Journal of Structural Biology 188 (2014) 61–70

Contents lists available at ScienceDirect

Journal of Structural Biology

journal homepage: www.elsevier.com/locate/yjsbi

# Visualisation by high resolution synchrotron X-ray phase contrast micro-tomography of gas films on submerged superhydrophobic leaves

Torsten Lauridsen [a], Kyriaki Glavina [a], Timothy David Colmer [b], Anders Winkel [b,c], Sarah Irvine [d,e,f], Kim Lefmann [a], Robert Feidenhans'l [a], Ole Pedersen [b,c,g,*]

[a] Niels Bohr Institute, University of Copenhagen, Universitetsparken 5, 2100 Copenhagen, Denmark
[b] School of Plant Biology, The University of Western Australia, 35 Stirling Highway, Crawley, 6009 WA, Australia
[c] The Freshwater Biological Laboratory, University of Copenhagen, Universitetsparken 4, 3rd Floor, 2100 Copenhagen, Denmark
[d] Laboratory for Macromolecules and Bioimaging, Paul Scherrer Institut, 5232 Villigen PSI, Switzerland
[e] Faculty of Biology and Medicine, University of Lausanne, 1015 Lausanne, Switzerland
[f] Laboratory for Dynamic Imaging, Monash University, 3800 VIC, Australia
[g] Institute of Advanced Studies, The University of Western Australia, 35 Stirling Highway, Crawley, 6009 WA, Australia

## ARTICLE INFO

*Article history:*
Received 25 April 2014
Received in revised form 8 August 2014
Accepted 15 August 2014
Available online 28 August 2014

*Keywords:*
Aerenchyma
Air film
CT scanning
X-ray phase contrast
Halophyte
Internal aeration
Leaf gas film
Hydrophobicity
*Spartina anglica*
Submergence tolerance
Tomogram
Wetland plant

## ABSTRACT

Floods can completely submerge terrestrial plants but some wetland species can sustain $O_2$ and $CO_2$ exchange with the environment via gas films forming on superhydrophobic leaf surfaces. We used high resolution synchrotron X-ray phase contrast micro-tomography in a novel approach to visualise gas films on submerged leaves of common cordgrass (*Spartina anglica*). 3D tomograms enabled a hitherto unmatched level of detail regarding the micro-topography of leaf gas films. Gas films formed only on the superhydrophobic adaxial leaf side (water droplet contact angle, $\Phi = 162°$) but not on the abaxial side ($\Phi = 135°$). The adaxial side of the leaves of common cordgrass is plicate with a longitudinal system of parallel grooves and ridges and the vast majority of the gas film volume was found in large ~180 μm deep elongated triangular volumes in the grooves and these volumes were connected to each neighbouring groove via a fine network of gas tubules (~1.7 μm diameter) across the ridges. In addition to the gas film retained on the leaf exterior, the X-ray phase contrast micro-tomography also successfully distinguished gas spaces internally in the leaf tissues, and the tissue porosity (gas volume per unit tissue volume) ranged from 6.3% to 20.3% in tip and base leaf segments, respectively. We conclude that X-ray phase contrast micro-tomography is a powerful tool to obtain quantitative data of exterior gas features on biological samples because of the significant difference in electron density between air, biological tissues and water.

## 1. Introduction

X-ray micro-tomography is a powerful non-destructive visualisation tool in biological sciences. Within plant sciences, visualisation of plant roots in soils *in situ* in pots is an example of application of X-ray micro-tomography to reveal 3D plant structures in an otherwise hidden environment (Mooney et al., 2012). Sand and clay soil particles are both highly X-ray attenuating as compared with the water-filled cells of roots, and so absorption-based X-ray micro-tomography shows contrast between roots and soil (Kuka et al., 2013; Tracy et al., 2010). X-ray micro-tomography has also been used to visualise porosity (gas volume per unit tissue volume) of plant tissues (Mendoza et al., 2007) where the contrast is achieved between water-filled cells and gas-filled intercellular spaces. 3D visualisation is required to model e.g., $O_2$ diffusion and distribution in tissues since gases diffuse 10,000-fold faster in air than in water; examples include studies of $O_2$ distribution in fruits (e.g., Ho et al., 2009) and in roots (e.g., Verboven et al., 2012). However in contrast to studies of 3D root architecture in soils, alternative techniques, such as conventional light microscopy, can also enable assessments of tissue structure to underpin modelling of $O_2$ movement in plants (e.g., Armstrong, 1979) although visualisation of light microscopy slides and their 3D reconstruction is a time-consuming process and lacks the sophisti-

---

[*] Corresponding author at: The Freshwater Biological Laboratory, University of Copenhagen, Universitetsparken 4, 3rd Floor, 2100 Copenhagen, Denmark.

*E-mail address:* opedersen@bio.ku.dk (O. Pedersen).

http://dx.doi.org/10.1016/j.jsb.2014.08.003
1047-8477/

cated software now available for the tomographic reconstructions and interpretations (Mendoza et al., 2007; Verboven et al., 2012, 2013).

In the present study, we used high resolution synchrotron X-ray phase contrast micro-tomography in a novel approach to visualise gas films retained on superhydrophobic leaves when submerged. In plant science, water repellence is characterised using the contact angle of a water droplet with the underlying surface (Adam, 1963; Brewer and Smith, 1997); contact angles above 150° implies superhydrophobicity (Koch et al., 2008; Neinhuis and Barthlott, 1997). The leaves of many wetland plants possess superhydrophobic leaf cuticles and during submergence, which can happen in the tidal zone (Winkel et al., 2011) as well as during overland floods (e.g., rice Colmer et al., 2014), such leaves are covered in a thin gas film (Pedersen and Colmer, 2012; Raskin and Kende, 1983). Leaf gas films greatly enhance the gas exchange between leaf surface and floodwater ($CO_2$ and $O_2$ in the light and $O_2$ during darkness) and hence, contribute to the flood tolerance of these wetland plants (Colmer et al., 2011; Verboven et al., 2014). During the day, the better $CO_2$ uptake from the floodwater results in enhanced rates of underwater photosynthesis (production of carbohydrates as well as $O_2$) and during the night, the better $O_2$ uptake sustains internal aeration of the shoot as well as of belowground tissues via internal $O_2$ movement via aerenchyma (Colmer and Pedersen, 2008; Pedersen and Colmer, 2012; Teakle et al., 2014; Winkel et al., 2011, 2013). Interestingly, the crop species rice (*Oryza sativa*) possesses superhydrophobic leaves (Pedersen et al., 2009; Winkel et al., 2013) and so do many wetland plants of ecological interest e.g., *Spartina anglica*, *Phragmites australis*, *Thypha latifolia*, and *Phalaris arundinacea* (Colmer and Pedersen, 2008; Winkel et al., 2011), so an in-depth understanding of the functioning and structure of leaf gas films is of importance to understand plant submergence tolerance in agricultural and ecological systems.

Characterisation of leaf gas film dimensions has previously been achieved using two contrasting approaches. The most accessible method involves buoyancy measurements of leaf segments before and after removal of the gas film; the hydrophobicity of the leaf surface can be greatly reduced by brushing it with a dilute detergent (Colmer and Pedersen, 2008; Raskin and Kende, 1983). The difference in buoyancy corresponds to the volume of gas film, and the average gas film thickness can then be estimated from the total area of the leaf segment – either for both surfaces (Pedersen et al., 2009) or one surface (Winkel et al., 2011), as appropriate. Application of the buoyancy method has shown that the average gas film thickness varies between 50 and 67 μm for the two species measured, *S. anglica* and rice (Pedersen et al., 2009; Winkel et al., 2011, 2013). However, an estimate of gas film thickness based upon this approach is unable to account for the surface microtopography, which likely results in great variation in actual gas film thickness. The spatial variation in gas film thickness can partly be captured using microelectrodes (Pedersen et al., 2009; Verboven et al., 2014). Pedersen et al. (2009) showed that gas film thickness of rice ranged from 10 to 140 μm by using $O_2$ microelectrodes to profile various locations on the leaf surface. Because of the high diffusivity in air, the gas film could be detected as a layer with no $O_2$ concentration gradient just above the leaf surface when submerged in darkness so that respiratory activity was consuming $O_2$ (Pedersen et al., 2009; Verboven et al., 2014).

In order to achieve improved characterisation of leaf gas films, here we used high resolution X-ray phase contrast micro-tomography to visualise the external gas layer on the superhydrophobic leaves of common cordgrass (*S. anglica*) when submerged. Using phase contrast micro-tomography instead of the conventional absorption-based methods resulted in much better contrast allowing for a more robust segmentation. Common cordgrass possesses a gas film only on the adaxial side of its leaves as the abaxial side is not sufficiently hydrophobic to retain a gas layer when under water (Winkel et al., 2011). The average gas film thickness on leaves of common cordgrass is approximately 50 μm (Winkel et al., 2011) so in order to capture any effects of leaf microtopography upon gas layer thickness, we aimed for μm resolution to match data obtained with $O_2$ microelectrodes from rice (see above). We hypothesised that with such a sample, despite relatively low differences in X-ray attenuation, the large difference in electron density between air and water would yield significant X-ray phase contrast to enable distinction between water, external gas trapped on the superhydrophobic leaf surface, the much denser cells making up plant tissues and the internal gas-filled spaces within the leaf tissues.

## 2. Materials and methods

### 2.1. Source of sample material

Turfs of common cordgrass (*S. anglica*, CE Hubbard) were collected from populations at the Bay of Ho, Denmark, at multiple time points during the summer months. For details regarding the natural growth conditions of this population of common cordgrass, see Winkel et al. (2011). The turfs were maintained outdoors in Hillerød, Denmark, under natural light and temperature conditions as waterlogged cultures in 5 L buckets until used in experiments. Specimens used for high resolution X-ray micro-tomography were gently washed out of the natural sediment, transported in moist paper towels to the facility in Switzerland and studied within 4 d.

### 2.2. Scanning electron microscopy

Scanning electron microscopy (SEM) was performed with a JEOL JSM-6320F scanning microscope in high vacuum. Leaf samples were dehydrated in Petri dishes at room temperature to avoid evaporation of water in the vacuum chamber and a subsequent build-up of charge. Moreover, the leaves were coated with gold to enhance the natural conductivity of the cuticle surface. Dry leaf segments were fixed using double adhesive carbon tape on top of a silicon wafer. Leaf segments from the lower third of the youngest fully expanded leaf (subsequently referred to as 'base') were coated with a layer of 20 nm gold and viewed at a voltage of 5 keV, whereas samples from the upper third of the leaf (subsequently referred to as 'tip') were coated with 15 nm of gold and viewed at 10 keV.

### 2.3. Contact angle and hysteresis

Contact angle of water droplets (1 μL droplet of deionised water; radius ~0.625 μm) was used to assess wettability of the leaf surface (Adam, 1963). On a rough and chemically heterogeneous biological surface, the Cassie and Baxter model predicts that water droplets are suspended across surface protrusions, and tiny air pockets are thus formed between water and surface of the material (Shirtcliffe et al., 2005). Using water to measure the contact angle between droplet and surface of material enables classification into hydrophobic (contact angle > 90°) or hydrophilic (contact angle < 90°). Koch et al. (2008) subdivided the two categories further and defined materials as superhydrophobic when the contact angle exceeded 150°.

A water repellent surface can also be categorised as self-cleansing when an applied water droplet rolls off the surface at a tilting <10° (Koch et al., 2008). Rolling of a droplet occurs when the hysteresis is small and is defined as:

$$\text{Hysteresis} = \theta_{\text{advancing}} - \theta_{\text{receding}} \quad (1)$$