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A Test of the Air-Seeding Hypothesis Using *Sphagnum* Hyalocysts[1]

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ABSTRACT

"Air-seeding" is a proposed mechanism for the initiation of water stress embolism in dead plant cells. During air-seeding, external air is drawn into the lumen of a dead plant cell through a pore or crack in the cell wall. The resulting bubble may expand to fill the lumen, thus embolizing the cell. The data presented confirm that *Sphagnum* hyalocysts can embolize by air-seeding when the pressure difference across the air-water meniscus is given by $\Delta P = 0.3/D$ (derived from the capillary equation), where ΔP is the pressure difference across the meniscus (megapascal), and D is the diameter (micrometer) of the pore through which the air bubble enters.

In 1983, Zimmerman (17) coined the term "air-seeding" to describe the hypothetical process in which an air bubble is pulled, under tension, into a lumen through a hole in the cell wall. At least since the early 1900s, botanists have discussed the possibility of cells embolizing by this method (1, 2, 4, 7, 11–13, 15). Verification of air-seeding in cells has proved difficult because of the difficulty of confirming the initiation of a small bubble (frequently less than 1 μm) in a relatively large cell (usually more than 1 mm in length), while eliminating the possibility of embolism from other sources. Renner (13) did conclude that pores in the outer wall of annulus cells on fern leptosporangia were approximately the right diameter (10 nm) to account for bubble entry through the walls at -30 MPa water potential as predicted by the capillary equation (Eq. 1). By indirect methods, he measured cell water potentials in this range at spore discharge which is caused by sudden embolism of the annulus cells. Evidence of air-seeding in tracheary elements is presented by Sperry *et al.* (14) who demonstrated that the pressure required to blow air through *Vitis* stems agrees with that predicted by the capillary equation for pore diameters previously measured in temperate hardwood species. They also found that very low pressure air could not be blown through *Vitis* stems until the stems were dehydrated on the countertop to water potentials that corresponded to the applied pressure differentials at which higher pressure air could be blown through the stems. They did not correlate their results with water potentials at which cavitation could be detected by other means. Crombie *et al.* (4) demonstrated that the pressure required to blow air through hydrated *Rhododendron* stems is approximately that at which cavitation occurs in its leaves and shoots.

An investigation into the mechanisms of embolism in *Sphagnum* was undertaken as part of a project to test more thoroughly the air-seeding hypothesis, and, in particular, to test the applicability of the capillary equation in describing the behavior of air-water menisci in plant cell walls. *Sphagnum*, commonly known as peat moss, is a nonvascular plant of wet places. It is especially appropriate for studies on embolism because it has a network of dead water storage cells in its leaves. The leaves are one cell thick, and events during dehydration and rehydration can easily be seen in the water storage cells, the hyalocysts, under a light microscope. Generally, each hyalocyst has an empty lumen, spiral thickenings on the inner wall surface, and one or more pores aligned along the margins of the abaxial cell surface and occasionally along the margins of the adaxial cell surface (Fig. 1). The pores are perforations in the cell wall communicating between the external air and the empty lumen (Fig. 2). These pores probably contribute to the ability of *Sphagnum* to recover and to rapidly restore photosynthetic processes after having been dehydrated to brittleness. As a hyalocyst dehydrates, a bubble pulls through a pore, filling the lumen with air; water is released and flows to the surrounding tissue. This paper presents the first direct measurements of lumen pressure at embolism, and by comparing the lumen pressures with pore diameters measured directly from the embolized cells, provides evidence that the lumen pressure measured when a bubble enters a hyalocyst agrees with that predicted by the capillary equation. Because the air-seeding hypothesis is based on the capillary equation, these experiments are the first direct test of the air-seeding hypothesis.

MATERIALS AND METHODS

Capillary Equation and the Air-Seeding Hypothesis. According to theory, when the meniscus first develops in a pore during air-

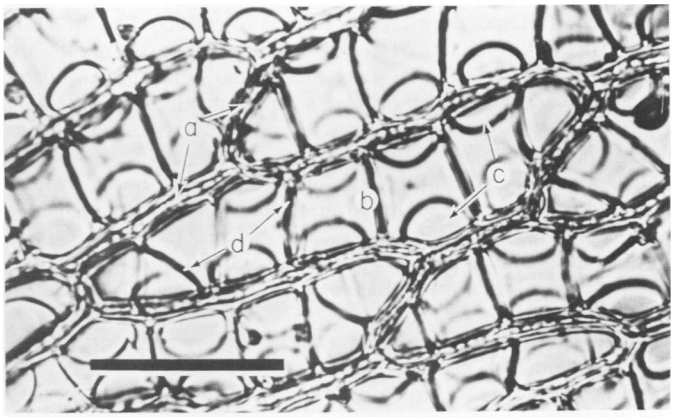

FIG. 1. Photomicrograph of abaxial surface of *Sphagnum* leaf showing chlorophyllous cells (a) and a complete hyalocyst (b) with hyaline pores (c) and spiral thickenings (d) on the interior of the hyaline wall. Scale = 50 μm.

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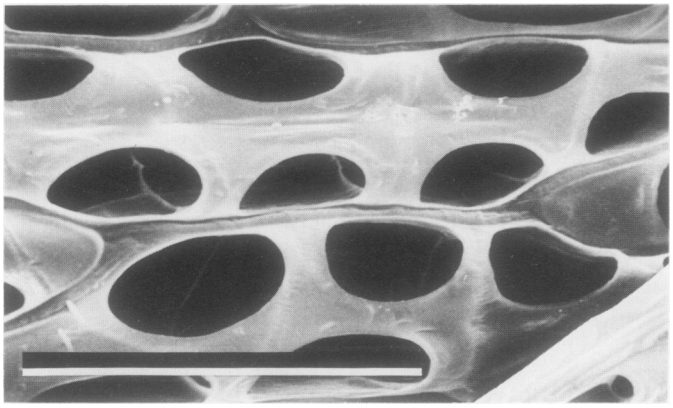

FIG. 2. Scanning EM of pores in *Sphagnum* hyalocyst. The pores are perforations in the cell wall which connect the cell's exterior to the lumen of the cell. A mature hyalocyst is dead; the lumen contains no protoplasm. Scale = 50 μm.

seeding, the meniscus radius is very large, much larger than the radius of the pore. As the tension in the lumen increases during dehydration, the pressure difference across the meniscus increases, thus decreasing the meniscus radius to approach the pore radius. When the pressure differential reaches a critical value, the meniscus radius equals the pore radius, and the resultant bubble penetrates the pore and expands under tension to embolize the cell.

The pressure exerted by a column of water in a capillary tube is described by

$$P = \frac{2T \cos \theta}{r} \quad (1)$$

where T is the surface tension at the meniscus, θ is the contact angle between the water and the tube, and r is the internal radius of the tube. The equation can be thought of as describing the behavior of a meniscus in an elongated pore as a function of the pressures involved. Eq. 1 is numerically

$$\Delta P \cong 0.3/D \quad (2)$$

where ΔP is the pressure difference (MPa) across the meniscus, and D is the pore diameter (μm) (see the "Appendix" for a more complete discussion). A hyalocyst should embolize when the difference between ambient and lumen pressure is equal to that described by Eq. 2 for the largest diameter pore in the cell.

Plant Material. Since the diameters of hyaline pores vary with species, several species of *Sphagnum* were collected at the Harvard Forest, Petersham, MA. The reported measurements are for *S. compactum* DC, *S. girgensohnii Russow*, and *S. palustre* L. Leaves were taken from branches in the apical tuft. The largest pore diameter in typical hyalocysts of tuft branch leaves ranged from 5 to 25 μm.

Observation of Embolism. Observation of embolism was facilitated by recording the dehydration and rehydration of hyalocysts on movie film at various speeds between 18 and 64 frames per second and on videotape at normal speed to allow repeated viewing of a particular sequence both at normal speed and in slow motion.

Lumen Pressure Measurements. Individual hyaline lumen tensions were measured using a pressure microprobe. The microprobe was developed originally to measure positive pressures in growing cells (8). It was modified by Cosgrove (3), who kindly assisted with further hardware and methodology alterations to permit measurements below atmospheric pressure. Nonami *et al.* (10) give an interesting account of the positive pressure microprobe method. The probe is essentially an oil-filled glass capillary with one end in contact with a pressure transducer and mounted on a micromanipulator (Fig. 3). The other end is inserted into a hyalocyst through a pore. The probed, hydrated leaf is held securely on a glass slide and viewed with a ×50 long working-distance objective (working distance = 6 mm) on a compound microscope with a stationary stage (Bausch and Lomb MicroZoom Microscope). Total magnification was ×750. In order to permit dehydration by evaporation, the sample was not enclosed in a humidity chamber, as is usual for positive pressure measurements. A Leitz micromanipulator improved the maneuverability of the probe. The maximum angle of inclination below horizontal (15°) of this manipulator was just sufficient to allow insertion of the probe through a hyaline pore of some hyalocysts without interference from the convex wall of an adjacent hyalocyst. The pressure transducer was calibrated at 14.27 mV/MPa.

The diameter of the capillary opening is critical. Small diameter probe tips clog with debris, or the oil-water meniscus fails to move freely when the interior wall of the tip is coated with debris from the water. A large probe opening results in difficulty controlling the oil-water meniscus because the resistance due to friction is too small and often leads to flooding of the sample with oil from the probe. Internal tip diameters of 2 to 3 μm were a good compromise for this study, and tips were replaced after every one or two probe attempts. Frequent replacement of the capillary tips also lessened the incidence of bubbles developing within the oil. Breaks in the integrity of the oil were only seen after debris had collected on the capillary wall or after a bubble was inadvertently allowed into the oil column.

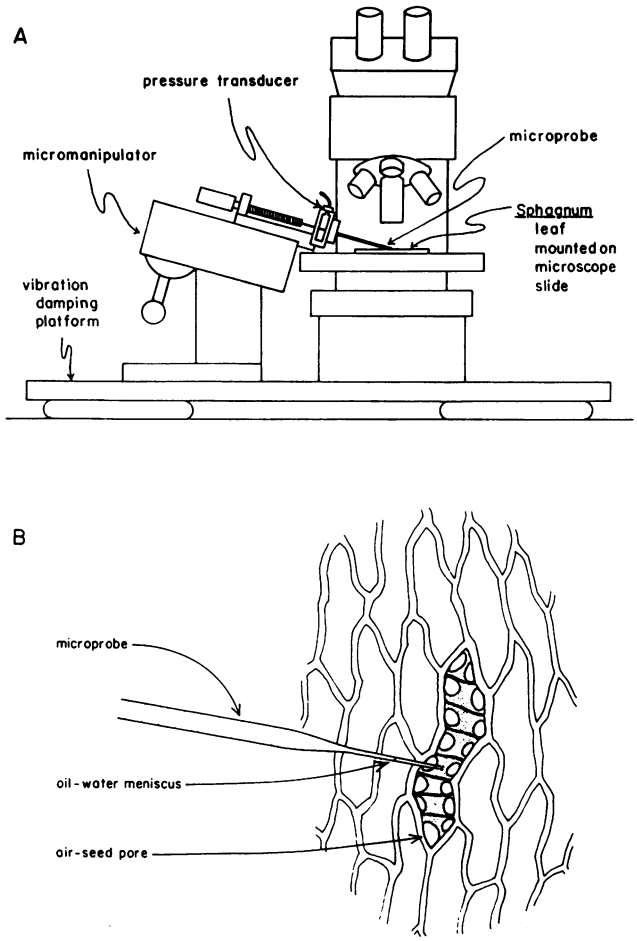

FIG. 3. Pressure microprobe setup. A, Microprobe apparatus; B, microprobe inserted into hyalocyst.

The breaking point of oil in a clean probe tip is not known but must occur at lower pressures than were achieved in the probed hyalocysts.

To ready the capillary tip for a probe, a small amount of lumen water is withdrawn into the capillary by suction created by backing off the thumbscrew control. For lumen pressure measurements, the visible oil-water meniscus must be held stationary during dehydration by manually manipulating the thumbscrew. The accuracy of a lumen pressure measurement depends in part on the experimenter's ability to maintain the position of the meniscus. Several factors cooperate to make that task difficult. The meniscus moves towards the probe tip as lumen pressure decreases, and vibration and minor fluctuations in ambient temperature cause the meniscus to vacillate. The difference between the pressure at full hydration and the pressure at bubble entry is ΔP.

A variety of other technical problems can occur during a probe (*cf.* 10). The difficulties of manipulating the oil-water meniscus and accidental flooding of the sample with probe oil have already been mentioned. Because of tissue movement during dehydration or because of vibration, the capillary tip can pull out of the pore or abut or push through the opposite hyaline wall. Any of these can occur at any time in the course of a probe and will adversely affect results. At least one of them usually did occur, so that the oil-water meniscus, the tip of the probe, and the air-water meniscus all had to be carefully monitored during a probe. Only those probes with no known complications were used in the analysis. This yielded 8 data points from about 50 measurements and as many aborted attempts.

An additional 4 measurements were taken from leaves that were wetted with a 0.1% (v/v) solution of the detergent Tween-80 in distilled water. The detergent should reduce the pressure differential required to pull a meniscus through a pore by lowering the surface tension at the air-water interface. When wetting a leaf with this solution, enough of the solution was applied to approximately double the volume of water already present in and on the leaf. Although the concentration of Tween-80 at bubble entry is not known, it should be close to that of the applied solution when enough water evaporates for menisci to develop in the pores of the hyalocysts. The surface tension of the 0.1% solution of Tween-80 was estimated by comparing its capillary rise in a clean glass tube with the capillary rise of distilled deionized water in the same tube. Assuming that all other parameters are the same (a close approximation), the ratio of the surface tension of 0.1% Tween-80 to the surface tension of water should equal the ratio of the respective capillary rises, and the surface tension of the Tween-80 solution can be calculated. The experimental value for distilled deionized water was less than 4% lower than the accepted value for the surface tension of water at the same temperature (16).

Pore Diameter Measurements. Following a lumen pressure measurement, the diameter of the pore through which the bubble entered was measured with a ocular micrometer. Most of the few elliptical pores were only slightly eccentric thus were taken to be circular. For more eccentric pores, the hydraulic diameter was used as a better approximation for the pore diameter in Eq. 2 ("Appendix").

RESULTS AND DISCUSSION

Excised leaves exposed to air dehydrate within a few minutes. Leaves which are rehydrated by the addition of water from a pipet or syringe return to almost complete hydration in seconds. All fully hydrated hyalocysts (*i.e.* those with no air bubbles visible in their lumens) embolized by air-seeding. The meniscus entered each lumen through the pore with the largest diameter unless that pore was obstructed. Figure 4 depicts an air-seeding sequence. The air-water meniscus in the pore at the arrow is just developing in Figure 4A as the leaf surface water dissipates. The meniscus is visible in the lumen in Figure 4B. Its diameter approximates that of the pore. In Figure 4C, the meniscus pulls into the hyalocyst and looks bubble-like within the lumen. In Figure 4D, the bubble expands to embolize the cell. When a meniscus pulled through a hyaline pore, bubbles in nearby cells contracted slightly, then continued to expand as evaporation proceeded. This localized relief of tension was common, but short-lived.

The only hyalocysts which did not air-seed had entrapped bubbles at the onset of dehydration. Figure 5, lower arrow, shows a bubble which begins to collapse when the leaf is wetted, but as dehydration begins before the bubble completely collapses, it expands, eventually filling the hyalocyst. This cell embolizes without air-seeding. On one occasion, a hyalocyst with an entrapped bubble air-seeded. In this case, the entrapped bubble was separated from the rest of the cell by a spiral thickening which closed the connecting passage to a diameter less than that of the pore through which the air-seed entered. Measurement of lumen pressures in hyalocysts with entrapped bubbles is not feasible with present techniques.

In Figure 6, lumen pressure measurements of air-seeded cells wet with tap water (solid dots) correspond well with the predicted curve (solid line). Those wet with tap water and Tween-80 (open circles) air-seeded at tensions lower than predicted by Eq. 2 because the detergent lowered the surface tension of the meniscus from 7.2×10^{-2} N/m (water at 20°C) to about 4.3×10^{-2} N/m at bubble entry, and thus lowered the pressure differential (dotted line) required to deform the meniscus enough to fit through the pore. The Tween-80 curve is intended to demonstrate the lowering of the surface tension, not to represent a curve that should fit the experimental data.

As Huber (7) suggested, *Sphagnum* hyaline cells may be a good model for the air-seeding of fern annuli. They may also be a good model for the air-seeding of tracheary elements, as Zimmermann (17) suggested. Perhaps the model is even more appropriate in the case of tracheary elements because both they and *Sphagnum* hyalocysts are no longer living and, when hydrated, contain water almost exclusively. The pores in the pit membranes of tracheary elements may be comparable to hyaline pores in that they prevent air from entering the lumen until a critical pressure differential across the pore is reached. There is evidence that *Sphagnum* hyalocysts are an appropriate model for xylem elements in other respects. In both hyalocysts and xylem elements, released water increases the hydration of adjacent cells (5). There is also mounting evidence that tracheary elements will air-seed more than once if completely rehydrated after drying, but that those elements with entrapped bubbles do not air-seed (AM Lewis, MT Tyree, unpublished data). But whatever the outcome of the xylem investigations, we now have confident measurements to indicate that the capillary equation does describe the behavior of air-seeding in plants, particularly in *Sphagnum*.

APPENDIX

In general, the capillary equation says that the upward component of force which raises the water in a capillary tube is equal to the weight of the water supported at equilibrium. The force acts on the water through the surface tension of the air-water meniscus as the water adheres to the tube wall. Thus, in the case of a circular tube of internal diameter a, $(\pi a)\ (T \cos \theta) = (\pi a^2/4)\ (h)\ (sg)$, where πa is the perimeter of the tube, $T \cos \theta$ is the upward component of the force exerted by the surface tension T through the contact angle θ between the water and the tube, and the weight of the supported water is given by the cross-sectional area of the tube $\pi a^2/4$ multiplied by the height of the water column h, the density of water s, and the acceleration due

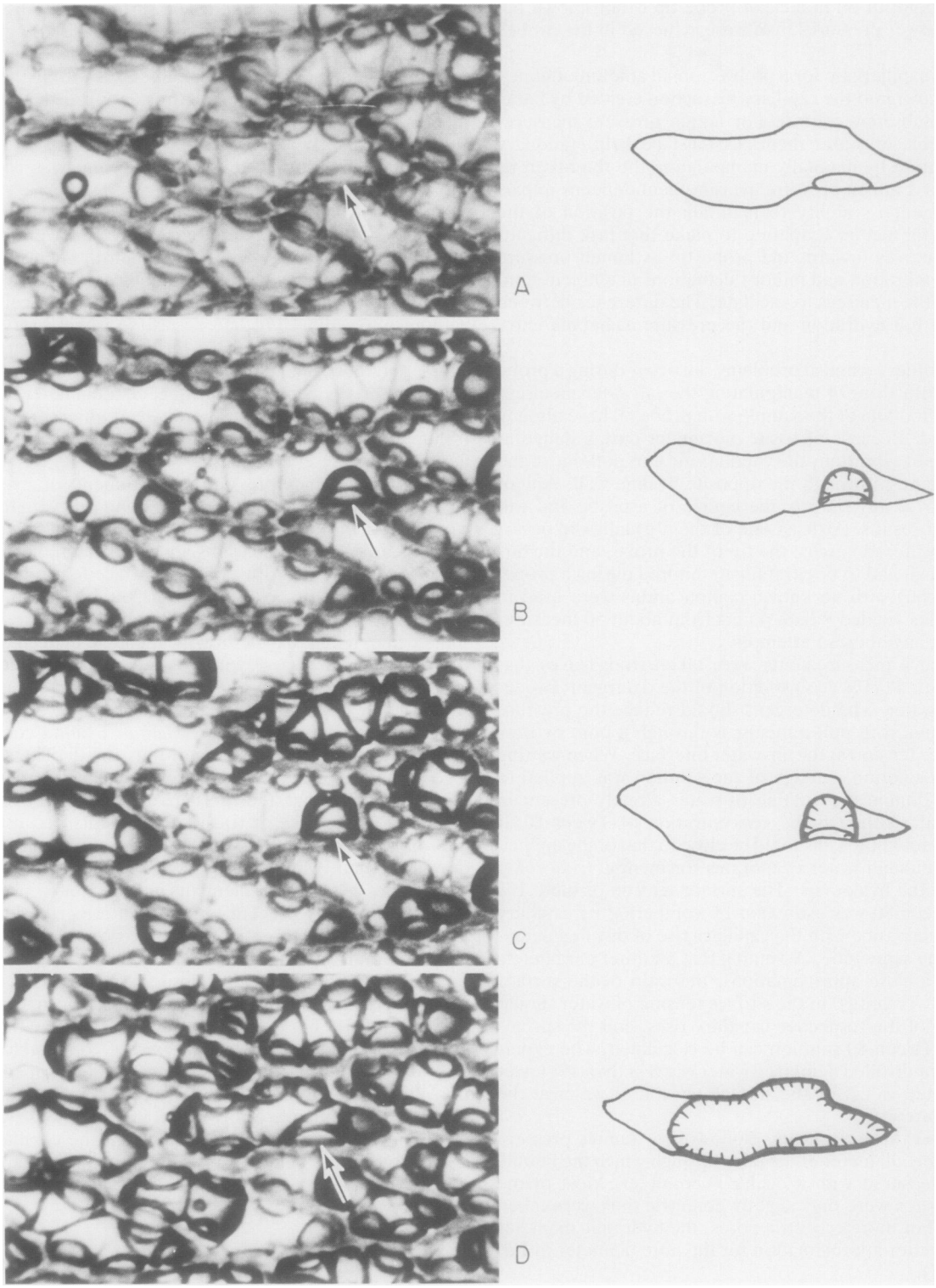

FIG. 4. Air-seeding hyalocyst. The photomicrographs are accompanied by line drawings of the hyalocyst for clarity. A, The pore through which the air-seed enters. B, The meniscus is visible in the cell lumen. The meniscus diameter has diminished until it approximates the diameter of the pore. C, The meniscus has pulled through the pore and begins expansion. After the diameter of the meniscus has decreased to that of the pore, allowing bubble entry, the meniscus increases its diameter. This expansion takes place within the hyalocyst lumen. The hyalocyst has air-seeded. Note that other hyalocysts are in different phases of air-seeding. D, The bubble expands to fill the cell. The cell is embolized.

to gravity g. Rearranging gives $hsg = (4T \cos \theta)/a$. Since T and $\cos \theta$ are constants and hsg is the negative of the pressure drop across the meniscus, the pressure differential Δp in the case of a cell dehydrating, $\Delta P = 0.29/a$ where ΔP is in MPa and a is in μm.

For a tube with rectangular cross-section, $2(a + b)\ (T \cos \theta) = (ab)\ (h)\ (sg)$ where a and b are the width and length of the rectangle. Rearranging gives $hsg = (4T \cos \theta)/(2\ ab/[a + b])$. Substituting the constants and correcting for units gives $\Delta P = 0.29/(2\ ab/[a + b])$. If $a = b$, then $\Delta P = 0.29/(2\ a^2/2\ a) = 0.29/$

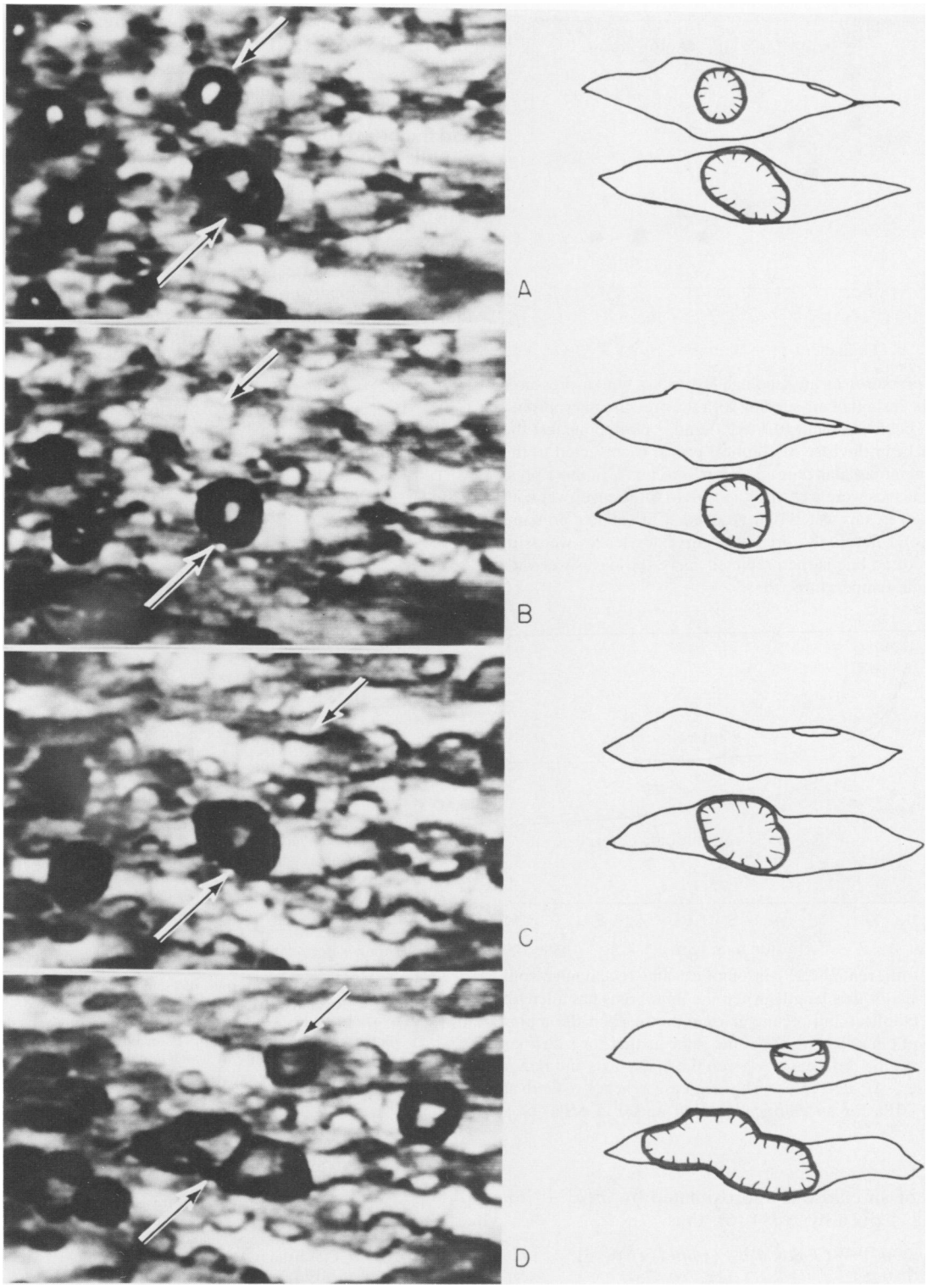

FIG. 5. Embolism by expansion of an entrapped bubble. In this sequence photographed from a video screen, the leaf has been rewetted after dehydration. A, Many hyalocysts contain entrapped bubbles formed during rewetting; B, the bubble in the cell indicated by the upper arrow has collapsed; the bubble in the cell indicated by the lower arrow shrinks, but does not completely dissolve before dehydration begins again; C, the bubble in the lower cell expands leading to embolism; D, the upper hyalocyst air-seeds.

a. The pressure required to pull the meniscus through a square tube of width a is the same as for a circular tube of diameter a. If $a << b$, then $\Delta P \cong 0.29/(2\ ab/b) \cong 0.29/2a \cong 0.15/a$ (Pickard's [12] equation for parallel plates). For wide parallel plates separated by distance a, the pressure required to pull the meniscus through the plates is one-half the pressure required to pull the meniscus through a circular tube of diameter a (Fig. 7).

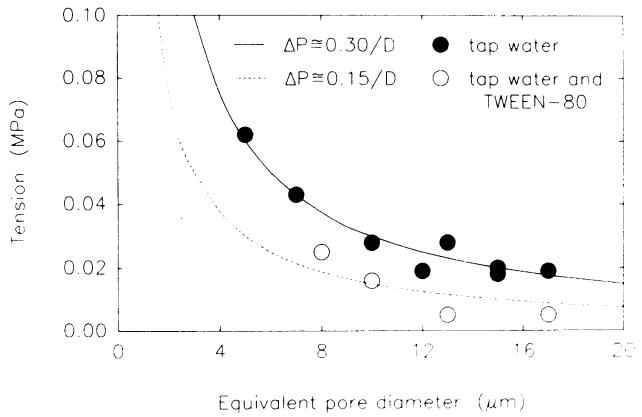

FIG. 6. Lumen pressures at air-seeding. Hyalocyst lumen pressures were measured at the instant of air-seeding with the pressure microprobe. Tension is pressure below ambient (0.1 MPa) and is plotted against the pore diameter, which, in the case of elliptical pores, is corrected to the hydraulically equivalent circular pore diameter (see text). In most pressure measurements leaves were wet with tap water (●). Leaves wet with 0.1% (v/v) Tween-80 in tap water (○) air-seeded at smaller pressure differentials. The solid line is the predicted curve for leaves wet with water at 20°C; the dotted line is the predicted curve for leaves wet with Tween-80 at the same temperature.

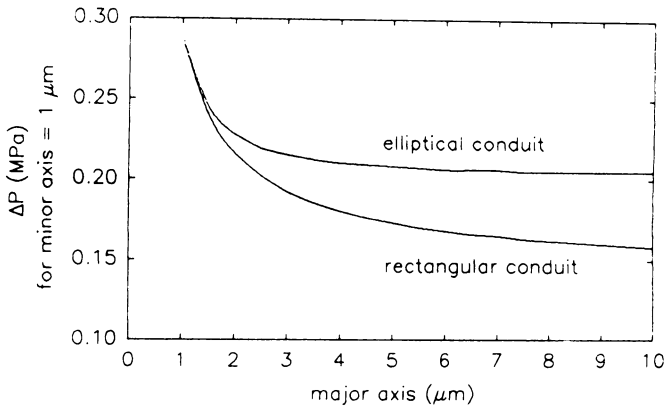

FIG. 7. Pressure differential (ΔP) in elliptical and rectangular conduits for a range of major axis lengths when the minor axis has a length of 1 μm. For both elliptical and rectangular conduits, when the major and minor axes are of equal length, ΔP is the same as that for a circular conduit, 0.29 MPa. As the difference between the major axis and minor axis lengths increases, ΔP decreases. The decrease reaches a limit at approximately 0.21 MPa for an elliptical conduit and 0.15 MPa for a rectangular conduit.

The perimeter of an ellipse is approximated by $\pi([a^2 + b^2]/2)^{1/2}$, and the area is given by $\pi ab/4$ (6), thus

$$(\pi[(a^2 + b^2)/2]^{1/2})\ (T \cos \theta) \cong (\pi ab/4)\ (h)\ (sg)$$

where a and b are the axes of the ellipse. Rearranging yields

$$hsg \cong (4T \cos \theta)/(2\ a^2b^2/[a^2 + b^2])^{1/2}$$

substituting for constants and correcting for units gives $\Delta P \cong 0.29/(2\ a^2b^2/[a^2 + b^2])^{1/2}$. If $a = b$, then $\Delta P \cong 0.29/(2\ a^2b^2/(2\ a^2))^{1/2} \cong 0.29/a$, the pressure required to pull a meniscus through a circular tube of diameter a. When $a << b$, $\Delta P \cong 0.29/(2\ a^2b^2/b^2)^{1/2} \cong 0.21/a$. In a tube with an elongated elliptical cross-section and minor axis of length a, the pressure differential required to pull the meniscus through the tube is about 7/10ths the pressure required to pull the meniscus through a circular tube of diameter a (Fig. 7).

The hydraulic diameter of a regularly shaped conduit is the diameter of the circular conduit that has the same rate of flow at the same pressure gradient (*i.e.* it is the geometric diameter of the hydraulically equivalent circular conduit), so that hydraulic diameter is calculated by $d = 4A/p$, where A and p are the area and the perimeter of the conduit (9, 18). Note that for a rectangular tube and for an elliptical tube, the terms $2\ ab/(a + b)$ and $(2\ a^2b^2/[a^2 + b^2])^{1/2}$, respectively, are the hydraulic diameters.

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