ASPERGILLUS NIDULANS SEPTIN ASPD IS INVOLVED IN NEW GROWTH EMERGENCE IN THE VEGETATIVE PHASE

by

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(Under the Direction of Michelle Momany)

ABSTRACT

Septins are novel cytoskeletal proteins found in animals, fungi and microsporidia. They are essential for orderly cell division and are major determinants of morphology. The model filamentous fungus *Aspergillus nidulans* has five septins: AspA, AspB, AspC, AspD and AspE. *In vivo* localization of the AspD-GFP fusion protein shows that *aspD* localizes to dormant conidia, isotropically growing conidia, germ tubes, hyphal tips, septa, along the hyphae, at the base of branches, at the periphery of branches, and at metulae and phialides in conidiophores. Deletion of the *aspD* gene resulted in early germination of conidia, multiple germ tubes emergence, hyperbranching, early septation, swollen conidia, hyphal splits and abnormal conidiophores. From this we suggest that *asp*D in *A. nidulans* influences germination timing, germ tube emergence, conidial and basal compartment morphology, hyphal tip integrity, septum formation timing and branch emergence. Overall, AspD is thought to be involved in new growth emergence in the vegetative phase of *A. nidulans*.

INDEX WORDS: *Aspergillus nidulans,* septins, AspD, germ tubes, septa, hyphal splits.

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DEDICATION

I would like to dedicate this thesis to my parents for their constant support and encouragement.

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INTRODUCTION

Septins were first identified by Hartwell in 1971 in the budding yeast *Saccharomyces cerevisiae* in screens for temperature sensitive mutants defective in cell cycle progression (Hartwell, 1971). They were observed to be at the mother bud neck in *S. cerevisiae* as a highly ordered ring of 10 nm filament subjacent to the plasma membrane (Byers and Goetsch, 1976). They were termed septins because of their role in septation and cell division. Septins are a family of novel cytoskeletal proteins found in animals, fungi and microsporidia. They have not been found in plants (Field and Kellogg, 1999; Pan et al., 2007). Septins are implicated in diverse cellular functions which include defining cell morphology, cytokinesis, establishing and determining polarity, apoptosis, organizing the cytoskeleton and functioning as scaffolds that recruit and tether other proteins to appropriate sites (Gladfelter et al., 2004; Larisch et al., 2000; Lindsey and Momany, 2006; Longtine et al., 1996).

Septins are GTP binding proteins. Septins purified from *Drosophila*, *Xenopus* and *Saccharomyces* have been shown to bind GTP or have GTPase activity (Field et al., 1996; Mendoza et al., 2002; Vrabioiu et al., 2004). However the biological significance of this is not yet clear. The typical septin structure comprises an N-terminal polybasic domain followed by a well-conserved GTPase domain (Pan et al., 2007). This GTPase domain is a common structural feature of septins in all organisms (Douglas et al., 2005). C-terminal to the GTPase domain lies the septin element that is conserved among many septins. At the C-terminus, most septins also contain a coiled coil domain needed for

interactions between certain septins (Pan et al., 2007). Septins polymerize into oligomeric multiseptin complexes that assemble into filaments (Versele and Thorner, 2005) in fungi and animals.

The number of septins varies in different organisms. The yeast *Saccharomyces cerevisiae* has seven septins, the fruit fly *Drosophila melanogaster* has five, the nematode *Caenorhabditis elegans* has two and humans have thirteen identified septins (Lindsey and Momany, 2006). In humans defects in septins have been linked to neoplasia and neurodegenerative disorders(Russell and Hall, 2005).

Recent studies have examined the localization of septins in a variety of patterns in many fungi and animals. Septins localize to projections at the projection base e.g. to the buds in budding yeast, or at the projection tip e.g. hyphal tips, a pattern common in fungi. Septins localize to partitions, e.g. to cleavage furrows in *Drosophila*, or at septa in *Aspergillus*, a pattern common in fungi and animals. Septins also localize to whole cells at the whole cell periphery, punctuate throughout, or as filaments throughout the cell e.g. in various mammalian cell types, a pattern common in animals(Lindsey and Momany, 2006).

Aspergillus nidulans is a multicellular fungus and is a classical and molecular model system. It is a model for septins in multicellular organisms. *A. nidulans* coordinates multiple compartments in different life cycle stages. *A. nidulans* has five septin genes: *aspA*, *aspB*, *aspC*, *aspD* and *aspE* (Momany et al., 2001). Preliminary protein sequence identities reveal that septin Asp*D* in *Aspergillus nidulans* is 51% identical to its human homolog SEPT9, whose levels are elevated in human tumor cells.

No other septin is reported to be closer to SEPT9 than Asp*D.* Asp*D* structure lacks the C-terminal coiled coil domain and is included in the Group1A of septins (Pan et al., 2007).

Previous work on septins in *Aspergillus nidulans* (Westfall and Momany, 2002), [Lindsey, Momany, unpublished], show that septins AspA, AspB and AspC participate in germ tube emergence, branching, septation and conidiophore development. We wanted to determine the role of AspD in *A. nidulans*. Here we show that AspD localizes to areas where new growth occurs suggesting that AspD in *A. nidulans is involved in* regulating new growth emergence.

MATERIALS AND METHODS

Aspergillus strains, media and growth conditions

All the strains used in this study are described in Table 1. Media used include minimal medium (MM) and complete medium (CM) which are standard for *Aspergillus nidulans* with appropriate supplements (Pontecorvo et al., 1953). MM was used for growing strains for observing the deletion phenotypes and the fluorescent tag. All the strains were grown at 30°C. Growth timing is indicated in each set of observations.

Construction of AspD-GFP and Δ*asp*D strains

AspD-GFP and Δ*asp*D strains were constructed by generating gene replacement constructs by fusion PCR (Yang et al., 2004)**.** Briefly, for constructing the AspD-GFP strain, a cassette of the fluorescent tag and the *pyr*G marker along with 2kb flanking gene target sequence was introduced in the Δ*nku* strain- TNO2A7, in which the gene for non-homologous end joining is deleted. For constructing the Δ*asp*D strain, a cassette of the *pyr*G marker, along with the 2kb flanking gene target sequence, was introduced in the TNO2A7 strain. These cassettes were obtained from the Fungal Genetics Stock Center.

Homologous recombination replaces the wildtype with the tagged allele at its endogenous locus, behind its own promoter. The strains were further crossed with wildtype (Table 1) to get the deleted nku gene (Nayak et al., 2006) back in the strain. Correct gene replacements were confirmed using PCR. The protocol for *A. nidulans* transformation was adapted from Osmani et al. (Osmani et al., 1987). Genomic DNA was extracted from ground, frozen mycelia using the DNeasy Plant Extraction kit (QIAGEN), as per manufacturers' instructions. AspD-GFP strain was observed under the DIC microscope and quantified to make sure it did not show any phenotypic variation from the wildtype.

Microscopic observations

Hyphae were prepared for microscopic observation as previously described (Momany, 2001). Briefly, ten milliliters of minimal medium was inoculated with 1×10^4 -5 $\times10^4$ conidia ml^{-1} and incubated in a small Petri plate containing a glass cover slip. The fungal hyphae attached to the coverslip were fixed with 3.7% formaldehyde, 0.2% triton X-100, 50mM phosphate buffer pH 7 for 30-45 mins. Coverslips were briefly rinsed in water and placed on mounting solution (50% glycerol, 10% phosphate buffer pH7, 0.1% n-propyl-gallate) on a slide. GFP strains were not fixed and viewed directly at the determined time-points. Differential interference contrast and fluorescence microscopy using the green filter were performed using the ZEISS (Thornwood, NY) Axioplan microscope at ×1000 magnification. Images were made using Photoshop CS version 8.0 (Adobe, Mountain View, CA).

Conidiophore study

Protocol for conidiophore development was adapted from Lin and Momany (Lin and Momany, 2003). Briefly, a small block of minimal agar medium with appropriate

supplements was placed on a sterile cover-slip kept on a water agar petriplate. Conidia were inoculated on the edges of the agar block, a sterile cover slip was placed on top of it and was incubated for 2-3 days at 30ºC. The conidiophores attached to the cover-slips were fixed and then mounted on a slide and observed microscopically.

Quantification of deletion mutants, wildtype and AspD-GFP

Δ*asp*D and AspD-GFP strains were incubated at 30°C for 5hrs, 6 hrs, 9 hrs, 10 hrs, 12 hrs, 21 hrs, and 3 days, as indicated by the life-cycle stages, in order to observe phenotypic variations. At the same time the wildtype strain was grown at the same conditions as a control. Phenotypes of the *asp*D deletion strains were observed and counted. A minimum number of 200 hyphae were counted for each characteristic. For counting germ tube emergence in the Δ*asp*D strain, conidia with more than three germ tubes were considered abnormal. All the images were taken at 1000X magnification.

RESULTS

Septins organize cell shape, determine cell morphology and have been shown to localize to the areas of active growth in other organisms. To determine the localization of AspD we constructed a strain in which the only copy of AspD was fused to GFP and was expressed behind its native promoter. To investigate the function of AspD, we constructed a strain in which the *asp*D gene was deleted. We compared the wildtype, AspD-GFP and the Δ*asp*D strains to check if AspD-GFP showed any phenotypic variation from the wildtype. We quantified morphological landmarks described in more detail below. We found that AspD-GFP behaved very similarly to wildtype in vegetative growth. Its phenotype in conidiophores was intermediate between wildtype and Δ*asp*D strains (Table 2). We observed the wildtype, AspD-GFP and the Δ*asp*D strains during early development.

AspD-GFP localizes as a dot in the dormant conidium, as filaments around the periphery of the isotropic spore and in germ tubes. The deletion mutant Δ*asp*D shows early germination and increased number of germ tubes. The dormant spore (conidium) breaks dormancy and starts growing isotropically after coming in contact with a carbon source. After a brief period of isotropic growth it switches to polar growth and sends out a germ tube and grows by apical extension(Adams et al., 1998). To determine if the septin AspD plays a role in the early developmental phase in the *A. nidulans*, we observed AspD-GFP and Δ*asp*D strains during the early developmental stages. The strains were grown in MM with suitable supplements at 30°C. AspD-GFP localized as a small dot in dormant spores. Isotropically growing AspD-GFP localized as filaments around the periphery of the spore (Fig. 1A). We focused microscopically up and down

and made sure that the filaments were not in the center of the spore. Wildtype *A. nidulans* spores do not send out germ tubes until 6 hrs of incubation under these conditions. 50% of the deletion mutants germinated early after only 5 hrs. of incubation (Fig.4A). In the wildtype, spores gave rise to two germ tubes and a few times three germ tubes were also seen. Occurrence of more than three germ tubes is hardly ever observed in the wildtype. When grown for 9 hrs, 18.5% of the deletion mutants had more than three germ tubes; typically they had four uncoordinated germ tubes (Fig. 4B, Table 2). Based on the localization of AspD-GFP to dormant conidia and the emergence of extra germ tubes in Δ*asp*D, it seems likely that AspD plays a role in preventing the emergence of extra germ tubes.

AspD-GFP localizes as a ring around the septa and the deletion mutant Δ*asp*D shows early septation. In the wildtype, septation starts to occur after three or four rounds of mitosis(Momany and Taylor, 2000), dividing the hypha into compartments (Momany and Taylor, 2000). AspD-GFP localized to the septa as rings around the hypha (Fig. 2A). After 11 hours of incubation, only 9.8% of the wildtype were septate, whereas 32.3% of the deletion mutants were septate (Table 2). Based on the localization of AspD-GFP in the septa and early septation in the deletion strain, it is likely that AspD plays a role in determining septation time in *A. nidulans*.

AspD-GFP localizes as a ring at the base of the branches and as filaments at the tips of hyphae. The deletion mutant Δ*asp*D shows hyperbranching, expanded conidial or basal compartment and hyphal end-splits. In wildtype, as the hypha grows branches emerge from the main hypha. Branches are a new axis of polarity. To determine if AspD has any role in branching and regulating hyphal morphology, we observed the

wildtype, the tagged strain and the deletion mutant at appropriate times. AspD-GFP localized as a ring to the base of the emerging branches (Fig. 2B). In the wildtype, one branch emerged from each compartment. However, 21.6% of the deletion mutants had increased branch emergence in hyphae often with two branches developing from the opposite sides of the compartment (Fig. 4C). Further, we observed that after 21 hours of incubation, 35% of the deletion mutants had an enlarged abnormally shaped conidial compartment or basal compartment, something that we hardly saw in the wildtype (Fig. 4D, Table 2).

Hyphae grow at their apical end. AspD-GFP localized as filaments to the tips of the hyphae (Fig. 1C, D, E). In wildtype *A. nidulans* there is usually a single hyphal tip. In the deletion mutant, we observed that 31% of the hyphal tips split dichotomously and sometimes even trichotomously (Fig. 4E, Table 2). Based on the localization of AspD-GFP at the hyphal tips and the occurrence of hyphal splits in the deletion mutant, we think that AspD plays a role in maintaining hyphal tip integrity.

AspD-GFP localizes in the metulae and phialides of the conidiophore. The deletion mutant Δ*asp*D shows disorganized conidiophores. In wildtype as the hypha matures, asexual reproductive structures called conidiophores develop from subapical compartments after two days of growth. Conidia are made by this specialized structure. The conidiophore is a highly organized structure developed from an aerial hypha. This hypha elongates by apical extension to form a conidiophore stalk(Adams et al., 1998). The tip of this stalk swells to form a vesicle. Metulae bud from the vesicle and phialides from the metulae as the conidiophore develops (Adams et al., 1998). Conidial chains extend from phialides. To determine if AspD plays a role in determining the conidiophore

structure, we observed AspD-GFP strain and the deletion mutant after conidiophores developed in these strains. AspD-GFP localized as filaments to the phialides (Fig. 3A) and metulae in the conidiophores (Fig. 3B). We observed that 14% of conidiophores in the deletion mutant were abnormal (Fig.4F, Table 2). They had disorganized layers and some of the conidiophores were pigmented. The deletion mutant did conidiate and the phenotypic variation of the conidiophore in the deletion mutant as compared to the wildtype, was subtle. Based on the localization of AspD-GFP in the conidiophores and the conidiophores of the deletion mutants, it appears that *asp*D might be a subtle regulator of conidiophore morphology.

DISCUSSION

There are five septins in *Aspergillus nidulans*. These septins are not completely functionally redundant, since they show different localization patterns *in vivo* and different deletion phenotypes (Lindsey, Rodriguez, Momany unpublished). However, none of the deletion mutant phenotypes are apparent in all cells in individual experiments. Double deletion of two septins showed increased occurrence of mutant phenotypes, both in the severity and the number of mutants (Lindsey, Momany unpublished). Hence we believe that in case of a deletion of one septin, the other septins can partially substitute for the missing septin. This is the reason we see less than 100% deletion phenotypes in the mutant strains. Δ*asp*D shows deletion phenotype in the range from 14% to 50%.

Deletion of *A. nidulans*, AspA, AspB, and AspC show a higher percentage of deletion phenotypes than AspD. We infer that these three septins have less overlap with other septins as compared to AspD. Septin AspE does not show any distinct localization pattern or deletion phenotype in the fungus.

Based on the AspD-GFP localization pattern and Δ*asp*D phenotypes, we infer that in *A. nidulans, asp*D is involved in new growth emergence in the vegetative stage. In a growing hypha, new growth occurs during germination of conidia, septation, branch emergence and apical extension of hypha, in the vegetative phase.

The fact that AspD-GFP localized to the dormant and isotropic conidia and the deletion mutant germinated earlier the wildtype, leads us to think that *asp*D is involved in germination in the organism in determining the timing of germ tube emergence. Also,

since the deletion mutant had an increased number of germ tubes emerging, we infer that a*sp*D is needed to control the number of germ tubes or determine their number.

Based on the AspD-GFP localization to the septa and early septation in the deletion mutant, we think that *asp*D plays a role in determining the timing for the occurrence of septa and hence its absence triggers septation earlier than in the wildtype. Thus it temporally regulates septum formation.

After 21 hours of incubation, the deletion mutant had enlarged, abnormally shaped conidial or basal compartments, leading us to infer that *asp*D is involved in spatially regulating the conidial and basal compartment morphology.

Because AspD-GFP localized to the base of the branches and in the branches, and the deletion mutant had increased branching, it seems likely that *asp*D restricts extra branch emergence. Further, from the AspD-GFP localization to the tips of the hyphae and the tip splitting in the deletion mutant, it appears that *asp*D is a required to maintain hyphal tip integrity and is a spatial negative regulator of hyphal tip integrity.

Taking into consideration the AspD-GFP localizations and the phenotypes of the deletion mutant, we infer that in the vegetative phase of the *A. nidulans* life cycle, *asp*D plays a role in suppressing unnecessary growth and conserving energy for the fungus to grow only in the right areas. Our data thus suggests that *asp*D is required for normal vegetative growth in *A. nidulans*.

In future studies, we would like to see the localization pattern of AspD in the other septin deletion backgrounds in *A. nidulans* as well as observe the localization pattern of other septins in Δ*asp*D background.

TABLE 1: *Aspergillus nidulans* strains used for this study

Original number	Relevant Genotype	Source
ATK03	pyrG89:wA; argB::trpC-B; methG1, pyroA4; lysB5; nicA2	Progeny from Momany lab
ATK40	pyrG89; argB::trpC-B; pyro A4	Progeny from Momany lab
AKK2	*aspD*::pyrG89;*wA;*argB; pyroA4	This study
AKK3	*aspD*::pyrG89;*wA;*argB; pyroA4	This study
ASH27	pyrG89::*aspD*-GFP, argB; chaA; riboB2	This study
AKK8	Diploid *aspD*-GFP, chaA	This study

Parental strains

Original number	Relevant Genotype	Source
A773	pyrG89, wA3, pyroA4	FGSC
ATK01	biA1, argB, meth, lysB, nicA	A850 x A495
A850	biA1;argB::trpC_B; veA1 trpc801	FGSC
A495	lys, nic, PA	FGSC
ARL148	aspD::pyrG89, pyroA, riboB2 TNO2A7 background	This study
TNO2A7	pyrG89; pyroA4; ; nkuA::argB; riboB2	Berl Oakley

TABLE 2: Frequencies[a] of different *asp*D deletion defects in the wildtype (wt), AspD-GFP and Δ*asp*D strains

	Early germination	More than 3 germ tubes emergence	Early septation	Expanded conidial/ basal compartment	Increased branch emergence	Split hyphae	Abnormal conidiophore
wt	0%	0.6%	9.8%	1%	0.5%	4.8%	1%
AspD-GFP	0%	0.5%	9.4%	1.4%	0.8%	1.9%	7%
Δ*asp*D	50%	18.5%	32.3%	35%	21.6%	31%	14%

[a]Quantification of early germination was 5 hours after inoculation, increased germ tube emergence 9 hours after inoculation, septation 11 hours after inoculation, swollen conidia, hyperbranching, split hyphae 21 hours after inoculation, conidiophores 3 days after inoculation.

FIG. 1. AspD-GFP localization in isotropically swollen conidia, germ tubes, hyphal tips. Top panel: AspD-GFP images, Bottom panel: corresponding DIC images. The strains were grown at 30°C in MM with suitable supplements. (A) AspD-GFP localizes to the periphery of the isotropically growing conidia (B) AspD-GFP forms filament like structure during germ tube emergence. (C, D, E) AspD-GFP localizes as filament like structures at the hyphal tip. All images magnified 1000X. Scale bar =5 μm

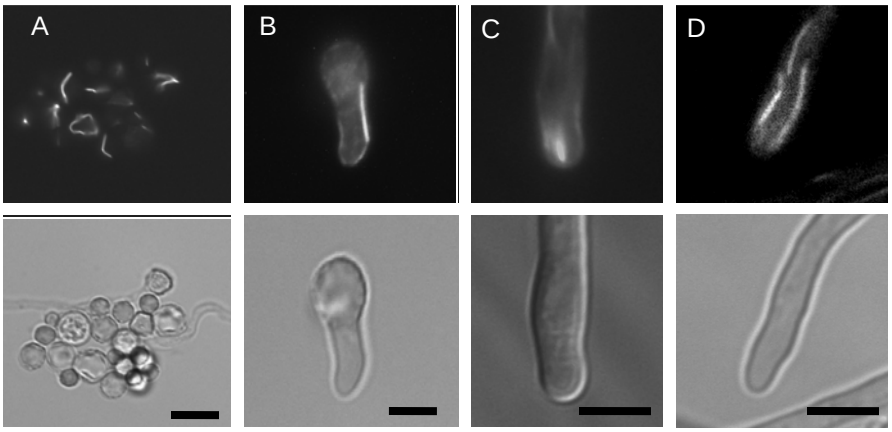
A
B
C
D

FIG. 2. AspD-GFP localization at the septum, base of branch, periphery of emerging branches. Top panel: AspD-GFP images, Bottom panel: corresponding DIC images. The strains were grown at 30°C in MM with suitable supplements. (A) AspD-GFP localizes to the septum (B) AspD-GFP localizes to the base of the branch (C) AspD-GFP localizes around a branch (D) AspD-GFP forms long filaments in hypha). All images magnified 1000X. Scale bar = 5 μm

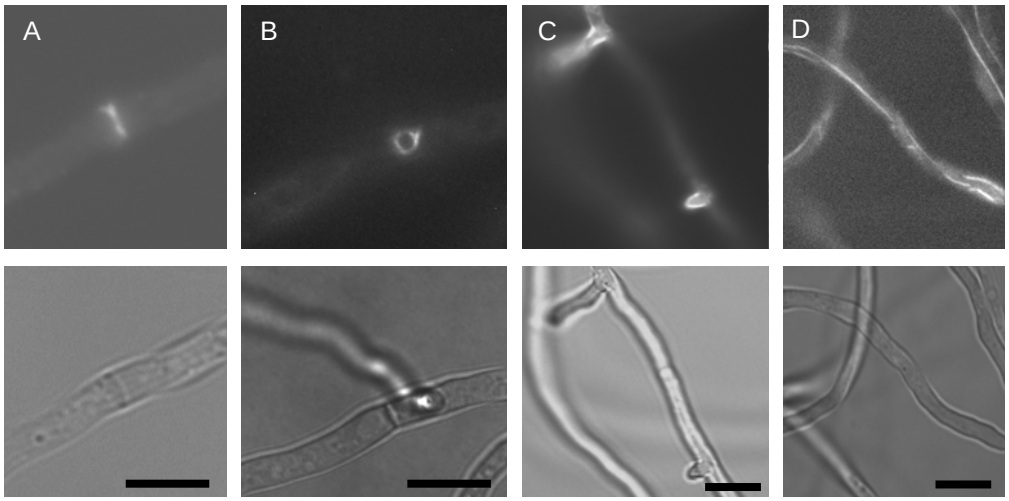
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FIG. 3. AspD-GFP localization to the conidiophores. Top panel: AspD-GFP images, Bottom Panel: Corresponding DIC images**.** (A) AspD-GFP localizes to the phialides in the conidiophores (B) AspD-GFP localizes to the metulae in the conidiophores. All images magnified 1000X. Scale bar = 5 μm

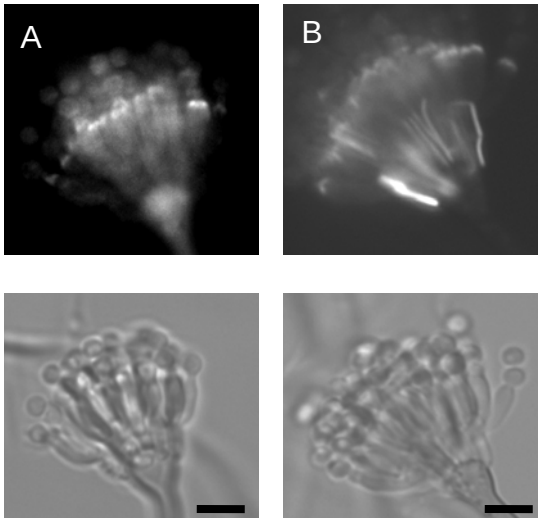
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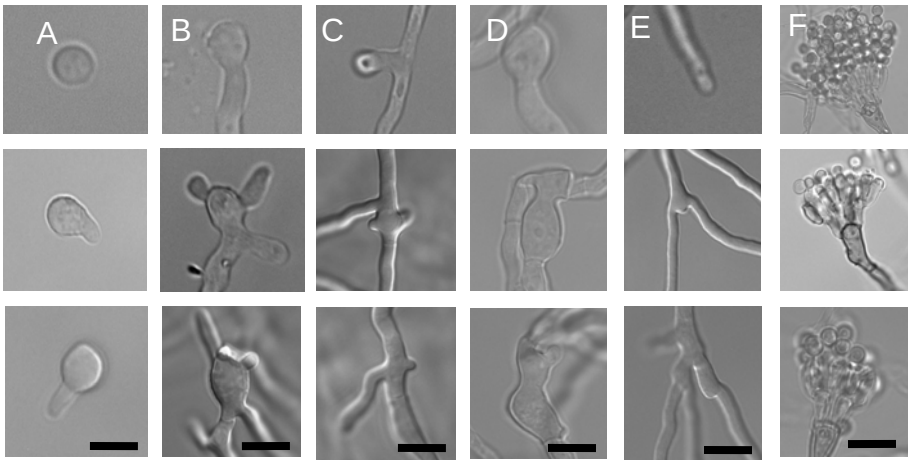
FIG. 4. *Asp*D deletion phenotypes. Top panel: Wildtype images, Bottom two panels: Δ*asp*D images of the corresponding life cycle stage. Deletion of *aspD* results in early germination of conidia, emergence of multiple germ tubes, hyperbranching, swollen conidium or basal compartment, split hyphae and abnormal conidiophores. (A) Early germination of conidia (image after 6 hrs. after inoculation), (B) Multiple germ tubes emergence (image after 9 and 18 hrs. of incubation), (C) Hyperbranching (image after 21hrs. of incubation), (D) Swollen basal compartment or conidium (image after 21 hrs. of incubation), (E) Split-hyphae (image after 21 hrs. of incubation), (F) Abnormal conidiophores (image after 3 days if incubation). All images magnified 1000X. Scale bar = 5 μm


A
B
C
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