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Surface characteristics of necrotrophic secondary hyphae produced by the bean anthracnose fungus, *Colletotrichum lindemuthianum*

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Abstract

During infection of bean (*Phaseolus vulgaris*), the hemibiotrophic anthracnose pathogen, *Colletotrichum lindemuthianum*, initially produces biotrophic primary hyphae that are large-diameter and entirely intracellular, followed by necrotrophic secondary hyphae that are narrower and either intercellular or intracellular. In the present study, transmission electron microscopy of infected tissues prepared by high-pressure freezing and freeze-substitution showed that secondary hyphae have much thinner cell walls (25–40 nm) than primary hyphae (100–130 nm) and are not surrounded by an extracellular matrix. Immunofluorescence labelling with a panel of monoclonal antibodies showed that glycoproteins which are present on conidia, germ-tubes, appressoria, primary hyphae and mycelium grown *in vitro* are absent from the surface of secondary hyphae. Chitin, detected with the lectin wheat germ agglutinin, was the only surface component shared by secondary hyphae and the other fungal cell types. The results suggest that the fungal cell surface becomes modified during necrotrophic growth, with none of the glycoproteins associated with earlier stages of the infection process being produced.

Introduction

Species of *Colletotrichum*, which cause anthracnose diseases on a wide range of host plants, utilise two main infection strategies: intracellular hemibiotrophy or subcuticular/intramural necrotrophy (Bailey et al., 1992; Perfect et al., 1999; O'Connell et al., 2000). In both cases, the initial stages of infection are very similar: conidia germinate on the plant surface, producing germ-tubes and appressoria which penetrate the plant cuticle directly. Intracellular, hemibiotrophic colonisation is exemplified by members of the *C. orbiculare* aggregate species, which includes the bean anthracnose pathogen, *C. lindemuthianum* (O'Connell et al., 1985; 2000). Following initial penetration, this fungus produces globular infection vesicles and then large-diameter primary hyphae which develop inside host cells, between the plasma membrane and cell wall, and feed biotrophically on living host cells. Subsequently, the pathogen switches to a destructive, necrotrophic mode of nutrition, associated with the production of narrow, secondary hyphae, which grow inter- and intracellularly and intramurally and ramify rapidly through the host tissues. At this stage, host protoplasts are killed, and host cell walls degraded, in advance of infection (O'Connell and Bailey, 1991; Perfect et al., 1999).

Electron microscopy and studies using monoclonal antibodies (MAbs) and lectins have revealed structural and biochemical differences between the cell surfaces of conidia, germ-tubes, appressoria and biotrophic intracellular hyphae of *C. lindemuthianum*

(O'Connell 1991; O'Connell et al., 1996; 2000; Perfect et al., 1999). A pre-formed fibrillar spore coat surrounds the spore cell wall and is involved in the adhesion of conidia to hydrophobic surfaces (O'Connell et al., 1996; Hughes et al., 1999). The MAb UB20 binds to several glycoproteins in the spore coat, including a major hydrophobic component at 110 kDa (Hughes et al., 1999). Germ-tubes and appressoria have similar surfaces, comprising hair-like fimbriae and a secreted extracellular matrix that mediates strong attachment of these infection structures to the substratum. Two MAbs, UB26 and UB31, label high molecular weight glycoproteins in this extracellular matrix and also label footprints of material that remain attached to substrata after removal of cells by sonication, which suggests that the MAbs recognise adhesive components (O'Connell et al., 1996; Pain et al., 1996). Mature appressoria of *C. lindemuthianum* are also characterised by the presence of melanin pigments in their walls (Bailey et al., 1992). Biotrophic intracellular hyphae are surrounded by an interfacial matrix that separates the fungal wall from the invaginated host plasma membrane (Pain et al., 1994a,b). A proline-rich fungal glycoprotein, designated CIH1, has been identified in this matrix using the MAb UB25 (Pain et al., 1994b; Perfect et al., 1998). In addition to labelling conidia and infection structures, all of the above MAbs label mycelia cultured *in vitro* on solid Mathur's medium (Mathur et al., 1950; Pain et al., 1992; 1996; O'Connell et al., 1996; Perfect et al., 2000).

In this paper, we extend the comparison of the cell surfaces of *C. lindemuthianum* infection structures, using immunofluorescence with a range of MAbs to examine the composition of secondary hyphae produced during the necrotrophic phase of infection. Previously, UB25 was shown to bind to primary hyphae but not to secondary hyphae (Perfect et al., 2000). Here, the affinity for secondary hyphae of the other *Colletotrichum*-specific MAbs described above was investigated, together with MAb UB7, which recognises *N*-linked carbohydrate side-chains on glycoproteins in a range of fungi (Mackie et al., 1991; Mitchell et al., 1997) and labelled mycelia of *C. lindemuthianum* grown *in vitro* (Pain et al., 1992). In addition, the ultrastructure of primary and secondary hyphae are compared using freeze-substitution transmission electron microscopy (TEM). Overall, the results show that the surface of the necrotrophic secondary hyphae is markedly different in both structure and composition from those of all other infection structures.

Materials and methods

Labelling of infected bean epidermal strips with MAbs and the lectin WGA

Cultures of *C. lindemuthianum* race kappa and seedlings of *P. vulgaris* cv. La Victoire were grown as described previously (Pain et al., 1992). Primary leaves were brush-inoculated with conidial suspension (1×10^6 conidia ml^{-1}) and incubated in humid boxes for three days. Abaxial epidermal strips were taken from infected leaves and transferred to the wells of a 72-well microtitre plate (Nunc, Denmark) containing 250 µl aliquots of all reagents. Fungal infection structures were labelled *in situ* by indirect immunofluorescence (IIF) of infected epidermal strips using a method adapted from Mackie et al. (1993). Strips were permeabilised in 0.1% (v/v) Triton X-100 in phosphate-buffered saline (PBS) for 10 min, washed three times in PBS and blocked in 10% (w/v) bovine serum albumin in PBS before incubation with antibodies or lectins. Samples were treated with undiluted tissue culture supernatants of MAbs UB7 (Mackie et al., 1991), UB20 (Pain et al., 1992), UB26 (Pain et al., 1996) or UB31 (O'Connell et al., 1996), followed by rabbit anti-mouse IgG antibodies conjugated to fluorescein isothiocyanate (FITC) diluted 1 : 50 in PBS. MAb UBIM22, which was raised to rat bone cells, was used as a negative control (Perry et al., 1990). The lectin wheat germ agglutinin (WGA), which binds to chitin in fungal cell walls (O'Connell and Ride, 1990), was used to directly label fungal infection structures within epidermal strips. The labelling protocol was as described above for MAbs except that WGA conjugated with FITC (0.1 mg protein ml^{-1}) was used in place of the primary antibody and the secondary antibody was omitted. Epidermal strips labelled with MAbs or lectin were mounted on multiwell slides in Vectashield antifade solution (Vector Laboratories, Peterborough, UK) and viewed using epi-fluorescence or differential interference contrast (DIC) microscopy (Pain et al., 1992).

TEM of infected bean tissue

For TEM, excised hypocotyl segments of *P. vulgaris* cv. Kievitsboon Koekoek were inoculated with droplets of spore suspension of *C. lindemuthianum* race gamma (Bailey and Deverall, 1971). At seven days after inoculation, strips of infected tissue (approx. 0.5 mm thick)

were removed from beneath the inoculation droplets and 2 mm diameter discs were cut from these with a cork borer. The tissue discs were vacuum-infiltrated with 8% (v/v) methanol in deionised water, frozen in a Balzers HPM 010 high-pressure freezing apparatus, freeze-substituted in acetone containing 2% (w/v) osmium tetroxide and embedded in Epon-Araldite resin (Knauf et al., 1989). Ultrathin sections were stained with uranyl acetate and lead citrate and viewed in a Hitachi H7000 TEM.

Results and discussion

TEM of infected bean tissue during the necrotrophic phase of infection showed that the primary hyphae of *C. lindemuthianum* were large (4–5 μm diameter) with relatively thick (100–130 nm) cell walls, whereas secondary hyphae were narrow (1.5–3.5 μm diameter) with very thin (25–40 nm) walls (Figure 1a,b). As noted by Heath and Skalamera (1997), the production of dimorphic mycelia, with wide biotrophic hyphae and narrow necrotrophic hyphae, is a characteristic feature of many hemibiotrophs, including *C. destructivum*, *C. truncatum*, *C. sublineolum* and *Magnaporthe grisea* (Wharton et al., 2001; O'Connell et al., 1993; Latunde-Dada et al., 1996; Heath et al., 1990). The larger surface area : volume ratio and thinner walls of the secondary hyphae would be advantageous for both efficient nutrient uptake and secretion of wall-degrading enzymes, and possibly toxins, during the necrotrophic growth of these fungi. On the other hand, large-diameter hyphae may help the pathogen avoid host recognition during biotrophic growth inside living host cells by minimising the area of contact with the host plasma membrane, while a thick hyphal wall may provide protection from host defences such as lytic enzymes.

During the necrotrophic phase of *C. lindemuthianum*, primary and secondary hyphae inside dead host cells were not surrounded by any extracellular matrix (Figure 1b). In contrast, an interfacial matrix separates primary hyphae from the plasma membrane of living host cells during biotrophic growth (Pain et al., 1994b), but this material disappears following rupture of the host plasma membrane at the end of the biotrophic

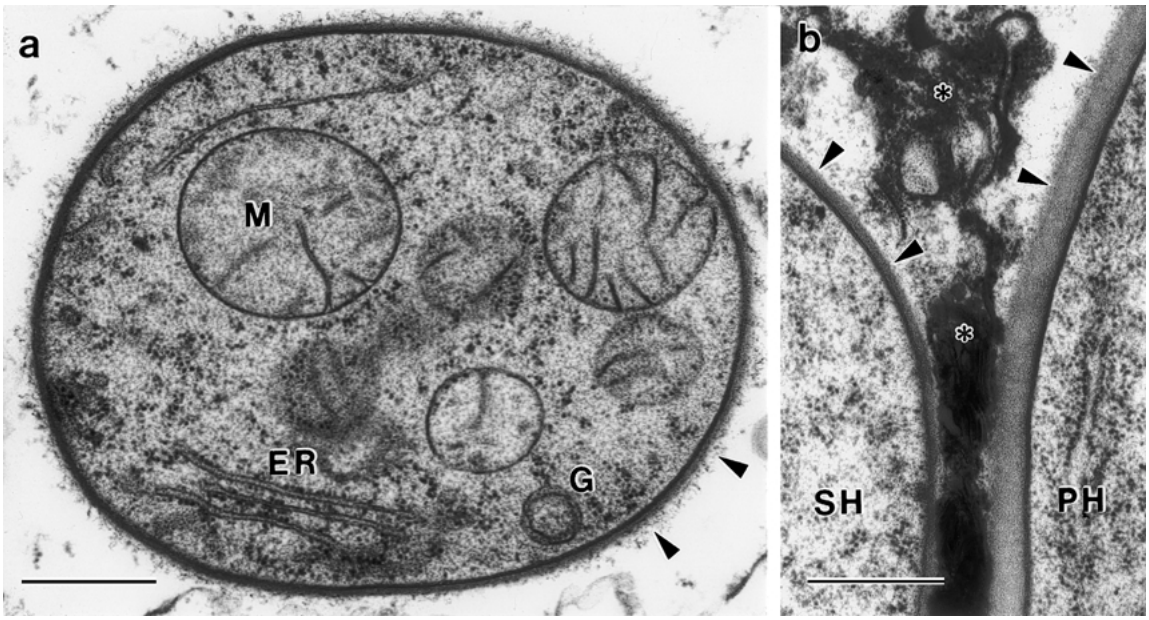

Figure 1. Transmission electron micrographs showing transverse sections through the primary and secondary hyphae of *C. lindemuthianum* during the necrotrophic phase of infection in hypocotyl tissue of *P. vulgaris* (seven days after inoculation). Samples were prepared by high-pressure freezing and freeze-substitution. Bars = 0.5 μm. (a) Secondary hypha inside a dead host mesophyll cell. Note the hyphal wall is thin and sparsely covered by electron-opaque, flocculent material (arrowheads). M: mitochondria; ER: endoplasmic reticulum; G: circular Golgi equivalent. (b) Part of secondary hypha (SH) and adjacent primary hypha (PH) inside a dead host mesophyll cell. Note the difference in thickness of the hyphal walls (arrowheads). Asterisk: electron-opaque host cell debris.

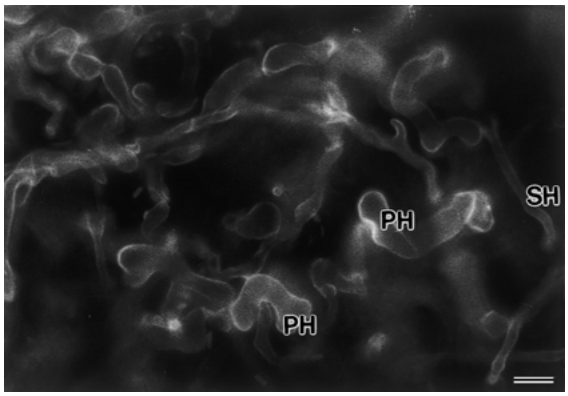

Figure 2. Infection structures of *C. lindemuthianum* in bean leaf epidermal cells labelled with the lectin FITC-WGA and viewed by epi-fluorescence microscopy. PH: primary hypha; SH: secondary hypha. Bars=10 μm.

phase (O'Connell et al., 1985). The interfacial matrix is rich in CIH1 glycoprotein, but primary hyphae in dead host cells continue to label strongly with MAb UB25 (Perfect et al., 1998), probably because CIH1 is also present in the cell walls of these hyphae (Pain et al., 1994b).

When infected epidermal strips were probed with the lectin WGA, primary and secondary hyphae were strongly labelled (Figure 2), indicating that the walls of both types of hypha contained chitin. Conidia, germ-tubes and appressoria were also strongly labelled by WGA, as reported elsewhere (O'Connell, 1991). The fact that labelling occurred at every stage of fungal development indicates that chitin is an important structural component in the walls of all infection structures, and is consistent with previous findings using colloidal gold-conjugated WGA for EM localisation of chitin (O'Connell and Ride, 1990). WGA labelling also demonstrated that the cell surfaces of all infection structures within bean epidermal strips were accessible to the MAb probes used.

Immunofluorescence on infected epidermal strips showed that MAb UB20 labelled conidia, germ-tubes, appressoria, infection vesicles and primary hyphae, as observed previously (Pain et al., 1992; Hughes et al., 1999), but did not label secondary hyphae (Figure 3a,b). UB26 labelled germ-tubes and appressoria, as found previously (Pain et al., 1996), but did not label either primary or secondary hyphae (Figure 3c,d). UB31 gave a similar pattern of labelling to that of UB26 (results not shown). UB7 labelled conidia, germ-tubes and appressoria, but did not label infection vesicles, primary hyphae or secondary hyphae (Figure 3e,f). UBIM22 did not label any structures in these samples (results not shown), indicating that the murine MAbs used in this study did not bind non-specifically to fungal cells.

The results of labelling *C. lindemuthianum* infection structures with MAb and lectin probes are summarised, together with earlier cytochemical data, in Table 1. It is apparent from this comparison that the only surface component shared by secondary hyphae and the other fungal cell types is the polysaccharide, chitin. The absence of labelling of secondary hyphae by all the MAbs (Table 1) shows that they do not contain any of the glycoproteins produced at earlier stages of the infection process, suggesting that the fungal cell surface becomes modified during necrotrophic growth. Although glycoproteins could not be detected on secondary hyphae with the available panel of MAbs, it is possible that the fungus produces a different set of surface glycoproteins at this stage.

The observed differences in surface composition are probably related to the specialised functions of each fungal structure in the infection process. Conidia, germ-tubes, appressoria, infection vesicles and primary hyphae all interact closely with host cells in processes such as adhesion, signalling, maintenance of host viability or avoidance of host recognition. These interactions may require specialised surface structures (e.g. the spore coat on conidia and fimbriae on germ-tubes and appressoria) or secreted matrices (e.g. the interfacial matrix around biotrophic primary hyphae). In contrast, the necrotrophic secondary hyphae are involved in rapid colonisation and complete utilisation of the host tissue as a nutrient source, using a battery of degradative enzymes to solubilise host wall polymers and disrupt host protoplasts (Wijesundera et al., 1989; O'Connell and Bailey, 1991; Centis et al., 1997). Mycelia growing *in vitro* are similarly involved in rapid colonisation and utilisation of a nutrient source. However, unlike secondary hyphae growing in host tissue, mycelia *in vitro* are labelled by all the MAbs (Table 1; Pain et al., 1992; 1996; O'Connell et al., 1996; Perfect et al., 2000). For these experiments, mycelia were cultured on a solid medium containing glucose as a carbon source and mycological peptone as an organic nitrogen source (Mathur et al., 1950). It is possible that the availability of such nutritional factors could affect the expression of surface glycoproteins by mycelia *in vitro*.

The start of the necrotrophic phase coincides with increased expression of wall-degrading enzymes such

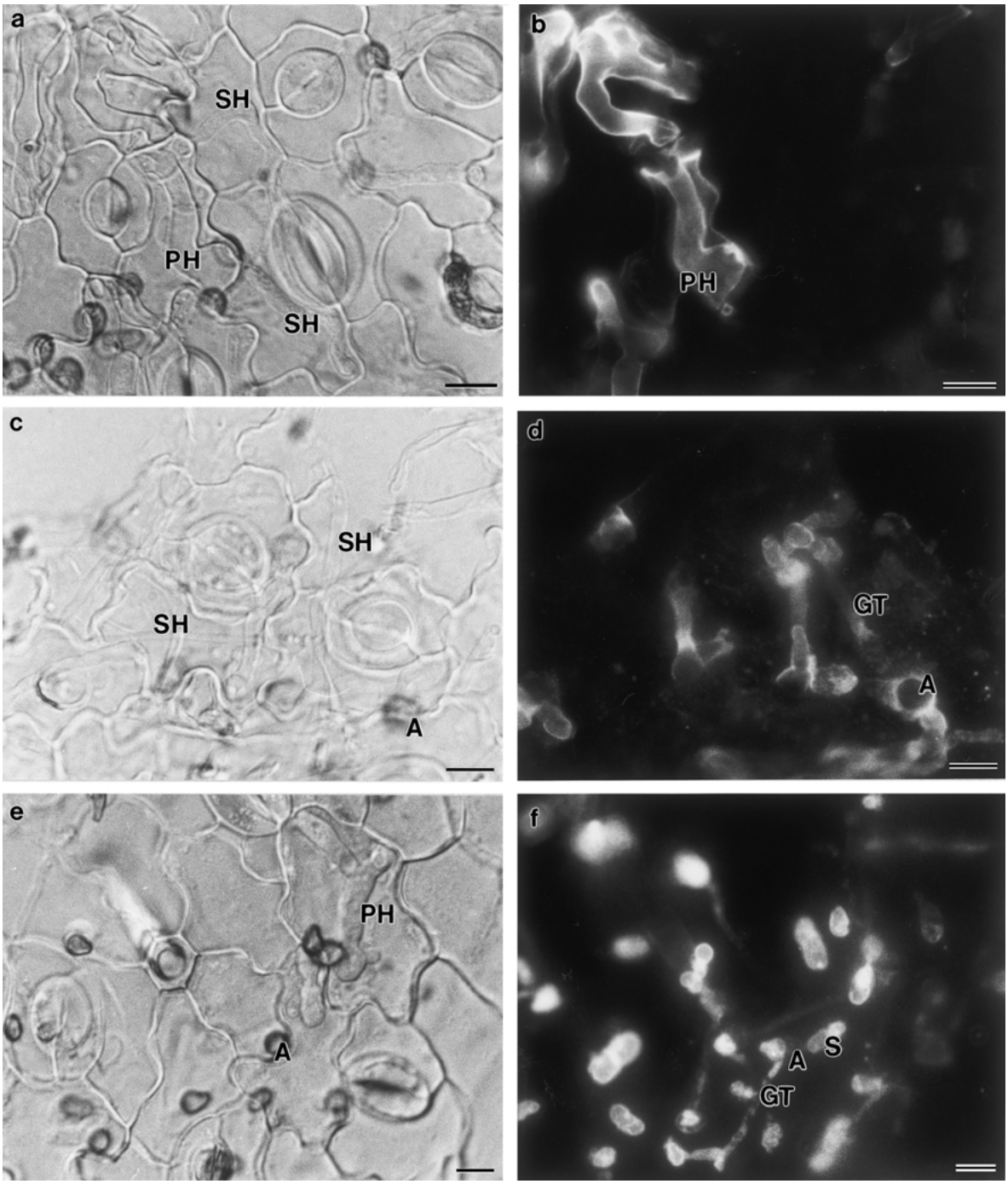

Figure 3. Infection structures of *C. lindemuthianum* in bean leaf epidermal cells labelled with MAbs and viewed with DIC microscopy (a, c, e) or epi-fluorescence microscopy (b, d, f). Infected epidermal strips were labelled with UB20 (a, b), UB26 (c, d) and UB7 (e, f). S: spore, GT: germ-tube, A: appressorium, PH: primary hypha, SH: secondary hypha. Bars = 10 μm.

Table 1. Binding characteristics of MAbs and the lectin WGA to the conidia, infection structures and *in vitro*-grown mycelium of *C. lindemuthianum*

Probe	Mycelia *in vitro*	Conidia	Germ-tubes	Appressoria	Primary hyphae	Secondary hyphae
WGA[1]	+	+	+	+	+	+*
UB20[2,3]	+	++	+	+	++	−*
UB31[4]	+	−	+	++	−	−*
UB7[2]	+	+*	+*	+*	−*	−*
UB25[5,6]	+	−	−	−	++	−
UB26[7]	+/−	−	++	+	−	−*

UB20, UB31 and UB7 recognise carbohydrate epitopes, while UB25 and UB26 recognise protein epitopes in cell surface glycoproteins.
References: 1: O'Connell and Ride (1990); 2: Pain et al. (1992); 3: Hughes et al. (1999); 4: O'Connell et al. (1996); 5: Perfect et al. (1998); 6: Perfect et al. (2000); 7: Pain et al. (1996).
*Results from present work, not previously reported.

Table 2. Comparison of the primary and secondary hyphae of *C. lindemuthianum*[1]

	Primary hyphae	Secondary hyphae
Nutrition	Biotrophic	Necrotrophic
Development	Intracellular	Intra- and intercellular, intramural
Diameter	4–5 μm	1.5–3.5 μm
Cell wall	Thick (100–130 nm)	Thin (25–40 nm)
Growth rate	Slow	Rapid
Interfacial matrix	Present	Not present
Surface glycoproteins	Present	None detected
Host wall dissolution	Highly localised	At a distance
Host wall penetrations	Hyphae constricted	Hyphae not constricted

[1]Compiled with data from the present study and O'Connell et al. (1985).

as endo-polygalacturonase (CLPG1) and endo-pectin lyase by *C. lindemuthianum* (Wijesundera et al., 1989; Centis et al., 1997). However, the distinct morphology and composition of the secondary hyphae reported here and in earlier studies (Table 2) indicates that many other changes in fungal gene expression occur during the transition to necrotrophy. Currently, we are screening cDNA libraries prepared from infected bean tissue to search for fungal genes that are differentially expressed in the necrotrophic phase. Ultimately, this may provide clues to the molecular regulation of shifts in the nutritional status of this hemibiotrophic pathogen.

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