Mapping the Growth of Fungal Hyphae: Orthogonal Cell Wall Expansion during Tip Growth and the Role of Turgor

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ABSTRACT By computer-enhanced videomicroscopy, we mapped the trajectory of external and internal cell surface markers in growing fungal hyphae to determine the pattern of cell wall expansion during apical growth. Carbon particles (India ink) were chosen as external markers for tip expansion of *Rhizoctonia solani* hyphae. Irregularities in the growing apical walls of *R. solani* served as internal markers. Marker movement was traced in captured frames from the videotaped sequences. External and internal markers both followed orthogonal trajectories; i.e., they moved perpendicular to the cell surface regardless of their initial position in the hyphal apex. We found no evidence that the tip rotates during elongation. The discovery that the cell wall of a growing hypha expands orthogonally has major repercussions on two fronts: 1) It supports the long-held view that turgor pressure is the main force driving cell wall expansion. 2) It provides crucial information to complete the mathematical derivation of a three-dimensional model of hyphal morphogenesis based on the vesicle supply center concept. In three dimensions, the vesicle gradient generated by the vesicle supply center is insufficient to explain shape; it is also necessary to know the manner in which the existing surface is displaced during wall expansion.

INTRODUCTION

The two-dimensional vesicle supply center (VSC) model of fungal morphogenesis has been a useful tool to analyze the role of the Spitzenkörper in generating a hyphal tube by apical growth (Bartnicki-Garcia et al., 1989). The VSC model predicts that the Spitzenkörper functions as a VSC responsible for the gradient of exocytosis in the apical region. In two dimensions (2-D), the polarized growth of a hypha can be explained by the linear displacement of the VSC, which creates a gradient of wall-building vesicles, which in turn determines the tubular shape of the hypha. The process and the resulting shape are described by the hyphoid equation [$y = x \cot (xV/N)$]. Several examples of fungal morphogenesis have been analyzed and found to comply with the predictions of the VSC model (Bartnicki-Garcia et al., 1995a,b; Reynaga-Peña et al., 1997; Riquelme et al., 1998, 2000).

Although the VSC concept can be used to compute the vesicle gradient required to produce a 3-D hypha, attempts to develop a 3-D VSC-based model ended up in a mathematical indetermination with an infinite number of solutions (Gierz and Bartnicki-Garcia, 2001; see also http://boyce3427.ucr.edu/the_3d_model.htm). Contrary to the 2-D model, the 3-D vesicle gradient alone was insufficient to explain shape generation. To resolve the indetermination it was necessary to specify the spatial pattern of surface expansion; in other words, it was essential to know how the new wall displaced the existing wall. Three different theoretical modes of cell expansion were formulated for a 3-D hyphoid tube. Each pattern can be visualized by following the trajectory of a point on the expanding cell surface (Fig. 1). In the isometric mode, newly inserted wall displaces existing wall evenly in all tangential directions. In the orthogonal mode, all points on the expanding surface move perpendicularly to the existing surface. In the rotational mode, the wall expands and maintains at all times the shape of a 2-D hyphoid rotated about its long axis.

The purpose of the present study was to determine, experimentally, the pattern of cell wall expansion in growing hyphal tips and compare them with the trajectories predicted by the theoretical models. Among a number of external markers tested, the best results were obtained by mapping the movement of carbon particles that became attached to the hyphal tips of *R. solani*. These results were complemented by following the displacement of cell wall irregularities in growing tips of the same fungus. These irregularities served as natural internal markers of wall expansion.

METHODS

Fungus cultivation

The strain of *Rhizoctonia solani* Kuhn employed for this work was obtained from E. E. Butler (University of California, Davis; culture 283) and maintained on 2% potato dextrose agar (PDA). Microscopic observation of internal markers was done by phase contrast microscopy with the fungus growing on a specially designed slide chamber (López-Franco, 1992). The growth medium was a very thin layer of 1.5% potato dextrose medium and 18% gelatin (Difco, Detroit, MI). The slide chambers were inoculated with small pieces of mycelium (3 × 10 mm) grown on 1.5% PDA (Difco). A coverslip attached along one edge of the slides with silicon sealer formed a flexible hinge. The coverslip remained propped up while the slide chambers were incubated in a large moist petri dish chamber for 48–72 h, until the new mycelium grew 1–1.5 cm (López-Franco et al., 1994). Before

Received for publication 3 May 2000 and in final form 7 August 2000.
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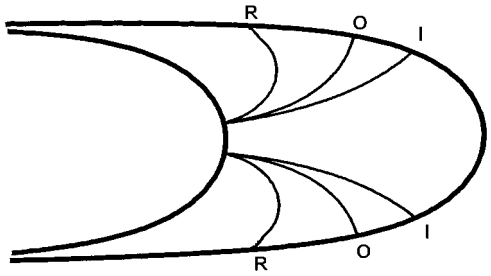

FIGURE 1 Idealized representation of the trajectories of two symmetrical points on the surface of a hyphoid growing from left to right. The three different trajectories for each point were calculated according to three different modes of surface expansion: rotational hyphoid (R), orthogonal hyphoid (O), and isometric hyphoid (I).

microscopic observation, inoculum pieces were removed and the coverslip was carefully lowered to contact the hyphae and the growth medium.

The experiments with external markers were done in a modified slide chamber in which the supporting strips of fingernail polish were replaced by strips of red lithographer's tape (item 616, 3M Scotch brand, 3M, St. Paul, MN). Because observations of external markers were done by differential interference contrast, gelatin was not needed to match refractive indexes and 2% PDA (Difco) was used. Before the chamber was closed, 5 μl of a suspension of carbon particles in potato dextrose broth (PDB; see below) was applied ~2–5 mm ahead of the growing hyphae. Observations started 2–10 min later to allow the hyphae to recover from any stress caused by the manipulation.

Computer-enhanced videomicroscopy

Observations were made with an Olympus Vanox microscope (model AH-2) equipped with a 100× oil-immersion objective lens (NA 1.25). External markers were observed with differential interference contrast optics. Internal markers were examined by phase contrast. Sequences were imaged through an enlarging eyepiece (25×) with a C-2400–1 (Chalnicon) video camera, processed with an Argus 10 real time digital image processor (Hamamatsu) and electronically zoomed 2×. Images were recorded at 30 fps on S-VHS NTSC videotape with a Panasonic video cassette recorder.

Preparation of carbon particles for use as external markers

One milliliter of Higgin's waterproof drawing ink (Black India ink 4415, Eberhard Faber, Inc., Lewsburg, TN) was centrifuged in an Eppendorf tube for 2 min at 10,000 × g and the supernatant was discarded (Grove et al., 1970). The pellet was resuspended in 1 ml of sterile distilled water and centrifuged again for 2 min. These steps were repeated at least 15 times and, finally, the carbon particles were re-suspended in 1 ml water and autoclaved 20 min at 121 C and stored. Before use, a small amount of the autoclaved carbon particles, usually 200 μl, was removed and centrifuged for 1 min in an Eppendorf tube at 10,000 × *g*. The supernatant was discarded and an equal volume of 7% PDB (Difco) was added to the pellet to serve as the stock suspension. Before use, samples of the stock suspension were diluted with two to three parts of oxygenated 7% PDB in an Eppendorf tube and vortexed briefly.

Image processing and analysis

Videotaped sequences were played on a variable tracking player (JVC model BR-S525U) and observed on a 13-inch, color monitor (Sony model PVM-1343). Individual images were captured from the videotaped sequences in 8-bit gray scale with an Imascan/Chroma frame grabber (Imagraph Corp., Chelmsford, MA).

Cell profiles (outermost boundary of the cell wall) and the positions of the surface marker were manually traced with the measurement option of Image Pro Plus Software for Windows (Media Cybernetics, Silver Spring, MD). Marker position was mapped on the digitized images. The *xy* coordinate values for profiles and markers were automatically collected into a text file with a Windows application program developed to interface with the Argus-10 analyzer (Bartnicki et al., 1994).

The traced profiles, designated here as median profiles, represent median longitudinal sections of the hyphae. We selected mainly external markers that became attached to the median profiles and remained on the same plane during the recorded growth period. For preliminary measurements, the center of each marker was estimated visually; for more precise measurements, the outline of the entire marker was traced and the center of gravity of the enclosed shape was then calculated and used as the center point.

Each sequence selected for analysis lasted ~2–3 min by which time the hypha had elongated ~6–18 μm. Cell profiles and marker positions were mapped at intervals of ~10–20 s, as needed. Each set began at the time, or moments before, the tip collided with the carbon marker particle and ended when the marker(s) had been displaced to the near-cylindrical subapex.

Data processing

The mapped data (text files) for the captured hyphal profiles and marker positions were imported into an Excel spreadsheet and processed as follows:

1) Because the origin (0,0) on a video screen is on the upper left corner, original ordinate values were all negative and thus were converted to positive values to avoid inverting the shapes.

2) A graphic template file was used to orient and normalize all profiles. The template was a hyphoid curve adjusted so that its maximum diameter was 2π. Except as noted, results are expressed in *d* values (d = distance between the VSC and the apical pole in the hyphoid equation; Bartnicki-Garcia et al., 1989).

3) The final cell profile in each sequence was scaled, rotated, and displaced until it matched the template. In this manner, the hyphal growth axis was oriented to coincide with the *x* axis and hyphal diameter was normalized to 2π. Because all profiles and marker trajectories data had been linked, the entire set of values was simultaneously processed and modified by the same factors.

4) Because hyphae do not grow perfectly straight, the axis of growth of each successive profile was independently aligned when necessary. This was done by repeating step 3, matching each hyphal profile with the template to locate the correct longitudinal cell axis and thus determine the exact position of the apical pole.

Theoretical trajectories

Routinely, three theoretical trajectories were calculated for each marker particle starting with the same initial (x,y) value corresponding to the position at which the carbon particle first attached to the cell surface. All subsequent points were calculated for the same times at which the position of the carbon marker was mapped. Theoretical trajectories were superimposed on the charts to compare them with the actual movement of marker particles.

RESULTS

Marking the surface of *R. solani* hyphae with carbon particles

Of all the external markers and fungi tested, the best results were obtained with small carbon particles from India ink

adhering to hyphal tips of *R. solani* (Fig. 2). The hyphae grew steadily and maintained a regular morphology in the slide chamber. As a hypha elongated, its tip occasionally collided with a suspended carbon particle, which became attached to the outer surface of the cell.

Median profile markers

For ease of mathematical analysis, particles attached to the median longitudinal profile of a hyphal tip were selected. In most instances, as the tip expanded, the attached markers

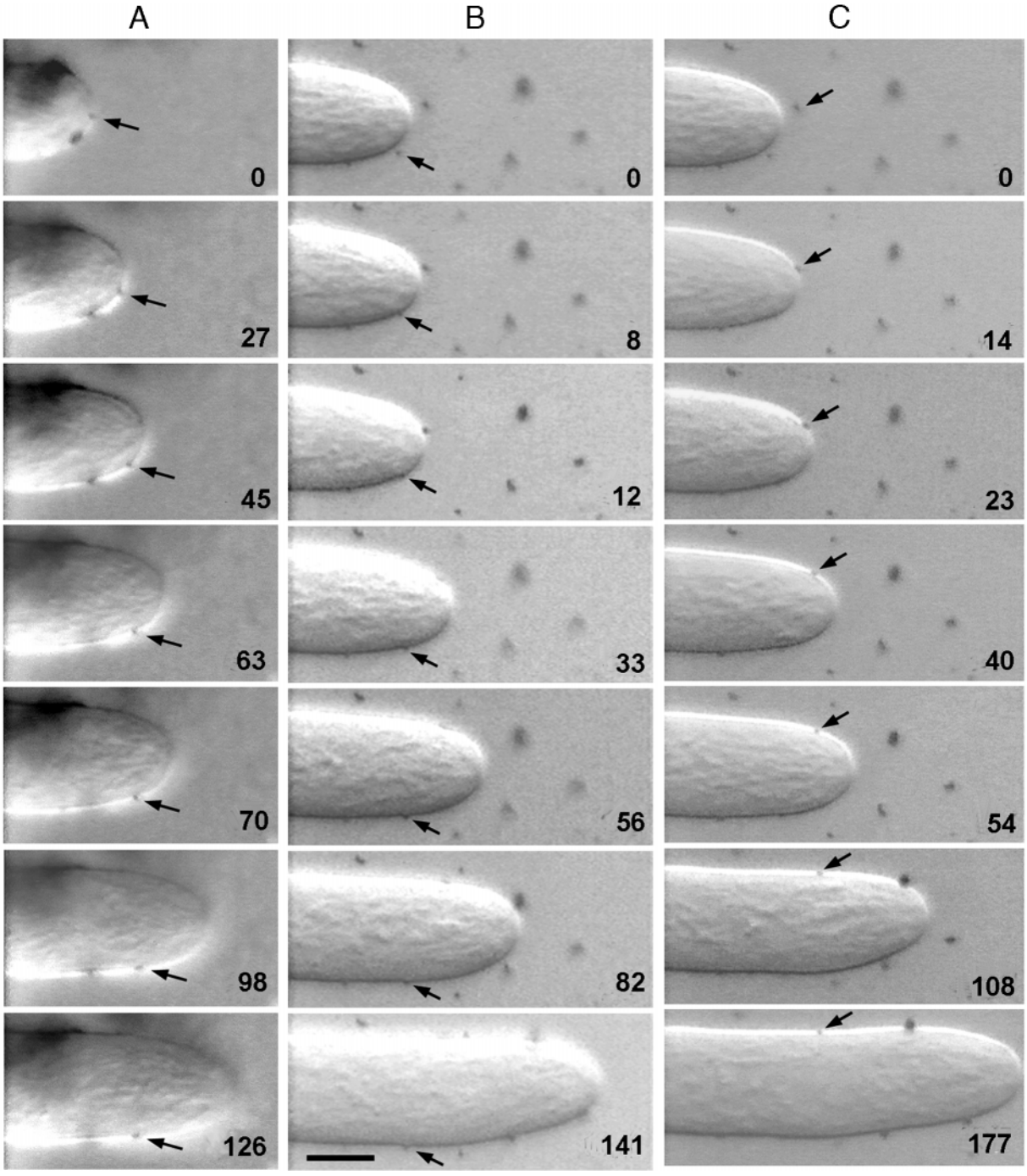

FIGURE 2 Movement of single carbon particles attached to the hyphal surface of *R. solani*. In each of the three columns *A* (left), *B* (center), and *C* (right), the arrows follow the displacement of the same marker. Columns *B* and *C* pertain to the same hypha but show different markers. In *A*, the hypha elongated at 5.4 μm/min, in *B* or *C*, at 6.2 μm/min. The trajectories of these markers were mapped and analyzed in Figs. 3 and 4. Numbers indicate time in seconds. Bar, 5 μm

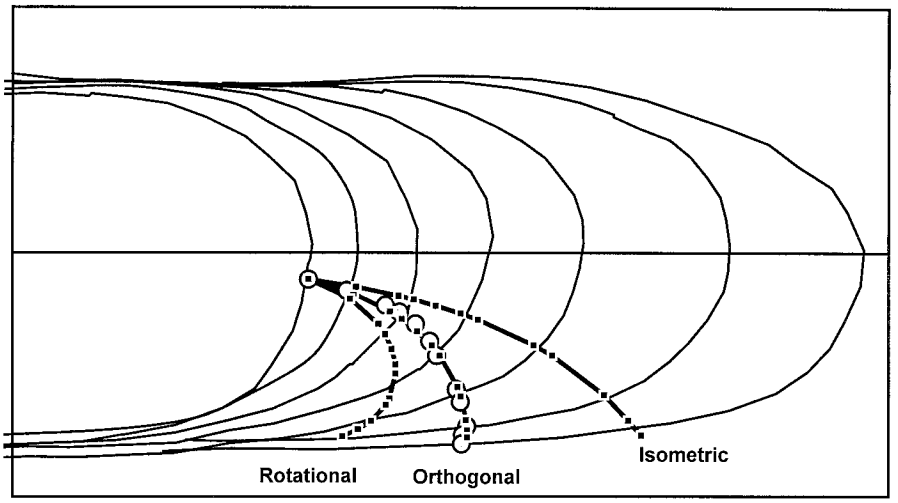

FIGURE 3 Analysis of the displacement of a surface marker shown in Fig. 2 *A* of a hypha of *R. solani* The marker (○) was mapped at variable intervals (4–41 s) during a 167-s period of growth after becoming attached to the hypha. Theoretical curves for the displacement of each marker were calculated for the three different modes of surface expansion. Points on each trajectory were calculated to correspond to the same mapped times for the carbon particle.

remained at the same focal plane, i.e., the plane of focus of the median longitudinal profile. Fig. 2 shows three examples of markers attached to the apical dome and their subsequent displacement. Marker position and cell profiles were mapped periodically for ~2–3 min. During such time, the hyphae elongated ~11–18 μm and the initial surface where the markers attached became transformed from apical dome into a tubular form (Figs. 3 and 4).

Fig. 3 shows the trajectory of a marker that became attached near the apical pole (Fig. 2 *A*); the marker moved both forward and sideways as the tip expanded and the dome surface became the near cylindrical surface of the hyphal tube. Fig. 3 also shows theoretical trajectories for this marker calculated according to three distinctly different modes of expansion for a 3-D hyphoid: isometric (I), orthogonal (O) and rotational (R). Remarkably, the carbon particle precisely followed the orthogonal trajectory, which differs substantially from the trajectories predicted for isometric or rotational expansion.

In all 18 hyphae analyzed, marker particles followed an orthogonal trajectory regardless of the exact point at which they became attached to the growing tip. Fig. 4 shows the trajectories of four different carbon particles that collided with the apical dome of the same hypha at different points. In all four instances, the markers followed the trajectory predicted for orthogonal displacement; i.e., the displace-

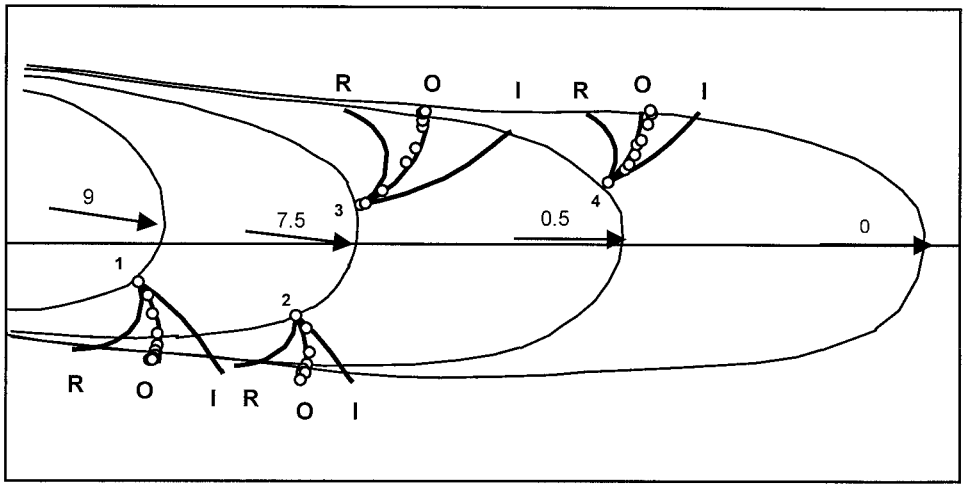

FIGURE 4 Analysis of the displacement of four surface markers during growth of a hypha of *R. solani*. Growth was followed for 219 s and each marker mapped at variable intervals after becoming attached to the hypha. Markers 2 and 3 correspond to those shown in Fig. 2, *B* and *C*. Marker 1 was attached at 0 s, markers 2 and 3 at 58 s, and marker 4 at 136 s. Theoretical curves for the displacement of each marker were calculated for three different modes of cell expansion (I, isometric; O, orthogonal; and R, rotational hyphoid). During this elongation sequence, there were some minor changes in the growth axis (*arrows*) that necessitated realigning the axis of the hyphal profile with the *x* axis (see Methods). The arrows, and values above them, show the actual growth axis for each cell profile and the angle relative to the *x* axis.

ment was always perpendicular to the expanding curved surface.

Nonmedian profile markers

Although median profile markers were the markers of choice for analysis because they were easier to interpret, we also recorded sequences in which the markers were not attached exactly at the median plane of a hypha and moved onto the upper surface of the hyphal tip. The best example was a cluster of particles that attached near the apical pole of a hypha (Fig. 5). Initially unresolved, the cluster separated into its three components as the apex expanded. Because the markers did not remain on the same focal plane, the analysis of their trajectory was more complex than that for median profile markers and involved calculations in the third coordinate (z). To better visualize the relative motion of the markers in 3-D, at each mapping time the three particles are shown linked into a triangular array (Fig. 6 *A*). This imaginary triangle expanded and turned as the cell grew, an indication that each marker moved independently of the others. Based on the final position of the three markers, calculations were made to predict the position of the triangles back to the start of the sequence. Displacements were calculated for the three types of expansion. There was good correspondence between the carbon particle markers and the triangles predicted for orthogonal expansion (Fig. 6 *B*), but not for triangles calculated to undergo isometric or rotational expansion (not shown).

Internal markers

In growing hyphal tips of *R. solani*, a useful natural marker for surface expansion was discovered. Occasionally, on the inner surface of the apical dome wall, a small phase-dark spot first appeared at the edge of the vesicle cluster of the Spitzenkörper and then gradually separated from the Spitzenkörper as the cell elongated (López-Franco, unpublished). The spots remained a stable feature of the cell wall. During the 2-min growth period shown in Fig. 7, four such spots appeared successively on the lower half of the cell, and a fifth appeared in the same manner but at the top of the cell. The nature of these spots or irregularities at the inner surface of the growing wall is not known, but they behaved as if they were an integral part of the expanding wall, and were thus considered useful internal markers of cell growth.

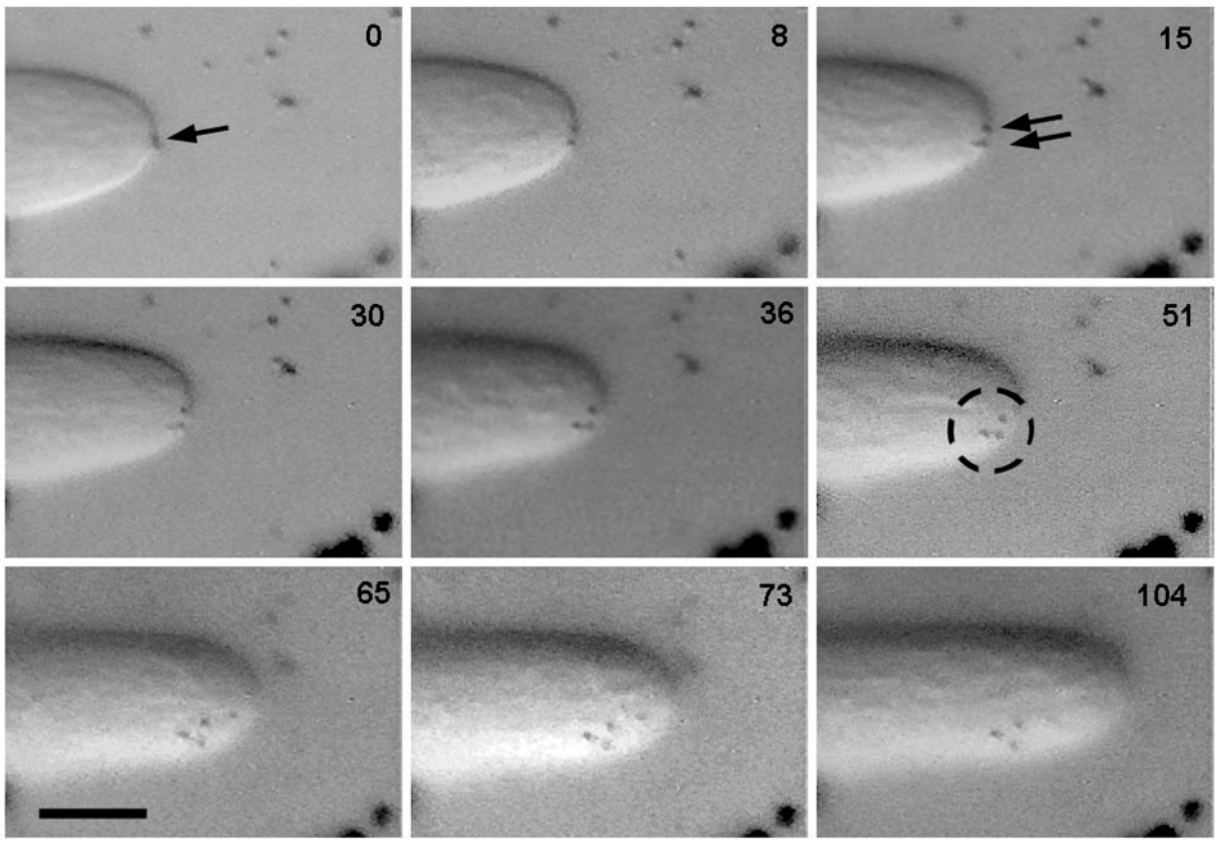

FIGURE 5 Movement and dispersal of a cluster of carbon particles attached to the hyphal surface of *R. solani*. A small unresolved cluster of carbon particles became attached to the advancing tip (*arrow*). As growth continued, the cluster started to separate (*double arrow*) until it dispersed cleanly into three individual particles (*dashed circle*). The trajectories of these markers are shown and analyzed in Fig. 6. Numbers indicate time in seconds. Bar, 5 μm

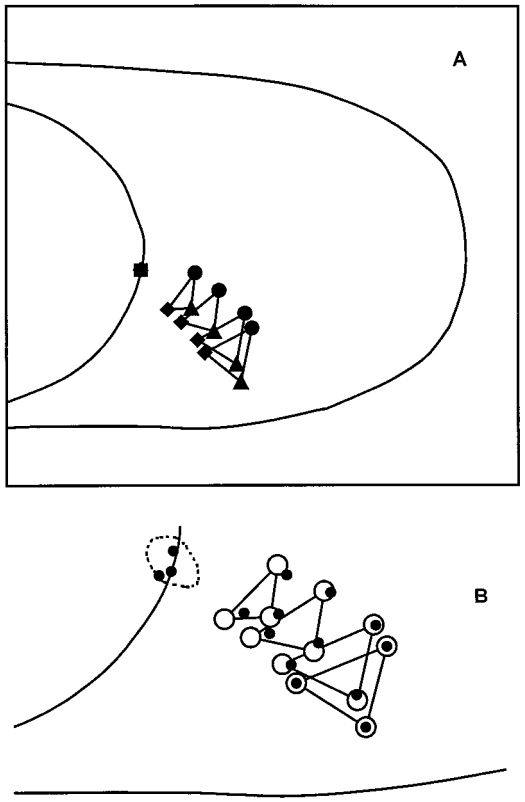

FIGURE 6 Analysis of the displacement and separation of the cluster of three marker particles shown in Fig. 5. The position of the particles was mapped at 0, 23, 36, 65, and 104 s. (*A*) The two cell profiles are at 0 and 104 s. Each particle is shown with a different symbol (▲, ♦, and ●). The triangular arrays connect the three particles at each mapping time. (*B*) Comparison of actual versus theoretical positions. The circles represent the actual positions of the markers in *A*. The black dots are the positions calculated from orthogonal displacement. At time 0, the exact position of the individual particles in the cluster could not be resolved; therefore, trajectories were calculated backward starting with the last positions.

When the trajectories of these internal markers were mapped and plotted (Fig. 8), they too closely followed the trajectories predicted for orthogonal displacement.

DISCUSSION

Orthogonal pattern of wall expansion

The mapped trajectories of external and internal markers make it clear that the cell surface in growing tips of *R. solani* expands orthogonally. Thus, anywhere in the growing region of a hyphal tip, surface expansion occurs perpendicular to the existing surface. This is true at or near the apical pole where expansion is maximal as well as in the more cylindrical portions of the hypha where expansion is minimal. Our findings provide the first direct evidence of orthogonal expansion in hyphal tips and rule out other plausible modes of wall expansion, namely, isometric and rotational (see Introduction). Orthogonal expansion is not a new concept. Following the work of Schwendener (1881) on various plant systems, Reinhardt (1892), with much intuition but little experimental evidence, used orthogonal projections to illustrate how the tip of a fungal hypha may expand.

Surface marker methodology

By dusting *Phycomyces* sporangiophores with starch grains, Castle (1958) pioneered the quantitative analysis of growth patterns in fungi. The photographic images, taken at 15-min intervals, clearly defined the topography of the apical growth zone of the sporangiophore. Other external markers, such as carbon particles (Grove et al., 1970) and polylysine-coated beads (Staebell and Soll, 1985; Merson-Davies and Odds, 1992) have been successfully employed to map growth zones of fungal hyphae. In the present study, we have extended the potential of this methodology by introducing computer-enhanced videomicroscopy to record images and image analysis to make measurements. This has made it possible to follow with high precision the trajectory of both artificial and natural markers of the cell surface. Displacements could be measured with an accuracy of ~2 pixels (0.1 μm) and at intervals as brief as 1/30 s.

Reliability of surface markers

The reliability of carbon particles from India ink as markers of cell expansion was an early concern in this study. Although the nature of the bond between the carbon particle and the cell surface is not known, the following observations support the conclusion that these markers were firmly attached and were thus bona fide landmarks for mapping cell surface expansion:

1) There was no evidence of erratic marker movement. In all of the 18 hyphal tips analyzed, the attached carbon particles followed orthogonal trajectories very closely. On rare occasions, during the initial encounter with the fungus, the particles moved or rolled erratically for a short distance before they became firmly attached.

2) The dispersal of the initial cluster of three carbon particles (Fig. 5) demonstrates that the bonding of each particle to the cell surface was stronger than the bonding of the particles to one another.

3) The fact that the internal markers also exhibited an orthogonal trajectory provides strong support for concluding that carbon particles are reliable markers of cell wall growth.

4) Because hyphal walls are often coated with an outer sheath (sometimes referred as an extracellular matrix), the possibility existed that the trajectories observed for the external carbon markers represented the expansion pattern of an outer sheath and not the underlying cell wall. How-

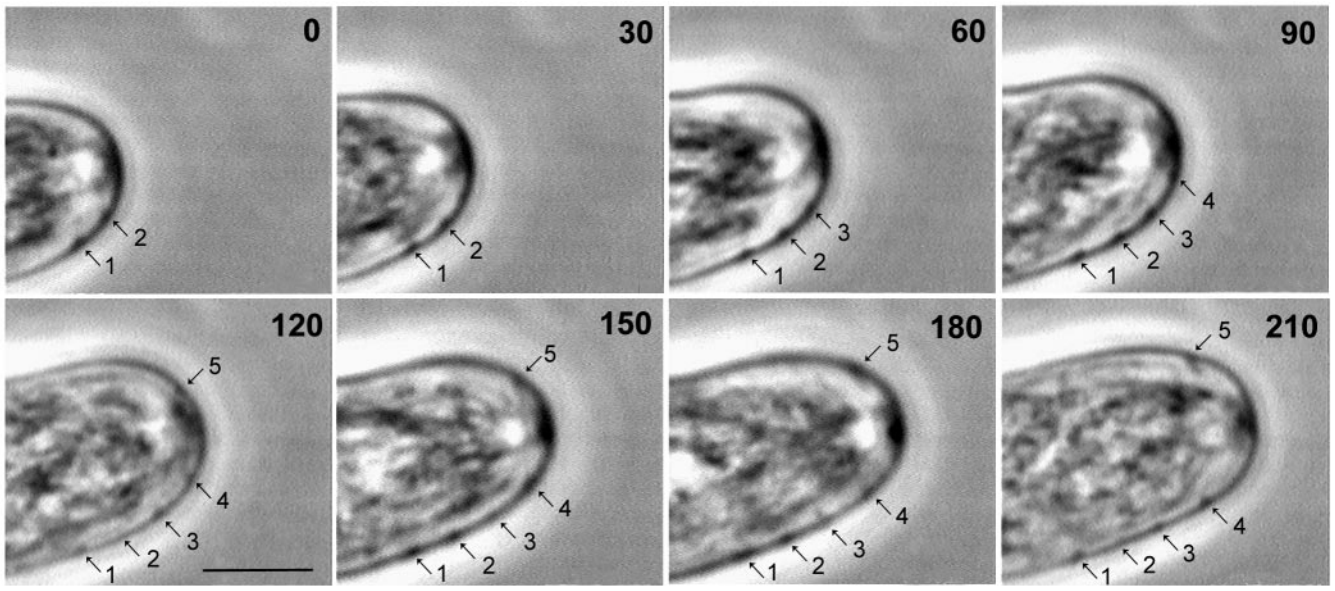

FIGURE 7 Development and migration of internal (cell wall) surface markers in a hyphal tip of *R. solani*. Arrows follow the movement of five different cell wall irregularities. Bold numbers indicate time in seconds. Bar, 5 μm.

ever, the finding that internal markers also moved orthogonally discounted this possibility. Clearly, if an outer sheath is present on the hyphae of *R. solani*, it expands as an integral part of the cell wall.

Turgor drives wall expansion

The finding that the cell wall of a growing hypha expands in an orthogonal pattern provides strong evidence to support the conclusion that turgor pressure is the physical force that drives cell wall expansion. We know of no other agent within a fungal hypha capable of providing an internal force oriented perpendicular to the cell surface over the entire growing region.

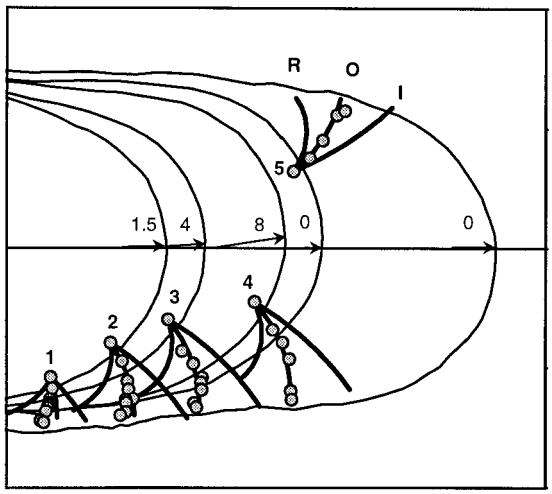

FIGURE 8 Analysis of the trajectories of the five internal markers of *R. solani* shown in Fig. 7. Each marker was mapped at 30-s intervals. Theoretical curves for the displacement of each marker were calculated for three different modes of cell expansion (I, isometric; O, orthogonal; and R, rotational hyphoid). The arrows show the growth axis for each particular profile. The values above each arrow are the angles of the growth axis relative to the *x* axis.

The role of turgor in the expansion of walled cells of both plant and fungi has long been debated (Reinhardt, 1892; Ray et al., 1972; Ortega et al., 1988; Cosgrove, 1987; Money, 1997). Our present findings provide experimental evidence against recent speculations proposing that the cytoskeleton, rather than turgor, supplies the driving force for hyphal tip expansion (Money, 1997; Heath and Steinberg, 1999). First, any pushing force (directed toward the outer surface of the cell) generated by elements of the cytoskeleton must be minuscule (Money and Harold, 1993) and therefore insignificant compared with the normal high turgor pressure of the fungal cytoplasm (Adebayo et al., 1971; Luard and Griffin, 1981; Eamus and Jennings, 1986; Money, 1994). Second, to provide a force for orthogonal cell expansion, cytoskeletal components would have to be deployed over the entire growing region in a cortical arrangement similar to the one diagrammed in Fig. 9. But there is no evidence to support such deployment in the literature on fungal biology.

Although Reinhardt (1892) invoked an orthogonal expansion pattern for the growing fungal apex, he did not favor the view that turgor played a role in expansion. He based this conclusion on the observation that hyphal tips burst not at the apical dome but behind it, in the subapex. He argued that if turgor were the cause of expansion it should have its greatest impact in regions of maximal growth, i.e., the apical dome. However, this purely physical interpretation of

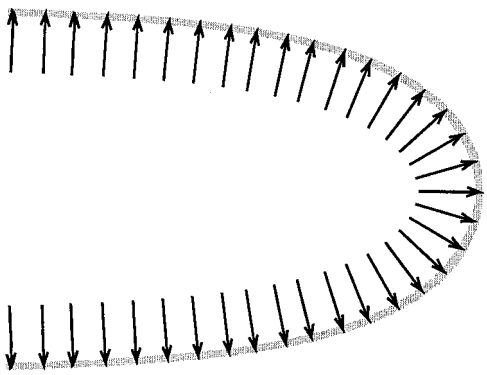

FIGURE 9 Schematic representation of the required deployment of forces needed to expand the cell wall of a hypha in an orthogonal pattern.

the bursting process led Reinhardt to an erroneous conclusion. As shown later (Bartnicki-Garcia and Lippman, 1972), hyphal tip bursting is caused primarily by a chemical and not a physical process. Consequently, the site of bursting does not provide evidence for or against the role of turgor pressure in wall expansion. Presumably, the shock treatments that elicit bursting disrupt the balance of wall synthesis and lysis responsible for cell wall extension (Bartnicki-Garcia, 1973). Bursting can occur anywhere in the apical region, where erratic vesicle discharge would weaken the wall beyond its breaking point.

Money (1997) argued that "turgor does not play any fundamental role in determining. . . the exquisite patterns of hyphal morphogenesis". We are in agreement that turgor is not the primary determinant of the shape of a hypha; the tubular shape is primarily established by the VSC-Spitzenkörper guided gradient of wall formation. However, our current findings indicate that turgor does have a fundamental role in determining how the wall expands in space, and this has an impact, albeit minor, in the final 3-D shape of a hypha. It is important to note that turgor does not enter in the formulation of the orthogonal VSC model (see Introduction), and beyond a minimum threshold value needed to expand the wall, the magnitude of turgor is not important.

Lack of cell rotation during tip growth

The fact that markers initially attached at the median longitudinal plane of the hyphae remained at this plane during cell growth yields another important insight into the process of tip growth, namely, that hyphae of *R. solani* do not rotate as they elongate. This is worth mentioning because in others fungal systems, notably the sporangiophore of *Phycomyces blakeesleanus*, tip rotation during the initial stage of elongation is well known (Roelofsen, 1950; Ortega et al., 1974). But sporangiophore rotation may not always occur. In *Pilobolus crystallinus* the young sporangiophore tip does not rotate (Ootaki et al., 1993). Others (Madelin et al., 1978; Trinci et al., 1979; Beever, 1980; Sherwood-Higham et al., 1994) have speculated that the spiral growth (coiling) of hyphae of several fungi may be a consequence of axial rotation of the extension zone. Our results with *R. solani* underscore the need to test these speculations experimentally with surface markers.

A 3-D orthogonal hyphoid model

For more than a century, researchers have sought to unravel the mathematical foundation for hyphal growth (Reinhardt, 1892; da Riva Ricci and Kendrick, 1972, Trinci and Saunders, 1977; Koch, 1982, 1994; Prosser, 1979, 1994; Heath and van Rensburg, 1996). The quest for a mathematical description of form development in a fungal hypha goes beyond being a challenging exercise in mathematical biology. By reducing the complexity of a hypha to its minimal expression, it was possible to deduce a probable mechanism for its morphogenesis (Bartnicki-Garcia et al., 1989). Accordingly, our original 2-D VSC model of fungal morphogenesis explained the generation of the gradient of exocytosis responsible for hyphal tip growth by invoking a VSC moving along a straight line while releasing wall-building vesicles in all directions. This feature has also been the basis for the development of a 3-D model. But in three dimensions, the action of the VSC alone cannot explain hyphal morphogenesis. It is also necessary to know precisely the spatial pattern of displacement of the expanding cell surface generated by the polarized gradient of exocytosis. This conclusion highlights the importance of surface expansion patterns in cell wall morphogenesis advocated by Green (1969). The evidence presented here showing that the cell surface expands in an orthogonal pattern is crucial information for constructing a realistic 3-D model of hyphal growth and morphogenesis. Accordingly, the proposed 3-D mathematical model of a hypha (Gierz and Bartnicki-Garcia, submitted for publication; see also http://boyce3427.ucr.edu/the_3d_model.htm) invokes an interplay between two key cellular ingredients: 1) the cytoskeletal elements that presumably displace the VSC and create a gradient of wall-building vesicles and 2) the turgor pressure that provides the physical force needed to expand the cell wall. The former generates the basic 3-D shape of a hypha; the latter modulates it.

The work described here required a combination of diverse skills and assignments. Authorship credit is given in alphabetical order. SBG coordinated the project and handled its publication. CEB supervised the videomicroscopy aspects. GG devised the mathematical solutions to interpret marker movement. RLF was responsible for the work on internal markers. HSL was in charge of the work on external markers. We thank Eleanor Lippman for assistance with data collection and processing.

Supported in part by grants from the National Institutes of Health (GM-48257) and the National Science Foundation (IBN-9204541 and IBN-9204628).

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