

Analysis of leaf surfaces using scanning ion conductance microscopy

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Abstract

Leaf surfaces are highly complex functional systems with well defined chemistry and structure dictating the barrier and transport properties of the leaf cuticle. It is a significant imaging challenge to analyse the very thin and often complex wax-like leaf cuticle morphology in their natural state. Scanning electron microscopy and to a lesser extent Atomic force microscopy are techniques that have been used to study the leaf surface but their remains information that is difficult to obtain via these approaches. Scanning electron microscopy is able to produce highly detailed and high resolution images needed to study leaf structures at the sub-micron level. It typically operates in a vacuum or low pressure environment and as a consequence is generally unable to deal with the *in situ* analysis of dynamic surface events at sub-micron scales. Atomic force microscopy also possess the high resolution imaging required and can follow dynamic events in ambient and liquid environments, but can over exaggerate small features and cannot image most leaf surfaces due to their inherent roughness at the micron scale. Scanning ion conductance microscopy (SICM), which operates in a liquid environment provides a potential complementary analytical approach able to address these issues and which is yet to be explored for studying leaf surfaces. Here we illustrate the potential of SICM on various leaf surfaces and compare the data to scanning electron microscopy and atomic force microscopy images on the same samples. In achieving successful imaging we also show that SICM can be used to study the wetting of hydrophobic surfaces *in situ*. This has potentially wider implications than the study of leaves alone as surface wetting phenomena are important in a range of fundamental and applied studies.

Introduction

The surfaces of all leaves are covered with a thin waxy layer known as the cuticle (Holloway, 1993). The cuticle is composed of a matrix of the biopolymers cutin and/or cutan with embedded waxes (Pollard et al., 2008). These waxes can be within the cuticle, called the cuticular waxes (CW) or form a layer above the biopolymer framework, called the epicuticular waxes (EW) (Jeffree 2006). The EW is known to form crystalline aggregates which can form different structures according to the chemical content of the waxes (Barthlott et al., 1998), and can be categorized in to six main groups (Jeffree, 2006); massive crusts, filaments, rods, tubules, plates and platelets. These crystalline structures range in size from 0.2 to 100 μm (Koch and Ensikat, 2008).

The role of the cuticle is to protect the leaf and to regulate transpiration (Riederer and Schreiber, 2001) and solute exchange (Richardson et al., 2007) between the leaf and its environment. The cuticle also protects the leaf from bacterial and fungal pathogens (Jenks et al., 1994), insect plant interaction (Kerstiens, 1996) and is the main barrier in agrochemical uptake (Santier and Chamel, 1998). Most studies of the cuticle have concentrated on the chemical constitution of the waxes and the cutin/cutan matrix in relation to the protective properties of the cuticle. Although, the chemical makeup of the cuticle is important, the material form (ie. crystalline, amorphous) and morphology of the EW waxes also provides protection for the leaf, but is generally understudied due to analytical challenges of imaging the native leaf surface without significant sample preparation (Long et al., 2003). It is worthy of note that a related area that has received significant attention recently is the phenomenon of super hydrophobic surfaces, many of which are based on studying the natural structures formed at leaf surfaces (Bargel et al., 2006, Marmur, 2004).

Imaging of leaf surfaces has mostly been achieved using conventional techniques and scanning electron microscopy (SEM) (Koch and Ensikat, 2008) in particular. SEM in its standard form typically requires samples to be dry, coated in a thin conducting film and imaged in vacuum. This fixes a sample and precludes studies on live leaves (Jeffree, 2006). Variable-pressure (VP) or Environmental SEM (ESEM) can address this issue to some extent (Stabentheiner et al, 2010) allowing imaging of some dynamic events, but this but still fails to provide access to imaging live samples (Koch et al., 2009) and apart from high performance field-emission systems tend to have lower spatial resolution than in standard vacuum SEM. Recent work (Neděla et al., 2014) has evaluated ESEM for use with delicate plant structures

showing improvements that can be made using low temperatures in terms of conserving and revealing micron-scale imaging of coniferous tissues. Cryogenic sample preparation can be a useful approach to preserving native structures for standard and VP SEM but again the sample is now fixed. An alternative is to use scanning probe microscopes, such as the atomic force microscopy (AFM). AFM has been used to image isolated cuticles (Canet et al., 1996), which showed AFM is comparable to SEM but with higher spatial resolution. Furthermore tapping mode AFM was used to image the surface of English Laurel (*Prunus laurocerasus*) (Perkins et al., 2005b) and the lotus leaf (Bhushan and Jung, 2006). Critically AFM of leaves has been carried out in ambient and liquid environments opening up the potential to view dynamic processes on live samples. AFM though is unable to image most leaf surfaces due to their microscale roughness (AFM has a limited ability to accommodate surfaces with large changes in height, changes above several microns prohibit imaging) and hence is limited to the few species with particularly flat cuticle surfaces. Another SPM technique, called scanning thermal microscopy (SThM) has been employed to study leaf surfaces. This utilises a tip that can be heated to record topographical data and can acquire local thermal analysis at a single point on a sample surface (Hammiche et al., 1996). This technique has been used to measure the plasticizing effects of non ionic surfactants on native leaf surfaces (Perkins et al., 2005a), to gain information on how chemicals affect certain areas of the cuticle. Another member of the SPM family is scanning ion conductance microscopy (SICM). This technique images surfaces within an electrolyte using a hollow nano-pipette (Korchev et al., 1997). SICM has to date been primarily used for imaging live cells at up to molecular resolution (Shevchuk et al., 2006, Liu et al., 2011). Unlike AFM, it is a true non-contact imaging technique (Korchev et al., 1997) and is able to deal with large variations in surface height, and hence has been shown to cause less damage to soft rough samples (Rheinlaender et al., 2011). (Rheinlaender et al., 2011). The resolution of SCIM, both lateral and height, are dependent, mostly, with the inner radius (r_i) of the pipette tip. The SICM best lateral resolution reported is between 3 – 6 nm from a tip with r_i of 6.25 nm (Shevchuk et al., 2006). With computational studies suggesting the lateral resolution to be $2r_i$, and the height resolution to be 5% of r_i (Del Linz et al., 2014). Self pulled tips can be made to fit the resolution that is required.

SICM hence represents a potentially valuable tool fitting between the versatility of SEM and the ultimate *in situ* resolution achievable by AFM on smooth surfaces and, in particular represents an interesting approach to those wishing to study live leaf surfaces. This paper

describes the use of SICM to study the leaf surfaces of English Ivy (*Hedera helix*), Strawberry (*Fragaria ananassa*), Oil Seed Rape (*Brassica napus*), Pea (*Pisum sativum*) and grass from the *Festuca* genus. The data is compared to comparable SEM and AFM studies. These species were chosen to illustrate different EW structures and hydrophilic and hydrophobic properties and to interrogate the ability of SICM to analyse such a variety of leaf surfaces and under what conditions optimal SICM data may be obtained.

Materials and Methods

Chemicals and materials

SICM nano-pipettes were pulled using P-97 flaming/brown micropipette puller (Sutter Instruments, California, US) from standard wall borosilicate tubes, with an outer diameter of 1mm and an inner diameter 0.5mm (Sutter Instruments). The pipette tip inner diameter was between 150 - 200 nm as determined by SEM imaging.

The imaging electrolyte solution was prepared with deionised water, resistivity of 18M Ω ·cm, obtained from a Milli-Q water purification system (Millipore Corporation, Massachusetts, US) and phosphate buffer solution (PBS) (Fisher Scientific, Loughborough, UK). AFM tips used were 0.01 – 0.025 Ohm-cm Antimony doped Si (BrukerNano, Coventry, UK), with a frequency between 347 – 393 kHz. Tween 20 (Sigma Aldrich, Missouri, US), solutions were made to a 10% w/w solution in the PBS electrolyte solution, for use on hydrophobic surfaces.

Leaf Samples

Leaves of the English Ivy and *Festuca* grass were harvested from plants continuously growing on the University of Nottingham's ground. Leaves of Oil seed rape and Pea were grown from seeds (Syngenta, Jealott's Hill, Berkshire, UK). Strawberry plant leaves were taken from plants locally sourced from a commercial retail supplier (Homebase Ltd). Leaf samples for the SEM were dissected and left to dry before coating with gold. For AFM entire leaves were used and imaged soon after picking. For SICM, the leaves were dissected and fixed, with double sided sellotape, in a Petri dish with the electrolyte solution covering the entire leaf segment. For SICM imaging of hydrophobic surfaces, first PBS was applied to the surface then Tween was added to make a 5% w/w droplet. For the study into how low concentrations of tween affect wetting, 4 ml of PBS was applied to the leaf surface then 0.5% w/w Tween solution was added to make a final concentration of 0.0006 % w/w Tween PBS solution.

Analytical Instruments

Scanning electron microscopy (SEM) images were recorded using a JEOL-JSM-6060Lv SEM (JEOL Ltd, Tokyo, Japan). Samples were coated in a layer of gold using a sputter coater EM SCD005 (Leica Microsystems, Illinois, US). Samples were sputtered for 300 seconds at 30mA.

AFM images were recorded using a D3000 AFM (BrukerNano, Coventry, UK). This instrument has a maximum scan size of 90 x 90 μ m with a maximum Z limit of 6 μ m. AFM images were processed using NanoScope Analysis (BrukerNano). SICM images were recorded using a commercial ScanIC SICM (Ionscope Ltd, London, UK) and operated in hopping mode, with a 512 x 512 pixel density. This instrument has a maximum scan range 90 x 90 μ m with a maximum Z limit of 25 μ m. The SICM probe consists of a nanopipette filled with electrolyte (PBS) and back inserted with a Ag/AgCl electrode with a Ag/AgCl reference electrode immersed in the electrolyte bath. SICM images were processed using SICM image viewer (Ionscope).

Hopping mode does not use a continuous feedback, but instead the probe approaches from above the sample, until the pipette reaches a current set point value, normally a reduction 1% of the value recorded with the probe far from a surface. Once the set point is achieved the z position is recorded and the tip retracted before being re-approached to the surface at the next measurement point (Novak et al., 2009)

Results and Discussion

Imaging of leaf surfaces

The adaxial surface of the English Ivy has regular undulating “hillock” morphology, as shown by the SEM image (Figure 1a). The shape of the cuticle is a result of the wax layer following the underlying epidermis cells, with the valleys mimicking anticlinal walls. SICM was also used to image the adaxial surface (Figure 1b). SICM was able to image the topology of the cuticle, showing the same undulating “hillock” structure as in SEM. The surface of the cuticle is smooth with variations in height of the “hillock” structures. The abaxial surface of the leaf is where a number of stomata are located. AFM and SICM were used to image

individual stoma. The stomata of the English Ivy consist of guard cells surround by cuticle wax (Figure 2).

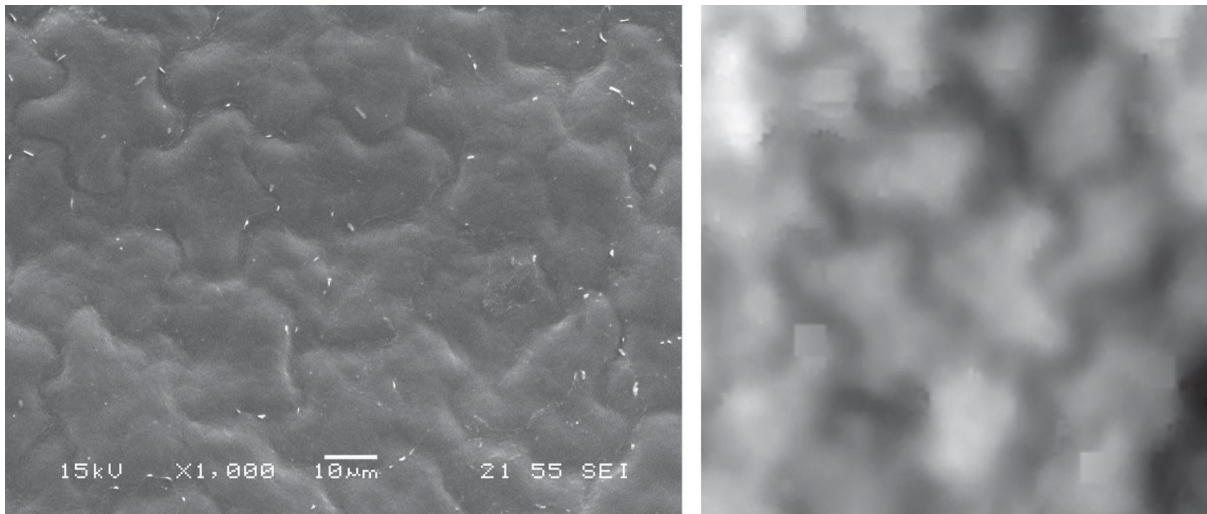


Fig. 1. To scale images of the adaxial surface of English Ivy (A) SEM image (B) SICM image.

AFM imaging clearly shows the guard cells, which are open, and the pore that the cells create. The guard cells appear to be similar in size and to have a relatively smooth surface. There is a wax chimney (Barthlott et al., 1998) structure that surrounds the guard cells and this is seen to be an elliptical structure. Also the AFM imaged the valleys before and after the wax chimney, but could not image further from the stoma. This was due to the increase of vertical (z) height of the surrounding cuticle, and the limited z range of the AFM. By comparison SICM was able to image the individual stoma, like the AFM, with the same features like the guard cells and the wax chimney being distinguished and was able to image the surrounding area of the guard cells with more detail. The wax chimney is shown to be a continues structure, as the suggested by the AFM data, but here the valley between the guard cells show a smaller inner chimney. The AFM was not able to image this inner chimney structure because the AFM tip was not able to penetrate into the valleys (Rheinlaender et al., 2011). A cross-section line trace of both the SICM and the AFM images show the differences between the AFM and SICM ability to penetrate into deep valleys on the cuticle surface (Figure 2c). The deepest point of the AFM, from the line trace, is the pore of the stoma, from the apex of the left guard cell to the bottom most point imaged, which is 2.5 μm . The deepest points from the SICM are the trenches beyond the wax chimney, with the deepest is 6.6 μm . This is because the SICM pipette tip approaches the surface from above but without interference from the pipette itself. As a consequence the SICM was able to

image beyond the stomata area (Figure 3), with this area having a similar “hillock” structure as the adaxial surface.

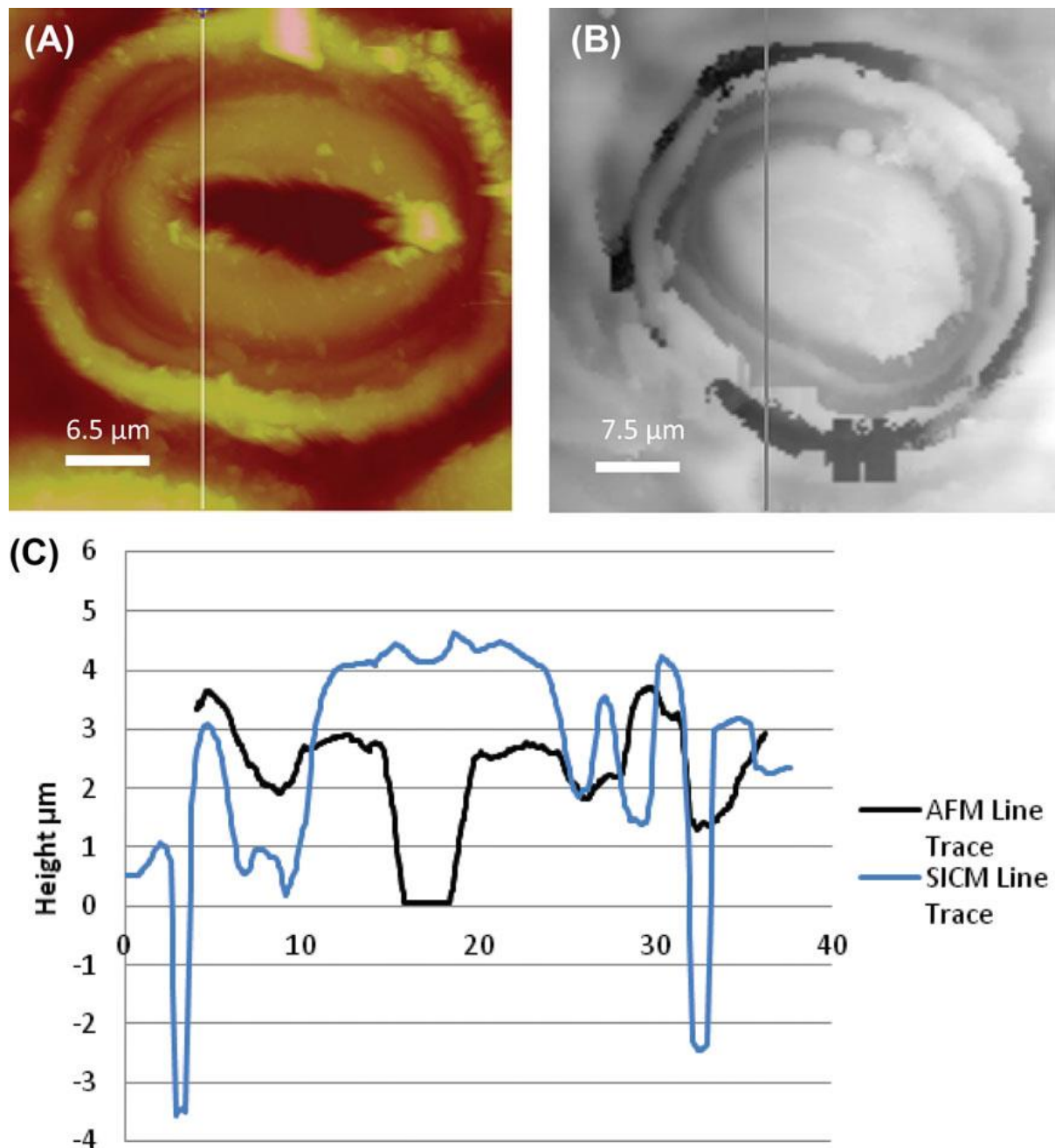


Fig. 2. Images of stomata from the adaxial surface of English Ivy. (A) AFM image, line shows the path of the line trace. (B) SICM image, line shows the path of the line trace. (C) AFM and SICM line traces of the stomata, showing changes in topography.

Another leaf species imaged with the SICM is the abaxial surface of Strawberry. SEM shows the surface to be highly detailed with a low density of filament features (Figure 4) (Mackerron 1976). The SICM was also able to image these filament features but without a metal coating and in the electrolyte solution. The images also provide additional information

on height, the features range from a height of 0.1 μm to 1 μm with a width of approximately 0.5 μm . The SICM also revealed that the surface comprises of even finer features between the main filament structures. These finer features are thinner, with larger filaments being approximately 2 μm in length and having a higher density on the surface of the cuticle. Where the finer features meet there appear to be “nodes”, potentially composed of wax, which are connected to many thinner filament features. This illustrates the spatial resolution of SICM and its ability to image delicate structures without damage. These fine features were not seen by SEM as carried out here, either due to a lack of resolution or more likely loss of structural details due to the sample preparation employed. AFM wasn't able to image the surface because of the trichomes, hairs, located on the surface; the trichomes would block the cantilever and prevent the tip from tapping the surface of the leaf. SICM is able to avoid these, and able to image the surface without hindrance.

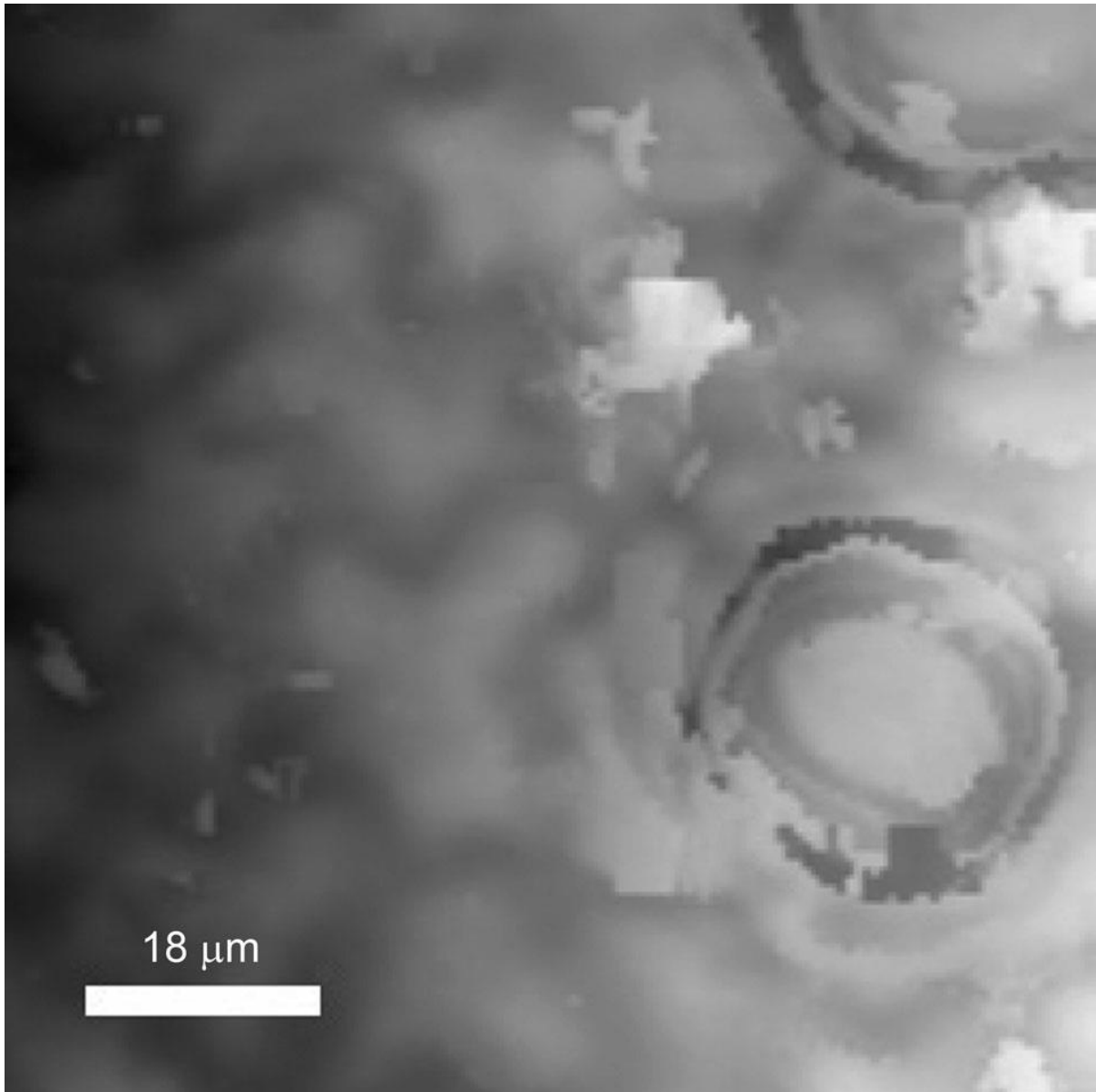


Fig. 3. SICM image of the adaxial surface of the English Ivy, showing stomata and the undulating hillock structure.

The use of SICM to image hydrophobic leaf surfaces

Leaf surfaces can be hydrophilic, hydrophobic, superhydrophilic and superhydrophobic (Bhushan and Jung, 2006, Koch and Barthlott, 2009), depending on the surface chemistry and structure. The previous SICM images were of leaf surfaces that are hydrophilic in nature; this allowed the electrolyte solution to have full contact of the leaf surface, which didn't restrict SICM imaging. Successful SICM imaging of a surface depends upon complete wetting of that surface by the electrolyte, as the probe must be immersed in electrolyte to detect an ionic current and hence image a surface. Hence hydrophobic and super hydrophobic surfaces present a potential challenge, particularly if they are rough as this can promote micro-bubble

formation at a surface (Feng et al., 2008). If this were to occur the surface would pose ‘hidden’ regions to the SICM due to air-electrolyte interfaces. Whilst a challenge to SICM imaging this also presents an opportunity to study wetting processes at surface *in situ* using the SICM to image liquid-air and liquid-solid interfaces as a solution wets a surface.

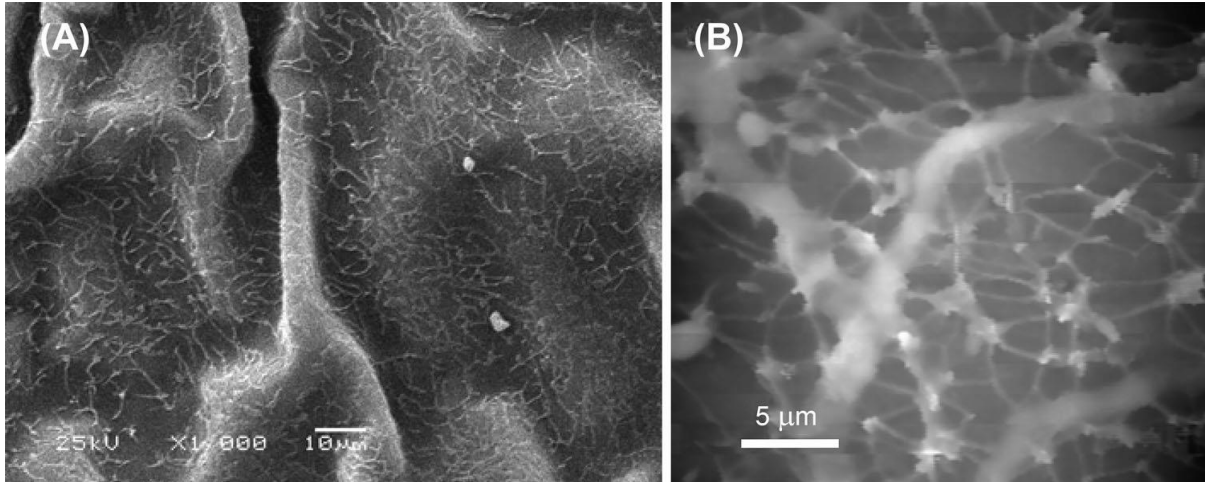


Fig. 4. Images of the abaxial surface of the Strawberry leaf. (A) SEM image showing the convoluted cuticle with filament features. (B) High-resolution SICM image of the filament features.

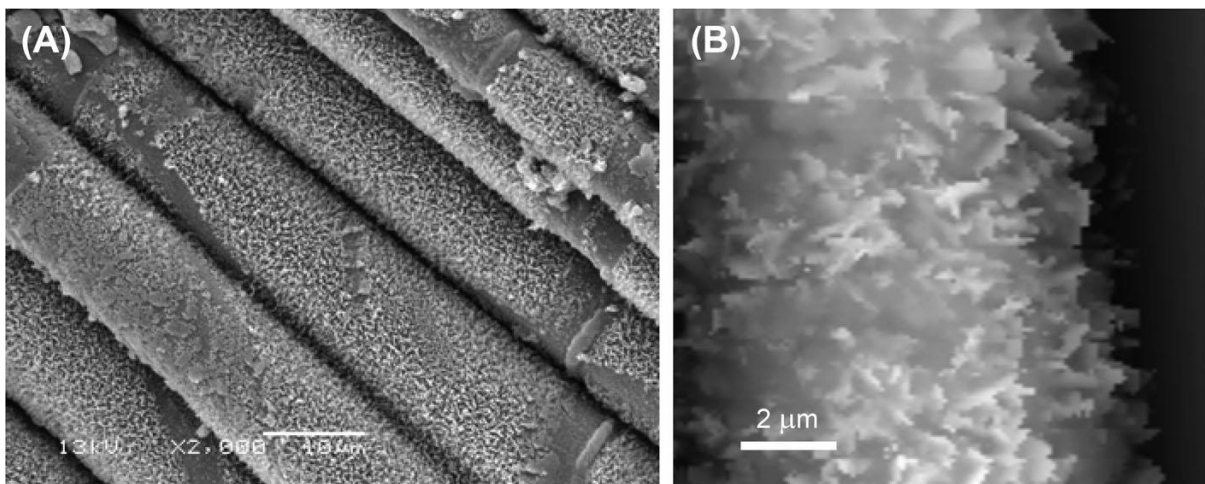


Fig. 5. Images of the abaxial surface of the Festuca grass (A) SEM images showing the platelet structure. (B) High-resolution SICM image of the platelet features, imaging above and the side of the features.

A grass leaf from the genus *Festuca* possesses a hydrophobic surface due to the density of the platelet structure (Holloway, 1969) (Figure 5). The SICM was able to image the surface of the leaf (Figure 5) showing the platelet structures. These structures have similar shape but vary in size and orientation. Since the electrolyte solution penetrated between the platelet structures it can be inferred that water was able to wet the surface between the platelet features. This regime of wetting does not prevent the SICM from imaging the surface, since

the electrolyte solution can be in contact with the entire surface and still allow conductance between the two electrodes.

The leaves of the Oil Seed Rape and Pea plants also possess a hydrophobic surface (Gniwotta et al., 2005). For such surfaces only a small fraction of the droplet is believed to be in actual contact with the surface due to trapped air pockets at the interface separating the drop and the leaf surface (Marmur, 2004). Oil Seed Rape and Pea have differing structures (Figure 6a and b). The Oil Seed Rape leaf surface is highly convoluted with the EW composed of tubule crystal features with amorphous wax between the features, Pea consists of a high density of crystal platelet feature, like *Festuca*, although the surface is not as convoluted.

For both leaf surfaces, when the electrolyte solution is applied alone the solution is not in contact with the entire surface of the leaves. This is evidenced by considering the SICM data in (Figure 6c and e). The images show that the SICM probe is not imaging the leaf surface and we propose that it is detecting the liquid/gas interface of micro air pockets trapped at the surface. For Oil Seed Rape (Figure 6c) this interface was smooth showing no protruding leaf structures. In contrast for Pea (Figure 6e), the SICM image shows some disruption of the interface, indicating that the apex of the platelet crystals are submerged in the electrolyte. This is consistent with a Cassie-Baxter wetting regime where only a relatively small fraction of the droplet is in contact with the surface of the leaf with small compartments of gas trapped under the liquid (Marmur, 2003).

To lower the surface tension of the electrolyte and promote complete wetting of the leaves a surfactant (Tween 20) was used (Lee et al., 2008). After adding the surfactant the electrolyte solution was in contact with the entire surface, which allowed the surfaces to be imaged. SICM images of Oil Seed Rape (Figure 6 d) depict tubules of the EW to be of varying size and shape. The SICM has also imaged into some of the tubule holes. Compared to the presented standard SEM image (Figure 6a) the SICM is able to image the tubule structures clearly showing the variations between size and shape of the crystals. For Pea the SICM (Figure 6e) clearly shows a large number of crystal platelet structures in a valley of the cuticle. Some of the platelets are imaged from above and some are imaged from their sides. The SICM shows the platelet structures clearly, which is comparable to SEM, the additional height information provided for the platelet structures show that the average height is approximately 1 μm .

Imaging of the interface of the liquid on the hydrophobic surfaces with the SICM allowed the study of how small concentrations of the surfactant Tween affect the wetting regime. Figure 8a shows the liquid/gas interface of Pea, after imaging the Tween solution was added to the PBS. After adding the tween the interface still remand for 5 minutes (Figure 8b), though there is a collapse in the interface while imaging. This collapse is seen again after 30 minutes of adding the surfactant, but this collapse resulted in the surface of the leaf being imaged. Clearly the introduced surfactant to the electrolyte solution has caused a reduction in surface tension and a collapse of the trapped microbubbles (Ying et al., 2005). In the future it may be possible to introduce this surfactant locally with the SICM pipette so as to initiate localised effects on trapped microbubbles and the underlying waxy cuticle.

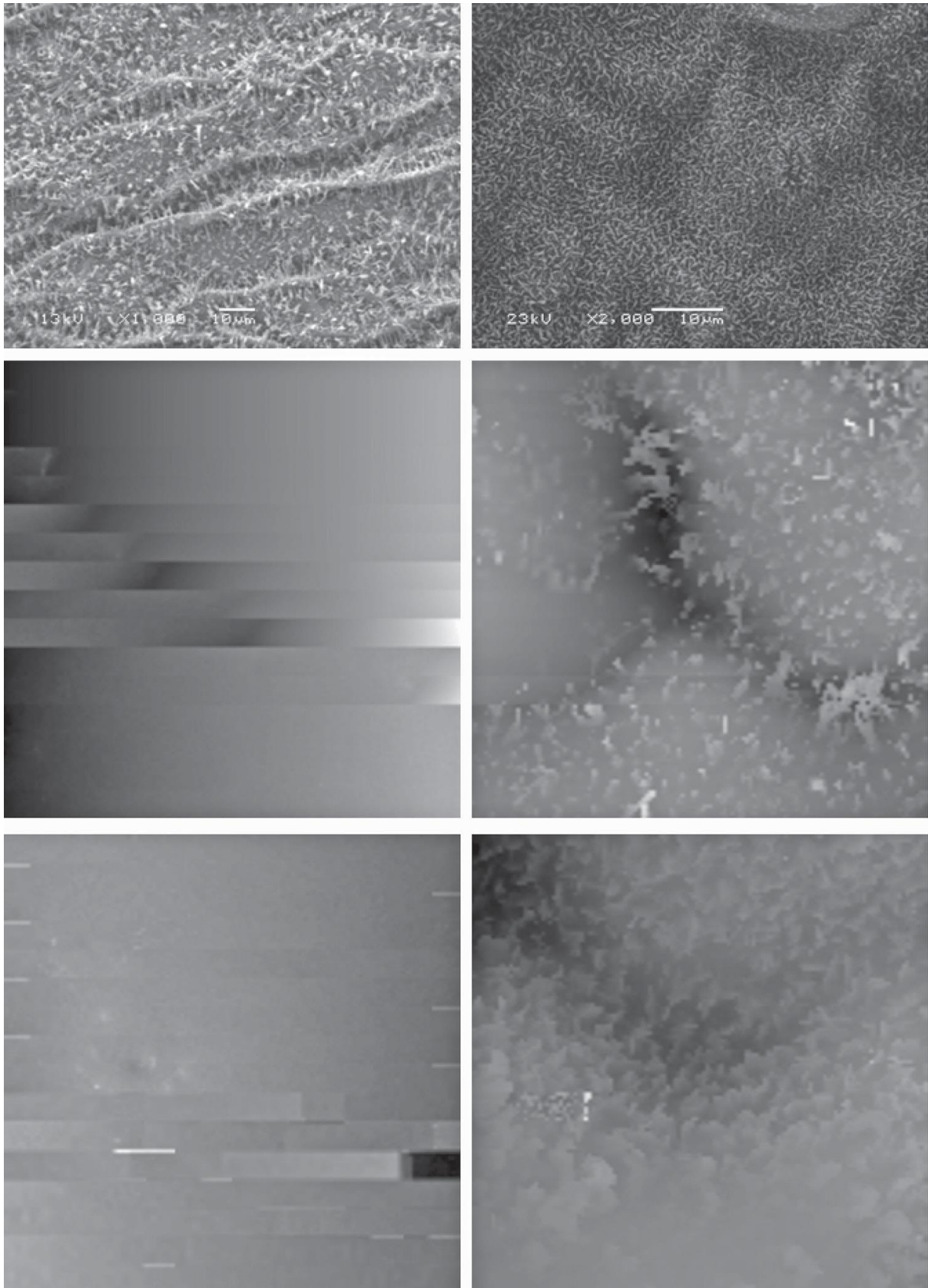


Fig. 6. Images of the abaxial surface of Oil Seed Rape and Pea leaves. (A) SEM of the surface of Oil Seed Rape. (B) SEM of the surface of Pea. (C) SICM image of the liquid–gas interface of the droplet on Oil Seed Rape. (D) SICM image of Oil Seed Rape after surfactant was added to the droplet. (E) SICM image of the liquid–gas interface of the droplet on Pea. (F) SICM image of Pea after the surfactant was added to the droplet.

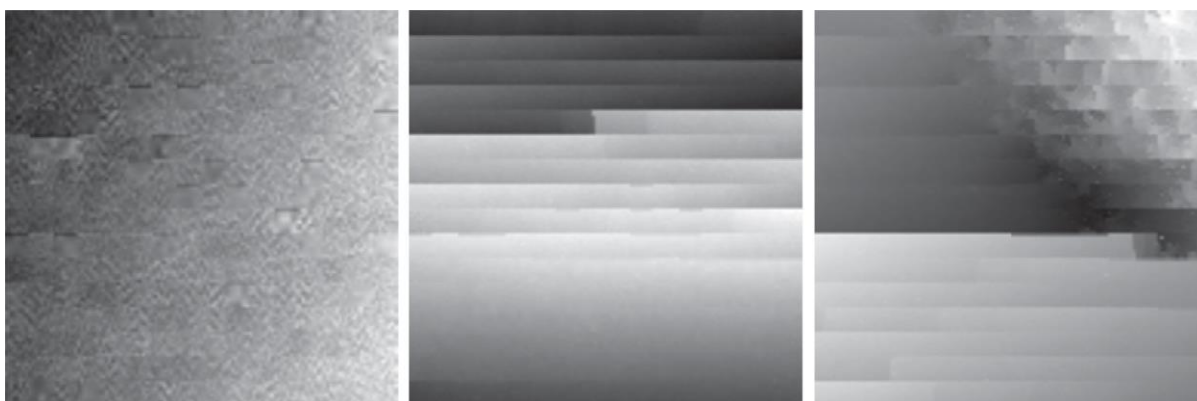


Fig. 7. SICM images of Pea leaf and the liquid–gas interface before and after adding small concentration of tween 20. (A) Interface before adding the surfactant. (B) Image after 5 minutes of adding the surfactant, showing a collapse in the interface. (C) Image after 30 minutes of adding the surfactant, showing another collapse and the leaf surface being exposed.

Conclusions

SICM was able to yield 3D images of a variety of leaf surfaces under electrolyte. Sample preparation simply involved inserting bare leaves into the SICM. Images are comparable to SEM (where samples normally need coating in a metal and imaging in vacuum) and AFM but provide complementary and additional utility, such as environmental flexibility compared to SEM (potentially facilitating a variety of in situ dynamic experiments) and an ability to image a wide range of rough leaf surface that cannot be imaged by AFM. The true non-contact nature of the SICM as compared to AFM is useful to image the fragile waxy features of leaf surfaces and clearly has the potential to image live leaf surfaces and hence processes that occur at the leaf cuticle. The rough hydrophobic surface of the leaves allowed wetting regimes of the surface to be studied. Trapped micro-air pockets at the surface, which inhibited SICM imaging were collapsed by adding a surfactant to lower surface tension. This has made it possible for SICM to image leaf surface that are hydrophilic, hydrophobic and superhydrophobic. This novel observation may not only open the way to achieving a greater understanding of wetting at leaf surfaces but also investigating other surfaces, like micro fluidic devices.

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