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Steven D. Harris
University of Nebraska-Lincoln, Steven.Harris@umanitoba.ca

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Steven D. Harris

Plant Science Initiative and Department of Plant Pathology, University of Nebraska, Lincoln, Nebraska 68588-0660

Abstract: *Fusarium graminearum* (teleomorph *Gibberella zeae*) is a significant pathogen of wheat and corn. *F. graminearum* forms multicellular macroconidia that play an important role in dissemination of the disease. The spatial pattern of morphogenesis in germinating macroconidia is described. Germ tubes preferentially emerge from the apical cells in a bipolar pattern that appears to be common to filamentous fungi. Chitin deposition occurs at two locations: the spore apices and cortical regions of macroconidial cells that subsequently produce a germ tube. The spatial pattern of morphogenesis requires the presence of functional microtubules, which may be responsible for the transport of key polarity factors to specific sites. These observations suggest that *F. graminearum* possesses a regulatory system that marks germ tube emergence sites. Perturbation of this system may represent an effective approach for inhibiting colonization of host plant surfaces.

Key words: cell polarity, *Fusarium,* microtubules, spore germination, macro conidia

INTRODUCTION

Spore germination represents a pivotal step in the colonization of new environments by filamentous fungi. Once dormancy is broken, spores undergo a defined set of morphogenetic events that lead to the formation of a polarized growth axis and the emergence of one or more germ tubes (reviewed by d'Enfert 1998 and Hardham 2001). The temporal and spatial regulation of these events typically reflects the biology and habit of a given fungus. For example germination of conidia produced by the saprophytic fungus *Aspergillus nidulans* is triggered by the presence of glucose and requires several hours for completion (Osherov and May 2000). By contrast, in the plant pathogen *Magnaporthe grisea,* conidial germination is relatively rapid and requires only the presence of water (Hamer et al 1988). In exceptional cases (i.e. *Colletotrichum gloeosporioides,* Barhoom and Sharon 2004), conidia display alternate patterns of morphogenesis depending on whether the fungus is initiating saprophytic or pathogenic growth. Although the mechanisms underlying the regulation of morphogenesis during spore germination are not well understood, it has been established that cAMP and MAP kinase signaling pathways have a general role in coordinating multiple events (reviewed by Xu 2000).

I and others have defined a series of morphogenetic landmarks associated with the germination of *A. nidulans* conidia (Harris et al 1994, Wolkow et al 1996, Harris et al 1999, Momany and Taylor 2000). These studies demonstrated that germ tube emergence and septum formation are subject to precise spatial controls and are tightly coupled to both growth and nuclear division. We have also shown that, in some cases, checkpoints enforce the temporal order of these events (Harris and Kraus 1998). In this study I sought to determine whether a similar set of landmarks could be defined during the germination of *Fusarium graminearum* (teleomorph *Gibberella zeae*) macroconidia. The study was initiated for two purposes. First, as the causative agent of Fusarium head blight (also known as wheat scab), *F. graminearum* is a plant pathogen of significant economic importance (McMullen et al 1997). The identification of landmarks associated with spore germination represents a preliminary step toward the development of tools that can be used to characterize early events in the interaction between the pathogen and its hosts. Second, it is not clear whether the landmarks identified in *A. nidulans* apply in general to other filamentous fungi. In particular, because *F. graminearum* macroconidia are complex multicellular structures, the morphogenetic patterns associated with germination may not be as simple as those observed in *A. nidulans*. However results presented here show that macroconidia display patterns of germ tube emergence and cell wall deposition that share some features in common with *A. nidulans*. These observations support the possible existence of a regulatory system that defines spatial features of the morphogenetic patterns associated with spore germination.

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[1] Corresponding author. E-mail: sharri1@unlnotes.unl.edu

MATERIALS AND METHODS

Strains and growth condition.—*Fusarium graminearum* (teleomorph *Gibberella zeae*) strain PH-1 was used throughout this study. Mycelia were grown on YMA plates (0.4% sucrose, 0.4% yeast extract, 0.4% malt extract, 1.5% agar) 3–4 d at 28 C, and macroconidia were harvested in sterile distilled water. In some cases macroconidia were prepared with liquid CMC media as previously described and recovered by centrifugation (Hou et al 2002). Macroconidia were used immediately after harvest.

For all growth experiments, macroconidia were germinated on glass cover slips arrayed on the bottom of 100 mm Petri plates (Harris et al 1994). A 20 mL suspension of macroconidia ($\sim 1 \times 10^5$/mL) in growth media was poured gently onto the cover slips, and the plates were incubated at 28 C. Growth media included YMA, YGV (2% glucose, 0.5% yeast extract and vitamins) and MNV (1% glucose, 5% nitrate salts, trace elements and vitamins). MNV-ethanol contained 1.2% ethanol in place of glucose. Phosphate-buffered saline (PBS) was used at 1%. Nitrate salts, trace elements, and vitamins were added to media as described in the appendix to Kafer (1977). Hydroxyurea (HU) was added to 20 mM from a 2 M stock solution made in sterile distilled water. Benomyl (Ben) and cytochalasin A (CA) each were added to 5 μg/mL from 1 mg/mL stock solutions made in DMSO.

Microscopy.—To monitor nuclear division and septation, cover slips with adherent macroconidia and germlings were fixed in 3.7% formaldehyde, washed in sterile distilled water and stained with Calcofluor (Fluorescent Brightener 28, Sigma) and Hoechst 33258 (Molecular Probes) as previously described (Harris et al 1994). To characterize patterns of cell wall deposition, macroconidia germinating on cover slips were incubated 5 min in growth media containing 5–10 μg/mL FITC-conjugated wheat germ agglutinin (WGA) or FITC-conjugated concanavalin A (conA). Cover slips were rinsed briefly in fresh, prewarmed growth media, then fixed and prepared for imaging. All cover slips were mounted in 10% phosphate buffer (pH 7.0), 50% glycerol, 0.1% n-propyl-gallate.

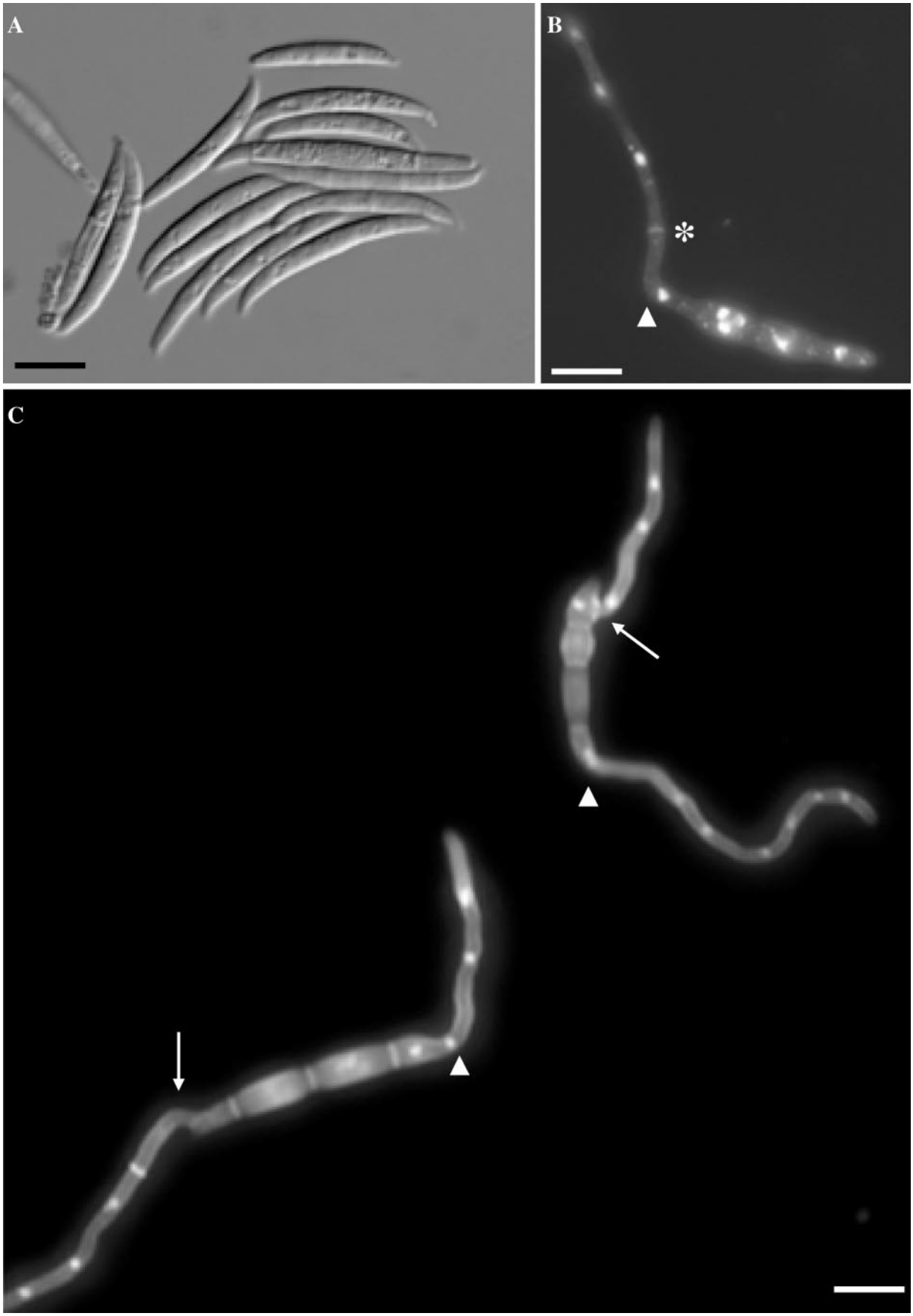
Slides were viewed with 60× or 100× objectives (Plan-Apo) of an Olympus BX51 fluorescent microscope. Images were captured with a Photometrics CoolSnap HQ CCD camera (Roper Scientific) and processed with IPLab software (Scanalytics Inc.). Images were converted from 16 to 8 bits and saved as TIF files. Additional processing was performed with Adobe PhotoShop 6.0.

RESULTS

Spatial pattern of germ tube emergence.—*F. graminearum* produces slender, elongated macroconidia that typically possess 3–5 cells demarcated by septa. The two apical cells possess obvious asymmetry (FIG. 1A), as each one gradually tapers toward a rounded end. By contrast the internal cells do not possess a definable pole (FIG. 1A) and in this context are considered symmetrically organized. Because a bipolar germination pattern has been observed in other filamentous fungi (Braun and Howard 1994, Harris et al 1999, Wendland and Philippsen 2001, Barhoom and Sharon 2004) it seemed reasonable that germ tubes preferentially would emerge from the apical cells of macroconidia. To test this idea macroconidia were allowed to germinate in rich media and the pattern of germ tube emergence was monitored. As expected there was a distinct bias toward the use of apical cells as the emergence site. In four independent experiments (n = 700 total), an average of 79.4% of germ tubes emerged from the apical cell of a macroconidium (FIG. 1B). This bias also was observed when the location of the first germ tube was scored in germinating macroconidia. In this case the first germ tube emerged from an apical cell 85.3% of the time (four experiments; n = 408 total). Notably approximately half of these germ tubes (49.3%) emerged from the extreme pole of an apical cell (FIG. 1C), whereas the remainder formed from the side of the cell (FIG. 1C). To determine whether the apical bias was affected by germination conditions, the location of the first germ tube was scored in macroconidia germinating in MNV-Ethanol, MNV with no added carbon source and 1% PBS. In each case the apical bias persisted (86, 89 and 87% of first germ tubes emerged respectively from an apical cell). However under these conditions the extreme pole of the apical cell was used more frequently than the side (80–93% of macroconidia, compared to 49.3% in rich media). Taken together these observations confirm that the apical cells of macroconidia are preferred sites for germination.

This analysis was extended to consider macroconidia that possess two germ tubes. In rich media 81.1% of macroconidia displayed a bipolar germination pattern (four experiments; n = 372 total) whereby germ tubes emerged from both apical cells (FIG. 1C). A further 15.6% of macroconidia possessed an apical germ tube and a second that emerged from an internal cell. Only rarely (3.2%) did both germ tubes originate from internals cells. The bipolar pattern also was observed to the same extent on poorer growth media (data not shown). Therefore, despite their multicellular nature, *F. graminearum* macroconidia exhibit the bipolar germination pattern characteristic of other filamentous fungi.

Distinct patterns of cell wall deposition in germinating macroconidia.—The plant lectin wheat germ agglutinin (WGA) has been used to label sites of polarized chitin deposition in filamentous fungi (Gooday 1971). To determine whether the observed germination pattern reflected an underlying asymmetry in the pattern of cell wall deposition, macroconidia ger-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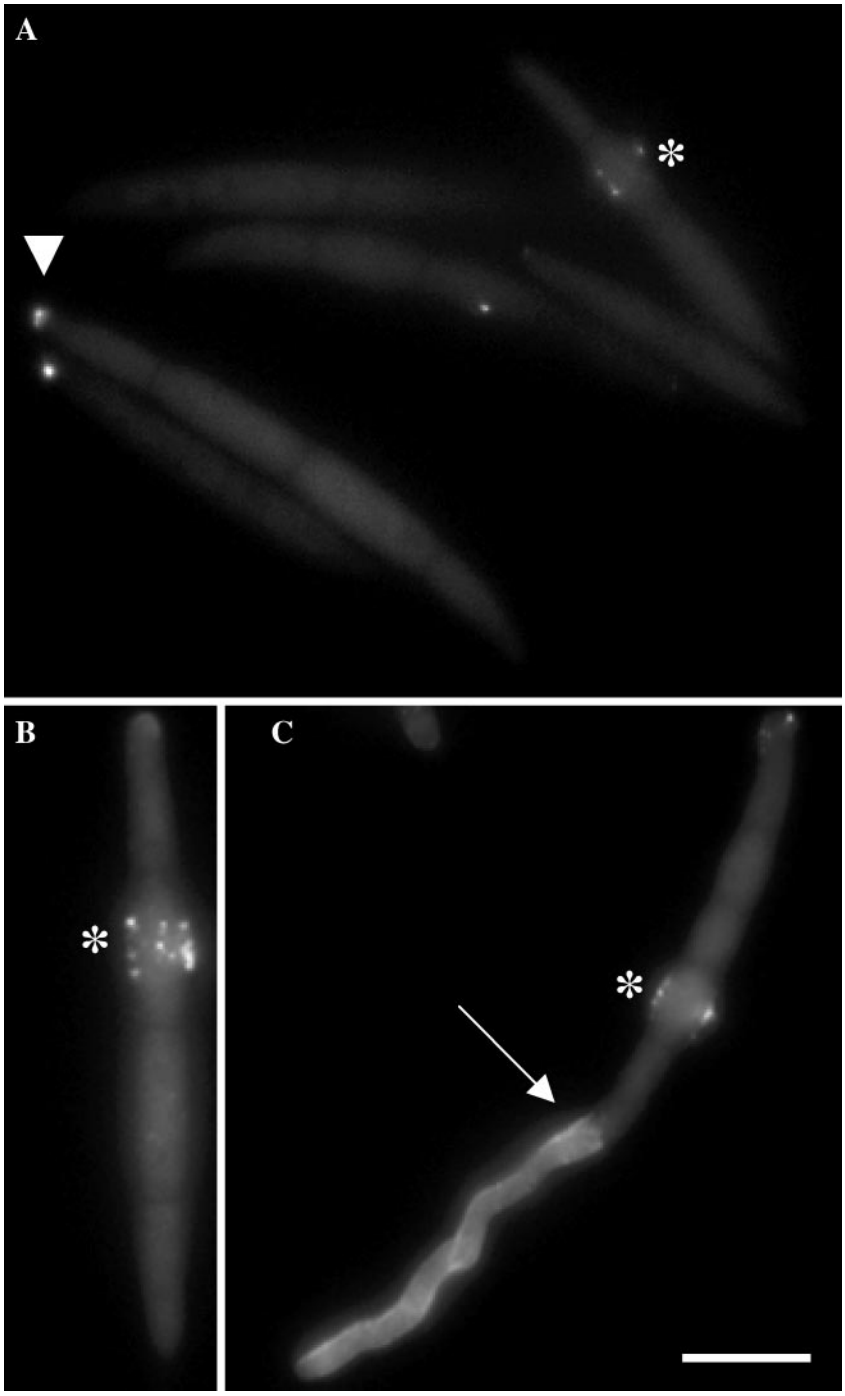

FIG. 2. Distinct patterns of chitin deposition associated with germination. Macroconidia germinating on cover slips were incubated 5e min in YMA media containing 5–10 μg/mL FITC-conjugated wheat germ agglutinin (WGA), then fixed and analyzed with fluorescence microscopy. Arrowhead, polar chitin patch. Asterisks, chitin patches on internal cells. Arrow, demarcation between chitin-rich and chitin-poor region of new germ tube. Bar = 10 μm.

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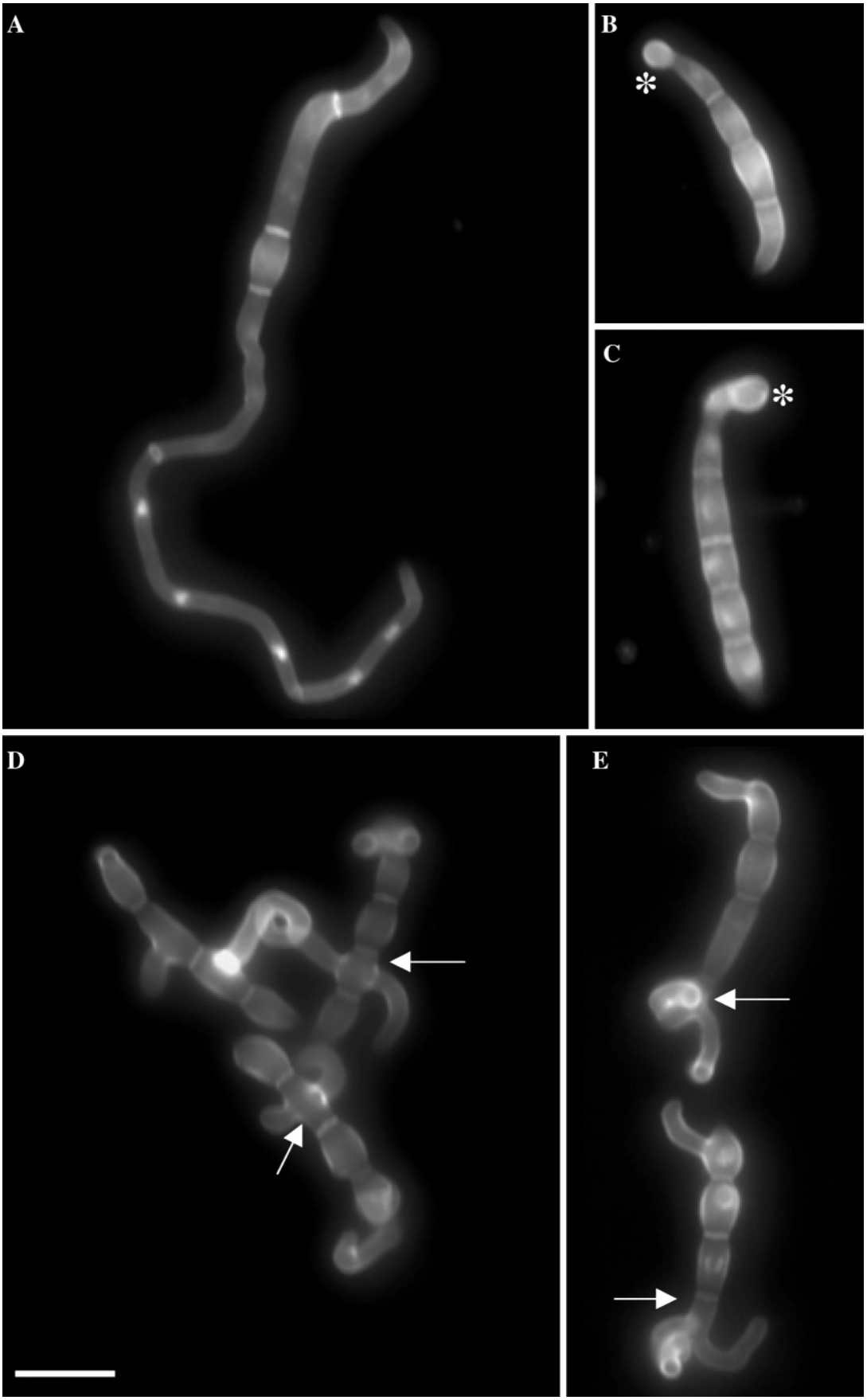
FIG. 1. The first germ tube preferentially emerges from apical cells. A. Freshly harvested macroconidia viewed with DIC optics. Note that apical cells are tapered toward the poles. B, C. Macroconidia germinating on cover slips were fixed, stained with Calcofluor and Hoechst, then analyzed with fluorescence microscopy. Arrowheads, germ tube emergence from the extreme pole of apical cells. Arrows, germ tube emergence from the side of apical cells. Asterisk, first septum in a new hypha. Bar = 10 μm.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minating in rich media were labeled with FITC-conjugated WGA. Different patterns of staining were observed (FIG. 2). In apical cells a prominent single spot occasionally was seen at the extreme pole (FIG. 2A). More often, in both apical and internal cells that were swollen, multiple bright patches were observed throughout the cortical regions (FIG. 2A, B). Notably, only one or two cells within a macroconidium appeared to be undergoing active chitin deposition. However once a germ tube emerged it displayed uniform FITC-WGA staining along its length (FIG. 2C).

The lectin concanavalin A (conA) can be used to detect cell surface mannoproteins. When germinating macroconidia were labeled with FITC-conA, only faint staining of swollen internal cells occasionally was observed (data not shown). However, like WGA, FITC-conA did uniformly label emerged germ tubes.

It seemed reasonable that swollen cells engaged in chitin deposition ultimately may produce a germ tube. To address this possibility, macroconidia were germinated 2 h in YMA, labeled with FITC-WGA and returned to growth media an additional 3 h. Of 82 cells that were labeled, 56 (68.3%) had produced a germ tube during the subsequent incubation period. These observations suggest that active chitin deposition is an early event associated with the germination of macroconidial cells.

Role of microtubules in regulating the germination pattern.—In the unicellular conidia of *A. nidulans,* the cytoskeleton plays an important role in establishing the bipolar germination pattern (Harris et al 1999). To determine if the cytoskeleton regulates the germination pattern, macroconidia were incubated in the presence of the microfilament depolymerizing agent cytochalasin A (CA) or the microtubule depolymerizing agent Benomyl (Ben) during germination. As expected germinating spores exposed to CA lost polarity and formed prominent tip swelling (FIG. 3A, B, C), however the germination pattern itself was not affected. Conversely, despite the delayed appearance of somewhat thicker germ tubes (FIG. 3A, D, E), Ben treatment did not prevent polarized growth. Still the resulting pattern was altered in two ways. First, macroconidia produced a greater number of germ tubes; in particular, 43% of treated macroconidia possessed three or more germ tubes, compared to only 12% of control untreated spores. Second, and more striking, the loss of microtubules appeared to permit the emergence of multiple germ tubes from a single cell (FIG. 3D, E). In the untreated controls no macroconidial cell produced more than one germ tube (n = 200). By contrast 41.5% of Ben-treated cells formed two germ tubes and 4% three tubes. These observations suggest that microtubules may play a role in establishing the asymmetrical pattern of macroconidial germination.

Relationship between germ tube emergence and nuclear division.—Apical cells of macroconidia contain a single nucleus (data not shown). To determine whether germ tube emergence is coupled to the division of this nucleus, macroconidia germinating in rich media were stained with Hoechst 33258. After 3 h 69% of macroconidia possessed a germ tube emerging from an apical cell, yet only 24% of these cells had undergone nuclear division. In addition 60% (n = 100) of apical cells possessing a germ tube were still uninucleate. Moreover, even when nuclear division was blocked upon exposure of macroconidia to 20 mM HU, which triggers arrest in S phase (Bergen and Morris 1983), germ tube emergence continued unabated (i.e. 74% of macroconidia possessed a germ tube). These observations suggest that, at least under ideal growth conditions, nuclear division is not required for the emergence of germ tubes from apical cells. By contrast, under similar conditions, conidial germination is tightly coordinated with nuclear division in *A. nidulans* (Harris 1999).

DISCUSSION

The ability of *F. graminearum* macroconidia to adhere to and germinate on host tissue presumably plays an important role in the localized dissemination of Fusarium head blight. As a first step toward understanding the morphological basis of the interaction with the host, the goal of this study was to characterize landmarks associated with the germination of macroconidia. The spatial patterns of germ tube emergence and cell wall deposition are described and shown to depend, in part, on the presence of functional microtubules. These observations highlight common morphogenetic features associated with spore germination in filamentous fungi and also

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FIG. 3. Regulation of the germination pattern by microtubules. Macroconidia were germinated on cover slips in YMA media for 4 h, fixed, stained with Calcofluor and Hoechst, then analyzed with fluorescence microscopy. A. Untreated controls. B, C. After 3 h, macroconidia were exposed to 5 μg/mL cytochalasin A. Astericks, swollen germ tubes. D, E. Macroconidia were exposed to YMA + 5 μg/ml Benomyl for 4 h incubation. Arrows, macroconidial cells from which multiple germ tubes have emerged. Bar = 10 μm.

identify subtle differences that may reflect variations in the lifestyle strategies of these fungi.

The morphogenetic program associated with the germination of macroconidia is relatively straightforward. As described in other plant pathogens the form multicellular conidia (Hamer et al 1988, Braun and Howard 1994, Hardham 2001), germ tubes preferentially emerge from apical cells. At this point it is not possible to determine reliably which apical cell is the foot cell that was attached to the phialide during conidiogenesis (Ohara et al 2004). This cell conceivably could retain a ''birth'' scar that biases germ tube emergence toward either the side of the cell or its extreme pole. Indeed the site of active chitin deposition observed with FITC-conjugated WGA might reflect the activity of such a landmark at the apical pole. On the other hand, chitin deposition may occur at the previously unattached apical pole and this site may correspond to the location of the carbohydrate-rich adhesive material found at the poles of other *Fusarium* species (Epstein et al 1994). An attractive model is that attachment to the host surface via this site may provide a spatial cue for subsequent germ tube emergence. The cue could be the physical process of attachment itself or, by analogy to bud site selection in yeast (Chen et al 2000), may consist of an extracellular matrix protein that acts as a spatial marker. In the latter case the marker still may serve as a spatial cue in the absence of host attachment (i.e. on a glass cover slip). By contrast there appears to be a general prejudice against the emergence of either of the first two germ tubes from internal cells. Moreover these cells display a different pattern of chitin deposition, which is characterized by a series of patches that accompany cell swelling. The internal cells may function as ''nurse cells'' that support germ tubes emerging from apical cells. However, under certain conditions (i.e. rich glucose media), they also may be capable of generating additional germ tubes.

In animal cells (Gunderson et al 2004) and certain fungi (i.e. *Schizosaccharomyces pombe*; Feierbach et al 2004), there is increasing evidence that microtubules play a role in specifying sites of microfilament formation. The observations presented here suggest that a similar mechanism may underlie the selection of germ tube emergence sites in macroconidial cells. In particular, treatment with a microtubule-depolymerizing agent delayed germ tube emergence and ultimately let multiple germ tubes emerge from a single cell. I propose that microtubules facilitate the delivery of microfilament organizing factors to specified sites that either are preselected (i.e. the extreme pole of apical cells) or stochastically determined. Possible organizing factors may include, for example, components of the fungal polarisome (Harris and Momany 2004). In the absence of microtubules, organizing factors may accumulate randomly at multiple sites over time. This could explain the delay in germ tube emergence while still accounting for germination from multiple sites within a single cell. Although this model is speculative, it can be tested by determining the location of polarisome components in macroconidia germinating under different conditions.

Despite their complex multicellular organization, *F. graminearum* macroconidia display a bipolar germination pattern similar to conidia from other filamentous fungi. This is not particularly surprising, because this pattern permits the most efficient exploration of the local environment, whether it is dead organic matter or the surface of a plant. On the other hand, certain features of germination-related morphogenesis differ from previous observations in the saprophyte *A. nidulans*. This includes the lack of coupling between germ tube emergence and nuclear division. The basis of this difference remains unknown but may reflect the alternative habits of pathogens and saprophytes.

In summary the spatial pattern of germ tube emergence from *F. graminearum* macroconidia has been characterized. The pattern suggests the existence of a mechanism that marks sites of germ tube emergence. Furthermore proteins that designate these sites may play an important role in the interaction with the host surface. Future efforts will be directed toward exploiting the recently completed *F. graminearum* genome sequence to identify candidate positional markers.

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