

Stage-specific cellular localisation of two hydrophobins during plant infection by the pathogenic fungus *Cladosporium fulvum*

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Abstract

Hydrophobins are central to developmental processes of filamentous fungi. HcF-1 and HcF-6 are two of the six hydrophobins identified in the plant pathogenic fungus *Cladosporium fulvum*. We have fused the viral epitope V5 to HcF-1 and HcF-6, introduced the recombinant genes into *C. fulvum* strains that lack the two genes, and localised the tagged proteins by immunofluorescence microscopy. HcF-1^{V5} is abundant on conidia and aerial structures formed in vitro and emerging from disease lesions on infected tomato plants. This is consistent with the proposed function of HcF-1 in aerial development and dissemination of conidia. HcF-6^{V5} is secreted onto the growth substrate by the hyphae and during invasion of plant tissues, which suggests a function in adhesion and infection. This was not supported by the phenotypic analysis of Δ HcF-6 strains. Hydrophobins may play distinct roles due to precisely regulated spatial localisation during infection-related development of *C. fulvum*.

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1. Introduction

Fungal hydrophobins are morphogenetic proteins implicated in a variety of developmental processes including sporulation, fruit body formation, and infection structure differentiation (W osten, 2001). Hydrophobins are small, secreted proteins that undergo spontaneous polymerisation at the interfaces between fungal cell walls and hydrophobic surfaces, or at water–air interfaces. In spite of their significance to fungal developmental biology, studies to date have been restricted to a relatively small number of hydrophobins, including, most notably, the Sc3 hydrophobin of the gill mushroom *Schizophyllum commune*. Sc3 is required for attachment to hydrophobic surfaces (W osten et al., 1994b), acts as a tensioactive protein that enables submerged hyphae to emerge into the air (W osten et al., 1999), it coats the surface of aerial hyphae (W osten

et al., 1994a) and it determines cell wall structure (van Wetter et al., 2000).

Fungal hydrophobins exist in two distinct forms that have been denoted Class I and Class II hydrophobins, based on their biochemical characteristics. Class I hydrophobins form very stable two dimensional films with a typical ultrastructure of a mosaic of parallel rodlets (W osten et al., 1993). These rodlets are composed of amyloid-like fibrils that are rich in β sheet structure (W osten, 2001). In contrast, Class II hydrophobins form aggregates that are easily dispersed, and do not have stable hydrophobic interactions between monomers. Little is known about why these two classes of hydrophobins exist. Complementation studies have shown that they are not direct functional equivalents (Kershaw et al., 1998). Another observation is that most fungi possess more than one hydrophobin in their genomes, indicating that hydrophobins may fulfil diverse functions during fungal growth and development (Whiteford and Spanu, 2002).

Pathogenic fungi such as *Magnaporthe grisea* use hydrophobins for infection structure differentiation.

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Mpg1, one of the hydrophobins of *M. grisea*, is formed during the early stages of infection and expression of the *mpg1* gene is regulated as part of the cyclic AMP-mediated morphogenetic programme for appressorium formation and plant infection (Soanes et al., 2002; Talbot et al., 1993). *M. grisea* mutants that lack the *mpg1* gene are unable to differentiate appressoria efficiently and are consequently reduced in their ability to cause rice blast disease (Talbot et al., 1996).

The plant pathogenic fungus *Cladosporium fulvum* causes tomato leaf mould disease and has at least six hydrophobins: four (HCf-1 to HCf-4) are Class I and two (HCf-5 and HCf-6) are Class II hydrophobins (Table 1) (Nielsen et al., 2001; Segers et al., 1999; Spanu, 1997). Two of these hydrophobins (HCf-4 and HCf-6) are bimodular protein and HCf-6, for example, has a long N-terminal extension that is 90% glycine and asparagine. The functions of the individual members of this diverse family of proteins in *C. fulvum* are not clear although the expression profiles of the genes encoding each hydrophobin vary significantly (Nielsen et al., 2001; Segers et al., 1999).

In this study we demonstrate that hydrophobins in *C. fulvum* exhibit stage-specific cellular localisation during the morphological transitions that accompany plant infection. Our results indicate that hydrophobins have evolved to allow fungi to grow and proliferate in intimate association with a wide variety of substrates, including the tissues of the host organisms that pathogenic species infect. Hydrophobins may therefore have been an important prerequisite for the spread of fungi to

diverse terrestrial ecosystems and in the evolution of fungal pathogenesis.

2. Results

2.1. Targeted deletion of HCf-6

HCf-1 has been deleted from *C. fulvum* as previously described (Spanu, 1998). The $\Delta HCf-1$ strain shows a marked reduction in the hydrophobicity of the conidia and a concomitant reduction in the ability of the spores to be disseminated by water (Whiteford and Spanu, 2001). The *HCf-6* gene was deleted from wild type *C. fulvum* strains by homologous recombination with the plasmid p $\Delta HCf-6LR$ in which the *HCf-6* open reading frame is replaced by a hygromycin resistance cassette derived from pAN7-1 (Fig. 1B) (Punt et al., 1987). Primary transformants were screened for loss of *HCf-6* by colony filter hybridisation followed by DNA-blot analysis of the genomic DNA to confirm loss of the gene (Fig. 1A). Three independent transformants that lacked an *HCf-6* gene were isolated. These $\Delta HCf-6$ strains all displayed similar growth rates, conidiation and pathogenicity as their wild type progenitor (not shown). These mutants were named $\Delta HCf-6$. In the DNA from wild type *C. fulvum* digested with *Bam*HI a single restriction fragment of 3.8 kb hybridises to the *HCf-1* specific probe. If the DNA is digested with *Xba*I a single fragment of 6 kb hybridises to the *HCf-6* specific

Table 1
Properties of the *C. fulvum* hydrophobins

Hydrophobin	Class	Domains	Expression (mRNA levels)	Phenotype of knockout	References
HCf-1	I	One module	Constitutive (high). Moderate induction following nitrogen or carbon starvation	Reduced hydrophobicity of conidia—reduced dispersal by water droplets	Spanu (1997), Spanu (1998), Whiteford and Spanu (2001)
HCf-2	I	One module	Constitutive (low). Moderate level of induction following nitrogen or carbon starvation	?	Segers et al. (1999)
HCf-3	I	One module	Constitutive (low)	?	Segers et al. (1999)
HCf-4	I	Bi-modular	Very low in vegetative mycelia. Marked increase in abundance during sporulation. Strong induction following carbon and nitrogen starvation	?	Segers et al. (1999)
HCf-5	II	One module	Non-detectable in vegetative mycelia. Marked increase in abundance during sporulation. Strong induction following nitrogen starvation	?	Segers et al. (1999)
HCf-6	II	Bi-modular (N-terminal module rich in glycine and asparagines)	High levels in vegetative mycelia. Marked down-regulation during sporulation and carbon starvation	?	Nielsen et al. (2001), this manuscript

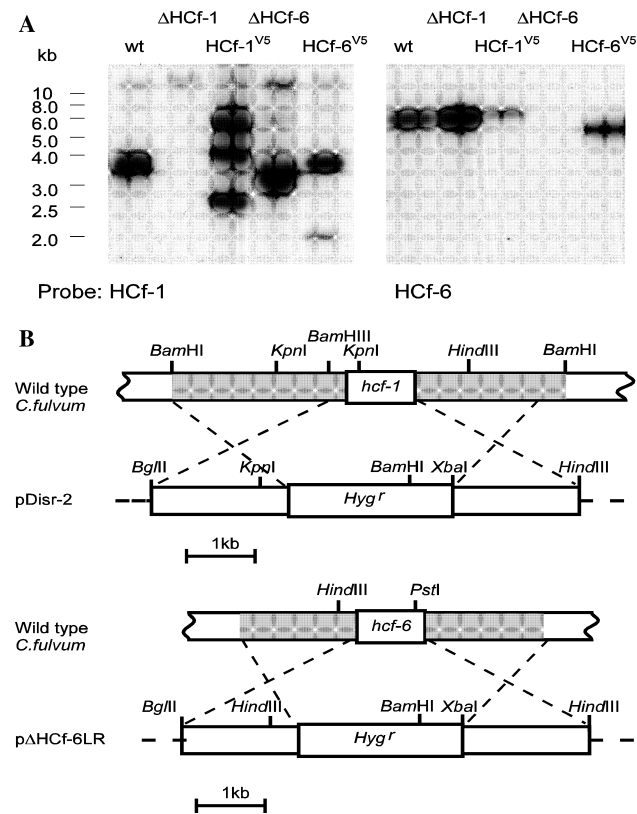


Fig. 1. (A) DNA blot analysis of the *C. fulvum* strains used in this work. Genomic DNA was digested with *Bam*HI (for probing with Hcf-1) or *Xba*I (for probing with Hcf-6). The fragments were separated by electrophoresis through 1% TBE/agarose. The blotted DNA was probed with either an *hcf-1* or *hcf-6* specific probe as indicated. (B) Map of the *hcf-1* and *hcf-6* loci and of the respective plasmids used for gene disruption. The hygromycin resistance cassettes displace *hcf-1* and *hcf-6* by homologous recombination.

probe. These fragments were absent in the DNA from the $\Delta Hcf-1$ and $\Delta Hcf-6$ strains, respectively.

2.2. *Cladosporium fulvum* hydrophobins are associated with different developmental stages

In order to localise the Hcf-1 and Hcf-6 hydrophobins of *C. fulvum*, we constructed recombinant hydrophobins fused to the V5 epitope tag. The resulting constructs were sequenced to confirm that the expected modifications were introduced in the plasmid DNA used for transformation. Plasmids encoding the tagged hydrophobins were introduced into *C. fulvum* protoplasts by co-transformation with pAN8-1, a plasmid vectors conferring resistance to phleomycin (Punt et al., 1988). We transformed the tagged hydrophobin genes into $\Delta Hcf-1$ and $\Delta Hcf-6$ strains. The isolation of the $\Delta Hcf-1$ strain has been previously described (Spanu, 1998). Preliminary experiments where the tags were inserted around the N-terminus resulted in removal of the viral epitopes from the mature proteins, possibly by proteolytic cleavage (results not shown). The epitope tags were

therefore introduced close to the C-terminus of the mature proteins. The colonies that grew on selective media were screened for presence of the tags by immunofluorescence labelling of fungal structures in mycelium grown in liquid medium in 96-well microtitre plates (not shown). In the case of Hcf-1, the transformants that showed a positive signal using the immunofluorescence assay were then tested for complementation of the reduced spore hydrophobicity phenotype (Whiteford and Spanu, 2001). These strains were named *Hcf1*^{V5} and *Hcf6*^{V5}.

DNA-blot analysis was hybridised with the gene specific probes generated by PCR as previously described (Whiteford and Spanu, 2001). An analysis of the selected strains (Fig. 1A) shows that DNA from the *Hcf1*^{V5} strain hybridises to three fragments of approximately 5.8, 4.0, and 2.6 kb. This indicates that *Hcf1*^{V5} has 3 copies of the plasmid integrated into the genome. The DNA from *Hcf6*^{V5} strain hybridises to a single restriction fragment of 5.2 kb pointing to a single integration event of the recombinant tagged Hcf-6^{V5}.

We used the V5 tag to localise the recombinant Hcf-1^{V5} by immunofluorescence microscopy. The primary anti-V5 and the secondary FITC labelled antibodies do not recognise any protein in the wild type *C. fulvum* or in the recipient hydrophobin gene replacement strains. Conidia of *Hcf-1*^{V5} were heavily labelled indicating that the hydrophobin accumulates in spores of *C. fulvum* (Fig. 2A). Optical sections of the labelled conidia using confocal microscopy revealed that the staining was localised most notably on the outer cell wall. When conidia germinated in liquid medium, newly formed germ tubes were completely devoid of Hcf-1: no labelling was observed on the nascent hypha at any stage (Fig. 2B).

To examine the spatial localisation of Hcf-1 in whole mycelium, we prepared sections through fungal colonies grown on agar medium and stained to detect Hcf-1^{V5} (Figs. 3A and B). In young colonies, prior to the onset of conidiation, the most evident label was observed on aerial hyphae (Fig. 3A): these appeared to be heavily labelled on the cell walls. At later stages, when conidia had formed, the cell walls of conidia were also strongly labelled (arrows, Fig. 3B). The hyphae growing in the agar medium and the hyphae that form a mat on the surface of the agar do not appear to have any detectable Hcf-1^{V5} antigen.

Cladosporium fulvum infects tomato leaves when conidia germinate and form germ tubes that enter the stomata. The deletion of Hcf-1 and the insertion of Hcf-1^{V5} have no effect on pathogenicity. In order to determine the spatial pattern of localisation of Hcf-1, we carried out plant infections and observed localisation of Hcf-1^{V5} during the course of infection of tomato leaves. Immunostaining Hcf-1^{V5} shows that Hcf-1 is present on the conidia, but not on the newly emerged germ tubes (Fig. 4A). Cross-sections through infected

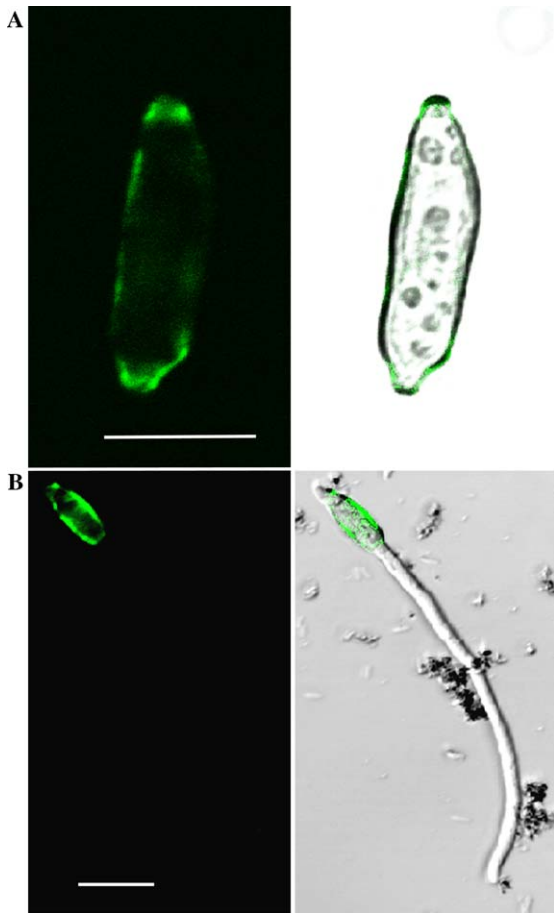


Fig. 2. Localisation of Hcf-1^{V5} on *C. fulvum* grown in vitro. Panels on the left show confocal optical sections of Hcf-1^{V5} labelled with anti-V5 antibodies. The panels on the right show bright field images of the same samples. (A) Ungerminated conidium; (B) conidium 24 h after germination.

leaves showed that no Hcf-1 can be detected on the hyphae that grow between the mesophyll cells (Fig. 4B). Two weeks after plant inoculation, hyphae emerged from the stomata (arrow) and develop into conidiophores (Fig. 4C). It was evident, from the labelling of these structures, that Hcf-1 appeared on the cell walls very shortly after emergence of the fungus from the stoma into the air (Fig. 4C). We conclude that Hcf-1 is associated with emergent structures of *C. fulvum* and does not accumulate on the surfaces of infectious hyphae within plant tissue.

2.3. Hcf-6 is present on *C. fulvum* hyphae

We used an Hcf-6^{V5} recombinant transgene to immunolocalise Hcf-6 and found no detectable levels of Hcf-6 on un-germinated conidia of *C. fulvum* (Fig. 5A). We observed a clear fluorescent signal on germinating hyphae about 20 μm from the germinating conidium (Fig. 5B). The label appeared to be somewhat diffuse around the hypha and the Hcf-6^{V5} protein seems to be

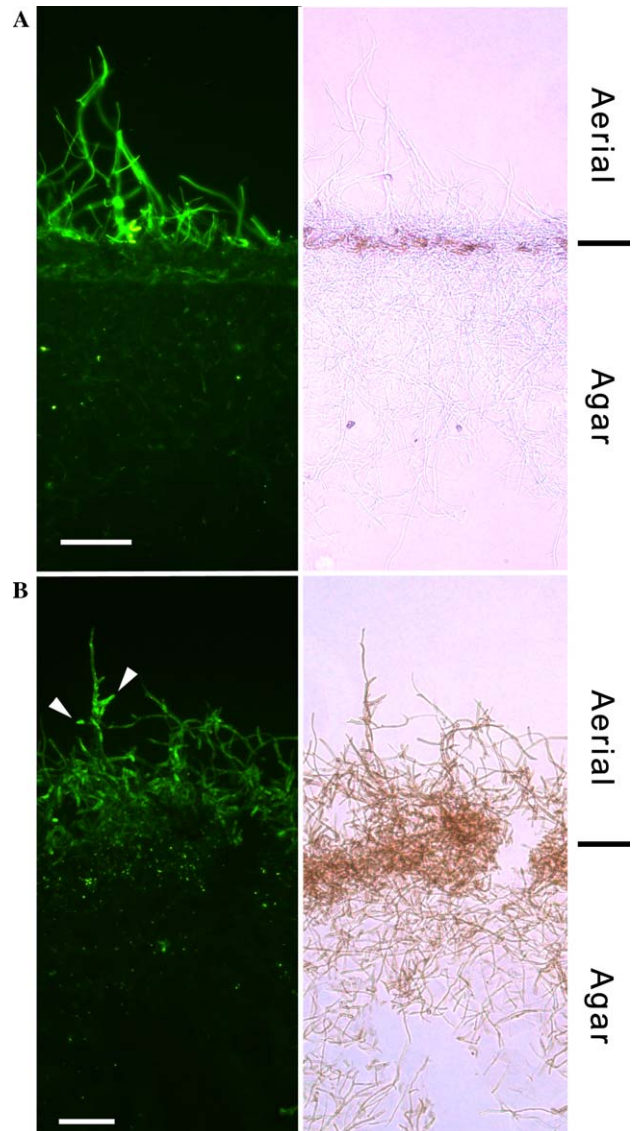


Fig. 3. Localisation of Hcf-1^{V5} on *C. fulvum* grown in vitro. Conventional fluorescence microscopy through colonies of Hcf-1^{V5} labelled with anti-V5 antibodies showing a section through (A) a 3-day-old colony (prior to sporulation) or (B) through a 5-day-old colony, at the onset of sporulation (bar: 50 μm).

secreted and deposited on the surface on which the fungus is growing. Immunostained cross-sections of colonies on agar show that Hcf-6^{V5} accumulates on a fairly discrete layer on the surface of the agar (Fig. 5C): the aerial hyphae, the submerged hyphae and the developing conidia are not stained, or are labelled at much lower levels. Interestingly, if conidia germinate in liquid medium at high concentrations (5×10^6 conidia/ml instead of 5×10^5 conidia/ml), Hcf-6^{V5} was detected after 24 h on the surface of the conidia as well as on the growing hyphae, indicating that the hydrophobin was deposited on the surface of these structures after secretion into the medium (Figs. 5D and E).

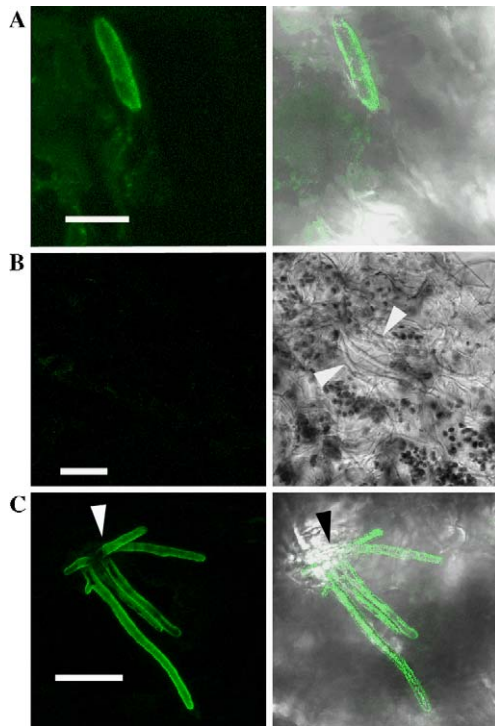


Fig. 4. Localisation of Hcf-1^{V5} on *C. fulvum* during infection. Panels on the left show immunofluorescence signals, panels on the right show bright field images of the same samples. Optical section through (A) a germinated conidium on the surface of a tomato leaf, (B) mesophyll of heavily infected tomato leaves showing intercellular hyphae, and (C) an optical stack of hyphae emerging from a stoma (bar: 20 μ m).

When the fungus is in intimate association with plant tissue during infection, Hcf-6^{V5} was absent from the conidia, and appeared on the germinating hypha about 20 μ m after emergence from the conidium, thus Hcf-6^{V5} was particularly prominent at the periphery of the hyphae during tissue invasion (Fig. 6A). Unlike the situation on glass slides though, the label does not diffuse around the hypha on the surface of the leaf. Cross-sections of leaves infected with Hcf-6^{V5} show that there is a visible, though not very intense, labelling of the intercellular hyphae growing between the mesophyll cells (Fig. 6B). The hyphae that emerge from the stomata are not labelled (not shown). Hcf-6 is therefore associated with infectious hyphae during their interactions with substrates, and completely excluded from the surfaces of emergent structures and spores.

2.4. Effect of hydrophobin gene deletion on conidial hydrophobicity, surface rodlets, and complementation

We investigated the effect of removing the *Hcf-1* or *Hcf-6* genes on the hydrophobicity of *C. fulvum* conidia and on the surface structures of conidial cell walls. The hydrophobicity of single celled micro-organisms such as *C. fulvum* conidia can be measured using a modification of the Microbial Adhesion To Hydrocarbons (MATH)

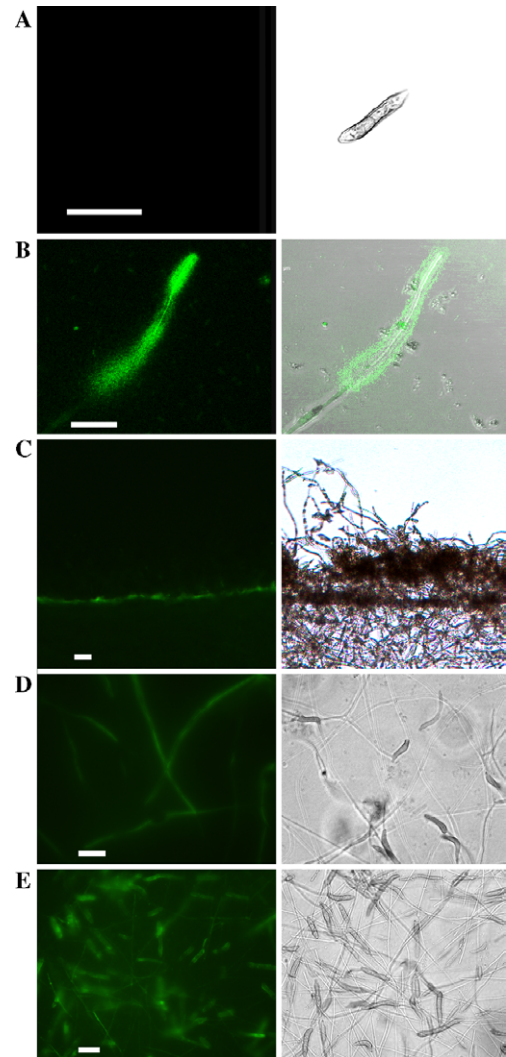


Fig. 5. Localisation of Hcf-6^{V5} on *C. fulvum* grown in vitro. Panels on the left show immunofluorescence signals, panels on the right show bright field images of the same samples. Confocal images of (A) an ungerminated conidium, conidium and (B) germ tube 24 h after germination (bar: 20 μ m). (C) Section through a mature colony of *C. fulvum* on agar (bar: 100 μ m). Conidia were suspended in liquid B5 medium at either 5×10^5 (D) or 5×10^6 (E) conidia ml^{-1} and then incubated for 24 h at 25 $^{\circ}\text{C}$. Hcf-6^{V5} was then localised by conventional immunofluorescence microscopy (bar: 20 μ m).

assay (Fig. 7) (Whiteford and Spanu, 2001). In this assay a suspension of conidia in buffer is mixed with oil (e.g., hexadecane). In the resulting emulsion, the conidia adhere to the interface between the oil droplets and the buffer. Addition of a detergent (Triton X-100) results in the conidia detaching from this interface and collecting in the lower (aqueous) phase. Titrating the amount of detergent needed to remove all the conidia from the water oil interface allows one to measure the hydrophobicity of the conidia. The conidia of the Δ *Hcf-1* strain are significantly more hydrophilic than the wild type strain (Fig. 7). Deletion of *Hcf-6* has no effect on conidial hydrophobicity. Re-introduction of an epitope

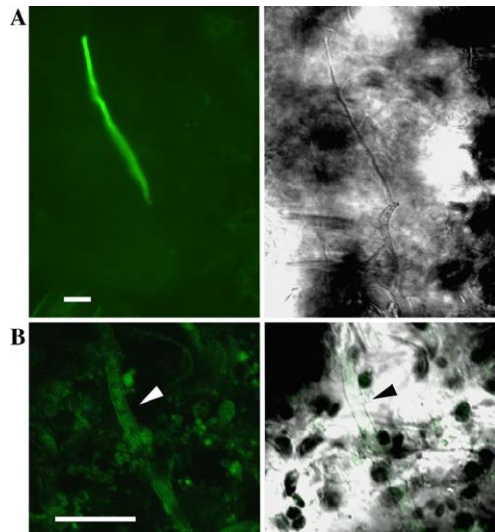


Fig. 6. Localisation of HCF-6^{v5} on *C. fulvum* during infection. Panels on the left show immunofluorescence signals, panels on the right show bright field images of the same samples. Optical section through (A) a germinated conidium on the surface of a tomato leaf and (B) mesophyll of infected tomato leaves showing intercellular hyphae (arrow; bar: 20 μm).

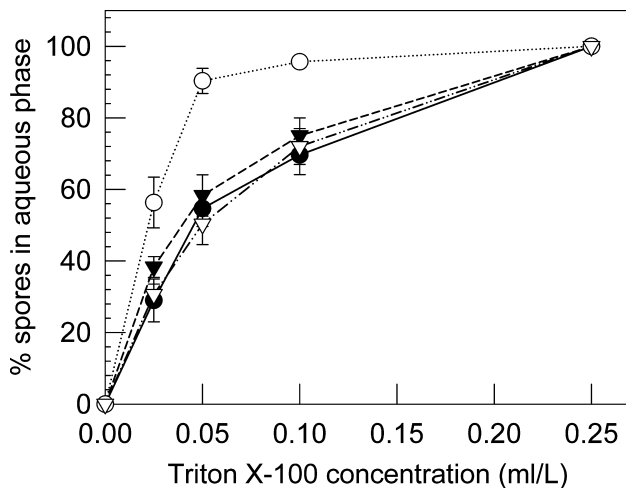


Fig. 7. Hydrophobicity of the conidia measured using a MATH assay. Conidia from the strains indicated on the graph were suspended in PBS/hexadecane and shaken vigorously. The number of conidia in the aqueous phase in the presence of increasing concentrations of Triton X-100 is shown (each point is a replicate of three replicates).

tagged HCF-1 (in *HCF-1^{v5}*) results in reversion of the conidial hydrophobicity to nearly wild type levels. Introduction of a tag in HCF-6 has no discernible effect on conidial hydrophobicity.

The aerial hyphae of all strains appeared morphologically similar under all conditions tested, including growth in conditions of high humidity. The hydrophobicity of the aerial hyphae of the Δ HCF-1 strain was slightly reduced, as previously described (Whiteford and Spanu, 2001); we

did not observe any changes in the hydrophobicity of the aerial hyphae in the Δ HCF-6 strains.

We compared the adhesion of the wild type *C. fulvum* and the Δ HCF-6 strains to its natural host plant or artificial solid substrates. Conidia were germinated on glass, GelBond, Parafilm, PTFE (Teflon) or tomato leaves. After 24 h incubation most of the germinated *C. fulvum* could be easily washed off all substrates by pipetting forcibly 1 ml water onto the droplet of medium. Only a few hyphae were visible on the surfaces after the wash. The deletion of HCF-6 did not cause differences to the adhesion of the fungus to any of the substrates tested (not shown).

To determine the role of HCF-1 in conidial hydrophobicity transmission electron microscopy of the surface replicas of *C. fulvum* spores was carried out, following freeze-fracture. Conidia of the wild type strain had large sheets of interwoven rodlets with a mean diameter of 8 nm (range 5–10 nm) as shown in Fig. 8. The

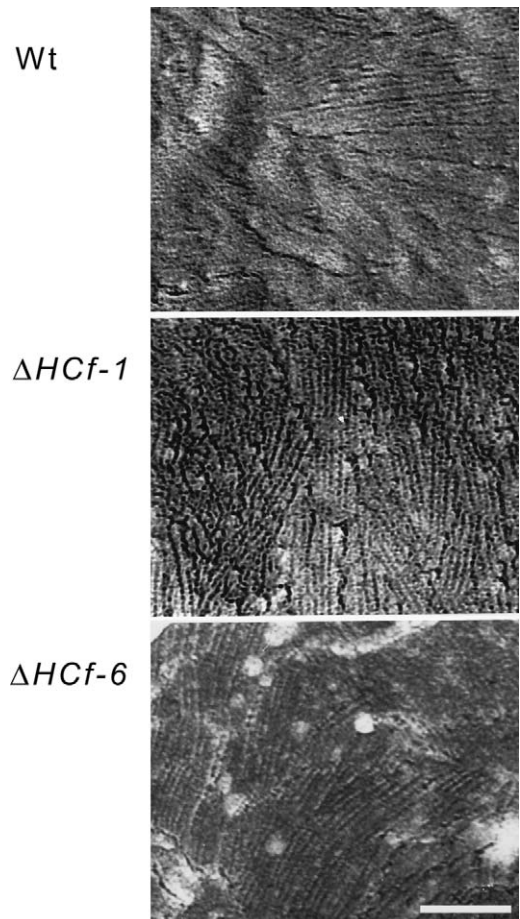


Fig. 8. Transmission electron microscopy of freeze-fractured conidium surfaces of *C. fulvum*. Conidial surface of a wild type *C. fulvum* (Wt) showing interwoven rodlets, which had a diameter of 8.0–10 nm. Δ HCF-1 mutant: rodlets were more sparsely distributed on conidia of this strain than the wild type, but were otherwise morphologically identical. Conidium surface of Δ HCF-6 mutant showing rodlets (bar for all panels is 100 nm).

$\Delta HCF-1$ strain, in contrast, possessed very few rodlets, which were found in dispersed patches. However, it was clear that $\Delta HCF-1$ mutants did still have the capacity to form rodlets, although, where present, the rodlets were less closely organised than in the wild type strain. We did not observe evident differences in the size of these rodlets. $\Delta HCF-6$ mutants possessed normal rodlets that were organised in the same way as the wild type and showed complete coverage of the conidial surface. The epitope-tagged strains also showed morphologically normal and intact conidial rodlet layers. We conclude that Hcf-1 forms a component of the rodlet layer, but that other hydrophobins must also participate in this process.

3. Discussion

Hydrophobins are morphogenetic proteins that are found only in the kingdom Eumycota and are restricted to filamentous species. They appear to be ubiquitous proteins in Ascomycetes and Basidiomycetes but are lacking in non-filamentous Ascomycetes such as the budding yeast *Saccharomyces cerevisiae* and *Ashbya gossypii*. In most fungi, it appears that each species has several hydrophobin genes that form small gene families (Whiteford and Spanu, 2002; Wösten, 2001). Each member of the gene family seems to play diverse roles in development, but it is far from clear why many hydrophobins are required for growth and development. For example, in *S. commune* SC3 is necessary for the formation and emergence of aerial hyphae while SC4 is required for the maintenance of unflooded air channels in the *S. commune* air channels (van Wetter et al., 2000). The different functions of hydrophobins, though, do not seem to be inherent in amino acid sequences of these proteins. The rice blast hydrophobin Mpg1 has been studied in detail and many hydrophobins, even those from different species, can partially complement a $\Delta mpg1$ mutant (Kershaw et al., 1998). It is thus possible that function depends on the timing of expression of hydrophobin genes and the precise spatial localisation of the proteins, in addition to specific structures of each hydrophobin.

Cladosporium fulvum, a biotrophic pathogen of tomato, has at least six hydrophobins. These include four Class I and two Class II hydrophobins. In order to clarify the function of individual hydrophobins within multi-gene families it is useful to observe their localisation during development and infection.

In this study we utilised a method for the tagging and localisation of Hcf-1 and Hcf-6 hydrophobins using the viral V5 epitope. This epitope is not present in *C. fulvum* and thus allow us to obtain clear immunocytochemical data. Our results show that it is possible to express recombinant Hcf-1^{V5} and Hcf-6^{V5} in *C. fulvum* when the epitope is inserted at or very close to the C-terminal end of the mature hydrophobins. The tag

can then be detected by immunofluorescence microscopy.

Hcf-1^{V5} is present most prominently on the surface of conidia. Hydrophobins are known to form self-assembled rodlets on the surface of many fungi. Patches of rodlets could be observed by transmission electron microscopy on the surface of *C. fulvum* conidia. The abundance of these rodlets was greatly reduced in the $\Delta HCF-1$ strain but was evidently restored to wild type levels on the conidia of Hcf-1^{V5}. Thus the localisation of Hcf-1^{V5} coincides with the rodlet patches. Moreover, we observe here that the Hcf-1^{V5} hydrophobin can effectively complement the reduction of the hydrophobicity of *C. fulvum* conidia caused by Hcf-1 deletion (Whiteford and Spanu, 2001). The fact that the $\Delta HCF-6$ strain showed no alteration in the conidial rodlet layer implies that this Class II hydrophobin is not involved in the formation of these structures in *C. fulvum*. Taken together, these results demonstrate that Hcf-1 is a major component of the *C. fulvum* rodlets, but clearly does not act on its own. In this way Hcf-1 resembles dewA, a hydrophobin from *Aspergillus nidulans* with the capacity to form rodlets, that has to act in concert with rodA in order to form the conidial rodlet layer of *A. nidulans* (Kershaw et al., 1998; Stringer and Timberlake, 1995).

The fact that hyphal septa were not labelled shows that these cell walls have a different constitution from that of the external wall. When *C. fulvum* is grown in vitro the only other place where Hcf-1 appears is on the surface of aerial hyphae on mature colonies. These hyphae are the emergent structures upon which conidia are eventually formed. It is interesting to note that neither the hyphae growing invasively in solid agar medium nor hyphae that form a mycelial mat on the immediate surface of agar are labelled. At present we do not know the nature of the signal required for expression of Hcf-1 in aerial structures, but such precise spatial localisation may contribute together with other hydrophobins to aerial development and sporulation.

When *C. fulvum* grows on and infects a tomato leaf the expression and localisation of Hcf-1 is excluded from invasive growth structures. Conidia are heavily labelled and germinate to produce hyphae that are not labelled. Remarkably, no further labelling is evident until the hyphae emerge from the stomata to produce conidiophores. The labelling of conidiophores indicates that Hcf-1 is secreted onto the surface of the hyphal cell wall very shortly after emergence from the stomata in the same way as aerial hyphae become labelled on colonies grown in vitro. It is unclear what might trigger the expression of Hcf-1 at this stage. The absence of a strong signal corresponding to Hcf-1^{V5} inside the plant may explain why the $\Delta HCF-1$ strain is as infective as wild type *C. fulvum*. However, the $\Delta HCF-1$ strain is affected in spore dispersal another essential virulence-associated trait of airborne foliar pathogens (Spanu, 1998).

The results from the localisation of the Class II hydrophobin HCf-6 reveal an entirely distinctive spatial distribution of the protein compared to HCf-1. HCf-6^{V5} is not visible on conidia or on the germinating hypha but is produced and accumulates on and around hyphae about 12 h after the conidia germinate. When grown in vitro, HCf-6^{V5} appears as a halo surrounding the hyphae and on the hyphal cell walls about 20 µm away from the germinated conidium up to the growing tips. Sections through mature colonies grown on agar reveal that HCf-6^{V5} forms a layer at the agar–air interface. If the conidia are germinated at high densities, all fungal surfaces are labelled. This suggests that growing hyphae secrete HCf-6V5. On agar plates the protein remains on the surface of the agar; when grown in a liquid droplet at low inoculum concentration, HCf-6^{V5} apparently dissolves in the medium and binds to surface of the hyphae that are secreting it. If the inoculum concentration is high enough, HCf-6^{V5} binds visibly to all fungal structures (including the conidial cell walls).

When *C. fulvum* infects tomato leaves, HCf-6^{V5} is visible on the hyphae 24 h after germination at a distance from where the hypha emerges from the conidium. The invasive hyphae in the leaf are also labelled but at a very low level which is just detectable above the general (auto)fluorescence of the stained leaf tissue. These results show that HCf-6 is associated with the interaction between nascent hyphae and the surface on which a fungus is growing, forming what appears to be a monolayer of protein between hypha and substrate. One possibility is that HCf-6 simply acts as an attachment factor. We therefore carried out a series of assays to test whether the adhesion of the fungus to various artificial substrates (glass, GelBond, PTFE, and Parafilm) and the leaf surface was affected in ΔHCf-6 strains (not shown). Hyphae did not appear less adhesive to any of the solid substrates tested, thus the role of HCf-6 is more likely to be as a primer rather than as a specific attachment molecule. In this capacity, HCf-6 may increase the wettability of a surface, allowing hydrophilic molecules to act more efficiently as attachment molecules. It is also possible that the hydrophobin layer contributes together with other hydrophobins to the ability of *C. fulvum* to invade plant tissue without eliciting overt disease symptoms during the initial stages of infection. This needs to be tested by deletion or down-regulation of more than one hydrophobin at the same time.

In conclusion, families of distinct hydrophobins may have evolved in fungi to allow them to contend with the diverse range of growth habits they encounter, including the internal tissues of the plants or animals that pathogenic fungi invade. *C. fulvum* is a biotrophic fungus that causes relatively mild disease symptoms despite extensive proliferation in the leaf. It forms an intimate association in the early stages of infection. We have shown that a class II hydrophobin HCf-6 is developmentally

controlled to be expressed specifically at the invasive stage of pathogenesis, while HCf-1 is expressed only after emergence of the fungus from the plant and generation of spores for the propagation of the fungus to new hosts. The action of diverse hydrophobins exhibiting such precise stage-specific localisation patterns may therefore be a fundamental characteristic of pathogenic fungi.

4. Experimental procedures

4.1. Fungal strains and culture conditions

Cladosporium fulvum race 4 was grown and maintained on solid potato dextrose agar (PDA) at 25 °C in constant darkness as previously described (Spanu, 1997). Liquid cultures were grown in B5 medium (Gamborg et al., 1968) supplemented with 2% sucrose as previously described (Spanu, 1998). The transgenic strains used in this work are listed in Table 2; these transgenics were maintained on PDA supplemented with either hygromycin (50 mg/L; Calbiochem) or phleomycin (50 mg/L; Cayla) as indicated.

4.2. Bacterial strains and plasmids

Escherichia coli strain XL-1 Blue (Stratagene) was used for production and maintenance of all plasmids. The plasmids used here are listed in Table 3. Plasmid pΔHCf-6 for the targeted deletion of HCf-6 was generated in the following manner. Primers HCL5 (5'ATCCGGATCC TTCCTTTCCTATC3') and HCL3 (5'TGGTGGGTA AGTGTATATGGGG3') were used to amplify a 1.5 kb DNA fragment downstream the HCf-6 open reading frame using *Pfu* DNA polymerase using conditions recommended by the manufacturer (Stratagene); a *Bam*HI restriction site was included in HCL5 (underlined). The resulting product was cloned into pAN7-1 digested with *Bg*III and *Stu*I to yield plasmid pHCF6-L. Primer HCR5 (5'ATCAGATCTGTGTATAAAACAGGC3') and HCR3 (5'ACGTAAGCTTCAGAAAGTGGTTG3') were used to amplify a 1.8 kb fragment downstream of the HCF-6 open reading frame using *Pfu* DNA polymerase; an *Xba*I site and a *Hind*III site were included in the HCR5 and HCR3 primers respectively. The resulting PCR product was digested with *Xba*I and *Hind*III and cloned into the *Xba*I and *Hind*III sites of pHCF6-L to generate the HCf-6 deletion vector pΔHCf-6.

4.3. Generation of V5 epitope tagged hydrophobins

The V5 epitope was inserted into the C-terminal end of the HCf-1 or HCf-6 proteins by PCR-mediated mutagenesis using pHCF1-3.8 and pHCF6-4.7 as templates, respectively (Table 3).

Table 2
Fungal strains used in this paper

Strain	Description	Antibiotic resistance marker	References
Wild type Race 4	Wild type <i>C. fulvum</i> able to overcome the tomato resistance gene Cf-4	None	Spanu (1997)
$\Delta Hcf-1$	Race 4 <i>C. fulvum</i> lacking the Hcf-1 hydrophobin	Hygromycin	Spanu (1998)
$\Delta Hcf-6$	Race 4 <i>C. fulvum</i> lacking the Hcf-6 hydrophobin	Hygromycin	This study
$Hcf-1^{V5}$	Race 4 <i>C. fulvum</i> derived from $\Delta Hcf-1$ with a transgenic V5 epitope-tagged Hcf-1	Hygromycin and phleomycin	This study
$Hcf-6^{V5}$	Race 4 <i>C. fulvum</i> derived from $\Delta Hcf-6$ with a transgenic V5 epitope-tagged Hcf-6	Hygromycin and phleomycin	This study

Table 3
Plasmids used in this paper

Plasmid	Description	Antibiotic resistance marker (organism in which this is active)	References
pHCf1-3.8	pBluescript KS(-) containing a 3.8 kb <i>Bam</i> HI fragment that encodes <i>Hcf-1</i>	Ampicillin (<i>E. coli</i>)	Spanu (1997)
pHCf6-4.7	pBluescript KS(-) containing a 4.7 kb <i>Bam</i> HI/ <i>Hind</i> III fragment that encodes <i>Hcf-6</i>	Ampicillin (<i>E. coli</i>)	Nielsen et al. (2001)
p $\Delta Hcf6LR$	Deletion construct in which the <i>Hcf-6</i> gene of pHCf6-4.7 is substituted with the hygromycin resistance “cassette” of pAN7-1	Ampicillin (<i>E. coli</i>); hygromycin (<i>C. fulvum</i>)	This study
pHCf-1 ^{V5}	Plasmid encoding a C-terminal V5 epitope-tagged Hcf-1 hydrophobin, derived from pHCf1-3.8	Ampicillin (<i>E. coli</i>)	This study
pHCf-6 ^{V5}	Plasmid encoding a C-terminal V5 epitope-tagged Hcf-6 hydrophobin, derived from pHCf6-4.7	Ampicillin (<i>E. coli</i>)	This study

pHCf-1^{V5}: Oligonucleotide H1cterA (GGTAACCG ATCCCGAACCCGCTGCTGGGTCTGGACTCCAC CGTCTCTGCTTAAGCCGCGCGCG) was synthesised to include the V5 epitope tag (underlined). HcterA was used with H1cterB (5' phosphorylated TGGGAT GCAGGAGACGTTGAT) in a PCR with pHCf1-3.8 as a template to produce a 6.8 kb product. Restriction enzyme *DpnI* was then added directly to the reaction mix and incubated for 2 h at 37 °C. *DpnI* digests any DNA methylated at its recognition site and therefore destroys the unmodified plasmid DNA. The enzyme was heat inactivated by incubating for 15 min at 75 °C. The DNA was then purified through a nucleotide removal column (Qiagen) following the manufacturers instructions. The mutated plasmid was ligated with T4 DNA ligase (NEB) and transformed into *E. coli*.

pHCf-6^{V5} was generated as described above was adopted using primers Hcf6cta (GGAAAGCCTAT CCCTAACCTAACCTCTCTAGGCCTCGATAG TACTTAAATGGATTCAGGTCCGCAA), which includes the V5 epitope tag (underlined), and Hcf6ctb (5' phosphorylated TGGGTTGCTGCAGAAGAGAC) and pHCf6-4.7 as template.

4.4. DNA extraction and DNA-blot analysis

Genomic DNA was extracted from *C. fulvum* mycelium ground to a fine powder in liquid nitrogen as pre-

viously described (Fulton et al., 1995). The DNA was digested with the appropriate restriction nucleases and fractionated by 1% agarose gel electrophoresis. The DNA was denatured and transferred to nylon membranes (Hybond-N, Amersham-Pharmacia Biotech) using standard procedures (Sambrook et al., 1987). Gene-specific probes were prepared by PCR amplification as previously described (Whiteford and Spanu, 2001). Radioactive probes were synthesised using the High Prime DNA labelling kit (Roche Diagnostics) and radioactively labelled [α -³²P]dCTP following the manufacturer's instructions. Hybridizations were carried out at 68 °C for 2 h in “PerfectHyb” solution (SIGMA, H7033) as described by the manufacturers followed by two washes in 2× SSC for 20 min each at 68 °C and two washes in 0.1× SSC for 20 min each at 68 °C.

4.5. Fungal transformations: targeted deletion of *Hcf-6*

Cladosporium fulvum protoplasts were generated and transformed with p $\Delta Hcf-6$ using protocols described previously (Marmeisse et al., 1993). The resulting transformants were screened for absence of the Hcf-6 open reading frame using a filter hybridisation assay as described (Whiteford and Spanu, 2001). Southern blot analysis of the strains that appeared to have lost Hcf-6 by targeted gene deletion was carried out to confirm that the gene was indeed missing.

4.6. Fungal transformations: selection of fungal strains with tagged hydrophobins

The strains *HCF-1^{V5}* and *HCF-6^{V5}* were generated by transforming a Δ *HCF-1* or a Δ *HCF-6* strain respectively with an equimolar mixture of pAN8-1 (conferring phloeomycin resistance) and pHCF-1^{V5} or pHCF-6^{V5}. The resulting transgenics were screened for insertion of the tagged plasmids by filter hybridisation (as described above) using the *HCF-1* or *HCF-6* gene specific-probes as appropriate. The strains that had incorporated one or more copies of the tagged constructs were then screened to select those that expressed a tagged hydrophobin protein detectable using immunofluorescence (see below).

4.7. Immunocytochemical localisation of tagged proteins

Samples to be analysed by immunofluorescence were fixed for 30 min in 4% formaldehyde. Conidia were germinated in 20 μ l B5 medium in the wells of Teflon-coated microscope slides. Cross-sections (50 μ m) of fungal colonies growing on agar, were prepared by sectioning colonies with a freezing stage microtome. The material was then washed three times for 5 min in phosphate buffered saline solution (PBS: 0.8% NaCl; 0.02% KCl; 0.115% Na₂HPO₄; 0.02% KH₂PO₄; pH 7.2). Infected leaves were first set in 1% agarose and the sectioned using the freezing stage microtome. The leaf sections were washed three times for 5 min in 0.05% w/v Tween 20 in PBS (PBST). All samples were then incubated for 1 hour in 0.5% w/v low fat skimmed milk protein (Marvel) dissolved in PBS. This was followed by three 5-min washes in PBST. The samples were then incubated for 1 h in 0.5 μ g/ml anti-V5 monoclonal antibody (Invitrogen) (in PBST). The material was then washed three times for 5 min in PBST and then incubated in 0.4 mg L⁻¹ FITC-conjugated anti-mouse IgG (Sigma, F9006). The antibodies were diluted in PBST as directed by the manufacturers. All samples were then mounted in Vectashield (Vectalab, H1000) mounting medium. The samples were observed either by conventional fluorescence microscopy (Zeiss, Axioskop 2 Plus) or by confocal scanning microscopy (Leica, TCS SPII).

4.8. Freeze-fracture and transmission electron microscopy of conidial surfaces

The surfaces of *C. fulvum* conidia were viewed as replicas made after freeze-etching. For freeze etching, conidia were fixed in 3% glutaraldehyde in 100 mM potassium phosphate buffer (pH 7.0) for 1 h and washed three times in the same buffer. Samples were cryoprotected by sequential infiltration with 10% (for 1 h) and 20% glycerol (overnight). Conidia were then frozen in Freon 22 (ICI, Runcorn, UK) and nitrogen slush.

Freeze-fracturing was carried out in a Balzers BA 301 (Balzers Pfeiffer GmbH, Lichtenstein) and conidia were then shadowed with carbon and platinum at 45 °C. A backing layer of pure carbon was added at 90 °C and the replicas floated onto distilled water. Replicas were cleaned overnight in 50% chromic acid and washed several times in distilled water before being picked up onto copper grids and viewed with a Jeol 100C transmission electron microscope (Jeol, Tokyo, Japan).

4.9. Infection of tomato plants

All infections were performed on 3-week-old tomatoes (cv Money Maker) as described (Spanu, 1998).

4.10. Hydrophobicity assays

The hydrophobicity of *C. fulvum* conidia was measured using a “microbial-adhesion to hydrocarbon” (MATH) assay, as previously described (Whiteford and Spanu, 2001).

4.11. Adhesion assays

We measured the adhesion of wild type and of the Δ *HCF-6* *C. fulvum* strains to a variety of solid substrates. Conidia were suspended in 20 μ l B5 growth medium and placed on glass microscope slides, Parafilm, PTFE (Teflon), GelBond (both the hydrophobic and the hydrophilic sides) and onto tomato leaf discs. The fungi were allowed to germinate and grow for 24 h at 25 °C in humidified chambers that prevented the medium from drying out. The germinated conidia were observed under the microscope and photographs were taken. We then pipetted 1 ml of water onto the position where the conidia had incubated and the surfaces and the conidia observed again with the microscope.

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