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Comparison of sample pre-treatments for laser desorption ionization and secondary ion mass spectrometry imaging of *Miscanthus* × *giganteus*

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ABSTRACT

Efforts to further the potential of the large perennial grass *Miscanthus* × *giganteus* as a biofuel feedstock would be aided by the ability to image the chemical species present during the fuel production process. Toward this end, two mass spectrometry imaging (MSI) approaches have been investigated here—laser desorption/ionization mass spectrometry (LDI-MS) and secondary ion mass spectrometry (SIMS). As a first step, cross sections of *Miscanthus* were subjected to a variety of sample preparation methods to optimize conditions for MSI. For LDI-MS, a thin metal coating (2 nm thick Au) provided high quality signals of saccharide-related ions. The traditional matrix-assisted laser desorption/ionization matrix, 2,5-dihydroxybenzoic acid, also showed high efficiency for the desorption of saccharide-related ions. In contrast, with α-cyano-4-hydroxycinnamic acid matrix, these ions were nearly absent in the mass spectra. Direct laser ablation of untreated *Miscanthus* sections was also performed. High resolution images, where the fine structure of the vascular bundle could be clearly visualized, were obtained using SIMS. Although coating the sections with a nanometer thick Au layer can greatly enhance the quality of SIMS images, the coating had limited effect on secondary ion signal enhancement. Using the optimized mass spectrometry approaches described here, information on the spatial distribution of several saccharides was obtained.

1. Introduction

The production of liquid fuel from renewable resources is a goal set forth in the national roadmap for renewable energy (Department of Energy, 2006). Ethanol derived from corn kernels dominates current biofuel production. However, future demand for liquid fuel, especially ethanol, will not be met by relying solely on corn. Consequently, lignocellulosic materials (LCMs), which include perennial grasses, wood chips and agricultural waste, are receiving significant attention as sources for biofuel (Ragauskas et al., 2006).

The conversion of biomass into cellulosic ethanol involves a complex chemical, physical and enzymatic treatment process—delignification, saccharification and fermentation. In addition, a variety of pre-treatment processes have been employed to break down the phenylpropanoid heteropolymers (lignin) and separate them from the fermentable cellulose/hemicellulose sequestered in the rigid cell wall structure of the plants. These methods include thermochemical, dilute acid and ammonia fiber expansion treatments (Alizadeh et al., 2005; Boateng et al., 2006; de Vrije et al., 2002; Michel et al., 2006; Sorensena et al., 2008; Szabo et al., 1996). Optimization of these pre-treatments requires knowledge of the spatial and temporal infiltration of the reagents into the plant cell wall structure, as well as their effect on the spatial and temporal profiles of compounds, such as lignin, present in the sample.

How can one obtain chemically-rich information on the processing of such materials? One option is via a new modality in mass spectrometry (MS) known as mass spectrometry imaging (MSI) (Belu et al., 2003; McDonnell and Heeren, 2007; Rubakhin et al., 2005). It combines the rich chemical/structural information inherent to MS with spatially resolved sampling. Thus, under carefully optimized conditions, distribution maps of individual chemical species in tissue sections can result. MSI also should be able to provide spatial and chemical information on the interaction between processing reagents and the plant cell wall structure during pre-treatment processes. This approach has advantages over more traditional biochemical imaging techniques because direct analysis of the tissue composition can be accomplished without analyte preselection or labeling, and multiple compounds can be detected simultaneously.

Before the full potential of MSI can be realized however, it is necessary to address several potentially problematic factors. One issue is that the efficacy of sample treatment and preparation procedures for MSI tends to depend both on the sample type as well as

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the MSI approach used. The two most frequently used MSI methods are laser desorption ionization mass spectrometry (LDI-MS) and secondary ion mass spectrometry (SIMS). LDI-MS has been used to visualize the distribution of various compounds, especially proteins and peptides, in tissue sections and in single cells (Andersson et al., 2008; Debois et al., 2008; McDonnell and Heeren, 2007; Monroe et al., 2008; Rubakhin et al., 2007). In LDI-MS, a focused laser beam is used to irradiate the sample surface. The analytes are often co-crystallized with a matrix to enhance analyte desorption and ionization. After the analytes are vaporized and ionized by a laser, they are detected by the mass analyzer. LDI-MS imaging is achieved by rastering the focused laser across the sample surface and correlating the detected ions with the location of the laser beam. The spot-size of the laser is typically on the order of 50 μm, which usually limits the spatial resolution of LDI-MS imaging to that size.

SIMS has also been widely used for the imaging of tissue sections and single cells (Fletcher et al., 2008; McDonnell and Heeren, 2007; Monroe et al., 2008; Parry and Winograd, 2005). In SIMS, a focused ion beam with a kinetic energy of 10–40 keV is used to bombard the sample surface. The diameter of the primary ion beam can be as small as 200 nm, thus allowing single cells or tissue sections to be imaged with sub-μm resolution. Furthermore, tissue sections can be directly analyzed with minimum pre-treatment, *i.e.* no matrix is needed. Due to the difference in ionization mechanism, SIMS typically detects mostly atomic, small molecular or fragment ions, precluding the measurement of large molecular weight species.

In summary, with SIMS imaging, the distribution of small molecular and fragment ions can be imaged with higher spatial resolution, while with LDI-MS, larger molecules, like peptides and proteins, can be imaged at lower spatial resolution. Therefore, to achieve optimal biological imaging and maximize the chemical information recovered from a single sample section, a combination of these two MSI methods is preferred.

Miscanthus × *giganteus* is a perennial plant that is suitable for bioenergy production (Clifton-Brown et al., 2007; Heaton et al., 2004, 2008). Advantages over other energy crops are its high biomass yield and low maintenance cost after establishment. *Miscanthus* has been widely investigated in Europe and pilot scale tests have been carried out in many testing fields throughout the state of Illinois. Here, we use both SIMS and LDI-MS imaging to identify and localize chemical species in *Miscanthus* stem cross sections.

As mentioned above, MSI of *Miscanthus* and other LCMs requires the development of sample preparation methods suitable for LDI-MS and SIMS. A variety of sample preparation methods have been used in LDI and matrix-assisted laser desorption/ionization (MALDI) MS imaging of plant tissues. In the simplest method, an infrared laser irradiates the plant tissue at ambient pressure, with native water in the tissue acting as the matrix to enhance the ionization of analytes (Li et al., 2008). Other methods include coating the tissues with conventional MALDI matrices, *e.g.*, 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), or 9-aminoacridine (Burrell et al., 2007; Ng et al., 2007; Shroff et al., 2007; Wu et al., 2007), as well as colloidal graphite (Cha and Yeung, 2007). In addition, coating the tissue section with a thin layer of metal has been effective in reducing surface charging and enhancing imaging quality (Altelaar et al., 2006). SIMS has also been used for the imaging of plant samples (Heard et al., 2002; Imai et al., 2005; Saito et al., 2005, 2008), however, not without several challenges, namely, low secondary ion yield and sample charging. Recent reports of SIMS imaging of biological samples have also used a metal coating to reduce surface charging and enhance the emission of secondary ions (Delcorte et al., 2002; Wehbe et al., 2008). A thin (nanometer) layer of Au or Ag enhances secondary ion yields several-fold, especially with atomic primary ion beams. A reliable, high-fidelity protocol for sample preparation and data collection for MSI of *Miscanthus* or similar LCMs has yet to be established. Here, using various sample preparation methods, we present MSI results obtained to visualize the distribution of chemical species in *Miscanthus* stems and assess the capability of these approaches in monitoring the biomass-to-ethanol conversion process.

2. Experimental

2.1. Sample preparation

Samples of *Miscanthus* × *giganteus*, grown in an experimental farm south of the University of Illinois at Urbana-Champaign (UIUC) campus, were collected in mid June (courtesy of Prof. Steve Long of the Crop Sciences Department, UIUC). Upon collection, the plants were plunged into liquid nitrogen and stored at –80 °C. Prior to mass spectrometric analysis, a small block of *Miscanthus* stem was embedded in ice and sectioned with a Leica CM 3050S cryostat (Leica Microsystems, Bannockburn, IL) at –20 °C. The thickness of the grass stem cross sections was 50 μm.

For LDI-MS experiments, *Miscanthus* sections were thaw mounted on either plain glass slides or indium/tin oxide (ITO)-coated glass slides. The sections were then dried in a vacuum desiccator and subjected to various treatments. For direct LDI-MS experiments, thin *Miscanthus* sections mounted on ITO slides were profiled without pre-treatment. For matrix-assisted LDI experiments, the sections thaw mounted on glass slides were coated with MALDI matrix—DHB or CHCA (Sigma, St Louis, MO)—with an airbrush (Badger, Franklin Park, IL). The concentration of the DHB solution was 20 mg/mL in 60:40 acetone and water solvent. The CHCA solution concentration was 20 mg/mL in 80:20 acetone and water. High purity N_2 at 15 psi was used as the nebulizing gas to drive the airbrush. The nozzle of the airbrush was placed ~20 cm away from the sample and moved slowly across the entire slide. Between each pass, the slide was allowed to dry (~2 min). Approximately 20 passes were made for each slide. After matrix coating, the samples were coated with a 1 nm Au layer using a Desk II TSC sputter coater (Denton Vacuum, Moorestown, NJ) to reduce surface charging. For Au-assisted LDI experiments, the *Miscanthus* sections were mounted on glass slides and coated with a 2 nm Au layer using the same sputter coater.

For SIMS experiments, the thin sections were thaw mounted on Si wafers and dried in a vacuum desiccator overnight. To reduce

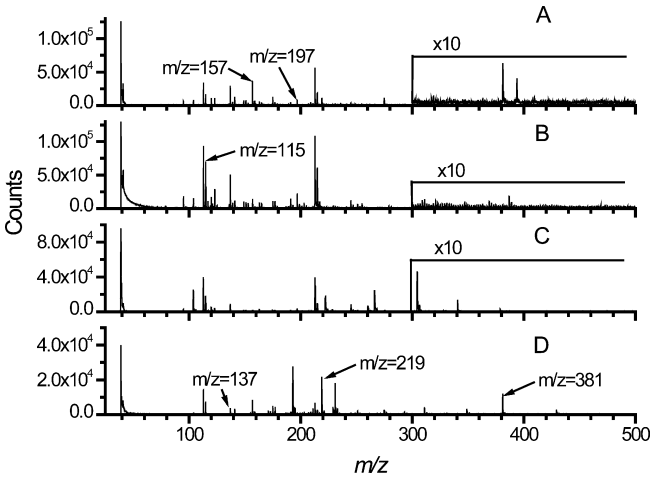

Fig. 1. LDI mass spectra of *Miscanthus* cross sections from: (A) Au-coated section, (B) ITO slide, (C) CHCA-coated and (D) DHB-coated section. The *y*-axis scale is magnified 10-fold as indicated to highlight low intensity peaks.