T in Citrus Seedlings

3.4.1 Zinkicide\textsuperscript{TM} Root Uptake

With a signature of the treatment, we investigated its uptake next. The root uptake of Zinkicide\textsuperscript{TM} TMN 110 in Citrus Seedlings was investigated using infrared spectroscopy and elemental analysis techniques in order to determine if it can enter the vascular system of the plant and reach where the bacteria reside. Following 24 h of uptake, the Raman signature of the extract inside the leaves (location of the cut shown in Figure 3.5 (c) in the methods section) reveal a
narrow and sharp peak at 1050 cm\(^{-1}\) (Figure 3.19) corresponding to nitrate which is one of the main reagents used in synthesizing Zinkicide\(^{TM}\) TMN 110.

![Average Raman spectra obtained from leaf extracts from different segments of the shoot system of the seedling following 24 h of uptake. Nitrate at 1050 cm\(^{-1}\), one of the components of Zinkicide\(^{TM}\), is present in all segments suggesting a movement of the treatment. The Raman spectra suggest that the reagents of Zinkicide\(^{TM}\) TMN 110 has penetrated the plant. However, we can only detect the signature of the reagents, and the presence of ZnO nanoparticles remains to be confirmed. This is important to determine whether the plant is only absorbing the reagents, or whether it also uptakes the nanoparticles. SEM imaging and EDS were employed.

Figure 3.19. Average Raman spectra obtained from leaf extracts from different segments of the shoot system of the seedling following 24 h of uptake. Nitrate at 1050 cm\(^{-1}\), one of the components of Zinkicide\(^{TM}\), is present in all segments suggesting a movement of the treatment. The Raman spectra suggest that the reagents of Zinkicide\(^{TM}\) TMN 110 has penetrated the plant. However, we can only detect the signature of the reagents, and the presence of ZnO nanoparticles remains to be confirmed. This is important to determine whether the plant is only absorbing the reagents, or whether it also uptakes the nanoparticles. SEM imaging and EDS were employed.
used to evaluate the elemental composition of the plant tissues (phloem and xylem). EDS spectra (Figure 3.20) reveal a peak at 1.01 keV in treated plants that is not present in untreated control. The peak at 1.01 keV correspond to the Lα transition in Zn$^{2+}$. Based on this, we could further investigate the composition of the xylem and phloem using SEM images and EDS localized spectra. We tested leaf midrib (Figure 3.20 (a)) and stem (Figure 3.20 (b)) cross sections obtained from all four levels of the seedlings following 24 h of uptake and found that the Zn peak at 1.01 keV is present in both the xylem and the phloem tissue of the leaf midrib (Figure 3.20 (c)) and stem (Figure 3.20 (d)) sections. In certain locations, a peak at ~ 2-2.1 keV was observed, which corresponds to Kα or Kβ transitions in phosphorus (P)$^{2+}$. The fact that the peak is observed in certain parts but not in others could be due to the localized nature of the measurement and the accumulation of phosphorus in certain parts of the plant$^{218}$. 
Figure 3.20. SEM images of a citrus leaf midrib cross section (a) and stem cross section (b). Xylem (red) and phloem (blue) regions are marked on both images. EDS spectra obtained from xylem and phloem regions in the leaves (c) and stem (d) from all segments of the shoot system after 24 h of uptake. The Zinc peak at ~ 1.01 keV is present in both the xylem and phloem in leaf and stem tissues compared to the untreated control (black).

Thus far the ability of the treatment to reach the phloem was demonstrated using SEM imaging combined with EDS localized measurements. However, it is still important to measure the
amount of Zn that the plant uptakes following treatment, and how fast the uptake occurs. For this, XRF was used to measure the Zn content in the leaves, stem, and roots for each uptake duration and for the control untreated plants. The same calibration procedure described in the methods section was used in order to obtain quantitative values for the Zinc content in each sample. The results (Figure 3.21) show that it is possible to track the uptake of Zinkicide™ in citrus seedlings by measuring the average Zinc concentration at different uptake duration time points. In this case, uptake durations of 6 h, 12 h, 18 h, and 24 h are tested. By measuring the Zinc concentration in different parts of the plant (as defined in Figure 3.5 (b)), it is possible to map the distribution of the Zn (Figure 3.21 (c)) in the seedling. The results of this measurement show that at the 6 h time point, the lower stem has significantly higher average Zn content than the rest of the seedling. The lower stem has the highest average Zn content compared to the middle and upper stems for all time points. In terms of the leaves, the earlier time points show that the upper leaves absorbed more Zn than the lower leaves. This is true until the 24 h time point where the distribution of the average Zn content is more uniform across the seedling.
Figure 3.21. Average Zinc concentration (in ppm) in leaves (a) and stem (b) measured by XRF. 
(c) Representation of Zinc distribution in a citrus seedling after 6, 12, 18, and 24 h of treatment uptake reconstructed using concentrations measured by XRF. The distributions show that at 12 and 18 h, the treatment reaches the leaves in the upper segments, before redistributing to lower leaves at 24 h.

3.4.2 MS3T and TSOL Foliar Uptake

MS3T was designed under the hypothesis that the antimicrobial components will be released from the deposited film on the surface of the leaves, then mobilize to their target regions. Fixed-quat will mobilize to the stomata (local subsurface) and the surface of the leaves and eradicate
microorganisms residing in these areas, while TSOL will mobilize systemically to the phloem and kill CLas, eventually metabolizing into useful plant nutrients. As a result, it is critical to understand the uptake and translocation of TSOL inside the plant following foliar spray of MS3T to confirm the hypothesis outlined above. To achieve this, the foliar uptake of MS3T and active ingredient TSOL was studied using IR spectroscopy (FTIR) and elemental analysis techniques (EDS and XRF).

Prior to analyzing the treatment uptake and effects, the residue of sprayed MS3T was studied on the surface of the leaf by SEM imaging and EDS mapping. Figure 3.22 (a) and (b) are SEM images of untreated and treated leaf surfaces, respectively. As shown in Figure 3.22 (b), MS3T forms aggregates on the surface of the leaf which cover the stomata. Figure 3.22 (c) shows smaller MS3T aggregates penetrating a stomatal opening, which suggests that stomata constitute an important path for uptake of the treatment. Elemental analysis was performed and confirms the composition of the features observed (data not shown here).
Figure 3.22 SEM Imaging of MS3T deposited on the leaf surface after spraying. (a) Untreated leaf surface showing stomatal openings. (b) MS3T aggregates forming on the leaf surface. (c) MS3T aggregates can penetrate the stomatal opening.
The foliar uptake of MS3T and TSOL (systemic Zn active) in citrus seedlings was first investigated using infrared spectroscopy to monitor the effect of the treatment and to determine whether we can detect its characteristic IR bands inside the plant tissues. The mean normalized FTIR spectra (Figure 3.23) exhibit IR bands that are attributed to the natural components of the leaves. The peaks observed at 1010 cm\(^{-1}\) and 1160 cm\(^{-1}\) are attributed to C-O-C stretching vibrations in cellulose\(^{219,220}\). The peak at 1100 cm\(^{-1}\) can be attributed to C-O stretching in cellulose and hemicellulose\(^{219}\). The peaks at 1515 cm\(^{-1}\) and 1600 cm\(^{-1}\) can be attributed to C=C and C-O stretching, respectively, in lignin\(^{219,221,222}\). The peak at 1730 cm\(^{-1}\) is attributed to C=O in hemicellulose\(^{220}\). The IR spectra alone show a possible variation at 1410 cm\(^{-1}\), attributed to C-H vibrations in cellulose and lignin, where the peak seems to be inhibited in TSOL and MS3T treated leaves after 12 h (Figure 3.23 (a)) and 24 h (Figure 3.23 (b)) of treatment, which suggests an effect of the treatment on the natural biopolymers of the plant.

**Figure 3.23.** Average mean normalized FTIR spectra obtained from untreated control leaves, TSOL treated leaves, and MS3T treated leaves at (a) 12 h, and (b) 24 h time points. The peak at 1410 cm\(^{-1}\) has a higher intensity in the untreated leaves than in TSOL or MS3T at 12 h and 24 h.
To validate the observations made in the FTIR spectra of the untreated and treated leaves, PCA was performed on the FTIR dataset. The results show that there is separation in the scores plot along the first principal component (PC 1) between treated and untreated leaves (Figure 3.24 (a)). PC 1 is responsible for 61% of variation in the 12 h time point data, and 42% of variation in the 24 h time point data. PC 1 loadings for both 12 h (Figure 3.24 (b)) and 24 h (Figure 3.24 (d)) treated leaves show significant variation in the bands at ~1010 cm\(^{-1}\) (corresponding to C-O-C stretching in cellulose) as well as ~1410 cm\(^{-1}\) (corresponding to C-H bending in cellulose and lignin). The results suggest that: 1) The treatment is uptaken by the leaves, or 2) The treatment is influencing the components of the plant cell wall inside the leaves. This necessitates further studies into the potential effects the treatments have on the plant. Separation between treated and untreated leaves was observed in the scores plots after 12 h (Figure 3.24 (a)) and 24 h (Figure 3.24 (b)) treatments, although the difference at 24 h is less significant. This will require further investigation.
Figure 3.24. PCA analysis of IR signatures obtained on untreated and treated citrus leaves after 12 h (a,b) and 24 h (c,d) of treatment with TSOL and MS3T. Scores plots for 12 h (a) and 24 h (c) treatments showed separation between treated and untreated clusters. PC Loadings for 12 h (b) and 24 h (d) are also shown.

The FTIR measurements show variation between treated and untreated leaves; however, it still cannot be concluded whether the treatment has entered the plant from this result alone. The changes might be coming from the treatment effects on the plant tissue. As a result, elemental analysis was used to monitor the Zn concentration levels in treated and untreated seedlings.
First, the rate of uptake of Zn active alone (TSOL) and Zn active + clay (MS3T) was compared by measuring the average Zinc content in the leaves and stems after 12 h and 24 h of uptake. For quantitative measurement, XRF was used with the same calibration method discussed in section 3.2. The XRF data show that foliar uptake of TSOL occurs faster than MS3T (Figure 3.25). For TSOL (Figure 3.25 (a)), the average Zinc concentration increases dramatically within 12 h of uptake. In the case of MS3T (Figure 3.25 (b)) however, the average Zinc concentration increases within 24 h, but not before 12 h.

Figure 3.25. Average Zinc concentration in citrus stems and leaves measured using XRF for (a) TSOL treated seedlings and (b) MS3T treated seedlings.

The next question we aim to answer is whether the treatment can reach the phloem of the citrus leaf vascular system. For this, SEM in combination with EDS was done on citrus leaf midrib cross sections obtained from the treated plants (Figure 3.26). For leaves treated with TSOL for 24 h (Figure 3.26 (a) and (b)), the characteristic peak for Zn Lα transition (~1.01 keV) is present in the phloem of the leaf midrib (Figure 3.26 (e), red curve). The peak was not observed in untreated leaf midrib cross sections (Figure 3.26 (e), black curve).
Figure 3.26. SEM images of treated and untreated leaf midrib cross sections. (a) Treated leaf midrib cross section. (b) Higher resolution SEM image of the treated leaf midrib cross section (marked by square in (a)), highlighting regions where EDS measurements were taken (marked by squares). (c) Untreated leaf midrib cross section. (d) Higher resolution image of the untreated leaf midrib cross section (marked by square in (c)), crosses mark regions where EDS measurements were acquired. (e) Average EDS spectra obtained from treated (red) and untreated (black) leaves to determine the presence of Zn (1.01 keV).
3.5 Summary

Plant diseases constitute a significant threat to food production at a time when world population growth and economic development necessitate more efficient and sustainable efforts in agriculture. Food availability and security is a major component of the food energy water nexus, and tackling the issue of rapidly spreading plant diseases is one of the major ways of supplementing the food sector.

In this chapter we focused on citrus greening disease, which is a systemic bacterial infection threatening citrus crops in Florida and other regions around the world with extinction. We examined two Zinc-based treatments developed for systemic activity, and demonstrated the uptake and the effects these treatments can have on citrus seedlings. For example, the molecular structure of the Zn active in MS3T was studied using infrared spectroscopy and our results indicate that the Zn ions bind to the oxygen atom in the carbonyl group in urea to form a Zn-urea complex which can be linked to its antibacterial activity.

The uptake and translocation of Zinkicide™ and MS3T is also studied using a protocol that combines infrared spectroscopy with elemental analysis techniques. We confirmed that the treatments can enter the plants through infrared spectroscopy techniques. We also demonstrated how fast the plants uptake the treatments through quantitative measurements of the average Zn content at different uptake durations using XRF. Finally, we proved that the active ingredients of Zinkicide™ and MS3T can reach the phloem where the pathogens responsible for HLB reside by combining SEM imaging with localized EDS measurements. This protocol combining multiscale characterization methods has the potential to aid future growers in designing efficient field application plans for novel treatments designed to overcome plant diseases and boost crop yields.
Several questions remain unanswered, however. The form that the treatments take inside the plant remains unknown, particularly for Zinkicide™ where it is important to know whether the Zn signal detected is coming from Zn\(^{2+}\) ions or the ZnO nanoparticles. It is also important to study the fate of the treatments following uptake: do they accumulate inside the plants, or do they decay with time? This is significant in terms of the safety to consume treated agricultural products. Currently, field trials of MS3T and TSOL are underway to answer this question.

Overall, this research is highly impactful in terms of sustainable agriculture as it aids in developing efficient treatments to combat plant diseases and enhance the efficiency of agricultural production to meet the demands of a growing world population and a rapidly developing world economy.
4 UTILIZING EXTERNAL STRESSES IN PLANTS FOR BIOMASS VALORIZATION – THE EXAMPLES OF BIOMASS FILMS AND GROWTH PROMOTERS

4.1 Background

Valorization of biomass is an increasingly growing field with the aim of putting waste and biomass processing byproducts into use in the synthesis of new carbon materials that are independent of the oil industry\textsuperscript{67,223,224}. The oil industry is currently the main source where chemical manufacturers obtain raw materials for synthesis of carbon-based products (plastics, toothpaste, tires, etc.)\textsuperscript{67}. Due to the impact on the environment, as well as rising costs and declining supply, it is important to find alternative sources for the raw materials that sustain these industries. As such, valorization of biomass waste into raw materials for these industries is quickly becoming a promising method to fill the role of petroleum.

We have illustrated in chapters 2 and 3 that the structure and composition of plants can significantly change when they are experiencing mechanical (chapter 2) and chemical (chapter 3) stresses. Hence, it seems acceptable to envision utilizing these concepts to move toward engineering of plants for future valorization of products from plant-derived materials. Such an endeavor would require careful design and engineering to obtain the desired effects in the plants. Mechanically stressed plants which exhibit favorable cellulose/lignin content for processing are a promising system. A previous study in which tension wood was induced by letting plants grow at a 45° angle, has already shown that it is possible to improve yield of glucose obtained by enzyme saccharification of the biomass using this approach\textsuperscript{225}. Improving the yield of cellulose conversion to glucose constitutes a significant step in making biofuel extraction from plants a more energy efficient process.
A significant part of improving cost effectiveness of biofuel production is the conversion of the biomass byproducts of the biorefining process into useful high-value products. This would constitute a major advance in developing a viable industry to reduce our current reliance on fossil fuels and instead focus on renewable biofuel\textsuperscript{226}. Examples of high value products that can be obtained from biomass include chemicals\textsuperscript{223,224,227,228}, carbon nanomaterials\textsuperscript{229,230}, carbon fibers\textsuperscript{231}, and biomass films\textsuperscript{232}.

Chemical stresses are often perceived as having negative effects on plants; however, if utilized in a controlled way, chemical treatments could increase crop yields and achieve more efficient agricultural production. The most common chemical treatment, nutritious fertilizers, has already shown significant increases in crop yields in recent decades\textsuperscript{143,147}. Nanoparticle-based treatments also show promise as inducers of chemical stress in plants to improve crop yield by enhancing plant growth\textsuperscript{15,17,18}. Figure 4.1 shows how more value can be obtained from agricultural products whether in terms of food production or energy and added value products.
In this chapter, we explore two examples of increasing the value of agricultural products. We first discuss the valorization of biomass using hybrid poplar as raw material to create lignocellulosic films with superior properties. Next, we discuss how novel nanoparticle-based chemical treatments can enhance the growth of tomato plants. The aim is to answer a set of scientific questions:

1) *Is it possible to tune new products properties based on the source of biomass used as the input?*
2) How does changing the solvent used in processing affect the composition of the properties of the final product?

3) Can we enhance food production using nanoparticle-based chemical treatments?

A set of characterization tools are used to answer these questions including Confocal Raman Mapping and advanced AFM-based methods such as Pulsed Force Mode AFM (PFM) (see the methods part in section 4.2) and nanoscale infrared spectroscopy (nanoIR).

4.2 Example 1: Biomass Films Produced from Lignocellulosic Biomass

4.2.1 Methods

Deconstruction and Processing:
Biomass films were synthesized at the Center for Renewable Carbon, University of Tennessee, Knoxville, Tennessee. After obtaining autohydrolyzed hybrid poplar by extracting the raw biomass (187 g) with DI water at 160 °C for 60 min in a Hastelloy C276 pressure reactor, the extracted material was dried at 40 °C until constant moisture was achieved, then milled using a Wiley mill equipped with a 40-mesh screen. The powder material was then dissolved in an ionic liquid (IL) ([C2mim][OAc]) by mechanically stirring at 100 °C in a 500 mL round bottle until total dissolution. Following dissolution, the film was created by directly casting from the solution while maintaining a temperature of 100 °C. Following casting, the films were submerged in a coagulant bath three consecutive times to completely remove the ionic liquid solvent. Three different types of coagulants were used: water, methanol, and a 50/50 by volume mixture of DMAc and water. A full description of the synthesis method can be found in the publication by Wang et al.232.
The main results of Wang et al. study are that the physicochemical properties of the films depend on the coagulant solvent used, and that this is due primarily to the solubility parameters of these solvents. The methanol film shows the highest Young’s modulus as well as tensile strength which is attributed to having the ideal compositional ratio of carbohydrates to lignin. Methanol films also exhibit homogeneous properties primarily due to slower diffusion of the ionic liquid used during immersion in the coagulant bath compared to DMAc/water and water. Overall, the results underline the promise of regenerating biomass films in a sustainable manner with significant implications to manufacturing of green biomaterials with properties that match or surpass current materials manufactured using synthetic non-biodegradable sources.

In this context, the measurements presented in the coming parts constitute a further step in analyzing the nanomechanical properties in order to gain a deeper understanding of how the processing parameters affect the final properties of these films.

**Characterization Methods:**

*Pulsed Force Mode Atomic Force Microscopy (PFM):*

PFM measurements were performed on a WITec Alpha 300 RA system. A cantilever with force constant of ~5 N/m (Mikromasch 14-series) is mounted on the AFM holder to perform the force distance measurements. In PFM, a force distance (or force time) curve is acquired at each point as the probe scans the sample. Figure 4.2 shows a typical force time curve obtained in this measurement. The slope of the curve is directly related to the stiffness of the sample at the point where the curve is obtained. Other information such as adhesion and the maximum force between the tip and the sample can be obtained from the curve as well.
Figure 4.2. Schematic representing a typical force time curve acquired during a PFM scan. Stiffness, adhesion, and maximum force are indicated.

Nanoscale Infrared Spectroscopy (nanoIR):

nanoIR measurements were performed using Anasys nanoIR2 system. A gold coated cantilever with spring constant of 0.1-0.6 N/m (Appnano, SICONGG-50) is used.

Lorentz Contact Resonance (LCR):

LCR measurements were also done using the Anasys nanoIR2 system. A thermalever probe (Anasys Instruments, AN2-200) with spring constant ~0.5 – 3.5 N/m was used. In LCR, the cantilever is driven by a magnetic force which results from an AC current that is passed through the cantilever in the vicinity of a magnetic pole piece (Figure 4.3). The frequency of the AC current is matched with the contact resonance of the tip-sample system (in this case 412 kHz), and the shift in this contact resonance frequency is recorded at each point while imaging the
sample in contact mode. A positive shift in contact resonance most commonly indicates higher local stiffness, while a negative shift indicates lower local stiffness. Following imaging, the probe was fixed at 15 random points on the sample and the cantilever driving frequency was swept between 200 and 600 kHz recording the amplitude at each frequency to obtain an LCR spectrum at each point. The average contact resonance was then calculated from these 15 points for each film.

Figure 4.3. Schematic of the setup of LCR. An AC current runs through a special probe which experiences an oscillating Lorentz force in the presence of an externally applied constant magnetic field.

4.2.2 Characterization of Biomass Films

The morphology of the films prepared using three different coagulants was measured using AFM (Figure 4.4).
Figure 4.4. AFM topography images of films prepared using methanol (a,d), DMAc/water (b,e), and water (c,f) as coagulants. Figure adapted from Wang et al.232.

The AFM images show significant variation in morphology between the films, with those prepared using methanol and DMAc/water exhibiting globular-like structures. PFM was used to test the stiffness and adhesion properties and identify any differences between the three films. In this measurement, images of 20x20 μm size and 512 x 512 points were acquired; therefore, a total of 262,144 points were tested on each film. The distribution of stiffness values obtained from the three films shows significant variation between the three (Figure 4.5 (a)), with the DMAc/water film having the highest stiffness, followed by the methanol film with a narrow stiffness distribution, and the water film having the lowest stiffness. The average force curve for each film was also plotted (Figure 4.5 (b)), and shows that the adhesion properties varied with the water and DMAc/water films being significantly more hydrophilic than the methanol film. This is in agreement with Hansen solubility data discussed by Wang et al.232. The PFM
measurements illustrate how changing one parameter of the processing conditions for the films, in this case the coagulant used to remove the ionic liquid solvent, can drastically alter the properties of the product obtained. This in turn shows the potential for materials design in biomass valorization leading to a wide range of applications. For instance, UV-vis spectroscopy measurements showed that the water films absorbed strongly in the UV range which could make them potentially useful in applications which require protection from UV radiation\textsuperscript{232}.

Figure 4.5. (a) Distribution of stiffness values obtained from the PFM measurements. (b) Representative PFM force curves for water (black), methanol (red) and DMAC/water (blue) films with the adhesion indicated in the red square.

In addition, when monitoring the frequency shift of the cantilever contact resonance with LCR, one can observe some differences in the mechanical properties of the films (Figure 4.6 (d-f)). The methanol films show the smallest domains and relatively smaller variation in the contact resonance frequency between the domains (Figure 4.6 (d)). This is in agreement with complementary results obtained by Wang et al\textsuperscript{232}. 

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Figure 4.6. Topography and LCR frequency shift maps of methanol films (a,d), DMAc/water films (b,e), and water films (c,f).

The average LCR contact resonance frequency obtained from each film show that methanol had the highest average contact resonance frequency (~ 414.5 kHz) and the smallest standard deviation (~ +/- 2 kHz) out of the three films, as shown in Figure 4.7. This agrees with the 5.9 GPa modulus of elasticity for the methanol film obtained by Wang et al.\textsuperscript{232} using Dynamic Mechanical Analysis (DMA), compared to 3.9 GPa and 4.5 GPa for the DMAc/water and water films, respectively. However, the nanomechanical behaviors of the DMAc/water and water films could not be differentiated with LCR due to the large variations in the measurements. This is likely related to the fact that LCR is only sensitive to surface properties as it does not displace any material unlike PFM or nanoindentation. In addition DMA measurements relate to the bulk properties on the material in a configuration that is different from the small volume displacement
(nm\(^3\) range) observed in PFM measurements. The variations in the measurements observed here are indicative of complex interactions that would be investigated further to grasp the connections between nanoscale and bulk mechanical measurements.

![Graph showing average contact resonance frequency for methanol, water, and DMAc/water films.]

Figure 4.7. Average Contact Resonance Frequency measured with LCR for the methanol, water and DMAc/water films.

Next, we performed nanoIR with the aim of identifying chemical variations that would be responsible for the nanoscale variations in mechanical properties resolved with PFM and LCR. For each film, four different 5 x 5 \(\mu\)m regions were tested, with 9 IR spectra acquired at different points within each region. The average spectra acquired at ambient conditions (50\% relative humidity (RH)) are presented in Figure 4.8. All three films exhibit characteristic bands that are
indicative of their composition. For instance, the bands observed at 1715-1730 cm\(^{-1}\) correspond to C=O stretching in hemicellulose\(^{219}\), while the band at 1650 cm\(^{-1}\) corresponds to adsorbed OH groups\(^{220}\). This band can also be attributed to ring-conjugated C=C in lignin. The band at 1715 cm\(^{-1}\) can also be attributed to unconjugated C=O groups in lignin\(^{220}\).

As it was difficult to resolve sharp IR bands under 50% RH, we repeated the measurements at 0% RH, obtained by purging the AFM chamber with nitrogen. The IR spectra show notable differences in the individual bands for each film. For example, a band at 1560 cm\(^{-1}\), possibly corresponding to lignin components\(^{233}\), has the lowest intensity in the methanol film, which is not in agreement with the result obtained by Wang et al. in that DMAc/water film has the lowest content of lignin and not the methanol film\(^{232}\). This is possibly due to the localized nature of this measurement compared to the FTIR measurement used by Wang et al. Moreover, the band at 1650 cm\(^{-1}\) becomes narrower in all three films possibly due to removal of adsorbed OH groups and being exclusively attributed to ring conjugated C=C in lignin at 0% RH.

With this configuration, we were able to measure the ratio of the intensity of the band at 1650 cm\(^{-1}\) to the intensity of the band at 1715-1730 cm\(^{-1}\). We found that it is highest in the DMAc/Water film. Wang et al. found that DMAc/water film has the highest carbohydrate to lignin ratio which might explain this variation. The band at 1650 cm\(^{-1}\) is most likely due to OH groups adsorbed to the films. Overall, the nanoIR spectra obtained at 50% RH showed a broad peak at 1650 cm\(^{-1}\) suggesting a high degree of adsorption of water molecules on all three films.
Figure 4.8. IR spectra obtained at 0% and 50% RH from (a) water film, (b) methanol film, and (c) DMAc/water film. Blue curves indicate the measurements performed at ambient conditions with 50% RH while red curves correspond to the measurements performed at low (close to 0%) RH.

4.3 Example 2: Improving Yields of Tomato Plants Using Quantum Dots Coated with Growth Promoters

Nanotechnology-enabled agriculture holds a promising future in one of three ways: internalizing growth promoters and pesticides in an efficient and cost-effective manner, enhancing the ability to detect crop diseases and conditions, and environmental stresses, and improving the plants resistance to these external stresses\textsuperscript{18,234}. In improving agricultural production, nanoparticles can act as fertilizers by supplying the plants with nutrients directly, or acting as the vehicle to deliver the nutrients in a more efficient way\textsuperscript{234,235}. These nanoparticle-based fertilizers (or “nanofertilizers”) offer several advantages including slow release of growth promoters as well as more efficient nutrient uptake\textsuperscript{18}. This minimizes the atrification and pollution caused by excessive use of fertilizers, which can contaminate ground water with excess nitrogen for decades\textsuperscript{236}.
Nanofertilizers can be grouped into two categories: macronutrient and micronutrient fertilizers\textsuperscript{235}. Macronutrients are those nutrients that are needed by the plants in large quantities (such as nitrogen, potassium, or phosphorous), whereas micronutrients are those that are beneficial to plants in relatively small quantities (such as zinc, manganese, or copper). The nanoparticles can be synthesized in such a way that they can be used to supply plants with both macronutrients and micronutrients to enhance growth and improve crop yields. The potential of nanofertilizers is already being demonstrated by numerous studies\textsuperscript{234,237-239}. For instance, Liu et al. synthesized hydroxyapatite nanoparticles intended to supply soybeans with phosphorus and observed a 33\% increase in growth rate as well as a 20\% seed yield\textsuperscript{240}. These effects were superior to regular phosphorus fertilizers.

One promising application is in improving the crop yield of tomato plants using Zn based nanoparticles (Nano-Zn) coated with growth promoters. Three different types of growth promoters were used: Urea (U, a source of nitrogen), N-Acetylcysteine (NAC, an anti-oxidant), and Sodium Gluconate (SG, a chelating agent).

4.3.1 Methods

Sample Preparations:

25-day old Tomato plants (Solanum \textit{lycopersicum}) were purchased from Home Depot. The plants were treated with 40 \textmu L/mL using the soil drench method. Following treatment, the plants were transferred to a growth chamber (Panasonic MLR-352H-PA), where the temperature is set to cycle between 20 and 35 \textdegree C, and the relative humidity kept at 75\% for 5 days. Control untreated plants were processed under the same conditions without treatment with any of the Nano-Zn formulations.
**Confocal Raman Spectroscopy:**

Confocal Raman spectroscopy measurements were performed using the WITec Alpha 300 RA system with a 20X objective (Carl Zeiss EC EPIPLAN). Each of the Nano-Zn formulations was tested by acquiring a 10 x 10 array of single spectra with an integration time of 1 s, and laser power of ~ 5 mW. Raman mapping of the tomato plant stem tissues was acquired by recording a single spectrum at 500 nm steps. A chemical map of the treatment distribution was then constructed by calculating the intensity of the characteristic Raman band at 1040 cm$^{-1}$.

**Scanning Electron Microscopy:**

SEM images were acquired using a Zeiss ULTRA-55 FEG system. The samples were coated with gold-palladium prior to loading into the microscope’s vacuum chamber. InLens detector was used with a working distance of 8 mm, a beam energy of 3 kV, and an electromagnetic lens aperture of 20 μm.

4.3.2 Results

First, the Raman signature of each formulation was tested. Raman spectra of Nano-Zn coated with SG exhibit a peak at 1040 cm$^{-1}$ attributed to C-O stretching in SG (Figure 4.9 (b)). Raman spectra of the plants confirmed the presence of the Nano-Zn coated with SG in stem cross sections based on the presence of the strong band at 1040 cm$^{-1}$. Furthermore, cube-like structures were observed in SEM images acquired from Nano-Zn-SG treated tomato plants (Figure 4.9 (d)), which could correspond to the effect of the Nano-Zn in the tissues, since the control experiment did not show any such features. In addition, the data acquired for the other treatment did not exhibit any such features.
Figure 4.9. (a) Optical image. (b) Raman spectra obtained from the stem cross section, bare Nano-Zn, and SG coated Nano-Zn. (c) Raman map of the intensity of the band at 1040 cm\(^{-1}\) acquired from the region marked by the white square in (a). (d) SEM image of a stem cross section of the tomato plant treated with SG coated Nano-Zn.

The effect of the Nano-Zn formulations on the physiological properties of the plants was also considered (Figure 4.10), in collaboration with Dr. Santra and Dr. Das. The relative water content (RWC) of the leaves was significantly enhanced in plants treated with all three coated Nano-Zn formulations (Figure 4.10 (a)). RWC is important in that it shows how much water the plants can retain under various conditions, and indicates that the Nano-Zn formulations can improve the ability of the plants to withstand external stresses such as drought or salinity. The
membrane stability index (MSI) is used to measure the integrity of cell membranes. In this case, the MSI values were higher for tomato plants treated with Nano-Zn formulations, indicating that the cell membranes are more stable (Figure 4.10 (b)). Root and shoot lengths were also improved in the Nano-Zn treated as compared to the untreated plants (Figures 4.10 (c) and (d)). The plants treated with NAC coated Nano-Zn exhibited the largest average root and shoot lengths among the three treatments. These results confirm the ability of Nano-Zn coated with growth promoters
to improve the yield of tomato plants, with strong implications for the future of using nanoparticles as vehicles for the efficient delivery of growth promoters to plants.

Figure 4.10. Comparison of the Nano-Zn-Urea, Nano-Zn-NAC, and Nano-Zn-SG treated plants with the control untreated plants. (a) Relative water content. (b) Membrane stability index. (c) Root length, and (d) Shoot length. Figure adapted from the study performed by Dr. Smruti Das [unpublished]²⁴¹.
4.4 Summary

Two examples of adding value to agricultural production are discussed, with impacts on the food energy water nexus. Biofuel production and biomass valorization, as well as enhancing crop yields for improved food production constitute important activities, in which nanoscale characterization has a major role to play. In the first example, the effect of using three different coagulants on nanoscale properties of high performance carbon films prepared by deconstructing hybrid poplar with autohydrolysis followed by ionic liquid pretreatment is studied. Nanoscale chemical characterization using nanoIR reveals a change in the relative intensities of characteristic peaks of the different films. However, our comparison to nanomechanical measurements (LCR, PFM) suggest that nanoIR remains limited in characterizing materials with high level of heterogeneity in their volume due to the large region excited by the IR laser focused on the sample inducing interference in the photothermal measurements. Nanomechanical with small volume displacement proved to be more sensitive to domains within the films of different local stiffness.

In the second example, efficient delivery of growth promoters using Nano-Zn to young tomato plants is demonstrated with positive effects on the plant physiology. Plants treated with all three Nano-Zn formulations exhibited longer roots and shoots, as well as better water retention capability and stronger membrane stability indexes. This shows that the treated plants can better survive conditions such as droughts or high salinity. More importantly, it demonstrates how nanoparticle based treatments can provide efficient delivery of growth promoters to plants, avoiding overuse of fertilizers and/or pesticides which can pollute the environment and clean water resources.
5 CONCLUSION

5.1 Overall Summary

In this dissertation, through various examples, the effects of mechanical and chemical stresses on the structure and composition of plant cell walls were studied, as well as the potential of using these phenomena to enhance energy and food production from the perspective of sustainable agriculture. The work presented highlights the importance of realizing how food, energy, and water security are interlinked in one nexus and their effects on each other must be considered in any policies designed to promote each individual sector.

The effects of gravity on the structure and composition of plant cell walls were found to be significant. Confocal Raman mapping combined with statistical analysis tools unveiled variations in the characteristic signatures of cellulose and lignin between tension, opposite, and normal wood. K-means clustering analysis made it possible to distinguish the different cell wall layers based on the Raman spectra acquired in the area of the cell. Beyond that, our multi-pronged approach facilitated a tailored selection of spectra from specific layers for further analysis. The S/G ratio, a critical measure of the molecular structure of lignin, was confirmed to be higher in tension wood than in normal wood. Cellulose crystallinity was measured for each layer in tension and normal wood. The G layer was found to contain the most crystalline cellulose microfibrils, as expected. However, we found that the middle lamella and S layer of tension wood cell walls were more crystalline than their respective counterparts in opposite and normal wood. Uncovering these subtle variations between tension wood and normal wood constitutes a significant step in understanding how plants respond to external mechanical stress at the cellular and subcellular levels.
Further, AFM based methods, namely AFAM and nanoIR, revealed variations within the cell wall layers that could not be resolved by confocal Raman microscopy or standard AFM topography mapping. AFAM phase mapping demonstrated local variation of 3° - 4° within 200 nm inside the S layer. These measurements were complemented with results from nanoIR to label the composition of the features resolved in AFAM, although much remains to accomplish to obtain a clear picture of molecular interaction in the plant cell wall.

The effects of chemical stresses in the form of treatments applied to citrus plants to combat citrus greening disease could be studied using a multiscale approach. Zinkicide™, a nanoparticle based treatment designed to systemically enter the plant and directly target the pathogens, was characterized using Raman spectroscopy and FTIR to identify the characteristic peaks, as well as to monitor its stability over time. The Raman signature of the formulation allowed us to study the uptake, translocation, and effects of Zinkicide™ on the plant through rapid screening of the extracts. Treatment translocated inside the plant following 24 hours of root uptake was identified. The distribution of Zn in the plant, though, required an elemental analysis. XRF and EDS combined with SEM imaging were found to be complementary method to confirm the uptake at the plant level but pinpoint the distribution of Zn in the vascular tissues such as phloem and xylem. Using this approach, the ability of the treatment to reach the phloem and xylem inside the leaves and stems of citrus plants was demonstrated. Overall, a robust protocol for tracking the uptake of new nanoparticle-based treatments designed to combat plant disease was developed and tested.

In addition to Zinkicide™, a different Zn-based treatment for slow release through foliar application, MS3T, was also studied using the aforementioned protocol. The characteristic
signature from the three individual components: clay, fixed quat and TSOL were obtained. As the molecular structure of TSOL was unknown, our infrared spectroscopy studies combined with molecular dynamics considerations (Dr. Loukas Petridis and his research group at the University of Tennessee and Oak Ridge National Laboratory) allowed us to confirm that Zn forms a chelate compound with urea by binding to the Oxygen atom in the carbonyl group. The foliar uptake of the systemic active TSOL was demonstrated using elemental analysis techniques (EDS and XRF) as well as FTIR combined with statistical analysis. It was shown that TSOL can penetrate the leaves and translocate to the vascular system within 24 hours of foliar spray, with the treatment deposited on the leaf surface for the duration of uptake. When combining TSOL with clay for slower release, the measurements suggest that Zn penetrates the plants at a much slower pace. These findings will be the subject of additional measurements in the near future. Our work and the protocol developed is expected to have significant impact on the agricultural industry, especially in supporting the design of new materials for tackling plant diseases and minimizing their effects on the food sector of the food-energy-water nexus. Further quantitative and specific monitoring of the treatment in the plant has the potential to support growers in adopting more efficient use of treatments by determining appropriate field application times and optimizing application frequency.

Lastly, we considered how tuning the properties of plants by application of external stresses could be utilized to trigger plant responses towards obtaining new eco-friendly products, as well as securing agricultural production efficiency. Valorization of biomass is sought after to improve the financial competence of biorefineries rendering biofuel production as a more attractive solution to the world’s impending energy crisis. One example of biomass products, namely lignocellulosic-based films, was studied. The effect of varying one processing parameter, the
coagulating agent, on the nanoscale mechanical and chemical properties was monitored using advanced AFM techniques. The stiffness and adhesion properties of the films were found to vary greatly, with the film prepared using methanol being the least hydrophilic and the film prepared using DMAc/water being the stiffest at the nanoscale. nanoIR measurements could not resolve nanoscale chemical composition variations within a film at this time. However, the effect of relative humidity on the films could be captured with nanoIR.

The last example focused on the effect of chemically induced stress from applying Nano-Zn formulations coupled with growth promoters on tomato plants. Evidence of increased shoot and root length in the treated plants could be reported, with the plants treated with Nano-Zn-NAC showing the greatest variation. The water retention capability, as well as the cell membrane integrity of the treated plants exhibited marked improvement compared to untreated plants. These results show the promise of using nanoparticle treatments in combination with growth promoting agents in enhancing the yields of important agricultural crops, which is of substantial importance in the food-energy-water nexus and in meeting the demands on the 10 billion population expected by 2050.

5.2 Future Directions

Our studies reveal interesting effects of mechanical and chemical stresses on the structure and composition of plant cell walls, sometimes hidden in the building blocks – cellulose, hemicellulose and lignin. However, the implications of these changes at the plant or population level and what they mean remain partly unanswered. For instance the arrangement of these biopolymers and their interactions is still not fully understood. With the advent of functional nanoscale characterization tools and rigorous statistical analysis, fundamental behaviors of the
complex heterogeneous systems and their ultrastructure will likely become uncovered in the near future. The long-term direction of this research involves designing new plant-derived materials, based on the knowledge acquired of their structure and composition at the nanoscale, with more favorable composition for the purpose of efficient biofuel extraction and added-value bioproducts manufacturing which can compete on the market with synthetics.

Significant progress has been made in answering questions regarding the uptake and translocation of key novel treatments in citrus plants to eradicate citrus greening disease. While it was shown that the treatment can reach the phloem of the plants, it is still unknown in what form the treatment exists inside the plant tissue. Are the nanoparticles still in their ZnO form? Or is the Zn detected in elemental analysis simply Zn$^{2+}$ ions? Different characterization methods such as X-ray Photoelectron Spectroscopy (XPS) and cathodoluminescence may be able to provide the answer. The mechanisms of entry in the plants also remain unclear. For instance, SEM images of the treated surfaces showed aggregates of the MS3T treatment inside stomatal openings suggesting that stomata could be gateways for the treatment to enter the leaves. However a lot of unknowns remain.

Finally, the protocols developed in chapter 3 are already being used to study the uptake of another copper-based treatment designed to tackle bacterial spot disease in tomato plants, highlighting the strong potential of using these methods in studying novel chemical and nanoparticle based treatments for plant diseases, and the potential broader impact this has on food-energy-water nexus research.

Overall we expect that multiscale characterization and more particularly studies at the nanoscale have an important role to play in meeting the demands of our growing world population.
APPENDIX A: BACKGROUND FOR RAMAN SPECTROSCOPY AND IMAGING
Raman spectroscopy\textsuperscript{114,242} is one of two infrared methods that are the most commonly used for plant research. In Raman scattering the incident light (generally in the visible range) distorts the electron clouds around the nuclei in the molecule, causing transitions to excited virtual states. A virtual state is short-lived before a photon is re-emitted as the electron transitions back to a lower state. In cases where the electron cloud is only distorted with little or no influence on the motion of the nuclei in the molecule, then the photon emitted presents little change in energy owing to how light the electrons are compared to the nuclei. This rather small change in energy of the scattered photons is called Rayleigh scattering.

When the interaction of the incident light with the molecules causes motion of the nuclei, Raman inelastic scattering takes place and some of the photons emitted have different energy. This is a weak process with the inelastically scattered intensity about $10^8$ times weaker than Rayleigh scattering. In the inelastic scattering process, energy can be exchanged between the molecule and the light. In the case where light gains energy, it is termed anti-Stokes scattering, while in the case where the molecule gains energy it is called Stokes scattering. Conventionally, this shift is represented by the change in wavenumbers (cm\textsuperscript{-1}) relative to the excitation wavelength. Figure A.1 below shows the basic setup of Raman spectroscopy measurement.
Figure A.1. Basic schematic representing Raman Spectroscopy. Inelastically scattered light is passed through a diffraction grating prior to reaching the spectrometer to analyze the shift in wavelength relative to the excitation laser.

Not all transitions are active in Raman spectroscopy. A basic selection rule in Raman scattering is that the incident light should change the polarizability of the molecule. The polarizability of a molecule is its ability to form instantaneous dipole moments as a result of an interaction with the electric field of the incident light. This change in polarizability occurs to the greatest extent when the molecule is symmetric, and therefore, symmetric molecules can give Raman peaks with strong intensities in the spectrum.
Spatially resolved chemical information is a great interest in heterogeneous samples. It can be obtained through IR or Raman imaging or mapping\textsuperscript{243}. In imaging, the light is focused onto the sample using appropriate lenses or objectives. Spectra are acquired for an array of points that will become the “pixels” of the image. Raman mapping is usually more attractive than IR since the wavelength of the excitation laser source in the visible range is almost 10 times smaller than the mid-IR range, leading to much higher spatial resolution according to the diffraction limit rule.

*Confocal Raman microscopy*\textsuperscript{242} is the most widely used method in chemical spectroscopic imaging due to the combination of confocal microscopy principles with Raman scattering to produce chemical images with resolutions down to around 250 nm. Modern systems also employ CCD detectors, which can make the spectral acquisition faster, greatly reducing the time needed to form the Raman map.

Although confocal Raman microscopy is advantageous in enabling faster acquisition of data, there are still some issues that present an obstacle to characterization such as the limited spatial resolution, the need to use higher integration times with samples which give lower intensity Raman signals (since larger integration times reduce the signal-to-noise ratio), and the presence of background fluorescence in many biological samples. Background fluorescence can be overcome by using FTIR, and this is another way both characterization techniques are complementary to each other.
APPENDIX B: BACKGROUND FOR FOURIER TRANSFORM
INFRARED SPECTROSCOPY
Fourier Transform Infrared Spectroscopy is the second type of infrared method commonly used to study plant materials. In this case, the sample is illuminated with a broad range illumination in the IR range. The detector is placed in the far field and records the spectrum to identify the bands absorbed by the material.

When infrared light is used to illuminate a material, the photons are absorbed when their energy matches the energy difference between the molecular vibrational states of the material following a set of selection rules. The absorption spectrum gives information about which specific frequencies of the infrared light (often expressed in wavenumber) are absorbed by the material. Mid-IR spectroscopy focuses on the 4000-400 cm\(^{-1}\) range. Since no two materials have the same set of absorption frequencies, an infrared spectrum is essentially a fingerprint of the material, especially in the 1600-500 cm\(^{-1}\) region.

Originally infrared spectroscopy was carried out using “dispersive” methods which involved shining the light at each frequency in the infrared range one at a time until a full spectrum was obtained. Fourier Transform Infrared Spectroscopy (FTIR) was introduced to accelerate data collection, by illuminating the sample with a broadband IR light: utilizing a Michelson interferometer, an interferogram containing the information of the sample can be acquired. The infrared spectrum is then obtained by taking the Fourier transform of the interferogram. Figure B.1 shows an example of the basic setup of an FTIR spectrometer.
Transmission techniques are the most popular ways to acquire FTIR spectra. The infrared beam passes through the sample and is detected on the other side. Advantages of transmission spectra include a high signal-to-noise ratio and the ability to scan a large variety of samples including solids, liquids and gases. However, as with many transmission based characterization methods, the “thickness” problem arises, and sample preparation for transmission based FTIRs is often strenuous and time consuming. In many setups, Potassium Bromide (KBr) pellets, ZnS or ZnSe plates, which are transparent to IR beams, are used as support and diluents for the samples.

Reflectance methods have the advantage of easier sample preparation but they also require an expensive setup to align the beam and focus it on the sample, and collect it at the detector.

Another disadvantage of reflectance based FTIR is the small depth of penetration. Reflectance based FTIR come in different varieties including: Diffuse Reflectance (DRIFTS) and Attenuated Total Reflectance (ATR-FTIR). FTIR systems which use specular reflectance are also common. DRIFTS is more suitable for solid and powdered samples, while ATR-FTIR is more suited for films and liquids. ATR-FTIR requires the use of a crystal to achieve total internal reflection of
the IR beam, and form the evanescent wave that will interact with the sample. Figure B.2 shows a schematic of how ATR-FTIR works.

Figure B.2. ATR-FTIR concept. The sample is placed on top of the crystal. An infrared beam is passed through the crystal and undergoes total internal reflection with the surface in contact with the sample. Evanescent waves from the beam are absorbed by the sample at the absorption wavelengths before the beam reaches the detector.

The most commonly used crystal is Zinc Selenide (ZnSe); however, it can only acquire spectra above 650 cm\(^{-1}\) which is a drawback for samples in which we are interested in observing absorption bands lower than this range.

In contrast to Raman spectroscopy, in mid-IR spectroscopy stronger absorption occurs when molecules have a larger change in their dipole moment. As such, the strongest peaks in an infrared absorption spectrum often arise from asymmetric molecules.

Hence, Raman scattering and IR absorption are complementary spectroscopic techniques and when used together can give a complete picture of a molecule’s vibrational modes and molecular composition.
APPENDIX C: BACKGROUND FOR ATOMIC FORCE MICROSCOPY
The invention of the atomic force microscope (AFM) triggered a revolution in our fundamental understanding of materials and living systems at the nanoscale\textsuperscript{246}. Nanoscale studies on a broad range of samples, from conductive materials to soft matter, became possible and a number of scientific questions could be unlocked by the great versatility of AFM in the ensuing decades. Since then, continuing developments of new functional configurations have been reported, showing that the full potential of AFM is still to be realized.

A basic AFM consists of a sharp tip, a diode laser (usually red) and a position sensitive detector (PSD) used in the read-out system to monitor the cantilever motion, a high resolution piezoelectric scanner (to enable movement in x, y, and z directions) with a feedback loop to control the tip-sample distance. In conventional AFM modes, microcantilever with a sharp tip is used as the probe. The read-out laser is aligned onto the back of the cantilever and its reflection is aligned with the center of the PSD. Figure C.1 shows the optical lever mechanism for detecting cantilever deflection.

![Figure C.1. Basic mechanism for detection of the cantilever deflection in AFM](image)

Upon variation in the tip-sample interaction (sample height change, electrostatic force, etc) the cantilever bends. For topography imaging, the vertical deflection of the cantilever is monitored
as it scans the sample. The topography image is created by displaying the voltage read out by the PSD at each point of the image and plotting the array in the 2D filled contour plot. However it is possible to monitor other traits of the tip-sample interactions to map properties such as mechanical, electrical, optical, thermal, and magnetic properties.

*AFM microcantilevers* shape, composition and tips should be selected with respect to the imaging mode and the material to be imaged. In particular, the cantilever stiffness and/or natural frequency, and its dimensions may play an important role in the quality of the images produced. In general, longer cantilevers have lower stiffness and therefore can vibrate with larger amplitudes during scanning. They are often preferred to image soft samples.

*AFM imaging modes* can be divided into two groups based on scanning dynamics: static and dynamic modes. Static modes involve no driving in the cantilever, while dynamic modes involve driving the cantilever at or near its resonance frequency.

Contact mode AFM is a static mode, in which the tip is engaged in contact with the sample. The force between the tip and the sample is kept constant and may be used in the feedback loop. Imaging is done in the repulsive regime of the interaction between the tip and the sample. The deflection of the cantilever at each position in the scan is measured as previously described, and gives topographic information on the sample. In addition to topographic information, friction between the tip and the sample can also be measured by sensing the torsional movements of the tip as it scans the sample. The force between the tip and the sample in contact mode AFM is relatively high and in the range of 1 – 100 nN. This commonly causes damage in soft samples.

In tapping (or AC) mode, the cantilever is driven at or near its resonance frequency, and the amplitude and phase of the cantilever vibrations change due to interactions with the sample. The
tip sample interaction forces are usually below 1 nN, which is advantageous for soft and biological samples. In tapping mode AFM, the tip-sample distance is larger and the interactions between the tip and the sample are governed by the attractive regime forces, such as vdW and capillary forces. The small attractive interactions dampen the oscillations of the cantilever, and give topographic information about the sample. In addition to topographic information, these dynamic modes of AFM can be used to study long range interactions such as electrostatic and magnetic forces.

Special consideration should be given to the force between the tip and the sample as it can reach up to 1 nN and also cause damage to the sample. In general, the ratio of the amplitude setpoint (defined by the user) and the amplitude of free oscillations is used to indicate the extent of interactions between the tip and the sample.

In addition to topographic information, tapping mode phase imaging has been shown to provide information about the stiffness of the sample as the phase shift in the cantilever oscillations is strongly related to the sample stiffness and adhesion. However, for polymeric samples, other factors can influence this kind of measurement such as the polymer’s viscoelastic response, capillary forces, and changes in the contact area, which is a critical factor in determining the stiffness of a material.

The power of AFM lies in its ability to scan samples under different environmental conditions with minimal sample preparation (as compared to characterization methods such as Scanning and Transmission Electron Microscopies), and achieve high resolution, with atomic scale resolution attainable under specific conditions.
APPENDIX D: COPYRIGHT PERMISSION FOR FIGURE 2.16
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