Microscope methods for observation of the phylloplane flora

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Abstract Microscope-based observation of surface microbes can support indirect techniques, such as culturing or DNA analysis of surface washings, by illustrating microbial distribution patterns, inter-relationships and the presence of unculturable or non-recovered organisms. Comparisons have been made between techniques of contrasting complexity. For example, surface replicas of the leaf made from transparent materials and scanning electron microscopy (SEM) were compared for their ability to present an accurate picture of the leaf surface and microbial populations. “Environmental” SEM (ESEM) and cryo-SEM minimise change and provide the most realistic and detailed images of the surface but have logistical difficulties. Conventional, critical point dried SEM samples, even with extra processing and some physical change, usually provided similar results and had advantages in handling. The simpler replica techniques retained microbial number and distribution when compared to ESEM but were poor with rough surfaces. Microbial material on replicas could be stained or labelled with antibodies to improve identification.

Keywords light microscopy, scanning electron microscopy, ESEM, cryo-SEM, surface replica, microbial ecology, immune-labelling, fungi, bacteria.

INTRODUCTION

A number of different techniques can be used to understand the extent and dynamics of populations of micro-organisms on plant surfaces. These can be divided into two approaches: those where the micro-organisms are removed from the surface and those where the surface is observed directly or indirectly. The former includes well-used techniques, such as washing micro-organisms from the plant surface and culturing or direct growth from the surface. Colony growth can be used to identify and enumerate (Lee & Hyde 2002) these micro-

organisms. Modern advances in DNA technology, for example DGGE (denaturing gradient gel electrophoresis) (Muyzer 1999) applied to such washings, obviate the need to culture and have the potential to illustrate the diversity of the population more clearly. However, washing techniques cannot provide information on spatial distribution on the surface or the interaction between micro-organisms, and are limited to those propagules that are removed by the technique and which are themselves culturable. Impression techniques, such as pressing the surface onto a growth medium (Bainbridge &

Dickinson 1972) or culturing microbes removed using adhesive tape (Langvad 1980), can provide information on spatial distribution but not their relationship with the topography of the surface; fast growing organisms can dominate and spatial resolution is rapidly limited as these colonies grow and merge.

Direct observation of micro-organisms on the living surface is the surest way to observe distributions and interactions between micro-organisms and with the plant surface (Lee & Hyde 2002). However, it has a number of drawbacks: limits to the resolution of instruments using incident or epi-illumination, the difficulty in visualising small or transparent bodies on often rough surfaces, the difficulty in identification, and time constraints when looking at ephemeral subjects. Visualisation can be improved by staining or fixing and staining. The latter also enables long-term storage, although such manipulation can result in movement of bodies that are poorly attached. Use of specific techniques, such as immuno-labelling and fluorescence *in situ* hybridisation (FISH), can enable identification (Andrews et al. 2002), although the increased manipulation of the sample can result in the loss or movement of microbial material. Natural autofluorescence from the plant may interfere with observation of labelled material. Micro-organisms transformed to express one of the various forms of fluorescence proteins (VandenWymelenberg et al. 1997; Poonguzhali et al. 2008) can be useful in following the fate of individual strains but this does not enable the tracking of micro-organisms in the wild. Fluorescence techniques can be particularly useful when combined with confocal microscopy, which provides clear images and the ability to visualise surfaces in three dimensions.

Scanning electron microscopy (SEM) of the leaf surface (Pathan et al. 2008) resolves many of the issues of visualising objects of various sizes and located on different planes because of its high resolution and large depth of focus. Techniques such as cryo-SEM (Falloon et al. 1989) and environmental SEM (ESEM) (Collins et al. 1993) offer unique opportunities to look

at unaltered surfaces. However, these methods involve the use of expensive equipment and in many cases may not be available for routine evaluations. In contrast, simple surface replica techniques have been successfully applied using only a light microscope and stain (Dickinson et al. 1974).

This paper describes studies that have compared a number of direct microscope observation techniques, surface replica techniques and scanning electron microscopy. The value of these techniques in assessing changes in surface microbial populations after various interventions and as support for more indirect enumeration techniques is discussed.

MATERIALS AND METHODS

Mature expanded apple leaves were taken from a pesticide-free block of 'Royal Gala' apple trees at the Plant & Food Research Mt Albert Research Centre in Auckland in late summer (February to March) between 2005 and 2010.

Scanning electron microscopy

For conventional scanning electron microscopy, 5-10 mm diameter pieces of leaf tissue were fixed with 2% fresh formaldehyde, 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 and stored at 4°C. Fixed tissue was washed in buffer, dehydrated with an ethanol series to 100% and then critically point dried using a BalTec CPD030 (Bal-Tec Inc Balzers, Liechtenstein) with CO₂ as the transitional fluid. Dried tissue was mounted using sticky carbon tape on aluminium specimen holders, gold coated using a Polaron E5100 (Polaron Equipment Ltd., Watford, UK) sputter coater and observed in a Philips PSEM 505 (Philips, Eindhoven, The Netherlands) at accelerating voltages of 10 kV to 15 kV.

Cryo-SEM was carried out on samples mounted on copper holders, frozen in liquid nitrogen and processed (ice sublimation at -80°C, sputter coating at -150°C) using an EMScope SP2000 (Emscope, Ashford, UK) sputter-cryo system (Falloon et al. 1989). Samples were immediately observed at -150°C or cooler on a cryo-stage in the Philips SEM.

Fresh un-dried, un-coated leaf samples were observed using the environmental (extended vacuum) mode (ESEM mode) (Stokes 2008) of a FEI Quanta 200 field emission SEM or Quanta 250 tungsten filament SEM (FEI Company, Hillsboro, OR) using water vapour as the environmental gas. Leaf samples were placed on a Peltier cooled stage with a few drops of distilled water. The chamber pressure was dropped to around 800 Pa, at a stage temperature of 3–5°C, to maintain humidity near 100%. Samples were observed at 20 kv in the Quanta 200 and 7.5–10 kv in the Quanta 250.

To assess changes in observable micro-organisms after critical point drying, the same areas of leaf samples were observed using ESEM then processed as above and re-observed using conventional SEM conditions. To estimate the effectiveness of leaf washing in removal of the micro-flora, samples of leaf tissue were observed using the ESEM before and after washing for 2 h using an orbital shaker.

Surface replication methods

Leaf surfaces were lightly coated with either a commercial clear nail varnish (for example, Clear as Nails®, Sally Hansen, Uniondale NY) or Shur/Mount™ slide mounting medium (Triangle Biological Sciences, Durham NC). Once the coating dried, it was carefully lifted from the leaf surface and either stained immediately or fixed with 2% freshly made formaldehyde, 0.1% glutaraldehyde in 0.1 M sodium phosphate buffer pH 7.2 and stored at 4°C for future staining and observation. To stain, washed fixed or un-fixed replicas were immersed in a 0.5% solution of toluidine blue in tap water for 30 min, washed and mounted in water and observed. Nail varnish replicas were also stained by immersing unfixed replicas in 1% cotton blue (aniline blue) in lactophenol then mounting in lactophenol (Dickinson et al. 1974). Observations were made using an Olympus Vanox AHT3 (Olympus Optical Co Ltd., Tokyo, Japan) microscope and imaged using a Photometrics CoolSnap camera (Roper Scientific Ltd, Tucson, AZ). To assess effectiveness of replicas in removal of surface

micro-organisms, adjacent samples of leaf tissue were observed before and after a replica was taken using cryo-SEM. Additionally, samples of leaf tissue were observed using ESEM, replicas taken, and the same area observed to examine the extent of microbe movement during replication.

Immuno-labelling

Two labelling methods were used to detect fungi on fixed replicas, using either the lectin wheat germ agglutinin (WGA - Sigma Chemicals, St Louis MO) to bind to chitin, followed by immune-labelling of the WGA, or direct immune-labelling of (1→3)- β -glucan in the fungal cell wall. Labelling with WGA was carried out after washing and incubating in blocking buffer containing 0.1% acetylated bovine serum albumin (BSA-C Aurion, Wageningen, The Netherlands) in phosphate buffered saline with 0.1% Tween 20 (PBS-T) for 15 min and followed by incubation in 10 μ g/ml WGA in blocking buffer for 60 min at room temperature. Subsequent immuno-labelling for both systems was carried out as described in Sutherland et al. (2009) using anti-WGA (1:1000 in blocking buffer) or anti-(1→3)- β -glucan antibody (BioSupply, Parkville, Australia) (1:100 in blocking buffer) incubated overnight at 4°C. After washing, samples were incubated for 1 h in Alexa 488 goat anti-rabbit (WGA) or Alexa 488 goat anti-mouse ((1→3)- β -glucan) (both Invitrogen, Eugene, Oregon) as the secondary antibody detection system. Epi-fluorescence observations were made using the Olympus Vanox AHT3 with the Olympus IB filter set.

Surface observation by light microscopy

Leaf tissue was cut into 5 to 10 mm diameter pieces that were fixed, stored, immuno-labelled and observed as above. In addition to the IB filter set, an Omega XF100-2 filter set (Omega Optical Inc, Brattleboro, VT) with a 535/45 nm band pass emission filter was used to reduce plant autofluorescence. Confocal microscopy was carried out using an Olympus FL1000 confocal microscope (excitation 488 nm, emission 520 nm).

RESULTS

Detailed images of smooth, rough and hairy surfaces and attached micro-organisms were possible using all SEM modes (Figure 1). ESEM was the most rapid method for sample preparation. Observation was possible within 20 min of the sample arriving in the laboratory. Clarity and resolution of ESEM samples was usually very good although variations in operation parameters or state of the leaf could result in the surface being masked by a water film (see for example Figure 3b where surface water has been retained after washing). In general, samples could be maintained without change for several hours of observation. If humidity

was allowed to drop below 90%, wrinkling occurred. A limitation of ESEM observation was the reduced area visible at lower magnifications, which limited the field of view to around 0.5 mm (Figure 1a). Samples for cryo-SEM could be stored under liquid nitrogen for prolonged periods but were, if necessary, observable within 1 h of receipt and produced results similar to or superior in detail to ESEM (Figure 1c). Critical point dried samples took 1-2 days to prepare but were able to be stored in fixative before drying and as dried coated samples for indefinite periods. Image detail was similar to that of cryo-SEM but alteration to the surface could sometimes be seen (Figure 1d). Comparisons of the same areas of leaf

Figure 1 Scanning electron microscopy of apple leaves. (a) low magnification and (b) high magnification of environmental (extended vacuum) scanning electron microscopy (ESEM) of fresh apple leaves showing good visualisation of surface features, fungal hyphae and spores. Inset in (a) shows restricted field of view in low magnification ESEM mode. (c) Cryo-SEM image of frozen apple leaf. (d) Critical point dried sample of apple leaf vein, arrow shows surface damage

viewed using ESEM then conventional SEM after critical point drying suggest that most micro-organisms remained in place (Figure 2). Counts for two separate experiments indicated retention in their original location of 80% of micro-organisms (fraction remaining 0.78, $SE \pm 0.11$, $n = 10$) and in a further experiment using older dryer

leaves, 98% remained (fraction remaining 0.98, $SE \pm 0.02$, $n = 18$). ESEM observation also showed that the fractions of hyphae, fungal spores/yeasts and presumptive bacteria removed by washing were 0.83 ($SE \pm 0.24$), 0.76 ($SE \pm 0.18$), 0.46 ($SE \pm 0.18$), respectively ($n = 8$) (Figure 3).

Figure 2 (a) Apple leaf surface viewed using environmental scanning electron microscopy. (b) The same area viewed using conventional scanning electron microscopy after critical point drying (CPD). Solid arrows show examples of material in the same location on both samples, open arrows show organisms not observable in CPD sample (i.e. lost) and arrow heads show displaced material (i.e. appearing in CPD sample but with nothing at same location in ESEM sample).

Figure 3 Loss and retention of micro-organisms on apple leaf samples visualised using environmental scanning electron microscopy (a) before washing and (b) after washing for 2 h. Arrows show material retained after washing.

Both nail varnish and Shur/Mount produced replicas that could be easily stripped from the surface. Both were readily stained with toluidine blue to show micro-organisms ranging in size from fungal hyphae and spores to bacteria (Figures 4a, 4b & 4d). However, undulations in the replicas due to the unevenness of the original surface meant that observation required continual changes in microscope focus and photography required two or more images at different focal planes. Micro-organisms buried within the replica (e.g. on leaf hairs) were not stained and occasionally it was difficult to distinguish stained debris from clumps of micro-organisms. Nail varnish replicas readily stained in cotton blue/lactophenol and could be stored for several weeks, but Shur/Mount replicas dissolved within 1 h. Both types of replica could be stored in fixative for 4 or more weeks without reducing staining, although nail varnish replicas often showed some cloudiness. Replicas were highly efficient at removing material from the leaf surface. For example, in one experiment there were 49 cryo-SEM fields of view containing hyphae and 50 containing spores from a total of 53, while after a replica had been taken there were 0 cryo-SEM fields of view containing hyphae and 4 containing spores out of a total of 80 fields of view. Comparison of ESEM fields of view with replicas of the same areas showed that most propagules remained visible and in the same location in the replicas (Figures 4c & 4d).

Detection and discrimination of fungal material was successful using both labelling techniques on leaf surfaces and on replicas (Figure 4e). Observation was relatively easy using replicas where optical techniques, such as differential interference contrast, could be used to visualise topography of the same surface (Figure 4f). However, plant autofluorescence obscured labelling on leaf surfaces unless a tight band pass emission filter was used. Even with this filter, observation was often difficult and the image hazy. Confocal microscopy provided much clearer images of the fluorescing material but obtaining a clear image on an undulating surface with sufficient resolution to discriminate

different types of micro-organism required collecting an image stack of often 25 or more for reconstruction, taking up to 15 min.

DISCUSSION

Direct observation of the interrelationships of the microflora (including pathogens) and the plant surface requires observation of microbes that might be only lightly attached, so methods are needed that can minimise loss, movement and distortion. Thus the primary aims of microscope-based methods of observation are to enable a good estimation of the numbers of different microbes present, their location and spatial relationships with each other. To achieve this, preparation methods must minimise disturbance to the surface. The ESEM mode of the scanning electron microscope, followed closely by cryo-SEM where the only manipulation is freezing of the tissue, satisfy these criteria. ESEM was originally proposed and developed in the late 1980s and has flourished over the last decades with three SEM manufacturers (FEI, Tescan and Zeiss) providing instrumentation (Danilatos & Postle 1982; Danilatos 1993; Stokes 2008). In ESEM the specimen chamber of the microscope is held at a relatively poor vacuum (in the region of 400–2000 Pa) by controlled introduction of a gas into the system. The column and electron gun of the instrument are held at much higher vacuums by the use of gas-limiting apertures and differential pumping; special electron detector systems are required. In most cases, particularly for biological systems, the gas used is water vapour which, particularly when combined with a temperature controlled stage, allows the sample to be observed in conditions around 100% humidity. Setting the exact pressure and temperature characteristics to enable this is critical and can be difficult in older instruments. If the humidity is too low the sample dries out and if too high the sample floods with water. The limited area that can be observed at lower magnifications is an inconvenience, making sample navigation difficult. However, the major disadvantage for studying the microbial ecology of surfaces is that fresh living tissue is used and

Figure 4 Surface replicas of apple leaves. (a) low magnification and (b) high magnification of Shur/Mount replica of lower surface of an apple leaf stained with toluidine blue. Uneven surface in (a) results in some areas being out of focus. Hyphae, spores and bacteria-like bodies are arrowed. (c) Apple leaf surface viewed using environmental scanning electron microscopy. (d) The same area as (c) from a nail varnish replica stained with toluidine blue viewed using light microscopy. Arrows show hyphae and spores retained in the same location in both samples. (e) Shur/Mount replica of upper surface of apple leaf labelled with anti-(1→3)- β -glucan visualised using Alexa 488. (f) the same surface as (e) visualised with differential interference contrast optics to show surface topography.

thus the number of samples that can be observed is severely limited, as they cannot be stored for later viewing. Cryo-SEM overcomes this latter difficulty as material can be collected, frozen and stored in liquid nitrogen for future preparation and observation. Samples are observed gold-coated using conventional vacuum and detection systems, with no degradation of performance. Disadvantages in terms of obscuration of the surface by ice have been reduced by much improved mechanisms for controlled ice sublimation. Thus for micro-ecological studies cryo-SEM would seem to be the best choice for detailed surface observation. Even though critical point dried samples do not show the surface with the fidelity of ESEM, the present results would suggest that they are a viable option, providing logistical advantages (bulk fixation and long-term storage) when large amounts of material have to be observed. Material can be observed in any SEM no matter how old or basic in configuration, including the new, relatively-low-cost desktop SEMs. Even the greatest loss of microbes using CPD was only 20% leaving 80% of the material in its original location, which, from the ESEM comparison, might be the recovery acceptable for washing techniques. The reason for the difference between the two ESEM/CPD comparisons may lie in the drier condition of the second sample; most propagules on the surface may be old and well established.

Replica techniques can be a viable low-cost alternative to SEM observation. The present comparisons show that replicas efficiently remove micro-organisms from the surface and can reproduce location with little or no movement. For relatively smooth surfaces, such as the upper lamina of an apple leaf or an apple fruit, they are likely to provide similar results to the SEM. However, observation of the leaf surface is not easy, requiring some manipulation of microscope optics. Two or more focal planes may need to be viewed or imaged to obtain results for a complete field of view. For rough or hairy surfaces, replica techniques will be less successful and in the latter case micro-organisms are likely to be encased in the replica

and not stained. Identification of the elements of the microbial flora is limited to visual physical characteristics. However, there is potential for this limitation to be remediated in replicas by using specific antibodies and possibly DNA probes *in situ*.

The difficulty in observing micro-organisms *in situ* on the leaf is only one of the issues for such microbial ecology. A second and equally important issue is the development of effective observation strategies that allow the extraction of usable data from surface observations without excessively tedious and impractical observation of entire surfaces. Samples need to be taken with some consideration of the statistical validity of the sampling pattern. Sample observations may need to be duplicated at different magnifications to collect information on organisms ranging in size from hyphae and large fungal spores to bacteria. The increasing automation of both light and electron microscopes (e.g. auto-focus and computer-controlled sample movement) may help to reduce the effort required to amass valid datasets (Inglese 2006; Henderson 2008). However, automated recognition of target micro-organisms in the complex environment of an SEM image of a leaf surface is not yet achievable.

In spite of these sampling issues cryo-SEM, in particular, has been used by the authors to assess spore distribution, germination and appressorial formation after inoculations with *Colletotrichum acutatum* and *C. gloeosporioides* (I.C. Hallett, unpublished data). A combination of SEM and replica techniques are currently being applied to determine changes in microbial populations, as compared with fungicide treatments, resulting from interventions with biocontrol agents and nutritional enhancement of beneficial organisms.

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