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Special Focus Issue – Mini Review

Synchrotron Radiation Sheds Fresh Light on Plant Research: The Use of Powerful Techniques to Probe Structure and Composition of Plants

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While synchrotron radiation is a powerful tool in material and biomedical sciences, it is still underutilized in plant research. This mini review attempts to introduce the potential of synchrotron-based spectroscopic and imaging methods and their applications to plant sciences. Synchrotron-based Fourier transform infrared spectroscopy, X-ray absorption and fluorescence techniques, and two- and three-dimensional imaging techniques are examined. We also discuss the limitations of synchrotron-based research in plant sciences, specifically the types of plant samples that can be used. Despite limitations, the unique features of synchrotron radiation such as high brightness, polarization and pulse properties offer great advantages over conventional spectroscopic and imaging tools and enable the correlation of the structure and chemical composition of plants with biochemical function. Modern detector technologies and experimental methodologies are thus enabling plant scientists to investigate aspects of plant sciences such as ultrafast kinetics of biochemical reactions, mineral uptake, transport and accumulation, and dynamics of cell wall structure and composition during environmental stress in unprecedented ways using synchrotron beamlines. The potential for the automation of some of these synchrotron technologies and their application to plant phenotyping is also discussed.

Keywords: FTIR spectroscopy • Microspectroscopy • Plant cell wall • Plant imaging • Synchrotron radiation • X-ray imaging.

Abbreviations: μCT, computed microtomography; EXAFS, extended X-ray absorption fine structure; FTIR, Fourier transform infrared spectroscopy; mid-IR, mid infrared; MRI, magnetic resonance imaging; SR, synchrotron radiation; STXM, scanning transmission X-ray microscopy; XANES, X-ray absorption near edge structure; XAS, X-ray absorption spectroscopy; μXRF, X-ray microfluorescence.

Introduction

Knowledge of the biochemical composition of plant organs, tissues and cells is often critical for both basic and applied plant research. Thus plant biologists routinely observe, dissect and disrupt plant organs to extract or modify biochemical compounds as an essential part of their research. The extracted compounds are often characterized by their spectroscopic characteristics such as absorbance and fluorescence emission. However, this classical method of obtaining chemical information also destroys valuable complementary information on spatial localization of those compounds at the tissue, cellular and subcellular level. Parallel developments in microscopy have in part overcome this challenge with histochemical, immunohistochemical and various other biochemical labeling techniques. Furthermore, modern microspectrometry techniques allow microscopes to localize some biochemical compounds in biological tissues using appropriate labels and marker compounds (Fernandez et al. 2010).

In this context, synchrotron-based technology affords researchers the ability to image and quantify specific chemical functional groups as well as trace metals directly at a tissue, cellular or subcellular level, making it a powerful tool in understanding the chemical processes of complex biological systems with minimal sample modification. This has generated critical insights into both agricultural and horticultural science in the areas of localization and quantification of seed mineral content and anti-nutritional compounds (Yu et al. 2003, Lombi et al. 2009, Iwai et al. 2012, Akhter et al. 2014, Kyriacou et al. 2014) as well as basic research related to nutrient starvation effects on algae, accumulation and speciation of toxic metals, and effects of acclimation on cell walls of plants (Heraud et al. 2005, Isaure et al. 2006, Goff et al. 2009, Tanino et al. 2013).

Despite the obvious advantages of synchrotron radiation (SR) in plant research, and the available synchrotron resources in most regions of the world, adoption of this powerful and versatile tool by the plant community has been slow. Therefore, the emphasis of this report is to highlight synchrotron applications in basic and applied plant science research while examining some of its challenges. It is our hope that this mini review will provide scientists with a brief overview of the capabilities of synchrotron-based research and an idea of how it could complement their research goals.

What is a Synchrotron?

Modern synchrotrons are powerful machines that accelerate charged particles such as electrons in a large ring-like trajectory at relativistic (near light) speed. The energy of these electrons is

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used to generate intense beams of photons or 'light' ranging in energies from infrared to X-rays, at very high intensities not obtainable from conventional sources (Kim 1989). There are currently 47 recognized synchrotron facilities in operation around the world (http://www.lightsources.org). Some synchrotrons are optimized for all light energies from infrared to soft and hard X-rays (Laboratori de Llum de Sincrotró, Barcelona, Spain; Advanced Light Source, Berkeley, USA), whereas others are optimized for hard X-ray techniques (SPring-8, Japan; Advanced Photon Source, Argonne, USA; European Synchrotron Radiation Facility, Grenoble, France). The hard X-ray-optimized synchrotrons are the largest in physical size, with a diameter of kilometers. At the Canadian Light Source (Saskatoon, Canada), electrons are generated using a hot tungsten plate (electron gun) and injected in bunches at nanosecond time intervals into a linear accelerator (LINAC) that uses radiofrequency waves to accelerate electrons to about 300 MeV. From the LINAC, the electrons are transferred to a circular booster ring that further accelerates the electrons to 2.9 GeV. Electrons are then injected into a larger storage ring (**Fig. 1**; http://www.lightsources.org/regions).

Radiant energy is emitted from the storage ring electron beam when it passes through: (i) bending magnets and (ii) other magnetic devices such as 'wigglers' and 'undulators' in the straight sections (**Fig. 1B**). Energy lost by the electron beam in this fashion is replaced by devices such as radiofrequency cavities that maintain the energy of the electrons in the storage ring for prolonged durations. In addition, electrons lost from the storage beam due to collisions with the walls of the storage ring and stray gas molecules are periodically replenished by fresh injections. However, photon beams of stable intensities can be delivered to users by maintaining near constant beam current by injecting fresh electrons at short time intervals, called the 'Top Up' mode of electron injection.

Three kinds of magnetic devices generate SR beams: bending magnets, wigglers and undulators (**Fig. 1C**). When electrons pass between the dipoles of a bending magnet, a flattened, fan-shaped electromagnetic radiation beam is emitted. Hard X-ray, soft X-ray, ultraviolet and infrared beams can be extracted from this radiation, by a combination of devices such as slits, monochromators and mirrors. The beams are usually collimated prior to being focused on samples in a microscope or spectrophotometer. A wiggler produces a beam similar to that of a bending magnet in its continuous spectral characteristics, but at several fold higher intensity (**Fig. 1C**). The arrangement of dipole magnets in an undulator is designed to generate

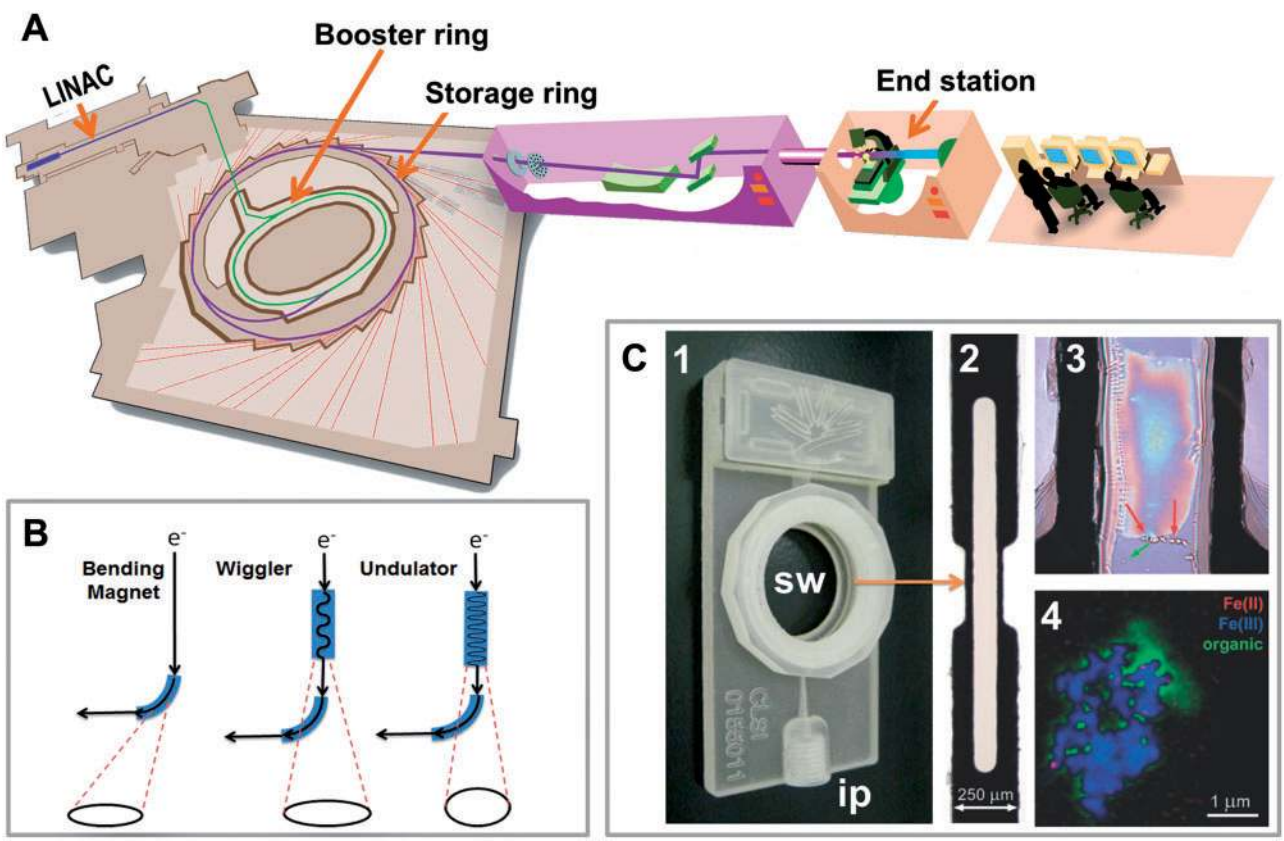

Fig. 1 Visual representation of a synchrotron facility. (A) Schematic of the synchrotron powering the Canadian Light Source, Saskatoon, Canada, and the representation of a typical beamline with its end station. (B) Pictoral representation of three different types of magnetic dipole devices used for generating synchrotron beamlines. Examples of hydrated sample cells used in (C-1) mid-IR and (C-2) soft X-ray microspectroscopy (Obst et al. 2008) of fresh biological samples. C-3 shows the optical image of the sample cell loaded with an iron-oxidizing bacterial sample (red arrows) and C-4 shows the chemical signature of bacterial activity showing iron III (blue), iron II (red spots) and organic compounds (green) generated using STXM image analysis.

interference patterns that result in emission of multiple discrete spectral bands of very high brightness (Sham and Rivers 2002).

The maximum possible number of beamlines in a synchrotron facility is physically limited by the number of bending magnets and the total number of linear segments available for insertion devices. In addition, a combination of the physical infrastructure, the scientific requirements, and availability of financial resources determine the mix of beamlines and end station facilities in a synchrotron. For example, the Canadian Light Source currently has 14 operational beamlines that harness far infrared/mid infrared (mid-IR), soft X-rays as well as hard X-ray beams for different types of spectrometry, microscopy and imaging applications designed to cater to users in the areas of material science, physics, chemistry and biology (**Table 1**; **Fig. 1**).

Individual 'beamlines' in a synchrotron carry the electromagnetic radiation from emission devices to 'end stations', where they illuminate sample material in a spectrometer or microscope. Sophisticated detector systems then collect signals emerging from samples and record diffraction, absorption and fluorescence spectra. Strategically positioned detector array systems can also be used to collect spectral data from a single point, a specific surface or volume of the sample to generate simple spectra, or two- or three-dimensional images, respectively (Miller and Dumas 2006). In addition, the polarization and nanosecond pulse properties of SR can be exploited for specialized experiments such as those involving circular dichroism measurements of protein folding (Tao et al. 2009) or investigation of submillisecond biochemical reaction kinetics (Graceffa et al. 2013).

Synchrotron Techniques and Their Applications to Plant Sciences

There are a number of sophisticated laboratory-based spectroscopic and imaging technologies available for studying and correlating the biochemical composition and structure of plants. They include optical infrared, X-ray and electron microscopes of different types, as well as absorbance, diffraction and fluorescence spectrometers in all these energy ranges. The advantage of using SR in plant research derives from the unique combination of extremely high brightness, cohesiveness, polarization and nanosecond pulse properties of the SR beam that is not matched by any conventional source (Miller and Dumas 2006). In addition, synchrotron light covers the entire spectral range from far and mid-IR to X-rays (Duncan and Williams 1983). While devices using conventional mid infrared light beams can achieve a spatial resolution of approximately 20 μm (Carr 2001), synchrotron light can attain spatial resolutions from 1 to 10 μm in mid-IR (Pellerin et al. 2004) to near 5 nm in soft X-ray devices (Shapiro et al. 2014).

For spectroscopy and imaging of plants, SR must be in the energy (spectral) ranges that will interact specifically with different components of the plant sample, but not drastically modify the physicochemical nature of the biological material during the time of observation. Secondly, the incident radiation must reach the targeted region of the plant sample, while the emergent radiation must reach the detectors without excessive distortions. For this to happen, incident radiation must penetrate the intact plant sample, or the target molecules should be exposed to the incident radiation through sectioning or homogenization. High quantum energies (X-rays) and intensities of light penetrate most plant tissue to considerable depth with minimal tissue destruction. Thus, synchrotron-based X-rays are ideally suited to detect, quantify and localize metals and other elements, and also ascertain their oxidation levels and chemical co-ordination in plant organs, tissues, cellular and subcellular preparations. In addition, X-ray, ultraviolet and infrared light can reveal the distribution of organic and inorganic compounds in tissues, at the cellular and subcellular level on carefully prepared samples. The development of ingenious sample cell configurations designed for use with both SR X-ray and infrared have further enhanced the potential of SR beamlines to reveal the dynamics of biochemical and developmental changes in real time at cellular and subcellular levels in situ (**Fig. 2**; Lee and Kim 2008, Goff et al. 2009, Wang et al. 2013).

Several excellent reviews on the application of SR techniques in plant, soil, environmental and animal sciences are available (Sutton et al. 1994, Salt et al. 2002, Lombi and Susini 2009, Neu et al. 2010, Pushie et al. 2014, Zhao et al. 2014) for those wishing to acquaint themselves with experimental details and scientific approaches developed for using SR in different fields of research. For example, Neu et al. (2010) compared the advantages and limitations of laser scanning microscopy (LSM), magnetic resonance imaging (MRI) and scanning transmission X-ray microscopy (STXM) regarding 'in situ analysis of the structure, composition, processes and dynamics of microbial communities'. It should also be noted that the publications discussed here are by no means an exhaustive list, and we hope this article will inspire interested readers to acquaint themselves with the much larger body of literature available on this topic. The reviews also clearly show that the best value of synchrotron-based methods for biological research are when combined with other non-synchrotron methods. In addition, excellent text books dealing with the basic science and technological complexities of using infrared and X-ray spectroscopy in biological sample analysis are also available (Schulze et al. 1995, Stuart 2000, Willmott 2011).

Synchrotron Radiation-Fourier Transform Infrared (SR-FTIR) Spectroscopy

SR-FTIR analysis can be used to quantify and measure the distribution of chemical constituents within intact plant tissue, tissue sections or homogenized material. While FTIR spectromicroscopy was developed with conventional light sources to study chemical constituents within plant cell walls (McCann et al. 1997) and whole tissues (Stewart 1996), it was only in the last few decades or so that SR-FTIR has been utilized in plant systems (Goff et al. 2009, Goff et al. 2013, Tanino et al. 2013, Xin et al. 2013). As an example, Tanino et al. (2013) conducted a non-destructive

Table 1 Characteristics and experimental parameters of synchrotron-based infrared and X-ray spectroscopy and imaging applications

Synchrotron beam	Energy range	Wavelength range	Target biomolecules	Modes of operation	Types of sample	Best spatial resolution	Reference
Mid-IR (bulk and imaging)	0.070–0.744 eV	17.7–1.7 μm	Organic compounds (proteins, carbohydrates, lipids, nucleic acids, etc.); some salts (phosphates, sulfates, nitrates); co-ordinated metal ions.	Bulk transmission	Thin samples (5–10 μm sections), compacted powder discs or small organisms	–	Lahlali et al. (2015)
				Transmission imaging	Thin samples (5–10 μm sections or small organisms)	MCT detector: 10 μm single point detection; FPA-array detector: 2.5 μm focal plane detection	Karunakaran et al. (2015)
				ATR	Surfaces of intact plant tissues (e.g. leaf cuticle)	Depends on the probe tip size, e.g. 3–100 μm	Miller and Dumas (2006)
Soft X-rays/ tender X-rays	80–4,000 eV	15.5–0.31 nm	Organic compounds (lignins, cellulose,pectins etc.); metals (K, Na, Ca); salts (phosphates, sulfates, chloride, etc.).	Bulk XAS	Powdered samples, intact tissues	–	Karunakaran et al. (2015)
				Bulk XRF	Powdered samples, intact tissues, thick tissue sections	–	Regvar et al. (2011)
				Transmission X-ray microscopy imaging	Thin sections (100 nm–5 μm)	20 nm	Andrews et al. (2011)
				X-ray fluorescence (XRF) imaging	Thick sections	20 nm	Regvar et al. (2011)
				X-ray computed tomography (CT) imaging	Thin sections (100 nm–5 μm)	20 nm	Schmid et al. (2014)
Hard X-rays	4,000–40,000 eV	0.310–0.031 nm	Metals only (Na, K, Ca, Fe, Zn, Cu, etc.)	Bulk XAS	Powdered samples, intact tissues, thick tissue sections	–	Liu et al. (2014)
				Bulk XRF	Powdered samples, intact tissues, thick tissue sections	–	Marguí et al. (2009)
				Transmission imaging	Intact tissue or thick sections (≤100 mm)	≥200 nm	Zhao et al. (2014)
				X-ray microfluorescence (μ-XRF) imaging)	Thick sections	≥200 nm	Singh et al. (2013)
				X-ray computed tomography (μ-CT) imaging	Intact tissues or plant organs	2 μm	Brodersen et al. (2010)

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PCP PLANT & CELL PHYSIOLOGY

investigation on cold acclimation-induced changes in intact single epidermal cell layers of the freezing-tolerant perennial Japanese Bunching onion (*Allium fistulosum* L.). They used SR-FTIR to map changes in the ratios of esterified to non-esterified cell wall carbohydrates, and α-helix to β-sheet protein secondary structure of the epidermis, and found that the most significant changes happen in the apoplastic space, during cold acclimation. It is important to note that these data were acquired with essentially no sample preparation and provided insights into the biochemical and structural changes that accompany and explain acclimation-induced freezing resistance of plants. Further insights were gained through complementary approaches of visible light- and X-ray based spectroscopy (**Fig. 2A–H**). Using standards of 85% methylated pectin and unmethylated polygalacturonic acid, the methylation levels of cell wall pectins were determined in these samples and spatially localized (**Fig. 2E**, **F**). The cold acclimation process appeared to be associated with a decrease in methylated pectins, with apparent shifts in spatial localization. Quantification of this response was performed using principal component analysis (Tanino et al. 2013). The mid-IR results confirmed the reduced immunofluorescent staining in acclimated samples using JIM 7, an antibody which binds to methyl esterified homogalacturonans (unpublished). Using SR X-ray absorption near edge structure (XANES) spectroscopy on the same samples, calcium was spatially localized with an apparent shift to the apoplast space in acclimated samples (**Fig. 2G**, **H**). This suggests a putative role for calcium in cross-linking demethylated carboxyl groups of pectin in acclimated tissue, thereby reducing cell wall porosity and suppressing the growth of ice crystals. As a first step, the ability to conduct spatial localization studies non-destructively in plant tissues using SR enabled development of specific hypotheses which could be further examined with SR and/or complementary techniques.

SR-FTIR mapping has also been used to quantify protein damage caused by frost (Xin et al. 2013) or fungal infection (Singh et al. 2011) in spring wheat (*Triticum aestivum* L.) kernels as well as to map differences in single-celled organisms in vivo (Heraud et al. 2005, Goff et al. 2009, Goff et al. 2013). Goff et al. (2013) could identify altered cell wall surface proteins in *Chlamydomonas reinhardtii* exposed to surfactant-mediated toxicity. In a similar system, Heraud et al. (2005) localized cellular chemical changes induced by essential nutrients in the medium. Withana-Gamage et al. (2013) could successfully identify the subcellular localization and characterize the differences

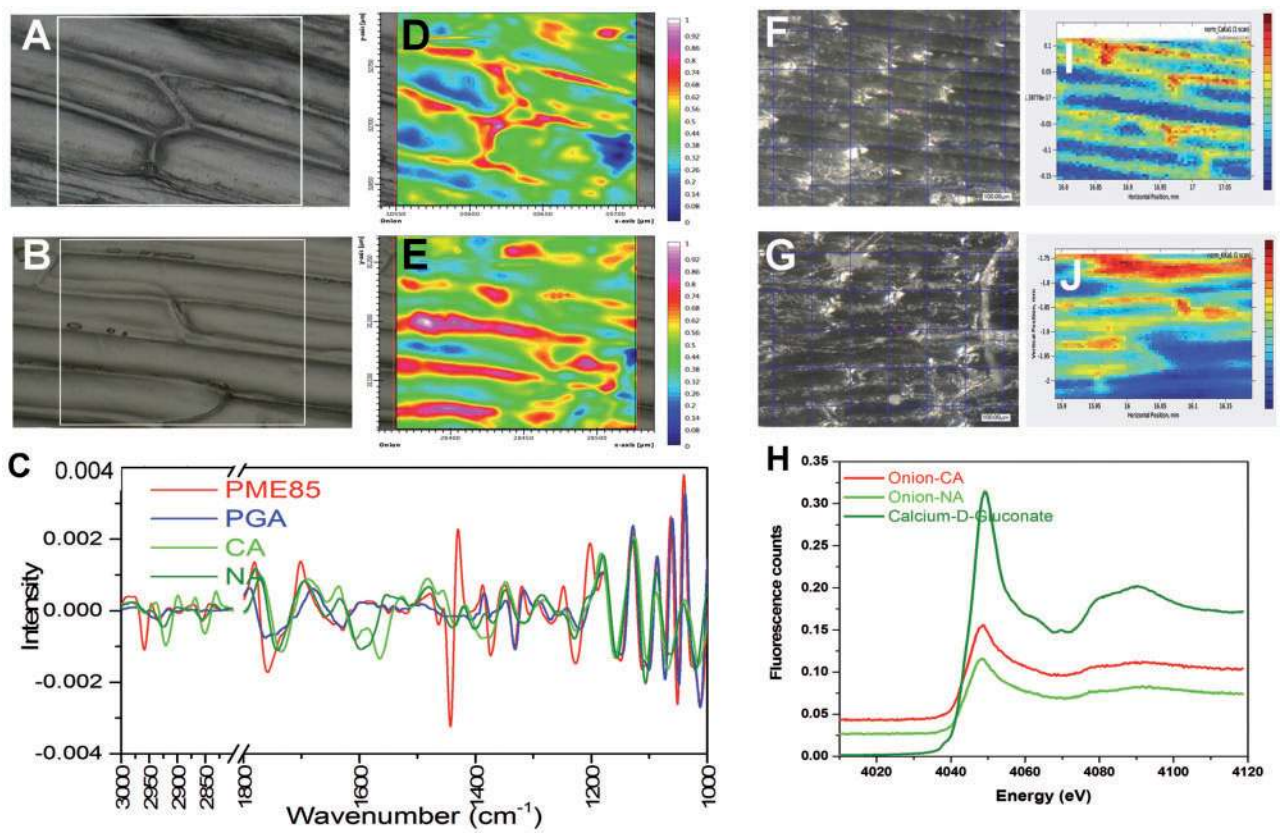

Fig. 2 Light microscopic (A, B, F and G), mid-IR (D and E) and X-ray (I and J) images of cold-acclimated (A, D, F and I) and non-acclimated (B, E, G and J) leaf epidermis of the freezing-tolerant Japanese bunching onion (*Allium fistulosum*). (C) The second derivative SR-FTIR absorption spectra of highly methylated pectin (PME 85), unmethylated polygalacturonic acid (PGA) and epidermal cell walls of cold-acclimated (CA) and non-acclimated (NA) plants. (H) Calcium XANES spectra of epidermal cell walls of cold-acclimated (CA) and non-acclimated (NA) plants along with that of calcium D-gluconate. The false color scheme of the mid-IR images show a greater concentration of methylated pectins (red) in the epidermal cell walls of non-acclimated plants. The false color scheme of the XANES images show an apparent shift of calcium (red) to the apoplast of acclimated plant epidermal cell layers.

in the secondary structures of seed proteins in the wild type and T-DNA insertion mutants of *Arabidopsis thaliana* cv. Columbia using SR-FTIR techniques without resorting to complicated cell biology and biochemical analysis.

Researchers at the University of Saskatchewan have tested and quantified differences in grain feed quality for livestock (Walker et al. 2009, Singh et al. 2011), and screened potential barley varieties for protein and carbohydrate improvements (Liu and Yu 2010) using SR-FTIR spectroscopy. Yu et al. (2003) exploited the synchrotron's high signal to noise ratios to generate data at ultraspatial resolutions (3–10 μm) to localize total lignin, protein, cellulose, carbohydrates and lipid distributions in the pericarp, seed coat, aleurone and endosperm of barley seed using 6–10 μm thin section of samples. Research on malting and feed barley varieties using these techniques has revealed relative differences in the proportion of protein α-helices, β-sheets, β-turns and random coils in seed proteins that have implications for their nutritional and processing quality (Yu 2006).

X-Ray Absorption Spectroscopy

Trace metals are difficult to quantify in biological systems because of their low concentrations and the overlap of the X-ray absorption bands of multiple elements (e.g. Pb, As and Se). Utilization of synchrotron light minimizes the errors due to these factors because of the greater resolution and sensitivity afforded by intense SR X-rays compared with conventional X-ray sources (Shen 2014). Scientists interested in studying trace metal accumulation and storage in plants can simultaneously identify, chemically characterize and localize minerals such as copper, potassium, manganese, magnesium, iron, zinc, etc. using SR X-ray analysis of intact plant tissues, with minimal sample preparation. Synchrotron X-ray absorption spectra of biological samples can be generated by detecting and recording: the transmission of an incident X-ray beam through the sample [X-ray absorption spectroscopy (XAS)], X-ray induced fluorescence photons from the surface of the sample [X-ray fluorescence (XRF)] or the electron current generated by X-rays in the sample that is in contact with its metal holder using a total electron yield detector. A XANES spectrum covers the leading edge of the absorption peak of an element including the pre-edge region at lower energies than the absorption peak, to approximately 40 eV beyond the peak, and can identify both the formal oxidation state and the co-ordination chemistry of the absorbing atom (Sarret et al. 2013). An EXAFS (extended X-ray absorption fine structure) spectrum includes the region from the absorption peak to 800 or 1,000 eV beyond the edge, and can be used to quantify the distances, co-ordination number and species of the absorbing atom's neighboring atoms. Therefore, these techniques have been used to analyze composition, modes of uptake, translocation and storage of metals in plants, as well as characterization of their chemical speciation in the context of plant nutrition, toxicity and environmental pollution (Isaure et al. 2006, Fukada et al. 2008, Harada et al. 2010, Terada et al. 2010, Maher et al. 2013, Ahkter et al. 2014, Kopittke et al. 2014).

SR-XANES- and EXAFS-based studies of kinetics of PSII photochemistry were instrumental in elucidating the mechanism of photosynthetic oxygen evolution of plants (Dau and Haumann 2003). Using PSII extracted from spinach leaves (*Spinacia oleracea* L.), Pushkar et al. (2007) detected the structure and orientation of catalytic manganese complex clusters involved in water splitting reactions using a combination of EXAFS and XANES spectroscopy. Similarly, iron XAS was used to infer transient protein interactions between PSI and Cyt *c* in the cyanobacterium *Nostoc* sp. (Diaz-Moreno et al. 2006).

In addition, EXAFS studies have provided insight into the plant–rhizosphere nutritional relationship in the context of agricultural and environmental research. Naftel et al. (2007) demonstrated that manganese and copper are present in their reduced form in the rhizosphere but in the oxidized ionic form within the roots of aspen (*Populus tremuloides* Michx) trees using XANES (Naftel et al. (2007). Using XANES, researchers have also identified the bioavailable form of arsenic in wheat (*T. aestivum* L.) and rice (*Orzya sativa* L.; Maher et al. 2013, Kopittke et al. 2014).

X-Ray Microfluorescence (μ-XRF) for Elemental Mapping in Plants

Judicious application of synchrotron techniques such as μ-XRF can help map the spatial distribution of metals within intact plant tissue by measuring the emission of X-rays (Vogel-Mikus et al. 2008, Moore et al. 2010). Since SR X-rays can be focused to a spot smaller than 1 μm, researchers have used it to localize essential nutrients (Takahashi et al. 2009, Iwai et al. 2012, Kyriacou et al. 2014) as well as to study the accumulation of non-essential elements such as arsenic (Lombi et al. 2009) in specific tissues of rice. Similarly, Akhter et al. (2014) quantified the amount of cadmium in the epidermis, cortex, endodermis and stele using a combination of μ-XANES and μ-XRF. They were able to determine the concentration of cadmium within the apoplastic and symplastic space in lettuce (*Lactuca sativa* L.) and barley roots (*Hordeum vulgare* L.) by combining scanning electron microscopy with wavelength dispersive spectroscopy. In the area of nutrition studies, Iwai et al. (2012) utilized XANES and μ-XRF to map changes in calcium, potassium, iron, zinc and copper concentrations during rice seed development and correlate with content of seed phytic acid—a compound capable of interfering with the bioavailability of mineral nutrients.

X-Ray Computed Tomography and Phase Contrast Imaging

SR X-ray phase contrast imaging and computed microtomography (μ-CT) are capable of generating virtual cross-sections of intact living plant organs, in real time spans of seconds to milliseconds, at spatial resolutions of 1–2 μm. These images are generated by measuring the absorption as well as the scattering characteristics of the X-rays passing through live plants. Pioneering work by Larabel and Gros (2004) and Parkinson

et al. (2008) revealed the possibility of obtaining high-resolution three-dimensional images of internal structures of living cells with minimal modifications using yeast cells as a model. These techniques have been further developed and used to study active physiological processes such as water translocation in plant tissues (Brodersen et al. 2010). In contrast, non-synchrotron techniques such as transmission electron microscopy cannot be applied to live plants, although they have better spatial resolutions (<1–2 nm).

Non-destructive analysis of water transport in plants has traditionally been carried out using MRI at low spatial resolution of hundreds of micrometers (Elliot and Dover 1982). However, Lee and Kim (2008) exploited the higher resolutions of SR X-ray microimaging to visualize the water refilling process in bamboo (*Phyllostachys bambusoides*) leaves at micrometer resolutions. Cloetens et al. (2006) investigated the three-dimensional apoplastic space of hypocotyls and cotyledons of Arabidopsis seeds to better understand the process of gas exchange and water uptake during imbibition. In the more applied discipline of dendrology, μ-CT has been utilized to study the fracture behavior of Scots pine wood (*Pinus sylvestris* L.; Forsberg et al. 2008) and to visualize microstructures such as tracheid diameter, cell wall thickness and pit diameter in a three-dimensional reconstruction of spruce wood (*Picea abies* L.; Trtik et al. 2007) at a resolution of 1.5 μm. Non-synchrotron X-ray microimaging, in contrast, is unable to resolve fine structures such as bordered pits essential for understanding water transport in and out of the vessels (Steppe et al. 2004).

Examples of SR X-ray phase contrast imaging in crop development include identification of barriers to the spread of infection in Fusarium head blight (*Fusarium graminarum*) in the spikelets of susceptible and resistant spring wheat cultivars (Lahlali et al. 2015). Paleobotanists interested in studying plant anatomy of fossilized specimens at a tissue and cellular level have also used SR X-ray phase contrast imaging to minimize the damage caused to the valuable fossil samples while imaging their internal microstructure (DeVore et al. 2006, Smith et al. 2009). They have exploited these techniques to correlate anatomical features of plant fossils to paleoecology.

Different combinations of synchrotron-based techniques such as FTIR, μ-CT, μ-XRF, XAS, XRF and XANES can be used to obtain greatly enhanced data sets to generate more comprehensive understanding of a biological system. For example, a combination of μ-CT and μ-XRF enabled Wang et al. (2013) to identify simultaneously the speciation, localization and concentration of nickel, zinc, manganese, copper, mercury, selenium and arsenic in roots of cowpea (*Vigna unguiculata* L.). Using a combination of μ-CT, μ-XRF and XANES with other non-synchrotron techniques, Punshon et al. (2003, 2005) estimated effects of historic environmental pollution on nickel accumulation in black willow (*Salix nigra* L.) annual rings. In a recent study, Karunakaran et al. (2015) used SR soft X-ray and FTIR absorption spectromicroscopy in a complementary fashion to map cell wall biopolymers in lentil (*Lens culinaris* L.) stem sections at 30 nm resolution.

Challenges in Use of Synchrotron Radiation in Plant Research

There are some challenges related to sample preparation in applying synchrotron-based techniques to research problems in plant science. The penetrating nature of X-rays and their ability to ionize elements can cause significant damage to the specimen during long exposures. Resulting artifacts can include changes in element distribution and chemical speciation. Soft X-rays may induce damage to samples due to disassociation of chemical bonds or by deposition of organics in the X-ray beam path on the samples. Radiation damage depends on the type of sample (elemental composition), sample state (e.g. wet or dry), rate of data collection, number of repeated scans (or spectral resolution) and the beam characteristics. It is known that photoreduction of redox-sensitive elements such as As and Se does occur during X-ray-based studies. However, such damage can be minimized by either reducing the radiation dose or using fast detector technologies and/or by cooling the samples to very low temperatures using liquid helium (Zhao et al. 2014). While the use of intact hydrated biological samples is ideal for gathering in vivo data and it is possible (Wang et al. 2013) for μ-CT, μ-XRF or XAS methods, the potential for sample damage needs to be considered (Scheckel et al. 2004, Lombi et al. 2010). This beam-related damage becomes even more acute in three-dimensional imaging experiments which require prolonged, repeated scanning of the same sample with X-ray beams. In such cases, tissue damage can happen during data collection due to the ionization and breaking of chemical bonds within the tissue, resulting in altered chemistry (Lombi et al. 2010). Additionally, sample heating and protein denaturation caused by X-rays, and the consequent potential for re-distribution of cellular components, also can introduce experimental artifacts. Such problems can be partially overcome by rapid scanning techniques as well as by the use of sealed sample cells, to minimize water loss and maintain the humidity of a specimen (Wang et al. 2013; **Fig. 1C**). Researchers have also been able to avoid tissue heating and water loss by analyzing frozen samples on cryogenic sample holders/stages (Rodriguez et al. 2014). Elaborately designed systems for humidity control of X-ray diffraction chambers have been successfully used (Hashizume et al. 1996) and can be adapted for use with diverse plant samples. These issues illustrate the importance of proper sample preservation for synchrotron experimentation and understanding the potential for sample alterations such as movement of compartmentalized trace metals while planning synchrotron-based experiments, especially with unfixed tissue. In our experience, judicious experimental strategies such as the use of fixed and unfixed samples in different X-ray and mid-IR, respectively, can also let researchers take advantage of the power of SR by circumventing the problems. During STXM imaging, radiation sensitivity of different biopolymers in the sample should be taken into consideration. For example, cellulose from pure cellulose acetate and wet bean chromosomes are prone to greater damage compared with oak cell wall cellulose and dried bean chromosomes, respectively. One way to detect radiation damage in biological

samples is to scan the same region after data collection and at energies where the damage is most visible (286.7 eV). Increases in the peak intensities of aromatic- and keto-enol-absorbing energy ranges (285 and 286.6 eV) of cellulose are a good indicator of radiation damage. Damage to samples can be limited by decreasing the dwell time (1 ms), limiting the number of repeated scans by selecting optimal spatial (100 nm) and spectral (0.2 eV) resolutions. In addition, reference spectra of samples can be collected by defocusing the beam, and an unwanted region of the sample can be selected for optimization to reduce the radiation damage dramatically (Karunakaran et al. 2015).

It is also important to be mindful of the physical parameters of the beam being used for spectroscopy and imaging purposes. For example, the cross-sectional dimensions of the mid-IR beam may vary from 3 to 13 μm. Naturally this beam can effectively illuminate only a thin rectangle of tissue surface at one time. In addition, the intensity of the beam may not be uniform across the cross-sectional area, and therefore the image and spectral data obtained from different areas of the sample may have to be corrected before analysis. If researchers are interested in mapping a larger region and synchrotron light uniformity is an issue, it is more effective to use a single point array detector with SR to collect individual spectra and then correlate this data with focal plane array mapping using more uniform non-synchrotron light (Chithra Karunakaran, personal communication).

Challenges in Sample Preparation

The success of a synchrotron-based experiment is dependent in large part upon the preparation of plant samples for analysis. Both the thickness of tissues and method of preparation can affect the quality and reliability of spectra collected from the samples. Intact samples or sections ranging in thickness from hundreds of nanometers to 5 or 10 μm are required for absorbance-based studies of biological material on soft X-ray and mid-IR beamlines, respectively. Thicker biological samples absorb most of the radiation in these energy ranges, and useful absorbance information cannot be gathered. However, in cases where it is not feasible to obtain very thin sections, X-ray fluorescence photons from the surface of thicker sections or intact organs can be used effectively (Pickering et al. 2000).

For most biological samples, three sectioning techniques are commonly employed by synchrotron researchers. They include fresh (Heraud et al. 2005, Goff et al. 2009, Goff et al. 2013, Tanino et al. 2013, Wang et al. 2013), cryogenic (Yu et al. 2003, Tappero et al. 2007, Walker et al. 2009, Singh et al. 2011, Liu and Yu 2010, Withana-Gamage et al. 2013, Xin et al. 2013, Yu et al. 2013, I.R. Willick unpublished) and resin-embedded sections (Heraud et al. 2007, Naftel et al. 2007, Akhter et al. 2014). Each method has its distinct advantages and limitations. Regardless of the sample preparation method used, the raw fluorescence or absorbance data from SR beamlines need to be corrected based on the thickness of the samples used and the background signals generated by the materials in the sample holder and chamber. Pickering et al. (2000) have outlined a standard technique to derive corrected X-ray fluorescence data based on careful calculation of absorbance, scattering and fluorescence data from the same sample to account for the sample thickness when detemining the absolute concentration of selenium in their plant samples.

Fresh samples are ideal for mid-IR research because of the minimal manipulation of the tissue and absence of interference from embedding materials. Individual spectra or images produced from such sections will most closely represent what is observed in the native sample (Heraud et al. 2005, Goff et al. 2009, Goff et al. 2013). Tissue samples such as epidermal peels (Tanino et al. 2013) and cell cultures (Goff et al. 2009, Goff et al. 2013) are best since they involve minimal sample processing and allow multiple non-destructive measurements of cellular structure/composition on a single sample. It should be noted that the large water content of fresh samples can cause interference and masking of mid-IR absorbance peaks of certain functional groups. In such cases, samples for mid-IR analysis may also be dehydrated or freeze-dried (Tappero et al. 2007).

However, in the case of metal speciation and localization studies, dehydration of tissues runs the risk of altering the in situ localization of dissolved components. Therefore, for X-ray studies, it may be advisable to maintain sample hydration of fresh samples even inside the vacuum chambers of the X-ray beamline instruments in specialized sample chambers (**Fig. 1C**), although that may exclude the use of X-ray fluorescence methods. For example, Wang et al. (2013) placed cut cowpea roots between 4 μm thick ultralene film to limit water loss. However, thick samples such as seed (Walker et al. 2009, Withana-Gamage et al. 2013), lignified tissue such as wood (Trtik et al. 2007, Forsberg et al. 2008, Harada et al. 2010) or cross-sections such as root (Akther et al. 2014) or cereal spikelets (Xin et al. 2013) require sectioning before SR soft X-ray spectroscopy and microscopy.

Cryogenic sectioning of biological samples can avoid the chemical fixation, dehydration and infiltration of embedding material into the tissue. It is conducted at low temperatures after embedding the tissue in an organic medium or flash-frozen ice with or without fixation. In ice-embedded, flash-frozen samples, the vitreous ice formed within the tissue acts as the supporting medium (Duncan and Williams 1983). While flash freezing and storage at −80°C is critical for avoiding tissue damage by intracellular ice crystal formation, long-term storage can generate artifacts in plant samples even at ultralow temperatures. Moreover, this method imposes limits on the size of the tissue sample ($< 2.5\ cm^2$) and section thickness (> 10 μm) due to the fragility of the embedded tissue unless very specialized techniques are used (Ian Willick, personal commication).

The next most common sample preparation technique involves tissue embedding either in a hard resin or in a soft paraffin wax. Resin embedding of fixed tissue has also been used to obtain ultrathin (100 nm to 1 μm) sections for elemental speciation (μ-XRF) and distribution (XAS) analysis of plant tissues (Naftel et al. 2001, Naftel et al. 2007, Akhter et al. 2014), although metal contamination in embedding media can introduce artifacts.

However, the fixation and dehydration process may cause relocation of trace minerals during processing, and both hard resin and paraffin embedding processes can interfere with the FTIR spectra if they is not carried out with great care. Use of formalin during fixation can oxidize double bonds of unsaturated hydrocarbon chains and cross-link primary and secondary amine groups of proteins, resulting in mid-IR artifacts. If paraffin is not completely removed during the tissue processing, it can produce spectral artifacts in the C–H (2,800–3,000 cm^{-1}) and a weaker 1,465 cm^{-1} peak of C–C vibrations (Miller and Dumas 2006). While it is possible to use ultrathin sections of paraffin-embedded plant material for SR-FTIR after paraffin removal using hexane (Heraud et al. 2007), there is considerable risk of residual paraffin masking sample signals. Therefore, in the absence of fresh samples, careful sample preparation using a cryogenic sectioning technique is advisable for SR-FTIR, while chemical embedding is preferred for metal speciation and localization studies in plant tissues.

Carefully designed sample cells are also available for analyzing live or hydrated preserved biological samples in X-ray (Wang et al. 2013) or mid-IR (Goff et al. 2009, Goff et al. 2013), while avoiding complications related to interference from the presence of water. These cells enclose the samples between X-ray or IR transparent windows, allow for injection of reagents for in situ experiments and may replace H_2O with less problematic D_2O in the case of mid-IR experiments (**Fig. 1C**). Silicon nitride windows are commonly used in STXM work for sample mounting, as wet cells or dehydrated materials or other sample handling for X-ray work, while the preferred window material for FTIR sample windows is calcium fluoride. Such windows have enabled observation of single cells and whole tissue samples in their native state and also monitoring of the effects of drugs or reagents in real time using SR.

Potential for High-Throughput Plant Phenotyping Using SR-Beamlines

Synchrotron science has a legacy that historically emphasized intensive research on topics in physics and material sciences. Therefore, most of the highly sophisticated synchrotron beamline instrumentation and the end stations are not currently designed to accommodate high-throughput sample handling and data collection. However, modern mutant screens, genomics and phenomics demand high-throughput data collection. In this context, it is noteworthy that demands for high-throughput protein structure determination using X-ray scattering and crystallography techniques from the health sciences has indeed resulted in successful automation of these processes in several synchrotrons (Snell et al. 2004). Examples of these are the construction of a robotic sample handling and data collection system for protein crystal structure installed at the Canadian Macromolecular Crystallography Facility beamline of the Canadian Light Source, and the automated sample handling system at the Small Angle X-ray Scattering beamline in the Australian Synchrotron (Melbourne, Australia). Importantly in both of these cases, the sample handling format and data collection protocols have been fully standardized and automated for high-throughput data collection without human intervention. In contrast, high-throughput analysis of live and fixed plant samples is not currently possible in most synchrotrons. However, in most synchrotron-based X-ray and infrared instruments, it is possible to mount multiple samples on a single sample holder, and to automate data collection. Health science researchers have indeed pioneered high-throughput analysis of bone structure, brain anatomy and embryo development on these instruments using prepared tissue samples (Mader et al. 2011). Implementing similar high-throughput synchrotron-based phenotyping platforms in plant research is also possible, provided sufficient scientific interest and financial resources for building such dedicated beamlines can be generated.

Conclusions and Prospects

Although SR techniques have been developed for several decades in the fields of physics, chemistry and medicine, it is only in the last 15 years or so that SR has been applied to plant systems (Naftel et al. 2001, Isaure et al. 2006, Fukada et al. 2008, Akhter et al. 2014). While continued advancements in hardware will improve resolution and sensitivity of synchrotron-based techniques, enabling higher quality imaging and spectroscopic analysis of plants, their greatest contribution to future plant research is likely to stem from their ability to generate vast structural and biochemical data sets that can complement and validate the results of modern transcriptomic, proteomic and metabolomic analyses of plants. Analyses of how plant systems respond to metal toxicity (Punshon et al. 2003, Punshon et al. 2005, Akhter et al. 2014), low temperature (Tanino et al. 2013, Xin et al. 2013) or pathogenic stress (Lahlali et al. 2015) are areas where SR is already shedding new light on our understanding of plant cellular and stress physiology. Similarly, a combination of multiple SR-based techniques has the potential to contribute significantly to crop improvement by helping to analyze seed phenotypes such as structure and nutrient composition, and mineral uptake. Although the potential for high-throughput plant phenotyping using these synchrotron techniques exists in most synchrotrons, development of standardized protocols for specific phenotypes and the commitment of a large enough user community and financial resources are required before this potential can be realized in practice.

Supplementary data

Supplementary data are available at PCP online.

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Disclosures

The authors have no conflicts of interest to declare.

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