Book of Abstracts

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Keynote Lectures

Auto-fluorescence based techniques in plant sciences

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A wide variety of plant compounds are fluorescent from cell wall components such as lignin to pigments such as chlorophyll. In plant science, autofluorescence has been used extensively for both fluorescent imaging and spectroscopy. Chlorophyll fluorescence, for example, is commonly used in the physiological assessment of plants involved in experiments and can easily be measured using portable sensors. Lignin fluorescence has been used as a measure of compression wood severity, in pulp and paper research, and in research on biomass and wood modification.

This talk will focus on applications of autofluorescence to microscopic imaging and microspectroscopy. I will give a general introduction to fluorescence spectroscopy including an overview of common plant fluorophores. I will then review work on the spectroscopic analysis of lignin fluorescence with particular reference to studies on reaction wood. In the second part of the talk, I will discuss autofluorescence of other fluorophores including cutin, ferulate, and suberin, which occur in cell walls, tannin's that accumulate in bark as defense compounds, and chlorophyll.

Lignin exhibits strong autofluorescence across the visible spectrum. There are two types of fluorophore, UV excitable blue fluorophores associated with phenolic structures, and blue excitable green or orange fluorophores associated with conjugated structures. The later fluorophores are of particular interest because they vary in intensity, emission spectrum and fluorescence lifetime in relation to chemical or physical treatments or to the molecular environment (pH), and hence offer opportunities for use in the characterization of experimental effects.

Cutin has a fluorescence spectrum similar to that of lignin but with stronger emission in the green and is of interest in studies of leaves particularly in relation to pathogens. Ferulates show an interesting pH dependent fluorescence with blue emission at low or neutral pH and green emission at high pH. Suberin has a strong blue emission when excited with UV but little or no emission when excited with blue light thus distinguishing it from lignin.

Finally I will deal with some applications of autofluorescence to measurement of wood cell wall properties including cell wall porosity by infiltration with lignin quenching agents and the use of FRET (Förster resonant energy transfer), signal colocalisation in studies of biomass deconstruction, spectral unmixing in lignin studies, and finally presenting some aspects of fluorescence lifetime spectroscopy (FLIM) as applied to wood cell walls.

Synchrotron Light for Spectral Imaging: Application to Plant Spectroscopy

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Synchrotron radiation is a source of light that offers a wide spectral "white" source ranging from infrared to hard X-rays. In the last decade, drastic improvements have been achieved in term of stability, brightness, low beam divergence and coherence. Therefore, numerous synchrotron beamlines are now dedicated to imaging. Imaging techniques using synchrotron radiation will be presented, both scanning imaging and full-field microscopies offers information and permit the mapping and free labeling of samples at high spatial resolution. At the SOLEIL synchrotron facility, particular attention is paid on coupling spectroscopies and imaging techniques. Indeed, it provides an efficient approach to study and characterize complex material as biological samples and plants. Coupling of multimodal, multi-resolution, and multivariate hyperspectral images by means of multiblock methods will be introduced. Various applications to plant science using synchrotron light will be presented with particular attention on acquiring *in-situ* data that can link molecular-scale to tissues all simultaneously in the same experiment.

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Chemometrics for hyperspectral images of biological samples

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Hyperspectral images of biological samples (plant or animal tissues) are unique measurements that provide information on the chemical structure of the samples preserving the original biological morphology. Yet, the interpretation of these measurements is not straightforward since spectra may be affected by intense artifacts, such as fluorescence contributions in Raman imaging or Mie scattering in FT-IR, that may affect the extraction of the relevant signal and, often, the spectral signature of different tissues or biological elements may be extremely similar.

Multivariate resolution (or unmixing) methods are powerful tools to recover the underlying model of the hyperspectral measurement, expressed as the sum of the signal contributions defined by basic spectral signatures and distribution maps of biological components. This chemometric tool provides *per se* relevant information, but the output provided can also be combined with other data analysis tools, such as segmentation or classification methods, for additional knowledge[1,2].

An important axis of multivariate resolution methods relates to the capability to analyze simultaneously several images with related information. This multiset analysis mode allows having more reliable results and a better description of the problem of interest. Examples can be the simultaneous analysis of images related to different individuals to characterize a biological population or to the fusion of images taken on the same sample with different spectroscopic platforms[3,4].

The power of multiset analysis and the potential of the connection among multivariate resolution and other data analysis tools will be shown using real examples of hyperspectral analysis of biological samples.

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Raman scattering and multimodal multiphoton microscopy for the characterization and identification of plant tissues

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Vibrational microspectroscopy has gained increasing importance in plant research, since obtaining molecular information with spatial resolution enables mapping of biochemical composition, structure, and interactions. On the one hand, vibrational spectra, such as Raman or infrared data contain detailed structural information, on the other hand, the wealth of molecules of the complex plant tissue results in characteristic spectral fingerprints that can be analyzed using pattern recognition techniques. We will discuss here the capabilities of Raman microspectroscopy for the characterization of different plant tissues, ranging from pollen samples [1] to sections of different plant organs. Aspects such as the heterogeneity of spectroscopic data due to histological origin of the samples, species, or variation of physiological conditions will be discussed, and their application to solve questions in spectral identification, e.g., in automated pollen warning will be illustrated.]2]

Since the microscopic and nanoscopic levels of understanding of the properties of plants are connected, description of the macromolecular, biochemical data from vibrational spectroscopy must be combined with morphological data, e.g., from different techniques in light microscopy or electron microscopy [3]. We will also discuss the possibility to combine different linear and non-linear microspectroscopic techniques, such as Raman scattering, second-harmonic generation, and multiphoton excited fluorescence, and illustrate their use in the investigation of the chemical and structural properties of the plant tissues.

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Acknowledgements

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Mass spectrometry imaging combined to in-situ enzymatic hydrolysis: a novel image of plant tissues

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Mass spectrometry imaging (MSI) has recently emerged as a new imaging method. MSI resolves, in intact tissue sections, both the spatial distribution of many types of molecules and their molecular structure [1]. After preparation of tissue sections, the instrument captures a series of mass spectra, each representing the mass profile of a specific region of the tissue. Abundant chemical and structural information at the whole tissue scale in addition to the fact that no specific probe is required to monitor the molecules give MSI a selective advantage and make the method attractive and complementary to other imaging techniques.

An overview of the technique and main fields of application of MSI will first be presented. We will then move to a more detailed description of some recent studies performed by MSI in our team, in the field of food science and agronomy. MSI was combined with enzymatic treatments and used to study the distribution and structural variability of the major cell wall polysaccharides of cereal grain endosperm, namely arabinoxylans (AX) and β-glucans (BG). The technique was applied at various stages of development of wheat endosperm, as well as in the mature endosperm of several varieties of barley and wheat [2,3] (Figure 1). Appropriate selection of enzymes provided a means of obtaining selective images of different classes of polysaccharides. The method has also been applied to establish the structural variation of hemicelluloses, lignins and hydroxycinnamic acids in the stems of maize genotypes of contrasted degradability.

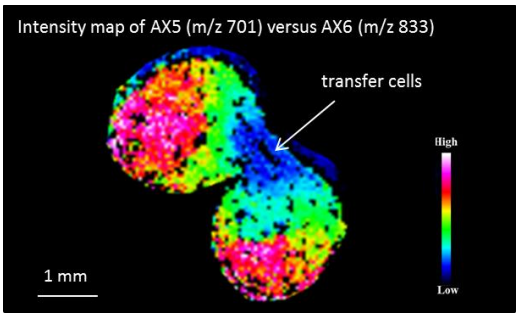

Figure 1. Distribution of a mono-substituted AX oligosaccharide (AX5) relative to a di-substituted AX oligosaccharide (AX6), as revealed by MSI, in the mature endosperm of bread wheat.

The presentation will thus highlight the potential of MSI, combined with enzymatic tools, to give a unique picture of the structural heterogeneity of major components of plant tissues. Complementarity to other spectroscopic or microscopic methods will be highlighted, as well as the future trends of this new imaging method.

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Analysis of plant raw materials and extracts applying various vibrational spectroscopy techniques - possibilities and limitations

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Quality control of cultivated plant species as well as products derived from them usually comprises correct botanical identification of the plant material as well as quantification of the individual active principles. Furthermore, residues (e.g. organic solvents) and contaminants (e.g. pesticides and heavy metals) are determined applying various sophisticated analytical techniques. For this, testing of plant material such as phytopharmaceutical products is usually performed in accordance with validated standard methods described in the Food Chemical Codex, the European Pharmacopeia, the United States Pharmacopoeia and others. Contrary to this approach, there is some need to apply also various rapid high-throughput methods aiming to characterise simultaneously several quality parameters and to reduce efforts for sample preparation to a minimum. In this context new vibrational spectroscopy methods (MIR, NIR and Raman spectroscopy) in combination with various chemometric algorithms are presented which allow efficient monitoring of numerous plant samples within a short time. Especially Raman spectroscopy has been found to be a reliable and non-destructive method for rapid discrimination of different plant species or chemotypes if characteristic key bands can be observed in the spectrum. But also NIR and ATR-IR spectroscopy has made the handling of powdered as well as liquid samples very quick and simple. Today portable IR and Raman spectrometer systems are available which only need sample amounts of a few microliters or milligrams for analysis. In most cases, vibrational measurements can be performed directly on plant tissues as well as on fractions isolated from the plant material by hydro-distillation or solvent extraction. Based on individual marker bands, spectroscopic analyses in principle allow the discrimination of different species, and even to classify chemotypes among the same species. Combination of vibrational spectroscopy and hierarchical cluster analysis provides a fast, easy and reliable method for chemotaxonomic characterization. The ability to rapidly monitor various plant components provide the possibility to efficiently select high-quality single plants from wild populations as well as progenies of crossing experiments. Today, vibrational spectroscopy is already introduced in industry in order to perform fast quality checks of incoming raw materials and continuous controlling of production processes.

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Session Lectures

Why one laser is not enough – a case study on cinnamaldehydes and the implications on Raman spectroscopy of plant material

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One of the most useful regions in the Raman spectrum of wood is ranging from 1660 to 1600cm^{-1}. It contains C=O, C=C and aromatic ring stretches. Wood spectra typically show a shoulder at 1620cm^{-1} which is almost always present and was attributed to the C=C stretch of coniferyl- and sinapaldehyde by Agarwal[1]. This assignment was questioned by Kihara et al.[2], who assigned it to the C=O stretch of these two substances. Here, we closer examine the substance class of cinnamaldehydes regarding their Raman and IR spectra. Assignments with emphasis on the 1660, 1620 and 1130 band are given. This substance class furthermore exhibits very strong Raman modes when excited at 532nm, while these modes are rather weak at 785nm. Raman spectra of two wavelengths (532 and 785) illustrate this effect. Additionally, a NaBH4-reduction experiment was conducted to show how the 1600cm^{-1} band both loses intensity and shifts towards higher frequency as a result of removing strong scattering chromophores. The results indicate that the 1660, 1620 and 1600 Raman bands of wood have significant contribution from aldehydic C=O, C=C and aromatic ring, respectively, therefore supporting the Agarwal assignment. While the 1140 band is also influenced by aldehydic C-C stretch, the lignin bands from 1500 – 1180cm^{-1} seem rather unaffected. Furthermore, the use of a second laser excitation wavelength or of complementary infrared spectroscopy is advisable for studying aromatic components of plant material, because only one wavelength might selectively enhance certain molecules, which in turn renders even semi-quantitative measurements invalid. On the other hand, the 532nm laser is very well suited for detecting even traces of π-conjugated aromatics like cinnamaldehydes.

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Autofluorescence variability in maize stems by multispectral image analysis of series of large images at the macroscopic scale

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The composition, structure and relative proportions of plant tissues are under biological control and determine the nutritional quality and use of the plant. In case of lignocellulosic biomass like maize stems, histological studies are of major importance for understanding the recalcitrance of plant material to enzymatic degradation[1]. In such plants, tissues can be observed without any labelling thanks to the autofluorescence of their cell wall components, i. e. lignin and hydroxycinnamic acids. Recent imaging equipments allows multispectral fluorescence imaging at the macroscopic scale with large fields of view (> 1 cm²) and a resolution below 3 µm per pixel. Such devices enable the study of large samples and take into account the statistical variability by analysing together sets of multispectral mosaic images.

In the present work, the variability of maize stem sections was investigated. Large images containing more than 4000 x 4000 pixels x 12 multispectral channels were acquired and usual chemometric approaches had to be adapted to account for the huge volume of data. Principal Component Analysis was implemented to assess common loadings from series of large images. An unsupervised multispectral image segmentation was retained to reveal fluorescence variability without any *a priori*. A multi-scale representation of images using image pyramids was combined with k-means clustering methods in order to take into account the whole volume of data[2].

We present here the analysis of a set of six cross sections of maize stem images taken from two stems showing different lignin fluorescences. The four first principal components revealed respectively fluorescent tissues, lignin and hydroxycinnamic acid fluorescences, the two phenotypes of lignin fluorescence, two regions of parenchyma that were related to different lignification. The multiscale k-means clustering model made it possible to segment the main tissues of the stem: sclerenchyma sheaths in vascular bundles in the pith and in the rind, epidermis, fibers of xylem, lignified and non-lignified parenchyma (Figure 1). A large variability in fluorescence properties was observed within and between stem sections.

The study demonstrate the potential of macrofluorescence imaging combined with appropriate chemometric methods to analyse together series of large samples. The method open the way to modelling fluorescence variability at the scale of the organ.

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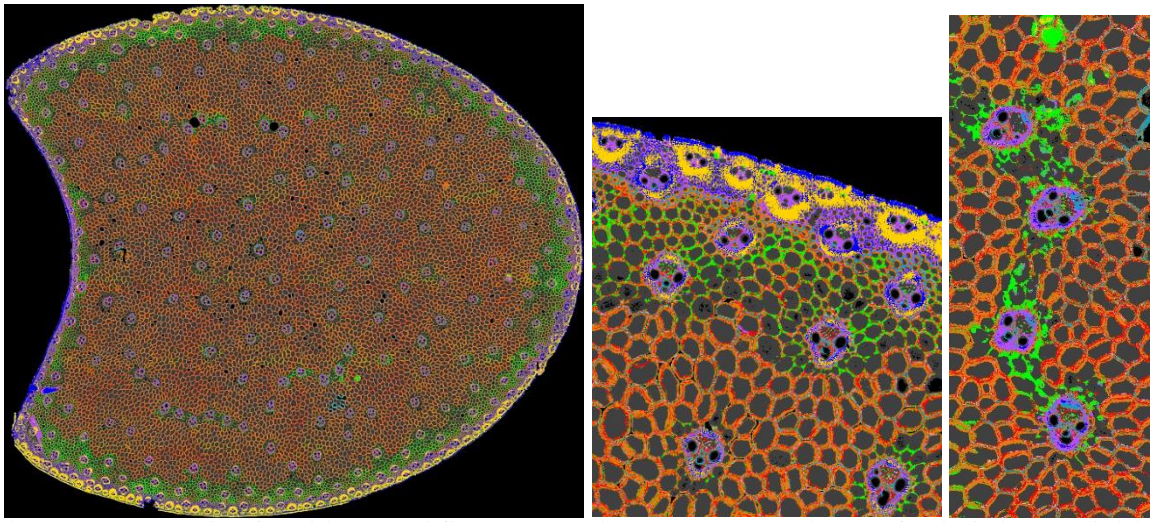

Figure 1. Example of multispectral fluorescence image segmentation. Left whole image (initial size 6397 x 5387 pixels ≈ 17 x 14 mm². Middle and right: selected regions: rind and centre of the stem. (700 x 800 pixels ≈ 1.9 x 2.1 mm² and 1000 x 400 pixels ≈ 2.7 x 1.1 mm²).

Multivariate analysis of Raman imaging data to study differences in plant organs

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Raman micro-spectroscopy is widely used for the investigation of plant tissues[1]. Using mapping approaches, a large amount of spectra from one sample can be obtained within short time. Chemometric methods, such as principal component analysis (PCA) or cluster analyses can be used in order to explore variances in the spectra from different plant tissues[2]. Often, such analyses are performed on averaged spectra or a few selected spectra. However, it would be very interesting to include information about the entire studied system, and it is desirable to avoid averaging of the spectra from different histological structures and substructures.

Here, we want to discuss characterization of the cross sections of xylem regions from leaves, stems and roots of plants with Raman micro-spectroscopy. Our goals include the comparison of cell wall structures of these different organs, as well as between different parts of the tissue. PCA were performed on tens of thousands of single spectra extracted from Raman imaging data. To achieve this, it is necessary to eliminate as many disruptive variances, e.g. between the different tissue components in each Raman map. We present a suitable extraction algorithm for spectra from the region of interest in the plant tissues, as well as an optimized pre-processing of the extracted data. In addition, considering the amount of spectra that will be analyzed together, the results need to be visualized and interpreted in an appropriate way.

The proposed analysis allows us to study variances in specific tissue regions, in most cases of the cell walls, of different tissues and in the same tissues in plants of varying phenotype. As an example, different environmental effects will be discussed. In the future, the developed procedure can be automated and may have impact in the broader applications of plant spectroscopy.

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Lignification of secondary cell wall characterized on a subcellular level: Implication to hygroscopic movement in the stork's bill's awn

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The awn in stork's bill (*Erodium gruinum*) seed dispersal unit coils as it dries. This hygroscopic movement promotes the dissemination and sowing of the seeds. The extent of the awn movement, driven by changes in the ambient humidity, is governed by its interaction with vapor water molecules. We aim to understand the movement, by correlating water dynamics within the awn to the spatial variation in the chemical composition of the awn's cell walls. We followed the hygroscopic movement visually and measured the kinetics of water in segments along the awn. We corroborated evidence from white light, fluorescence, and Raman microscopy, and MALDI-FTICR MS microanalysis to characterize the micro chemical makeup of the awn. We calculated the spatial distribution and relative amounts of total lignin and ester vs. ether-bound ferulic acid. We found that the coil's top segment is more sensitive to humidity changes than the awn's base. We correlated the higher sensitivity at the top to higher concentrations of aromatic materials. At the top part of the coil, lignin modified by cinnamaldehyde and ferulic acid rendered the cell walls hydrophobic. Ferulic acid residues at the middle lamellas, apparently bound the cells strongly together. In comparison, the base part of the awn contains smaller concentrations of aromatic residues, including ferulic acid, at both the cell walls and middle lamellas. We suggest that the ferulic acid residues contribute weak water binding sites to the tissue. The hydrophobic nature of the aromatic materials at the top part of the coil allows faster water molecule dynamics. This induces fast reaction to the ambient humidity. Strong cell-cell attachment in this region creates a durable tissue required for the awn's repeated movement induced by the diurnal humidity cycles.

Epithelium defense mechanism in conifers: Current challenges and methods to unravel the native state of extractives

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From the historical point of view, conifers are much older than deciduous trees and have a homogeneous anatomical cell structure of mainly traicheids. Additionally parenchyma cells are found in rays and special defence structures, called resin canals. Normal resin canals are found not in all, but some species (e.g. spruce, pine, larch, douglasie) in axial and radial direction. Additionally so called traumatic resin canals can be formed in tangential bands as a response to environmental abiotic and biotic stressors like drought and mechanical wounding (abiotic) or fungi and bark beetles (biotic). Most of the research on resin canals is done by light microscopy (fluorescence, staining) and extraction. Though, changes during sample preparation and extraction give not the true and complete picture of the native resin canal [1].

Therefore the aim of this work was to investigate the feasibility of Confocal RAMAN Microspectroscopy (CRM) to characterize extractives in native wood, especially in resin channels. Due to the fact that extractives often include phenolic compounds problems with fluorescence and high background have been expected. To overcome the fluorescence problems laser power, integration time as well as the used excitation wavelength have to be adjusted carefully[2].

To elucidate the chemical micro-distribution together with the ultrastructure of a resin canal, CRM was combined with Scanning Electron Microscopy (SEM) (Fig 1 A and B). Changes in chemistry on the micron level have been visualized by Raman images based on band integration. Furthermore the most pure extractive components have been revealed with the help of vertex component analysis (VCA). This enabled for the first time to acquire RAMAN spectra of native resin canals, which can assist in understanding the biosynthesis of these compounds.

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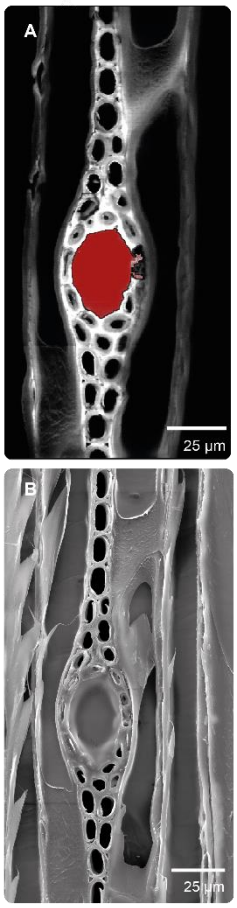

FIGURE 1 | (A) RAMAN overlay image by integrating the phenolic compounds (bright lignin and red resin) in a radial resin canal of spruce. (B) SEM image of the same resin canal.

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Unravelling hierarchical microstructure and chemical composition of hazelnut (*C. avellana*) shells

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Nutshells stand for a compact, hard to crack and highly resistant biological system protecting a kernel for the next tree population. A knowledge gap on the micro- and nano level of the structure and chemistry of nutshells implies the need for distinct scientific methods to answer the question on how nutshell design is optimized to bring up that fascinating life protecting materials. In this study extensive characterization of the hazelnut shell was carried out by applying micro-spectroscopic techniques such as Raman and infrared (IR) microscopy as well as scanning electron microscopy (SEM) and micro-computer tomography (CT) for 3D material characterization. Revealing intrinsic morphological principles helped to highlight the exact dependencies between various tissues, composed of different sclerotic cells, arranged in a specified manner. The identification of chemical compounds within different tissues and cells was performed by applying univariate and multivariate data analysis methods. With the help of cluster analysis similar spectra from the whole data set were grouped to clusters, which differentiated cell wall, compound middle lamella, pits and lumens and the corresponding average cluster spectra their chemical composition. Additionally unmixing multivariate data analysis approaches, such as non-negative matrix factorization (NMF) and vertex component analysis (VCA) have been applied to find the most significant chemical components and their distribution throughout the measured area. As a result, calcium and magnesium oxalates accumulation spots were found in close proximity to cribovascular bundles. In addition the presence of randomly distributed kaolinite, clay mineral, was unravelled. These findings were supported by the infrared spectral data, serving as a complementary technique to Raman spectroscopy. Our results clearly indicate the potential of Raman and infrared microspectroscopy to gain new insights into chemical identification of the nutshell microstructure of different nut species. In the long term we aim at revealing important structure-function relationships of the nutshell design during development to result in possible applications in biomimetic research.

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Synchrotron time Lapse imaging of lignocellulosic biomass hydrolysis: enzyme autofluorescence and infrared microspectroscopy of cell walls modifications

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The enzymatic conversion of lignocellulosic materials for the production of chemicals in place of petroleum feedstock is a very promising approach due to high enzyme selectivity and mild use conditions. However, in absence of pretreatment, the efficiency of enzymatic degradation of plant cell walls is low. In the last decade, many efforts have been devoted to the understanding of biomass recalcitrance to enzymatic degradation but no consensus has been reached on which cell wall features are the most important. This is due to the large diversity of biomass and to the fact that it is often considered as a bulk material, not taking into account the diversity and heterogeneity of plant cell walls in general, and the dynamic of enzymatic degradation.

In the present work, the objective was to develop time-lapse imaging to track both enzymes and changes in cell wall composition according to cell types on the course of degradation. Maize stem was considered as a model of grass lignocellulosic biomass and a commercial enzymatic cocktail with both cellulase and xylanase activities was used. The experiments were carried out at synchrotron SOLEIL. The DISCO beam line allowed visualizing both enzyme and cell walls without labelling, by exploiting specific auto-florescence properties of enzymatic proteins and of cell wall phenolic compounds. The change in cell wall composition was tracked by FT-IR micro-spectroscopy on the SMIS beam line using a dedicated microfluidic cell. Image analysis and chemometry allowed the quantification of the modifications during degradation and the comparison of cell types.

A contrasted affinity of enzymes according to cell types was evidenced from the beginning of the reaction. Combining the fluorescence and FT-IR information, we demonstrated that enzymes were absent from lignified cell walls and that these cell walls were not modified during the reaction. Enzymes concentrated on non-lignified cell walls. Consistent variations of enzyme concentration were found locally during the degradation with a decrease of the amount of enzyme in cell lumen together with an increased amount of enzymes on the surrounding cell walls. Different rates of polysaccharide degradation were found depending on cell types. In all cases, hemicellulose degradation was found to occur prior to cellulose degradation. An unexpected variability was found in enzyme localisation, initial biochemical composition and degradation pattern highlighting micro-domains in the cell wall of a given cell by fluorescence multiscale imaging and FT-IR microspectroscopy.

The vascular tissues in *Arabidopsis thaliana* floral stem: High spatial resolution using Synchrotron infrared, Raman and deep UV spectroscopy

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In higher plants, a network of vascular tissues (the phloem and the xylem), which supply water, minerals and other nutrients, connects the different organs and provides mechanical support for the aerial organs. The xylem and the phloem are two highly specialized conductive tissues. The cell wall surrounding the vascular cells is reinforced by a complex matrix of sugar-derived compounds (i.e.: cellulose, hemicellulose, pectins) and lignin, whose compositions depend on the cell type and developmental stage. For example low lignin and high hemicellulose content characterize phloem cells, while lignin and xylans are enriched in xylem cells. The supply and synthesis of the cell wall precursors take place in specialized parenchyma and fibre cells, which are responsible of the thickening of the associated conductive cell walls. As a consequence phloem and xylem cells represent a sink for consumption and sequestration of carbohydrates, with a competitive allocation of carbon pools between lignin and cellulosic compounds, which leads to a negative correlation between biomass production and lignin content.

To improve our knowledge on these processes, we focus on the carbohydrate components deposited during cell wall formation of phloem and xylem cells using the *Arabidopsis thaliana* floral stem as a model for carbon allocation. Our investigations, by label-free Synchrotron FT-IR, Raman and deep ultra violet microspectroscopies, of the cell wall composition and the cytosolic content of the different xylem and phloem cell types will be presented, using the floral stem of both *Arabidopsis* wild-type plants and mutants affected in sugar homeostasis.

Plants facing fire: Insights into *Banksia* seed pods by using *in situ* Raman and FT-IR spectroscopy

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In fire-prone ecosystems, many plants employ heat triggered fruit opening and seed release as a strategy to maximize seed dispersal, germination rates and seedling establishment after fire. The timed release of mature seeds from the plant canopy upon specific external stimuli is not only an important driver for vegetation dynamics in these regions, but it is also a source of inspiration for materials scientists.

This presentation reveals some of the basic principles that govern the long-term dimensional stability and opening mechanism in *Banksia* seed pods (Fig. 1). The species of this ancient[1] Australian plant genus open their woody fruits (follicles) in response to highly specific temperature stimuli. Since the mature follicles are composed of dead tissue, the differences in their ecological behavior can only arise from different material properties. Based on the existing intra- and interspecific differences in fire sensitivity[2,3], we studied the follicle tissue of selected species with a variety of techniques in order to identify and characterize the properties that enable long-term dimensional stability along with temperature sensitivity. Our results show that the follicles are a fine-tuned multi-parameter system, in which the geometry, chemical composition and mechanical properties are equally important for their functioning. The insights provided by utilizing *in situ* Raman and FT-IR spectroscopy techniques are explored in detail; revealing a temperature dependent, wax based self-sealing mechanism and the presence of heat-insulating[4] tannins in the follicle tissue.

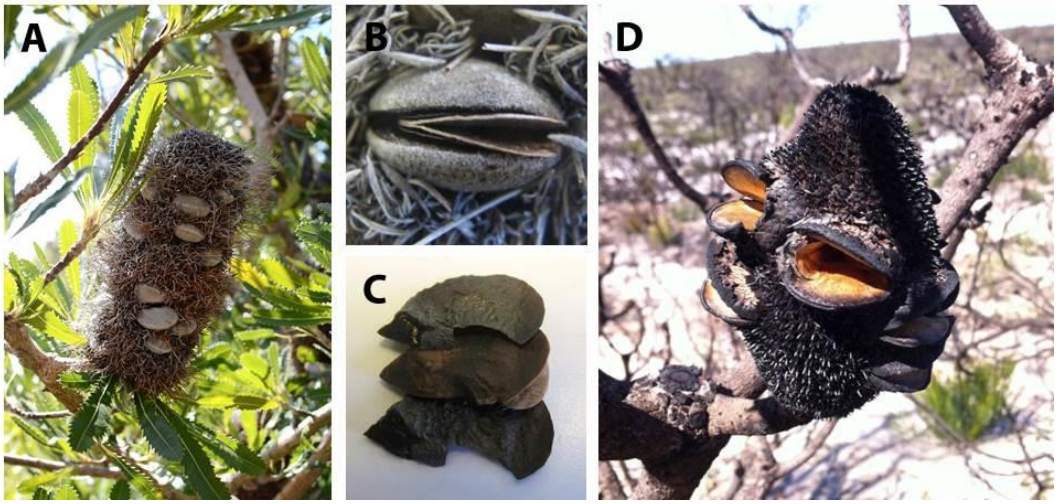

Figure 1. Typical features of *Banksia* fruit cones. (A) Woody fruit cone of *B. serrata* with closed follicles, (B) open individual follicle, (C) content of one follicle: two winged seeds separated by a hygroscopic plate, (D) cone of *B. attenuata* after fire and seed release.

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Potassium silicate maintains optimum cellular sodium and chloride homeostasis in wheat (*Triticum aestivum* L.) cultivars as monitored by dual-wavelength photometry

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Salinity is an environmental stress with adverse effects on crops[1]. Plant resistance to salt stress is a complex phenomenon and involves reactions causing a rebalancing of the physiological homeostasis of the cytoplasm[2]. We investigated the effect of silicon (Si) on cytosolic concentrations of sodium, $[Na^+]_{cyt}$, and chloride, $[Cl^-]_{cyt}$ by using dual-wavelength photometry, an efficient tool for monitoring ion concentrations in plant cells. Mesophyll protoplasts were isolated enzymatically from wheat seedlings of cvs. S-24 (salt tolerant) and Vinjett (salt sensitive). For $[Na^+]_{cyt}$ measurement the protoplasts were loaded with the sodium-specific dye, SBFI, and for $[Cl^-]_{cyt}$ measurement with the anion-specific MQAE dye. The protoplasts were subjected either to NaCl (0, 50 or 100 mM) followed by Si (1 mM as K_2SiO_3) or to Si (1 mM as K_2SiO_3) followed by NaCl (0, 50 or 100 mM). Additionally, both the cultivars were grown in nutrient solutions with, or without, 1 mM Si and the isolated protoplasts thereafter were subjected to 0, 50 or 100 mM NaCl to investigate the Si effects on $[Na^+]_{cyt}$, and $[Cl^-]_{cyt}$. The cultivars exhibited differential response to salt stress in terms of $[Na^+]_{cyt}$, irrespective of Si treatment. The $[Na^+]_{cyt}$ significantly increased only in cv. Vinjett, while in cv. S24 $[Na^+]_{cyt}$ it remained low even at the higher NaCl level applied. Significant reduction in $[Na^+]_{cyt}$ was observed for both the cultivars when treated with Si followed by NaCl additions as compared to NaCl treatment alone. However, when the protoplasts were subjected to NaCl prior to Si addition, the $[Na^+]_{cyt}$ exhibited a steady increase with time in both the cultivars. The $[Na^+]_{cyt}$ was significantly reduced when NaCl was added to protoplasts of both cultivars pre-treated with 1 mM Si during cultivation as compared to those without Si pre-treatment. The pharmacological results indicated that Na^+ uptake in cv. Vinjett was mediated by both non-selective cation channels, NSCCs, and K-specific channels, but in cv. S-24 mainly by NSCCs. In cv. S-24, Si inhibited Na^+ uptake via the K-channels while in cv. Vinjett the uptake was negatively influenced via both types of channels. Silicon inhibited the cytosolic uptake of chloride in both cultivars when Si was added prior to NaCl addition. It can be concluded that the salt tolerance of cv. S-24 depends on less uptake of sodium and chloride and that the uptake was inhibited by Si. Further research is needed to identify the role of Si on Na-uptake channels under saline conditions.

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Fluorescence lifetime imaging of plant cell wall

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Autofluorescence of plant cell wall originates from fluorophores such as aromatic phenolic molecules mainly contained in lignin (Ralph 2010). Thus it is commonly used to investigate architecture of plant cell wall by fluorescence microscopy. But fluorescence is also a complex measurement which is highly sensitive to its environment (temperature, pH, interactions,…) so that any correlation between fluorescence and biochemical cell wall property can be difficult to establish.

Fluorescence of plant cell wall is usually defined by its properties to be excited by a given wavelength light (λ_{EX}) and then to emit a higher wavelength light (λ_{EM}), both at given intensities, all these parameters being routinely measured by spectroscopy. Another relevant parameter less often used is fluorescence lifetime (Coletta 2013, Donaldson 2013), which represents the average lifetime of the fluorophore in the excited state (Paës 2014). Lifetime measurements techniques can be classified into time-domain and frequency-domain methods (Becker 2012), with the possibility, when imaging is carried out with a fluorescence confocal microscope, to measure the lifetime in the whole image or for each pixel (Figure 1).

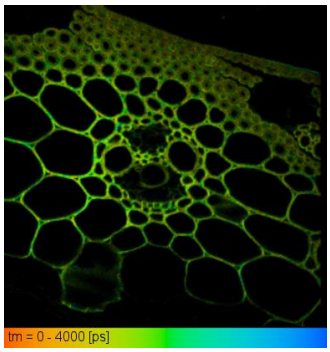

Figure 1. Fluorescence lifetime imaging of a 30 µm section of wheat straw treated with chlorite.

In the frame of lignocellulose biorefinery to assay the effect of pretreatment and the efficiency of enzymes during saccharification, lifetime measurements can successfully be used to:

- map the physical state of lignin at the cellular scale, thus providing information on the effect of biomass pretreatment;
- evaluate the condensation degree of lignin contained in steam exploded biomass samples, to be correlated to the amount of β-aryl ether linkages;
- measure quantitative Förster Resonance Electronic Transfer between lignin and fluorescent probes such as labelled enzymes directly in biomass samples, thus revealing strength of non-specific interactions at the molecular scale.

Perspectives related to the possibility of performing medium to high-throughput lifetime measurements will also be discussed.

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Understanding the formation of highly durable heartwood in Teak by use of Raman hyperspectral imaging and multivariate resolution techniques

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Wood is the most important renewable and natural material for structural applications (buildings, furniture etc.). Being a biological material, wood is susceptible to degradation by fungi and insects, which is as unwanted during the service life of such structures as it is for the living tree.

In this project we study Teak (*Tectona grandis*) as an example of a tree species with high natural durability. In Teak and in other durable tree species, the formation of heartwood plays an important role in the resistance against degradation due to the high natural abundance of extractives deposited in the cell walls and voids. Understanding the exact chemistry and the spatial distribution on the micro-scale involved in heartwood formation will in the long run make it possible to design protection systems that can increase the natural durability of wood from less durable species.

Confocal Raman Microscopy (CRM) is a powerful tool for providing spatial and spectral information of the constituents present in the cell walls during heartwood formation. From the data analysis point of view, wood tissue images analysis calls for the application of multivariate resolution techniques.

The aim of hyperspectral image resolution methods is to provide distribution maps and pure spectra related to image constituents of a sample based on the information contained in the measured raw image. Multivariate Curve Resolution-Alternating Least Squares (MCR-ALS) is an iterative resolution method oriented to recover the underlying spectroscopic bilinear model, i.e., concentration profiles (folded back into distribution maps) and pure spectra, by applying constraints related to chemical or mathematical properties of the profiles to be resolved. Single image analysis by MCR is often used, but it may be easily extended to the analysis of multiset structures formed by several images[1,2].

The study shows how CRM and MCR-ALS multiset analysis can be used to understand the chemical composition and deposition pattern on the microscale during heartwood formation in Teak.

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Analysing the mercerisation of dissolving cellulose pulp by Raman spectroscopy and multivariate data analysis

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We present a quantitative study of the degree of activation, denoted by DoA, of softwood sulphite dissolving cellulose pulp by aqueous sodium hydroxide. We base our quantitative analyses on Raman spectroscopic data combined with partial least square (PLS) regression modelling. Results are confirmed by X-ray diffraction and ^{13}C CP/MAS NMR spectroscopy.

Mercerisation of cellulose pulp by alkali treatment is the most common procedure used to activate cellulose fibres into many commercial cellulosic materials. During mercerisation, the NaOH solution enters the cellulose fibres, transforming them into a swollen and a highly reactive material called alkali cellulose (Na-Cell). In case NaOH is washed out of the cellulose structure, Na-Cell turn into Cellulose II (Cell II) upon drying. Based on this transformation, we defined the amount of Na-Cell as the amount of Cell II after washing and referred to this as DoA.

The technique to quantify the DoA from softwood sulphite dissolving cellulose pulp (Cell I) to Cell II was based on Raman spectra studies[1] on Cell I and Cell II and included a multivariate analysis approach based on Schenzel et al [2]. This Partial Least Squares (PLS) technique allows distinguishing between Cell I and II in the whole spectral region of approx. 327 – 1495 cm^{-1} simultaneously. The distinction is chemically explained by different conformations of molecular chains in the two polymorphs.

A calibration model, that allow the quantification of DoA, was built using PLS on Raman spectroscopic data. The calibration set samples consisted of a mixture of dissolving cellulose pulp (DoA = 0%) and mercerised material of the same origin (DoA = 100%), with their exact amounts known. This model can both explain (R^2) and predict (Q^2) 99 % of the variation in the samples (see Fig.1.). Once the model was built, quantitative predictions of DoA for samples mercerised at industrial relevant conditions for viscose fibres[3] and cellulose ethers[4] was made using average Raman spectral mapping data for each sample. The predicted DoA of the mercerised samples was in agreement with the DoA observed in the X-ray and ^{13}C CP/MAS NMR spectroscopy.

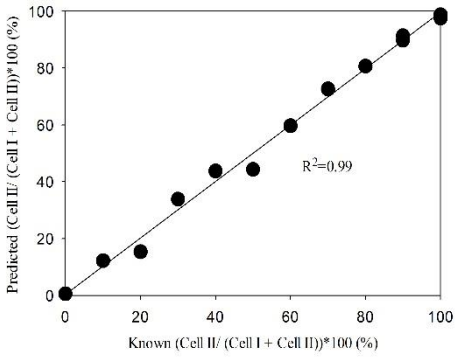

Figure 1. Calibration model built on the Raman spectra data. Predicted DoA % (Cell II/ (Cell I + Cell II)) vs. the known % (Cell II / (Cell I +Cell II)) of calibration samples.

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Dynamic FTIR for assessing lignin interaction in wood

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The complex heterogeneous structure of the wood cell wall has always provided for a challenge with regard to the understanding of how the wood polymers are organized and how they interact with each other to provide for the excellent mechanical properties of wood fibers. In particular the organization and contribution of the amorphous, matrix polymers of hemicelluloses and lignin has been debated. With the advancement of spectroscopic techniques, it is now possible to obtain a deeper understanding of this structure. One such technique is dynamic FTIR or 2D-FTIR which has the possibility of detecting the degree of interaction between the constituents within a composite material. For lignin, its contribution to strength properties in wood fibers has not been easily addressed, mainly due to the dominating effects of the cellulose. Earlier studies have not been able to detect any contribution from lignin during straining of wood fibers as viewed by the absence of changes in the lignin peaks in IR or Raman spectra coupled to molecular deformations[1, 2]. However, by utilizing dynamic FTIR it is now shown possible to follow the response of lignin during mechanical straining of the wood fiber material. Using sufficiently large deformations, above the elastic limit, the contribution to the stress transfer of lignin in wood samples may be shown[3]. By analyzing the dynamic coupling in the 2D-FTIR experiments it is clear that, although molecular lignin deformations are detected, the lignin do not directly contribute to the stress transfer in the wood fiber structure. The lignin is only showing a time delayed response. By removing hemicelluloses to different extent by chemical processing, where sufficient amounts of lignin still remain in the cell wall, the interaction between lignin and cellulose may be altered. Thus, for such cases the dynamic FTIR signals show that the lignin has becomes more involved in the load bearing and interacts more directly with the cellulose, i.e. it deforms simultaneously with the cellulose. This is particularly so in the case of pre-hydrolysis, where the high temperature of 170 ^{o}C with water present leads to a lignin flow and redistribution. By analyzing these changes due to the structural alterations of the wood polymers, a plausible picture of the structural arrangement of lignin in the cell wall is proposed.

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Dual labeling by chemical reporters allows visualization of lignification dynamics in plants

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Lignin is a major structural component of certain plant cell walls and plays an important role in mechanical support, water transport and protection against pathogens. Together with cellulose/hemicelluloses it constitutes lignocellulosic biomass utilized in a variety of industrial applications (e.g. timber, paper, biofuel, fibers). Lignin is a phenolic polymer composed of monomers (monolignols) that are assembled by a radicalar polymerization process initiated by two enzymes, laccases and/or peroxydases, during lignification. In angiosperms, three main monolignols (*p*-coumaryl alcohol, coniferyl alcohol and sinapyl alcohol) are oxidized giving rise to the H (*p*-hydroxyphenyl) units, G (guaiacyl) units and S (syringyl) units of the lignin polymer[1]. If the biosynthesis of monolignols is relatively well understood, the dynamics of lignification at the cellular level is still unclear and a better understanding of when, where and how these monomers are incorporated into the polymer is necessary to improve our knowledge of lignin formation and the valorization of lignocellulosic biomass.

The recent emergence of a chemical approach to visualize biomolecules in their native systems is helping to resolve these questions. In this strategy, an analogue of the biomolecule of interest modified with a biocompatible chemical function is metabolically incorporated into the target biomacromolecule and functions as a 'chemical reporter'. The incorporated chemical reporter is then visualized by fluorophore tagging initiated by a bioorthogonal chemistry reaction. A few years ago, analogs of coniferyl alcohol tagged with clickable azide or alkyne functions were incorporated into plant cell walls and labeled with fluorescent dyes to study lignification in Arabidopsis[2,3,4,5]. However the lignin polymer consists of more than one monolignol and a more accurate picture of lignin formation requires the simultaneous detection of multiple chemical reporters.

Here we present the recent development of a "Bioorthogonal Labeling Imaging Sequential Strategy" (BLISS) to visualize and analyze the incorporation of both *p*-hydroxyphenyl (H) and guaiacyl (G) units into flax (*Linumusitatissimum* L.) lignin *in vivo* by using a combination of strain-promoted and copper-catalyzed azide-alkyne cycloadditions (SPAAC/CuAAC)[6]. We designed a new azide-tagged monolignol reporter for H-units in metabolic lignin engineering and used it in conjunction with an alkyne-tagged G-unit surrogate to study lignification dynamics in flax by confocal fluorescence microscopy. Here, we show that BLISS provides precise spatial information on the zones of active lignification.

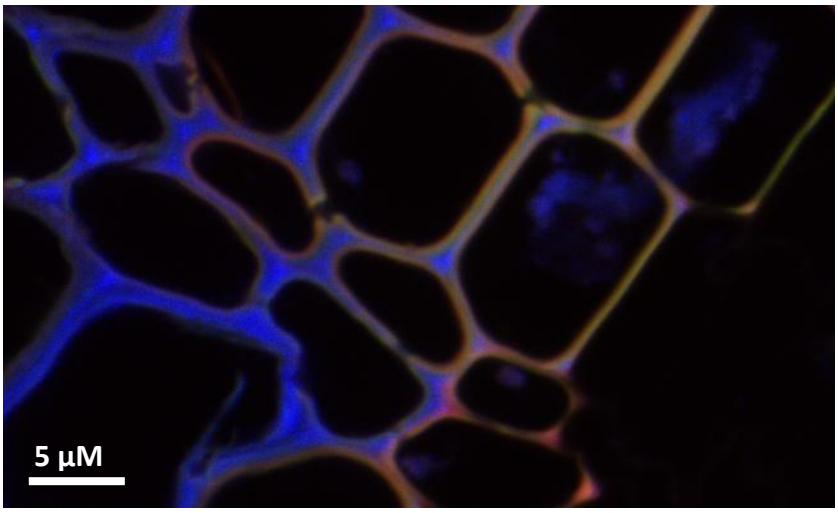

FIGURE 1. CHEMICAL REPORTER DUAL LABELING OF FLAX XYLEM BY BLISS

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Multimodal structural and functional analysis of sorghum tissues and sorghum biosilica

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The ability of silicon to reduce biotic and abiotic stresses in plants is well documented.[1] It is deposited in the form of solid amorphous silica, $SiO_2 \cdot nH_2O$, in the cell walls improving plant fitness.[2] The formation of biosilica has been widely studied, however, the biochemistry and physiology of plant silica deposition is not yet fully understood.[3,4] We intend to elucidate the chemical interactions of the plant cell with silica by addressing the effect of cell wall composition and the role of organic compounds on silica deposition. With this purpose, plants of *Sorghum bicolor* (L.) Moench (wild type; line BTX-623) and *sblsi1* mutant deficient in silicic acid uptake were grown either in soil or hydroponically with diverse amounts of silicon supplementation in the form of sodium silicate.

To relate the organic compounds involved in silicification with the silica properties, phytoliths from mature wild type plants were extracted using different approaches and analysed using solid state 29Si Magic Angle Spinning NMR, Raman spectroscopy and Synchrotron X ray diffraction. Raman imaging and SEM-EDX were used to obtain information on the cell wall composition and to locate organic compounds and elements in plant tissue cross sections. We identified differences in protein, aromatic phenol and cellulose composition in plants with and without silica. We also found signatures of organic compounds in the extracted silica that may be involved in the silicification. Our results indicate that cells with different morphologies deposit hydrated silica in uneven amounts (Figure 1).

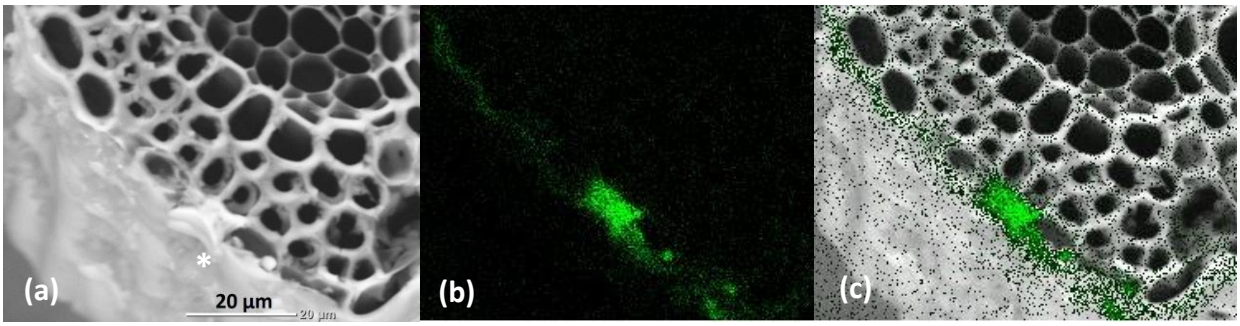

Figure 1. a) Scanning electron micrograph of a sorghum leaf cross-section, with a silica cell marked with *. b) Silicon map of the same region obtained by energy dispersive X-rays showing the spatial distribution of silicon, the silica cell heavily silicified and silica deposits in epidermal cells. c) Overlay of the two images.

We also detected that extraction methods of phytoliths alter the silica molecular structure and its condensation degree. Silicification in grasses is a spatially and temporally controlled process, however, there is no agreement as to whether it is a consequence of an active or passive mechanism.

As we observed heterogeneity of the silica distribution in plant tissues, we hypothesize that silica deposition may involve both active and passive mechanisms, which likely are tissue and functionally dependent. In future research, we aim to address this problem in depth.

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Vibrational spectroscopy of pollen

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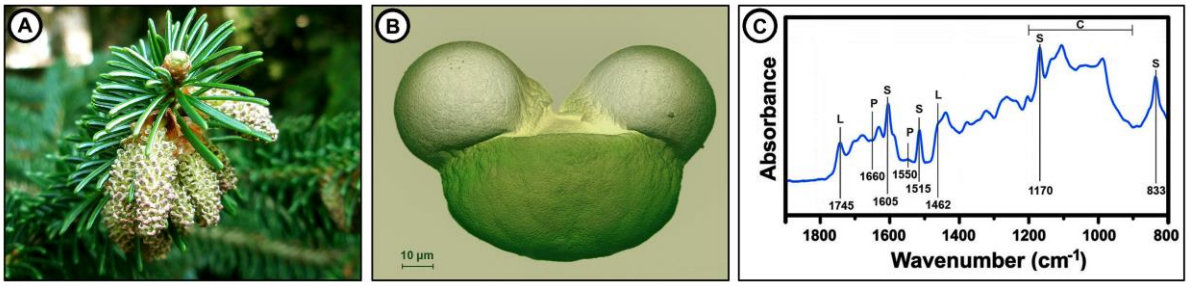
Pollen is a key contributor in one of the most fundamental processes in plant biology: fertilization. However, male function in plants is much less known than the female function due to methodological constraints in evaluating pollen quality. In addition, characterization of pollen provides useful information in ecology and palaeoecology, allergology, forensics, stratigraphy and environmental archaeology.

In the recent years, the group at NMBU has been at the forefront of pollen research by vibrational spectroscopy, offering novel insights to functional and evolutionary perspectives as well as developing powerful tools for analysis of aeroallergenic pollen.[1-8] Vibrational spectra of pollen contain abundant information on the biochemical composition of a pollen grain, with specific signals related to lipids, proteins, carbohydrates, pigments, and complex constituents, such as grain wall sporopollenins. Our studies have shown that vibrational spectroscopy offers a rapid and economical measurement of pollen for identification and classification purposes via taxon-specific spectral fingerprints.[1-4] Moreover, spectral signatures offer characterization of phenotypical differences and biochemical interpretation with respect to environmental stress, for example temperature.[5,6]



Abies cephalonica (Greek fir): **A**) Branch with male cones (strobili) during pollination. **B**) Scanning electron microscope image of pollen grain in equatorial view. **C**) FTIR spectrum of pollen; the marked vibrational bands are associated with lipids (L), proteins (P), sporopollenins (S) and carbohydrates (C).

Vibrational spectroscopy of pollen is extremely versatile, and encompass a broad range of measurement techniques, such as FTIR and FT-Raman bulk measurements (10^4-10^6 pollen grains per measurement),[1,3-6] FTIR and Raman microspectroscopies on single pollen grains,[2,3,8] and imaging of grain substructures by µRaman and synchrotron-based µFTIR.[7] For example, we have introduced recently a new spectroscopic method for high-throughput FTIR characterization of pollen, thus allowing for fast, simple and cost-effective measurement of hundreds of samples in environmental studies.[6] Furthermore, microspectroscopy of single pollen grains face some specific challenges, such as strong Mie scattering in FTIR microspectroscopy that results in anomalous spectral features.[2,7,8] We have recently demonstrated that scatter-free FTIR spectra can be obtained by using an embedding matrix, and thus achieving identification of single pollen grains with unprecedented accuracy.[2] In general, our fieldwork and greenhouse studies on over 500 plant species by wide range of techniques have shown that spectral data offers unique perspective on plant acclimation and adaptation, and can be used for a wide range of research, from biology, ecology, agronomy, and forestry, to medicine, forensics, geology and archaeology.

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Special Issue

Selected Papers from the 1st International Plant Spectroscopy Conference

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Deadline for manuscript submissions:
31 December 2017

Message from the Guest Editors

The 1st International Conference on Plant Spectroscopy (http://www.conference.plantspec.org/) organized by the International Society for Plant Spectroscopy (www.plantspec.org) and the Vibrational Spectroscopy Core Facility at Umeå University (http://www.kbc.umu.se/english/visp/), will take place in Umeå, Sweden, 29–30 August, 2017.

The aim of conference is to bring spectroscopy to plant scientists and plant sciences to spectroscopists, and a broad range of topics and techniques will be covered, from roots to pollens and wood, from basic research to industry, from vibrational spectroscopy to autofluorescence, NMR to mass spectrometry and synchrotron based techniques, as well as data analysis.

The Special Issue will include selected papers by authors who were selected, by the scientific organizing committee, to presented at the conference, after successfully finalizing the peer review process of the journal with the aim of rapid and wide dissemination of research results, developments, and applications.

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