Methods

Frozen in time: a new method using cryo-scanning electron microscopy to visualize root–fungal interactions

Steve Refshauge[1,2], Michelle Watt[1], Margaret E. McCully[1] and Cheng X. Huang[3]

[1]CSIRO Plant Industry, GPO Box 1600, Canberra, ACT 2601, Australia; [2]School of Biochemistry and Molecular Biology, The Australian National University, Canberra, ACT 0200, Australia; [3]Research School of Biological Sciences, The Australian National University, Canberra, ACT 0200, Australia

Summary

Author for correspondence:
Michelle Watt
Tel: +61 26246 4902
Fax: +61 26246 5399
Email: michelle.watt@csiro.au

• A new method of sample preparation for cryo-scanning electron microscopy was used to visualize internal infection of wheat (*Triticum aestivum*) roots by the pathogenic fungus *Rhizoctonia solani* AG-8. The new method retained fungal hyphae and root cells *in situ* in disintegrating root tissues, thus avoiding the distortions that can be introduced by conventional preparation by chemical fixation, dehydration and embedding.
• Infected roots frozen in liquid nitrogen were cryo-planed and etched (sublimed) at −80°C for a critical length of time (up to 9 min) in the microscope column to reveal plant and fungal structures in three dimensions.
• Root and fungal structures were well preserved irrespective of infection severity. Root and hyphal cell walls were clearly seen and hyphal architecture within and between root cells was preserved.
• This rapid method permits three-dimensional *in situ* visualization of fungal invasion within roots and has broad application for examination of diseases caused by other necrotrophic fungi.

Introduction

Studies of plant–pathogen interactions are often hampered by difficulties in obtaining undisturbed internal views of infected material. This is particularly true for roots infected by necrotrophic fungi as they rot and disintegrate. *Rhizoctonia solani* AG-8 is a necrotrophic fungus that infects the roots of cereals, causing them to rot at infection sites, resulting in reduced shoot growth and yield (MacNish & Neate, 1996). Root tissues heavily rotted by this fungus are delicate and difficult to section by hand or with a vibratome as tissues readily fragment. Consequently, there is a paucity of detailed information on how and where *R. solani* spreads within cereal roots.

Observing where pathogenic fungi grow inside plant organs has traditionally been achieved with tissue sections following chemical fixation, dehydration and stabilization in wax or plastic. These procedures can drastically damage the structure and arrangement of both plant and fungal cells, particularly during removal of water and soluble components, and during exchange of solvents with the embedding matrix. Damage is particularly serious in diseased tissues because agitation during the preparative steps often results in disintegration of rotted tissue. Our initial attempts to fix and embed *R. solani*-infected wheat roots resulted in the loss and collapse of cortical tissues and hyphae (Fig. 1). Similar tissue preparations of *R. solani*-infected sugar beet (*Beta vulgaris*) (Ruppel, 1973), alfalfa (*Medicago sativa*) and red clover
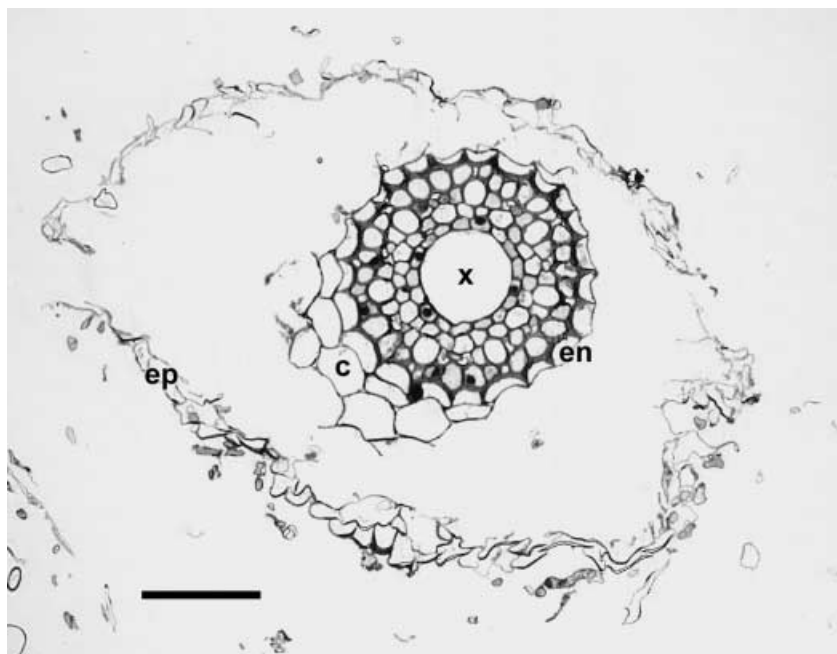
Fig. 1 Transverse section of a wheat (*Triticum aestivum*) root 12 d after inoculation with *Rhizoctonia solani*. The rotted epidermis (ep) and cortex (c) collapsed and fragmented during the agitation caused by chemical fixation, dehydration and embedment. No hyphae are visible. The sample was prepared by fixation in 3% glutaraldehyde, serial dehydration in ethanol and embedment in LR White resin (London Resin Co. Ltd, Berkshire, UK), and was sectioned at 2 µm and stained with toluidine blue O. Bar, 100 µm. en, endodermis; x, late metaxylem vessel.

(*Trifolium pratense*) (Chi & Childers, 1964) exhibited disintegration and collapse of root cells possibly caused by the preparatory steps rather than by the fungal pathogen.

Cryo-fixation (freeze-fixation) is an alternative to chemical fixation for the observation of diseased tissues. Because cryo-fixation involves plunging fresh, hydrated samples into liquid cryogen, the detrimental effects of chemical fixation are overcome because tissue is not dehydrated and specimen components are rapidly immobilized. Cryo-fixation is commonly followed by freeze-drying (Walker & Powell, 1979), critical point-drying (Jones *et al.*, 1987), freeze substitution (Hoch, 1991) or direct observation of the fully hydrated frozen tissue (Grabski *et al.*, 1987; Jones *et al.*, 1987; Jansson *et al.*, 2000). Freeze-drying and critical point-drying introduce severe artefacts (such as cell wall collapse, movement of cell contents and damage to delicate structures) that render these methods unsuitable for detailed visual examinations (Sargent, 1986b). In particular, critical point-dried material can undergo collapse and wrinkling akin to chemically fixed, dehydrated and embedded tissue. Freeze-substitution effectively preserves cell ultrastructure, but necessitates the substitution of water and soluble compounds with an organic solvent. A further weakness is the lengthy preparation required, ranging from 1 to 3 d (Hoch, 1991). Many authors who have carried out studies involving fungi concur that, among these preparatory techniques, direct observation of hydrated frozen material consistently produces superior preservation of plant and fungal material (Beckett *et al.*, 1982; Sargent, 1986a; Allan-Wojtas & Yang, 1987; Jones *et al.*, 1987; den Belder *et al.*, 1993). Key advantages include retention of water and solutes, and rapid immobilization of cell structure compared with chemical fixation. Thus, material is preserved *in situ* and retains a life-like

appearance. Simplicity and speed of preparation (in the order of minutes) are additional benefits of observing hydrated frozen material over other freezing techniques. Read & Jeffree (1991) noted the value of hydrated cryo-fixation for soft, necrotic tissue that is readily distorted and sloughed away by alternative methods. Similar benefits apply to delicate plant structures such as root hairs and thin cell walls (Sargent, 1986b; Huang *et al.*, 1994) and fungal structures such as hyphae, conidiophores and spores (Jones *et al.*, 1987; Sargent, 1988).

The advantages of cryo-scanning electron microscope (cryo-SEM) observation of fully hydrated cryo-fixed specimens for morphological studies have been frequently documented (Beckett *et al.*, 1982; Sargent, 1986a,b; Allan-Wojtas & Yang, 1987; Grabski *et al.*, 1987; Jones *et al.*, 1987; Sargent, 1988; Read & Jeffree, 1991; Read, 1991; den Belder *et al.*, 1993; Jansson *et al.*, 2000). To observe anatomical details it is necessary to expose internal surfaces. This can be done in two ways: freeze-fracturing or cryo-planing. Huang *et al.* (1994) highlighted the shortcomings of fracturing, particularly uncontrolled points of fracture and uneven fracture surfaces, and presented cryo-planing as an alternative method (for a review, see Nijsse & van Aelst, 1999). Cryo-planing is predominantly a preparatory step for X-ray microanalysis, but is also useful for anatomical studies (Van Cauwenberghe *et al.*, 1993; Walther & Müller, 1999; McCully *et al.*, 2000; Ryan *et al.*, 2003). A disadvantage of examining planed surfaces, however, is limited contrast which can make cell structures difficult to identify. Additionally, three-dimensionality is lost, and thus arrangement of structures in the vertical axis cannot be ascertained.

Now we have extended the advantages of cryo-planed specimens to allow three-dimensional observations of

structural features by deeply etching planed surfaces under the controlled conditions of a cryo-SEM.

Materials and Methods

Infection assay

Four square bio-assay dishes (245 × 245 × 20 mm; Nunc A/S, Roskilde, Denmark) were prepared for plant culture by aseptically boring three equally spaced holes (6 mm diameter) through one edge of each dish to allow shoots to emerge. Sterile agar [2% weight/volume (w/v); Sigma-Aldrich Pty Ltd, Sydney, Australia] containing 1/8 strength Hoagland's solution was then poured into the dishes to a depth of *c*. 4 mm. Wheat (*Triticum aestivum* L. cv. Janz) seeds were surface-sterilized [1% bleach (w/v) for 5 min, rinsed in sterile water 3 times] and a single seed was placed on the agar adjacent to each hole 2 cm from the edge of each dish. Seeds were fixed to the agar surface with a drop of molten agar. Dishes were sealed using surgical tape (Micropore™; 3M, St. Paul, MN, USA), covered with aluminium foil (leaving gaps for shoots to pass through) and leaned at an angle of *c*. 70° to horizontal (to keep roots growing along the surface of the agar) in an incubator at 15°C under fluorescent lights (*c*. 100 µmol m^{-2} s^{-1} at shoots).

Seven days after planting, seedlings were aseptically inoculated with *Rhizoctonia solani* AG-8 (isolate 08RW11) or with a sterile inoculum (control). Inocula of *R. solani* consisted of 3-mm-diameter plugs removed with a sterile corer from the edge of an actively growing culture on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI, USA). Control plants were inoculated with sterile PDA plugs. Plugs were placed immediately adjacent to two seminal root tips (roots *c*. 10 cm long) of each plant. Three dishes (nine plants) were used for the *R. solani* treatment, and one (three plants) for the control. All dishes were again sealed, covered and returned to the incubator.

Tissue preparation and microscopy

Inoculation sites were sampled at 6 and 12 d postinoculation by excising a 1-cm root segment, the mid-point of which was adjacent to the inoculum plug. At the time of each sampling, root tips had grown beyond the site of inoculation. Root segments, three at a time, were quickly put into sterile distilled water, then transferred to a dry watch glass with forceps, gently gathered into a bundle that was held together by surface tension, and then plunged into liquid nitrogen (N$_2$). Bundling of samples allowed three roots to be prepared and examined at the same time. The frozen root bundles were bisected transversally under liquid N$_2$ with a scalpel, thus exposing the interior of roots at the inoculation site. Each half was then quickly mounted on aluminium stubs (with the inoculation site exposed) using tissue freezing medium (TBS™; Durham,

NC, USA), and immediately returned to liquid N$_2$. Each mounted bundle was planed at −100°C with a diamond knife in a cryo-ultramicrotome (Ultracut E, Reichert-Jung (Leica), Nussloch, Germany) to produce a smooth transverse face. Planed samples were transferred under liquid N$_2$ to a cryo-transfer unit (Oxford Instruments, Eynsham, UK), then to the stage of a cryo-scanning electron microscope (JEOL 6400; JEOL Ltd, Tokyo, Japan). The sample was then observed at an accelerating voltage of 2 kV while the surface was etched by subliming ice at −80°C for 2–9 min after cell outlines appeared. The sample was then re-cooled to −180°C and returned to the cryo-transfer unit where samples were sputter-coated under vacuum with high-purity gold for 4–6 min (duration increased with increasing depth of etching) in an argon atmosphere. Samples were then returned to the microscope column and viewed at an accelerating voltage of 15 kV. Images were recorded using a digital image recorder (ImageSlave; OED Pty Ltd, Hornsby, Australia). Samples etched for 9 min were imaged by recording several images at different focal planes which were then stacked to produce a single focused image using image analysis software (Auto-Montage Essentials; Synoptics Inc., Frederick, MD, USA).

Results

All control specimens examined displayed well-preserved root cells with the geometry of turgid tissue (Fig. 2a). Cryo-planing produced a smooth, flat surface that etched uniformly and enabled rapid identification of cell types. Longer etching allowed observation of structures deep within roots with no apparent change in their shapes or positions.

Hyphal walls were preserved with a smooth, uncollapsed form (Fig. 2b). The location and spatial arrangement of the fungus within host root tissue were easily observed. This contrasts with previous unsuccessful attempts to visualize hyphae in disintegrating root cells using conventional embedment and sectioning (Fig. 1). Our technique also revealed structural detail within both root (Fig. 2a–d) and hyphal (Fig. 2c, inset) cells. Despite the delicate nature of the most necrotic roots (12 d postinoculation), epidermal and cortical cells surrounding sites of severe damage were retained intact (Fig. 2c).

Vacuoles in cells of uninoculated roots contained striations which are the sequestered solutes formed during freezing (Fig. 2a). In inoculated plants, root cells invaded by hyphae contained few or no striations, while those without hyphae were often striated (Fig. 2b). Six days postinoculation, hyphae were generally confined to the epidermis, the outer two layers of cortical cells, and intercellular spaces of these cell layers (Fig. 2b). Occasionally, cell walls penetrated by hyphae were revealed, particularly in samples etched beyond 2 min. At this time, little destruction of root cell walls was evident. Six days later (12 d postinoculation), hyphae were often found in deeper layers of the root cortex, and occasionally in the endodermis. At this advanced stage of infection, the presence of hyphae often was
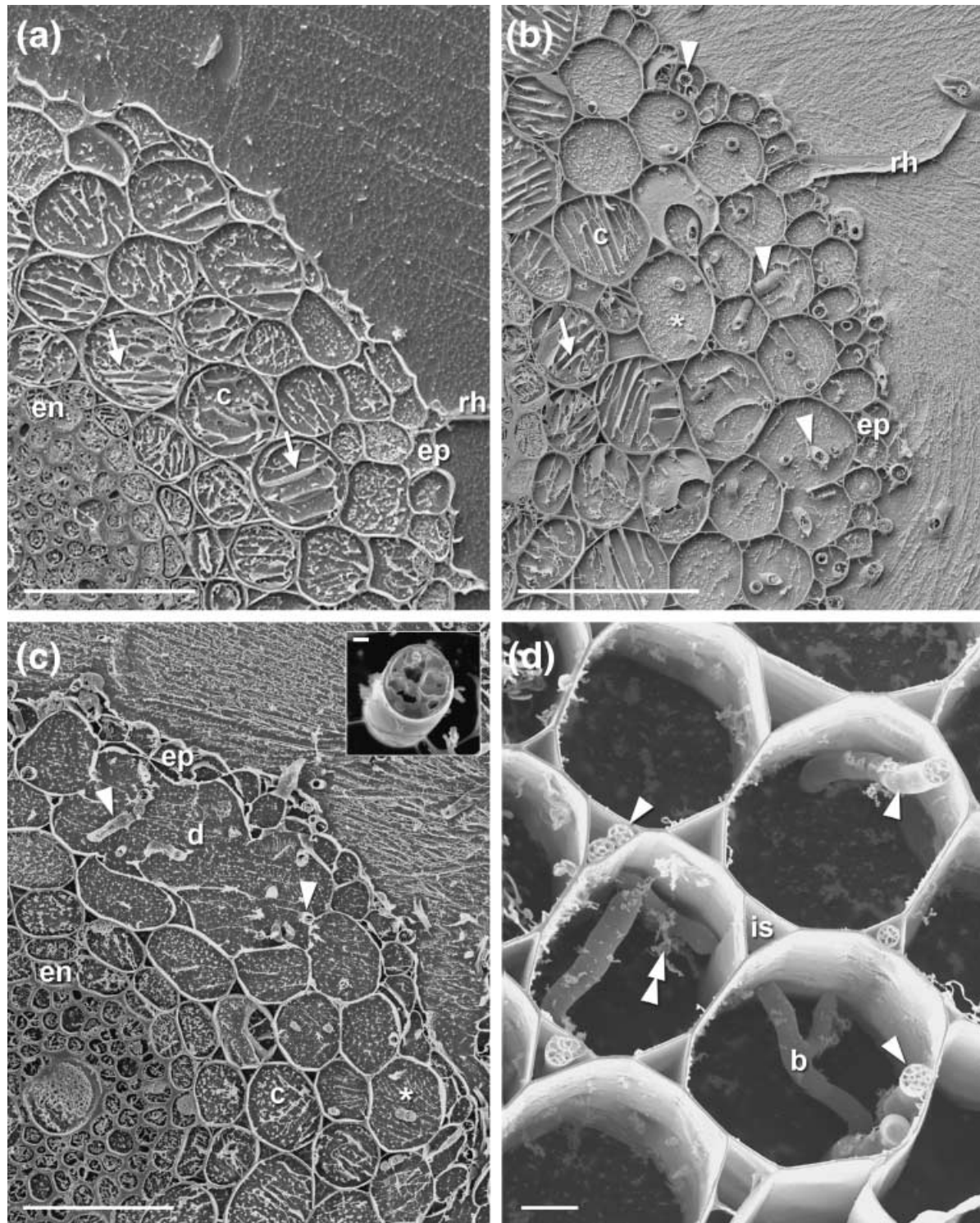
Fig. 2 Cryo-planed, etched transverse faces of wheat (*Triticum aestivum*) seminal roots viewed using a cryo-scanning electron microscope. (a) An uninoculated control root. Striations (arrows) formed by sequestered solutes remain in cells that were living when frozen. (b) Six days postinoculation, hyphae of *Rhizoctonia solani* (arrow heads) are clearly seen within epidermal and cortical cells. Walls of these cells remain intact but the lack of striations (asterisks) indicates those cells were not living when cryo-fixed. (c) Cortical cell wall degradation is visible 12 d postinoculation. Inset: hyphal walls did not collapse and unidentified structures within hyphae are revealed. (d) Deep etching reveals hyphal arrangement, branching, passage between root cells and unknown debris (double arrow heads). This image is a stack of three images taken at different depths. Bars: (a–c) 100 µm; (d) 10 µm; (c inset) 1 µm. ep, epidermis; c, cortical cells; en, endodermis; rh, root hair; d, damage caused by infection; is, intercellular space; b, hyphal branch.

associated with deterioration or absence of cortical cell walls (Fig. 2c). At both sampling times, most hyphae were aligned with the root vertical axis (Fig. 2b,c), although deeper etching revealed radial growth and branching (Fig. 2d).

Discussion

We successfully obtained three-dimensional, internal views of fungal-colonized roots *in situ* by deep etching of cryo-planed tissues and avoided the distortions produced by alternative procedures, as in the example in Fig. 1. The success of this technique depends on the combination of fixing structures in a hydrated state *in situ*, planing for an even fracture face and etching to reveal three-dimensionality.

During cryo-planing, delicate structures within the fully hydrated root cells were supported by ice and remained *in situ*. Cryo-planing overcame difficulties associated with cryo-fracturing and, following etching, enhanced cell identification. A significant advantage of deep etching after planing is the depth of field achieved, and thus the need for time-consuming serial sectioning or planing is eliminated. Deep etching revealed the three-dimensional spatial organization of hyphae within root tissues as well as the structural integrity of necrotic root cells. The pathways by which hyphae penetrate cell walls, modify their structure and migrate throughout the host become apparent, particularly when duration of etching is extended.

After deep etching, structures protruding from the remaining ice are not collapsed. This is probably a result of the retention of unfreezable (also known as 'hydration' or 'bound') water (Read, 1991). Such water is tightly bound in membranes and cell walls by interactions with macromolecules (Bachmann & Mayer, 1987; Wolfe *et al.*, 2002). Prolonged etching can cause shrinkage in some instances (Read & Jeffree, 1988; Read, 1991; Jansson *et al.*, 2000), but we did not observe shrinkage of structures after etching for 9 min. Additionally, no visible damage resulted when gold coating was extended to compensate for increased surface area. When examining etched roots, we did not observe damage arising from the freezing process. The small (9%) volume increase produced by freezing is considered a minor artefact of frozen hydrated tissue that is acceptable for low-resolution cryo-SEM, such as that in the present study (Read & Jeffree, 1988; Sargent, 1988).

It is important to note that the maximum depth of etching is limited by: (i) the depth at which cell end-walls appear; (ii) the depth at which gold coating becomes ineffective; and (iii) the depth at which secondary electrons can be adequately detected. We did not experience these limitations in samples etched for 9 min, although electron emission at depth was diminished.

Our detection of hyphae inside and between cells of wheat roots 6 d postinoculation is analogous to the findings of Hall (1987), who observed hyphae of *Rhizoctonia* inside wheat roots 7 d after inoculation. Hall (1987) used a cryostat to obtain cross-sections of infected roots, but reported difficulty when sections were < 16 µm thick as a consequence of the fragility of the rotted material, particularly beyond 14 d postinoculation. Our technique enabled examination of root tissue 12 d postinoculation despite significant degradation of cell walls where hyphae were present. At this stage, we observed hyphae as deep as the endodermis. Noteworthy is the apparent resistance of the young epidermis to digestion by the fungus (also seen in Fig. 1). Also evident 12 d post-inoculation was hyphal growth through root cell walls and vertically. The tendency of hyphae to colonize roots in the direction of the root vertical axis is possibly a result of the long, tubular structure of cortical cells (Wenzel & McCully, 1991), thereby confining hyphae in the vertical direction or providing a path of least resistance.

The sequestration of solutes by ice crystal formation (thus forming the striations observed in the frozen vacuoles) is a common artefact of cryo-fixation of living cells (Canny & Huang, 1993; Huang *et al.*, 1994), and their presence can be used to suggest cell viability. The absence of sequestered solutes in root cells containing hyphae of *R. solani* suggests that the fungus had killed the invaded cells, digested membranes and organelles, and absorbed or released solutes. The reverse also was true, with hyphae never seen in cells that retained striations (unlike that of biotrophic fungi observed by Ryan *et al.*, 2003). An important finding revealed by the presence of sequestered solutes in Fig. 2(b) and (c) is that some cells that have retained no solutes (and thus presumably have lost membrane integrity) are adjacent to cells containing solutes, suggesting that *R. solani* may kill cells very locally.

We conclude that our technique has potential for improving our understanding of the structural aspects of plant–fungal interactions. This technique is not limited to root-invading pathogens, however, as studies of stem and leaf invasion by fungi (and potentially bacteria) also will benefit from this approach, particularly if material is delicate and susceptible to damage.

References

Allan-Wojtas P, Yang AF. 1987. Solutions to difficulties encountered in low-temperature SEM of some frozen hydrated specimens: Examination of *Penicillium nalgiovense* cultures. *Journal of Electron Microscopy Technique* **6**: 325–333.

Bachmann L, Mayer E. 1987. Physics of water and ice: Implications for cryofixation. In: Steinbrecht RA, Zierold K, eds. *Cryotechniques in biological electron microscopy.* Berlin, Germany: Springer-Verlag, 3–34.

Beckett A, Porter R, Read ND. 1982. Low-temperature scanning electron microscopy of fungal material. *Journal of Microscopy, Oxford* **125**: 193–199.

den Belder E, Boekestein A, van Esch JWJ, Thiel F. 1993. Low-temperature scanning electron microscopy in fungus–nematode interaction. *Scanning* **15**: 37–42.

Canny MJ, Huang CX. 1993. What is in the intercellular spaces of roots? Evidence from the cryo-analytical-scanning electron microscope. *Physiologia Plantarum* **87**: 561–568.

Chi CC, Childers WR. 1964. Penetration and infection in alfalfa and red clover by *Pellicularia filamentosa*. *Phytopathology* **54**: 750–754.

Grabski C, Guggenheim R, Lüönd G, Düggelin M, Müller T. 1987. Plant pathogenic fungi observed in SEM equipped with the SCU 020 cryopreparation unit. *European Journal of Cell Biology* **44** (Suppl.): 19.

Hall G. 1987. SEM studies of sterile fungi on roots of sterile wheat seedlings. *Transactions of the British Mycological Society* **88**: 549–553.

Hoch HC. 1991. Preservation of cell ultrastructure by freeze-substitution. In: Mendgen K, Lesemann DE, eds. *Electron microscopy of plant pathogens.* New York, NY, USA: Springer-Verlag, 1–16.

Huang CX, Canny MJ, Oates K, McCully ME. 1994. Planing frozen hydrated plant specimens for SEM observation and EDX microanalysis. *Microscopy Research and Technique* **28**: 67–74.

Jansson HB, Persson C, Odeslius R. 2000. Growth and capture activities of nematophagous fungi in soil visualized by low temperature scanning electron microscopy. *Mycologia* **92**: 10–15.

Jones D, McHardy WJ, Alexander C. 1987. Low-temperature scanning electron microscopy of fungi. *Scanning Microscopy* **1**: 1423–1429.

MacNish GC, Neate SM. 1996. Rhizoctonia bare patch of cereals – an Australian perspective. *Plant Disease* **80**: 965–971.

McCully ME, Shane MW, Baker AN, Huang CX, Ling LEC, Canny MJ. 2000. The reliability of cryoSEM for the observation and quantification of xylem embolisms and quantitative analysis of xylem sap *in situ*. *Journal of Microscopy, Oxford* **198**: 24–33.

Nijsse J, van Aelst AC. 1999. Cryo-planing for cryo-scanning electron microscopy. *Scanning* **21**: 372–378.

Read ND. 1991. Low-temperature scanning electron microscopy of fungi and fungus–plant interactions. In: Mendgen K, Lesemann DE, eds. *Electron microscopy of plant pathogens.* New York, NY, USA: Springer-Verlag, 17–29.

Read ND, Jeffree CE. 1988. Common artifacts associated with biological material examined by low-temperature scanning electron microscopy. *Institute of Physics Conference Series* **93**: 17–18.

Read ND, Jeffree CE. 1991. Low-temperature scanning electron microscopy in biology. *Journal of Microscopy, Oxford* **161**: 59–72.

Ruppel EG. 1973. Histopathology of resistant and susceptible sugarbeet roots inoculated with *Rhizoctonia solani*. *Phytopathology* **63**: 123–126.

Ryan MH, McCully ME, Huang CX. 2003. Location and quantification of phosphorus and other elements in fully hydrated, soil-grown arbuscular mycorrhizas: a cryo-analytical scanning electron microscopy study. *New Phytologist* **160**: 429–441.

Sargent JA. 1986a. Cryopreservation for scanning electron microscopy avoids artifacts induced by conventional methods of specimen preparation. *Tissue and Cell* **18**: 305–311.

Sargent JA. 1986b. Cryo-preservation of roots for scanning electron microscopy. *Annals of Botany* **58**: 183–185.

Sargent JA. 1988. Low-temperature scanning electron microscopy – advantages and applications. *Scanning Microscopy* **2**: 835–849.

Van Cauwenberghe OR, Newcomb W, Canny MJ, Layzell DB. 1993. Dimensions and distribution of intercellular spaces in cryo-planed soybean nodules. *Physiologia Plantarum* **89**: 252–261.

Walker GD, Powell CL. 1979. Vesicular-arbuscular mycorrhizas in white clover: a scanning electron microscope and X-ray microanalytical study. *New Zealand Journal of Botany* **17**: 55–59.

Walther P, Müller M. 1999. Biological ultrastructure as revealed by high resolution cryo-SEM of block faces after cryo-sectioning. *Journal of Microscopy, Oxford* **196**: 279–287.

Wenzel CL, McCully ME. 1991. Early senescence of cortical cells in the roots of cereals. How good is the evidence? *American Journal of Botany* **78**: 1528–1541.

Wolfe J, Bryant G, Koster KL. 2002. What is 'unfreezable water', how unfreezable is it and how much is there? *Cryoletters* **23**: 157–166.