FULL PAPER

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Effect of nutrient availability on hyphal maturation and topographical sensing in *Aspergillus niger*

Abstract Topographical sensing (thigmotropism) is an essential component of efficient fungal growth. It is an important element in the complex pathway of sensory and mechanical elements that drive and control the growing hyphal tip, a fuller understanding of which will bring the mycological community a step closer to complete comprehension of the hyphal growth mode. Previous work has led us to hypothesize that the stress induced by nutrient deficiency causes structural changes in the hyphal tip that induces a thigmotropic response in *Aspergillus niger*, a soil fungus that does not display thigmotropism under normal conditions. In this study, we have sought to identify some of the factors that influence this induction of thigmotropism using a novel combination of microengineered substrates and imaging and analysis techniques to quantify thigmotropic behavior in complex hyphal systems. We have shown that the sensitivity of fungal contour sensing appears to be directly linked to nutrient availability and hypothesize that this may be caused by a stress-induced flattening of the tip and increased immaturity of the hyphal apex.

Key words *Aspergillus niger* · Hyphal growth · Hyphal tip · Nutrient availability · Thigmotropism

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Introduction

The growth of fungal hyphae is an extraordinary process. It requires a complex balancing of internal pressure, cell wall flexibility, and cytoskeletal support to allow the extension of the hyphal tip without compromising the integrity of the fungal cell. One of the defining characteristics of fungal growth is its unidirectional nature. In contrast to spherical bacterial cells, which may theoretically extend in any direction, hyphal growth is extremely polar, with the vast majority of cell wall deposition localized within a few micrometers of the growing tip (Sietsma and Wessels 1994; Harold 2002; Bartnicki-García 2003). This polarized extension is driven by internal hydrostatic pressure (turgor), which may apply a force equivalent to several atmospheres against the surrounding cell wall. However, the fact that dramatic variations in turgor pressure can have little or no effect on fungal growth rate (Harold et al. 1996) means that the exact function of turgor pressure is still uncertain. It is thought that local regulation of the cell wall and cytoskeletal properties control both hyphal growth rate and orientation (Money and Harold 1992; Money and Hill 1997). Actin filaments in particular have been shown to fulfil a major role in tip extension and support, as numerous studies have reported hyphal growth defects in response to anti-actin drugs (Geitmann and Emons 2000; Heath et al. 2000). The microtubular component of the cytoskeleton plays a less well-defined role in tip support because growth is able to continue despite microtubule depolymerization, although tip structure is often distorted (Heath et al. 2000; Horio and Oakley 2005).

This cytoskeletal scaffold supports the continual deposition of cell wall and membrane, forming building blocks at the apex of the hypha (Gooday 1994; Bartnicki-Garcia et al. 2000). This region is extremely fluid because of the presence of these immature, non-cross-linked polymer chains, although as maturation progresses, covalent bonds form between the β-1,3-glucan and chitin polymers to cross-link and rigidify the cell wall, thus strengthening it against turgor pressure (Ma et al. 2005). This action creates a solid

base at the foot of the apical dome and allows mature hyphae to act as stable conduits for nutrient transfer to the mechanically unstable growing tips.

Although it is important to understand the mechanisms behind hyphal tip extension, it is also crucial to investigate the way in which external stimuli influence these systems. Detailed information on the perception of extracellular influences and their exact effect on hyphal growth is relatively scarce. However, several factors have been identified that influence the guidance of the fungal hypha, including electrical fields (galvanotropism) (Lever et al. 1994; Gow 2004), chemical factors (chemotropism) (Fomina et al. 2000; Sbrana and Giovannetti 2005), and topographical sensing (thigmotropism) (Watts et al. 1998; Apoga et al. 2004). Thigmotropic reactions are thought to be important in human and plant pathogenesis and have been studied in rusts, cereal pathogens, and *Candida albicans* (Perera et al. 1997; Watts et al. 1998; Tucker and Talbot 2001; Jaffe et al. 2002; Apoga et al. 2004). Nevertheless, it can be of little doubt that topographical sensing is important in more general aspects of growth in addition to these specialized cases. Current theories regarding topographical sensing mechanisms center on stretch-activated calcium channels that are located in the fungal cell membrane and react to its deformation (Watts et al. 1998; Shaw and Hoch 2000; Silverman-Gavrila and Lew 2002). The positioning and specific mode of action of these Ca^{2+} channels, however, is the subject of debate.

We believe an understanding of the mechanisms behind fungal growth and growth control will provide valuable information on both hyphal structure and development. We have created a system to examine the complex thigmotropic reactions of the ubiquitous fungus *Aspergillus niger* using a combination of microscopy and microengineering. We have investigated the effect of nutritional stress on topographical sensing by *A. niger* and have provided some novel conclusions on the significance of nutrient availability to both thigmotropic sensing and hyphal tip extension.

Materials and methods

Channel slide preparation

Clean quartz microscope slides (38 mm × 26 mm) were spin coated with S1813 photoresist at 25 g for 4 s and then 900 g for 20 s to ensure a uniform thickness. The slides were then placed on a hotplate at 90°C for 10 min to harden the resist before exposure. Samples were then irradiated for 3 s using UV light (365 nm) through a mask patterned with 20-μm lines that were aligned with the short side of the slide. The resist was developed using CD26 developer for 15 s, washed with deionized water, dried using nitrogen, and then postbaked on a hotplate for 20 min at 120°C. Using the patterned resist as a mask, the slides were placed in buffered HF etch for 20 min. After removal, the remaining resist was stripped using acetone. The slides were finally washed and dried as before to produce the structure seen in Fig. 1A.

Fungal growth analysis

A channel slide was placed on top of four plain microscope slides to allow sufficient elevation for a reasonable depth of agar (~1 cm) around them. Slides had previously been washed in detergent overnight, autoclaved, and dried in an oven at 100°C for 24 h to sterilize them and remove any moisture from their surfaces. The stack of slides was then placed in a Petri dish and surrounded with malt extract agar up to the level of the top slide (Fig. 1B). No agar was allowed onto the channel slide to ensure effective separation of thigmotropic reactions and chemotropic responses during the experiment. The dishes were then inoculated with *Aspergillus niger* van Tieghem (ATCC 201373) at sites surrounding the channel slide at a distance of ~1 cm from the edge of the slide and incubated at 27°C for 3 days. Control experiments used an identical setup but substituted the channel slide for a blank microscope slide.
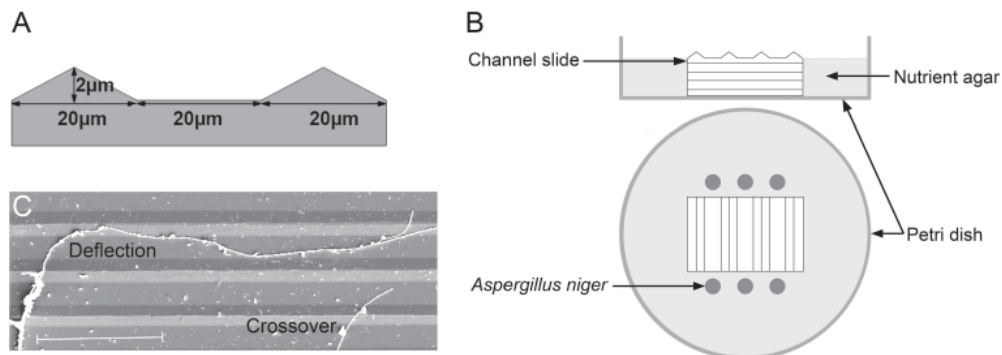

Fig. 1. Experimental design. The microengineered substrate was created by etching a repeating pattern of 20-μm channels with edges angled at 11.54° into a quartz matrix (**A**). These slides were placed into a Petri dish on top of four plain microscope slides to allow a sufficient depth of agar. Nutrient agar was added up to the level of the top slide but not allowed to encroach onto it to preserve a separation of thigmotropic and chemotropic reactions. *Aspergillus niger* was inoculated around the edges of the slide and allowed to grow for 5 days before analysis (**B**). Two parameters (crossovers and deflections) were used to estimate the levels of thigmotropic behavior (**C**)

Time-course experiments

The microcosms were set up as already described and inoculated with *A. niger*. Two different areas of colony development were observed over a 17-h period, both at the interface between the agar and the channel slide (high nutrients) and further onto the slide (3.5 mm), where lower levels of nutrients would be available.

Spore analysis

Analysis of the thigmotropic responses of *A. niger* spore germination was carried out using a channel slide placed on top of four plain microscope slides that were sterilized as previously. The slides were placed in a Petri dish and surrounded by sterile cotton wool soaked in autoclaved ddH$_2$O to provide a moist atmosphere. A solution of spores in sterile ddH$_2$O was then inoculated on top of the channel slide, and the Petri dishes were placed in an incubator at 27°C for 12 h before visualization.

Imaging and image analysis

Time lapse and spore growth imaging was carried out using a Leica DMLB microscope and a Nikon Coolpix 4500 digital camera. Time-lapse photography also utilized a Nikon MC-EU1 remote unit that obtained images every 10 min over a 17-h time period. All images were processed using the ImageJ analysis software package (available at http://rsb.info.nih.gov/ij/). Imaging for the other experiments was carried out using a Leica DM 4000 M microscope and the "Capture" image capture package: $300 \times 300\,\mu m$ sections on the outer edge of the channel slide were chosen at random, and images were taken on a direct line inward up to the tip of the growing mycelia. Pictures taken were analyzed using ImageJ image analysis software and a specially designed Excel spreadsheet. In all cases, levels of thigmotropic growth were quantified using two simple, but important, parameters (Fig. 1C):

1. Hyphal deflections away from ridges (thigmotropic behavior) were classed as a hyphal movement during which the tip entered onto a ridge, did not pass the halfway point, and then turned to emerge from the same side of the ridge (Fig. 1C).
2. Hyphae crossing over ridges without deflection (no thigmotropism).

The percentage of hyphae that deflected on contact with a ridge provided an approximation of the degree of contour-following behavior exhibited in any given area of the fungal colony. This percentage was calculated as follows: *% deflection = (number of hyphal deflections / total number of interactions) × 100%.*

Scanning electron microscopy

Samples were grown on microengineered slides for 3 days as previously described. The channel slides were then carefully removed from the agar using a sterile scalpel and forceps. The channel slides with fungal biomass attached were air-dried for 48 h, coated with 30-nm Au/Pd using a Cressington 208 HR sputter coater, and examined using a Philips XL30 environmental scanning electron microscope (ESEM).

Cryo-scanning electron microscopy

Small drops of malt extract agar were positioned in the center of glass coverslips cut to 0.25 cm^2. The drops were inoculated with *A. niger* spores, and these were allowed to grow overnight in a humid environment until the hyphal tips were protruding onto the glass surface. The glass squares were then positioned in the jaws of a sample holder. Liquid nitrogen was placed under vacuum in a freezing chamber (Alto2500) until it became semisolid (slush). The chamber was then brought back to atmospheric pressure, allowing the sample to be plunged into the chamber and the vacuum immediately reapplied. The sample was withdrawn from the chamber just before the nitrogen resolidified and transferred under vacuum to the preparation chamber. The sample was warmed to −95°C for 5 min to remove the surface water. After sublimation, the sample was cooled to −115°C before coating with approximately 5-nm Au/Pd. Samples were examined using a Philips XL30 environmental scanning electron microscope (ESEM).

Results and discussion

Variation in the intensity of thigmotropic response with nutrient availability

Previously we have observed that the level of thigmotropic response appeared to increase as the fungal colony grew further from the nutrient source onto the channel slide. Figure 2A demonstrates this phenomenon by showing that the percentage of hyphae that reorientated their growth on contact with a channel wall showed a general increase with increasing distance from the slide edge (nutrient source). This observation established that the sensitivity of fungal topographical sensing was not constant as the leading edge of the colony moved away from a nutrient source.

There are two possible explanations for this variation in the level of topographical response. First, this increase in the level of sensitivity to topography may be caused by an increase in hyphal stress as it moves away from its sole nutrient source. Although it is difficult to make detailed predictions about the specific mechanism from these data alone, it seems feasible that decreasing nutrient concentrations could influence some mechanical properties of the hyphal structure (a topic discussed later in this article) or the signaling processes that interpret topographical information.

A second, equally plausible, theory is that the shift in thigmotropic levels may simply be the result of advancing, heterogeneous colony development. Exploratory hyphae
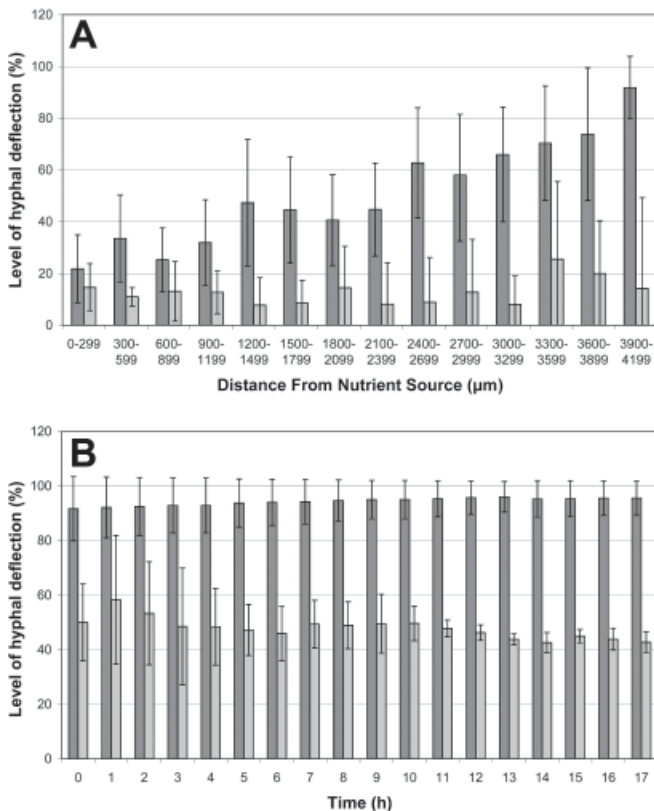
Fig. 2. Variation in thigmotropic response with distance from nutrient source. As the fungus penetrated onto the channel slide, the levels of thigmotropic response increased from approximately 20% to almost 100% over a distance of 4.2 mm. The *dark-shaded bars* show measurements from growth on slides etched with 20-µm channels; the *light-shaded bars* illustrate growth on unetched slides as a control (**A**). The experiment was repeated three times, and a total of 11 sections were examined, representing the analysis of 3504 hypha–ridge interactions. A time-course investigation of these observations revealed that there were significant spatial differences in the thigmotropic response, but no significant variations over time (**B**). The *dark-shaded bars* are time-course measurements taken 3.5 mm from the nutrient source; the data shown by the *light-shaded bars* were collected 0.3 mm from the nutrient source. The experiment was repeated three times for each of the two different areas. *Error bars* for both graphs indicate standard deviations

might possess a higher sensitivity to topography to enable energy-efficient exploration, but once an area has been colonized secondary hyphae with a lower sensitivity to contours could act to form a hyphal network to allow more efficient nutrient transport. Therefore, the apparent spatial variation in thigmotropism may in fact be a temporal difference as this secondary colonization has yet to occur at the leading edge.

Time-course investigation of thigmotropic fluctuations

To determine which of these mechanisms occurred in *A. niger*, we observed hyphal growth on the microengineered substrates over a period of 17 h. Two different areas were examined, and the levels of thigmotropic behavior were quantified over time (Fig. 2B). The first zone was at the outer edge of the slide where nutrients were readily avail-
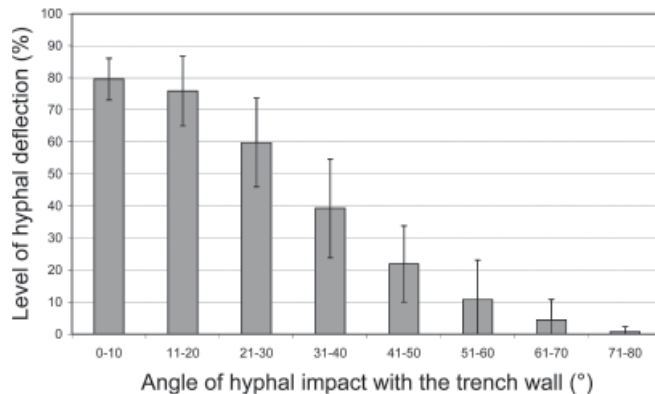

Fig. 3. Variation in thigmotropic response with changing angles of impact. The strength of the reorientation response also varied depending on the angle of hyphal contact with the ridges. At angles of interaction greater than 60°, the response was essentially zero, but as the contact angle decreased, the levels of reorientation increased to more than 80% at angles less than 10°. *Error bars* represent standard deviations

able. The second sector (3.5 mm from the slide edge) was isolated from the nutrient source, meaning that the fungus would be under increased stress from the lack of available resources. We found that although there were significant differences between the thigmotropic sensitivity of the inner and outer areas of the slide, there was no significant change over time. This observation suggested that nutrient availability was the main cause of the variations in thigmotropic behavior. If the fluctuations were an artifact of advancing colony development, then levels of thigmotropism would be expected to decrease over a given time period as the exploratory hyphae branch to produce a more dense network. We did find some examples of this occurring during the course of our observations, but it appeared that the thigmotropic variations we observed were more prominently caused by nutrient deprivation.

Variation in thigmotropic response with angle of interaction

Previous work by Watts et al. (1998) and our own experimental work has demonstrated a variation in the levels of thigmotropic sensitivity based on the angle at which the hyphae impact the channel wall. It was observed that the sensitivity of the thigmotropic response fell as the angle of interaction increased (Fig. 3). Contour-sensing responses are believed to be controlled by stretch-activated calcium channels in the cell membrane that are stimulated by the deformation of the cell wall (Money and Harold 1992; Silverman-Gavrila and Lew 2002). This deformation signal is transferred to the calcium channels via Hectian strands (Bachewich and Heath 1997) that connect the cell wall and plasma membrane. The integrity of the cell wall, cell membrane, and the connections between them are therefore extremely important for the transformation of topographical information into a growth response via these calcium channels. We hypothesize that the immature wall and membrane material continually deposited at the hyphal apex

would be too fluid to efficiently transmit stretch responses and that the plasma membrane–cell wall links might not be fully formed. A previous study (Bachewich and Heath 1997) found varying membrane–cell wall adhesion patterns throughout the hyphal tip with broad, irregular connections at the apex, regular and continuous distribution throughout the transition zone, and an infrequent set of connections in the mature regions of the hypha. This finding suggests that the transition zone has the optimum level of cell wall connections while the apex and mature trunk of the hypha have lower levels of links. Also, it appears possible that the calcium channels themselves might not be present, or active, in the areas where the cell membrane is under construction. Theoretically, therefore, the least sensitive areas of the hyphal tip are at the apex in the most immature region and in the trunk of the mature hypha where the cell wall is fully developed, inflexible, and infrequently linked to the cytoplasm; this would explain the decrease in thigmotropic sensitivity at increasing angles of impact. At the maximum angle of impact (90°), the area of contact with the obstacle would be at its closest point to the tip. As the angle of impact decreases, the point of contact moves further back from the hyphal apex into the maturing area of the tip.

Using cryogenic scanning electron microscopy (cryo-SEM), we visualized the apical profiles of *A. niger* hyphae grown under conditions of nutrient stress (Fig. 4A,B). We observed that the hyphal tip appeared flattened from the side, a morphology that is very similar to that shown by plant pathogenic fungi in their thigmotropic growth phase. This morphology is thought to allow close contact between the substrate and the topography-sensing mechanisms in the tip, making it essential for efficient thigmotropism. The flattened tip shape of stressed *A. niger* hyphae is significantly different from that displayed under high nutrient conditions (Fig. 4C) when no thigmotropism seems to take place. These tips are twice the diameter of those under stress and exhibit a gently rounded apex. When viewed from above, it became apparent that the cause of the flattened profile was a ridge of material around the base of the hypha (Fig. 4D). The response of the *A. niger* cell wall to chemical stress has been investigated (Ram 2004), and it was found that the fungus responded by increasing the levels of chitin in the cell wall to maintain the integrity of the hyphae. This excess deposition of cell wall material, combined with a deflation of the hyphal body, would explain the novel morphology we have observed.

Effect of nutrient levels on the efficiency of thigmotropic sensing

To investigate the effect of varying nutrient levels on the thigmotropic sensing mechanisms, we monitored the germination and growth of spores on channel slides under nutrient-free conditions. Figure 5A shows a comparison between the thigmotropic response during spore germination and normal hyphal growth under high nutrient conditions. It seems that low nutrient levels serve to exaggerate the pattern of angular variation as the responses from 0° to 40° were all increased compared to those grown under high nutrient conditions. However, above 40°, the thigmotropic response of the germinating spores fell to zero. The effects of nutrient levels were also seen by analyzing data taken from fungi grown under normal experimental conditions on high nutrient agar. As the hyphae penetrated further onto the nutrient-free surface of the channel slide, a change in

Fig. 4. Hyphal tip morphology. Hyphal tip morphology was observed under conditions of nutrient stress using cryo-scanning electron microscopy (cryo-SEM). Hyphae were examined in profile with both gently sloped (**A**) and "micro-tip" (**B**) morphologies in evidence. These stress-induced tip structures were significantly different from the larger, rounded tips found when colonies were grown under optimal nutrient conditions (**C**). When observed from above, the hyphae appeared flattened at the edges, with excess wall material appearing to form a "foot" around the base of the hypha (**D**). This foot is what appears to form the micro-tip in the hyphal tip profiles
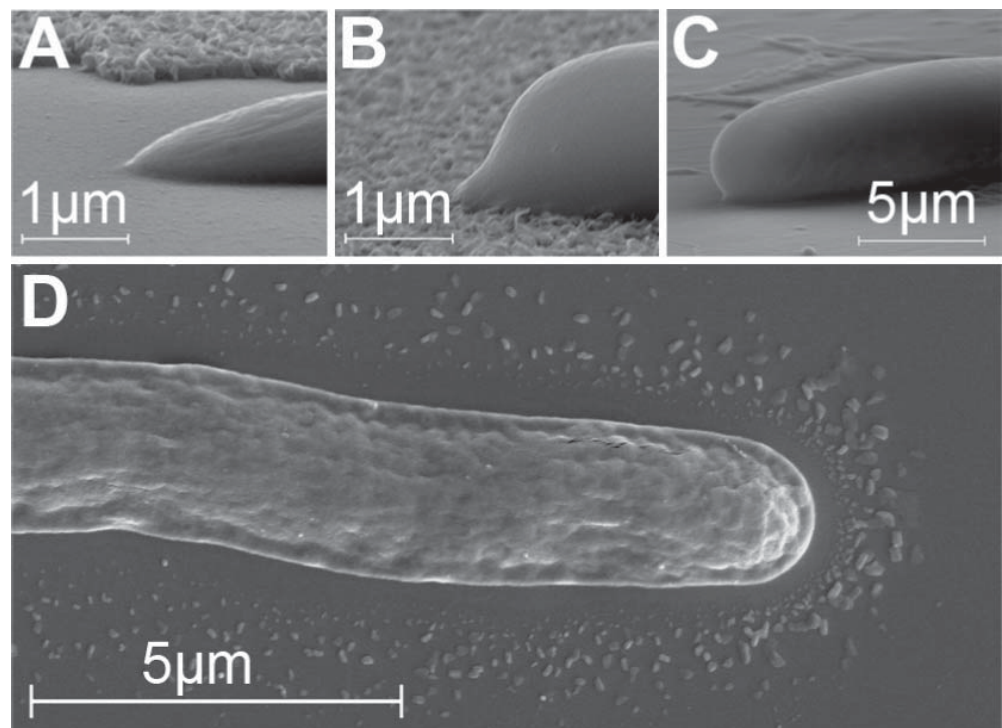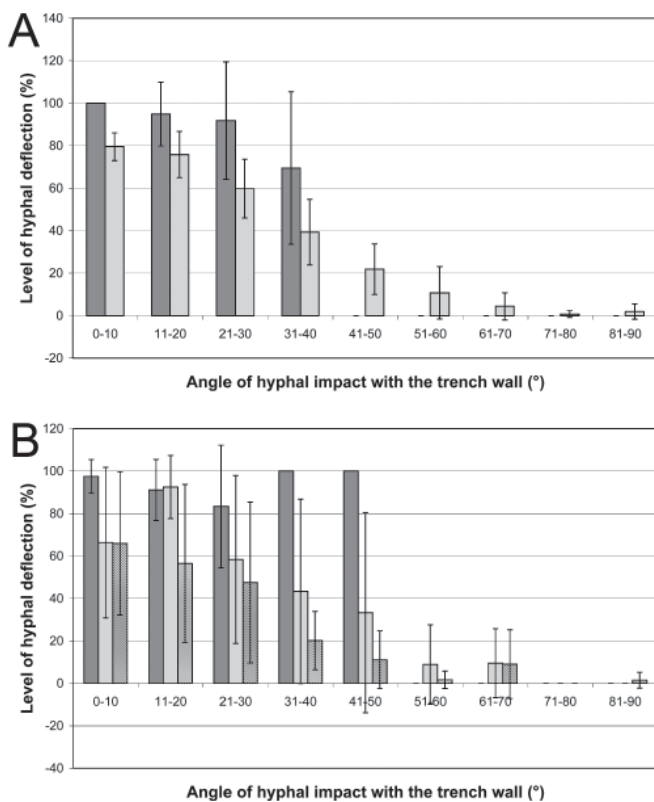
Fig. 5. Variation in thigmotropic response with nutrient availability. A comparison of spore growth in nutrient-free conditions (*dark-shaded bars*) and normal hyphal growth at high nutrient levels (*light-shaded bars*) (**A**) showed significant differences in the reaction to varying angles of hyphal interaction with the substrate. This pattern was repeated when analyzing fungal growth at increasing distances from the nutrient source (**B**), and therefore decreasing nutrient availability, although the trend is not as clear (*dark-shaded bar* = 4 mm from nutrient source; *light-shaded bar* = 1.5 mm from the nutrient source; *cross-hatched bar* = 0.3 mm from the nutrient source). *Error bars* represent standard deviations

Fig. 6. Structural changes in the A. *niger* hyphal tip both induce and inhibit topographical sensing. Our results indicate that thigmotropism is initiated in A. *niger* under conditions of nutrient stress. We believe that this is the result of the flattened morphology (when viewed in profile), which allows a greater area over which to sense topographical variations and that will amplify any response produced (**A**). We have also shown that the hyphal tip is insensitive to topological variations over a 60° (*light shading*) arc (when viewed from above) at the apex under relatively high nutrient conditions, but that this blind spot increases to an arc of ~100° (*dark shading*) as nutrient levels drop. We propose that this is because the newly deposited cell wall (*short dashed line*) is too immature, both in its lack of connections to the cell membrane and physical fluidity, to transmit thigmotropic cues. The plasma membrane (*light dashed line*), too, will be in a state of flux near the apex as vesicles from the Spitzenkörper constantly introduce new components into the membrane. The combination of these two factors would create an area in which topographical signals could not be sensed or transmitted. As nutrient levels decrease, this area would increase, as the building blocks become scarce and delay the maturation of the cell wall and membrane

the response to varying contact angles was observed (Fig. 5B). At the nutrient boundary, thigmotropic responses were lower at all angles of interaction and occurred in significant numbers up to 70°. At 1.5 mm onto the slide, the levels of thigmotropic behavior increased while the maximum angle of response remained around 70°. However, by the time the colony had penetrated 4 mm onto the slide, the maximum angle of response had reduced to 50°.

Both these sets of data show that nutrient depletion appeared to cause decreased thigmotropic sensitivity at higher angles of hyphal impact with obstacles, and this in turn suggested that the area of the "blind spot" in topographical perception caused by the immature cell wall and membrane had increased. This result supports our theory that stress-induced structural changes in the hyphal tip cause thigmotropic sensing. Previous results indicate that increases in abnormal tip structure correlate with increasing distance from the nutrient source, whereas these data show that the blind spot increases with decreasing nutrient concentrations. It seems likely that these two factors are linked and that declining nutrient levels disrupt the supply of cell wall-
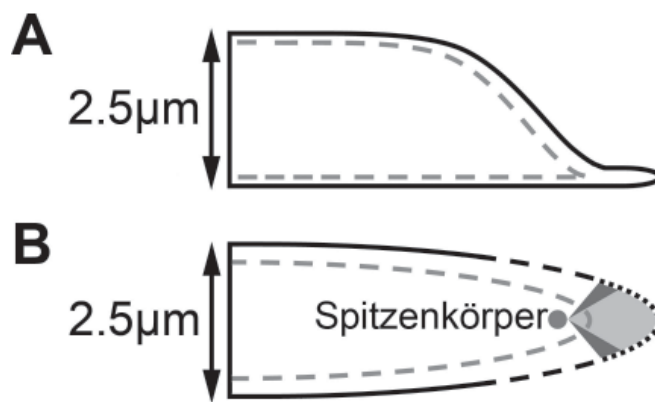
and membrane-building blocks. Thus, a larger area of the apex would be undeveloped and unlikely to be able to respond to thigmotropic signals. The combination of flattened morphology, which allows closer contact with the substrate and a wider area over which to sense topographical changes (Fig. 6A), and the increased blind spot (Fig. 6B) create a hyphal tip that is more sensitive to obstacles in regions toward the trunk of the hypha, but blind to those at the apex.

In conclusion, we have shown that the levels of thigmotropic behavior appear to vary with nutrient availability and have put forward a hypothesis to explain these observations. We believe that these data provide an important advance in understanding the interplay between environmental and physiological factors in fungal thigmotropism. The study of these poorly understood mechanisms of hyphal control could provide glimpses into the structure and development of the hyphal tip and further understanding of this fascinating area of fungal biology.

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