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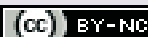
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Negative correlation between phospholipase and esterase activity produced by *Fusarium* isolates

K. Ishida¹, D.S. Alviano³, B.G. Silva^{1,3}, C.R. Guerra¹, A.S. Costa¹,
M. Nucci², C.S. Alviano³ and S. Rozental¹

¹Laboratório de Biologia Celular de Fungos, Instituto de Biofísica Carlos Chagas Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

²Departamento de Clínica Médica, Serviço de Hematologia, Hospital Universitário Clementino Fraga Filho, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

³Departamento de Microbiologia Geral, Instituto de Microbiologia Prof. Paulo de Góes, Universidade Federal do Rio de Janeiro, Rio de Janeiro, RJ, Brasil

Abstract

Fusarium species have emerged as one of the more outstanding groups of clinically important filamentous fungi, causing localized and life-threatening invasive infections with high morbidity and mortality. The ability to produce different types of hydrolytic enzymes is thought to be an important virulence mechanism of fungal pathogens and could be associated with the environment of the microorganism. Here, we have measured the production of two distinct lipolytic enzymes, phospholipase and esterase, by sixteen *Fusarium* isolates recovered from the hospital environment, immunocompromised patients' blood cultures, foot interdigital space scrapings from immunocompromised patients, and foot interdigital space scrapings from immunocompetent patients (4 isolates each). Fourteen of these 16 isolates were identified as *Fusarium solani* species complex (FSSC) and two were identified as *F. oxysporum* species complex (FOSC). Some relevant genus characteristics were visualized by light and electron microscopy such as curved and multicelled macroconidia with 3 or 4 septa, microconidia, phialides, and abundant chlamydospores. All *Fusarium* isolates were able to produce esterase and phospholipase under the experimental conditions. However, a negative correlation was observed between these two enzymes, indicating that a *Fusarium* isolate with high phospholipase activity has low esterase activity and vice versa. In addition, *Fusarium* isolated from clinical material produced more phospholipases, while environmental strains produced more esterases. These observations may be correlated with the different types of substrates that these fungi need to degrade during their nutrition processes.

Key words: *Fusarium*; Hospital isolates; Phospholipase; Esterase

Introduction

Fusarium species are important pathogens causing invasive fungal disease in severely immunocompromised patients, especially those with hematologic malignancies (1). More than 50 species of *Fusarium* have been identified, including plant and animal pathogens, but only few species are related to human disease. The more frequent species causing human disease are *Fusarium solani*, *F. oxysporum* and *F. moniliforme* (1). Traditionally, the identification of *Fusarium* species has been performed by observation of the macro- and micromorphologic characteristics of the mycelium and reproductive structures such as colony

pigmentation, banana-shaped or oval conidial morphology, and the presence of multicellular macroconidia with a foot cell at the base. However, these methods are laborious, time-consuming and require a qualified professional. More recently, molecular techniques have replaced morphological ones and are considered to be gold standard procedures for fungal identification (2).

The major portal of entry for *Fusarium* spp in human disease is the inhalation of air-spread aerosols of *Fusarium* conidia, followed by skin tissue breakdown. The hospital water system may serve as a reservoir for *Fusarium* spp

Correspondence: S. Rozental, Laboratório de Biologia Celular de Fungos, Instituto de Biofísica Carlos Chagas Filho, UFRJ, Av. Carlos Chagas Filho, 373, Bloco C, Sala C0-026, 21941-902 Rio de Janeiro, RJ, Brasil. Fax: +55-21-2280-8193. E-mail: rozental@biof.ufrj.br

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and showering may be an efficient mechanism for the airborne dispersion of fungal conidia and its transmission to immunocompromised hosts in the hospital environment (3).

The clinical forms of fusariosis depend largely on the immune status of the host and the portal of entry of the infection. Keratitis and onychomycosis are the most frequent diseases in immunocompetent individuals, whereas disseminated (and frequently fatal) disease predominates in severely immunocompromised patients (1). Several fungal virulence factors are associated with the pathogenesis of fusariosis, such as the ability to adhere to prosthetic material, secretion of mycotoxins (trichothecenes), which suppress humoral and cellular immunity and may cause tissue breakdown, and production/secretion of extracellular enzymes including proteases, collagenases, phospholipases, and esterases (4).

In general, the lipases, including phospholipases and esterases, are enzymes that hydrolyze phospholipids to fatty acids. Lipases have an important function in fungal infection as they hydrolyze fat and provide fatty acid residues for fungal nutrition, mainly in the subcutis and cuts (5). Depending on the different and specific ester bonds cleaved, these enzymes have been classified into phospholipases A, B, C, and D. Phospholipase production is associated with host cell-membrane damage, which can promote cell damage and/or expose receptors to facilitate adherence and fungal invasion (6). Esterase (or monoacylglycerol lipase) hydrolyzes monoacylglycerol with a long-chain fatty acid (C_{12} or more).

The aim of the present study was to determine the production of extracellular enzymes, such as phospholipase and esterase, by *Fusarium* species isolates recovered from biological material from immunocompetent and immunocompromised patients, and from the hospital environment.

Material and Methods

Fungal isolates

We selected 16 *Fusarium* isolates recovered from the wall and water of the bathroom of the intensive care unit (IA-1, IA-2, IA-3, and IA-4), immunocompromised patients' blood cultures (H-1, H-2, H-3, and H-4), foot interdigital space scrapings from immunocompromised patients (RTMO-1, RTMO-2, RTMO-3, and RTMO-4), and foot interdigital space scrapings from immunocompetent patients (RIC-1, RIC-2, RIC-3, and RIC-4). Fourteen of these 16 isolates were identified as *F. solani* species complex (FSSC) and two were identified as *F. oxysporum* species complex (FOSC) by sequencing as previously described (2) and reported elsewhere (7). *Fusarium* sp isolates were cultivated on Sabouraud dextrose agar (SDA) plates for 10 days at 28°C and subcultures were then taken at the same time and under the same growth conditions.

Morphological analysis

Light microscopy. Mycelial structures of each isolate were carefully examined in the preparations obtained from microcultures inoculated onto SDA medium between glass slides and coverslips. Briefly, this technique is performed using a sterile 15-cm diameter Petri dish whose bottom is covered with filter paper or cotton mesh, and 2 glass slides are placed onto a U-shaped glass rod. SDA medium was cut with a sterile scalpel blade into 1-cm² squares, which were placed on each microculture chamber slide. Each fungal colony was inoculated onto the 4 sides of each culture medium square and a sterile coverslip was placed over the medium. The microculture chamber was maintained at 28°C for 7 to 10 days under humid conditions for the observation of colony growth and conidiation. The vegetative and reproductive mycelia of *Fusarium* sp adhered to the coverslips were stained with lactophenol cotton blue, dripped onto the glass slides and examined with light microscope (Axiostar plus, Zeiss, Germany).

Scanning electron microscopy. *Fusarium* sp isolates obtained by microculture as described previously were fixed in a solution of 2.5% glutaraldehyde and 4% freshly prepared formaldehyde in 0.1 M cacodylate buffer, pH 7.2, for 2 h at room temperature. After fixation, the isolates were post-fixed for 2 h in 1% osmium tetroxide containing 1.25% potassium ferrocyanide and 5 mM $CaCl_2$ in 0.1 M cacodylate buffer, pH 7.2, dehydrated in ethanol, critical-point dried in CO_2 , coated with gold, and then observed with a JEOL JSM-5310 (Japan) scanning electron microscope.

Phospholipase assay

Phospholipase production was determined using egg yolk agar plates (SDA medium supplemented with 1 M NaCl, 5 mM $CaCl_2$, and 8% sterile egg yolk emulsion) as described by Price et al. (8). In this method, egg yolk digested by phospholipase produces precipitation around fungal colonies. Ten microliters (1×10^7 conidia) of a suspension of each strain was placed in the center of the egg yolk agar plate (9-cm diameter Petri dishes). After incubation at 37°C for 15 and 30 days, the colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured with a digital pachymeter.

Esterase assay

Esterase production by *Fusarium* was assayed using SDA medium, consisting of 10 g peptone, 5 g NaCl, 0.1 g $CaCl_2$, 15 g agar, and 1000 mL distilled water, with pH adjusted to pH 6.5 (9). The medium was autoclaved, cooled to about 50°C, and 5 mL autoclaved Tween 80 (Sigma, USA) was added. The suspension of 1×10^7 conidia (10 μ L) was inoculated and plates were incubated at 37°C up to 30 days. The colony diameter (a) and the diameter of the colony plus the precipitation zone (b) were measured with a digital pachymeter.

Enzymatic score and statistical analysis

Phospholipase and esterase activities are reported as Pz value (a/b) as described by Price et al. (8). According to this definition, low Pz values indicate high phospholipase or esterase production and, conversely, high Pz values indicate low enzymatic production. The Pz value was scored into four categories: high Pz values of 1.0 were scored as negative enzymatic production, Pz values between 0.999 and 0.700 as low enzymatic production (+), Pz values between 0.699 and 0.400 as moderate enzymatic production (++), and Pz values between 0.399 and 0.100 as high enzymatic production (+++). All experiments were repeated at least three times and all systems were performed in triplicate.

Data were analyzed statistically using the GraphPad

Prism program version 4.0 and a P value <0.05 was considered to be significant. The Pearson test was performed to correlate phospholipase and esterase activities and the Student *t*-test to compare the enzyme production by the fungal isolate group (IA, H, RTMO, and RIC).

Results and Discussion

Typical morphological characteristics of the *F. solani* and *F. oxysporum* species complex (FSSC and FOSC, respectively) were observed independently of the site of fungal isolate, as visualized by light and scanning electron microscopy (Figure 1). The 14 FSSC isolates presented characteristic structures of this group such as curved and

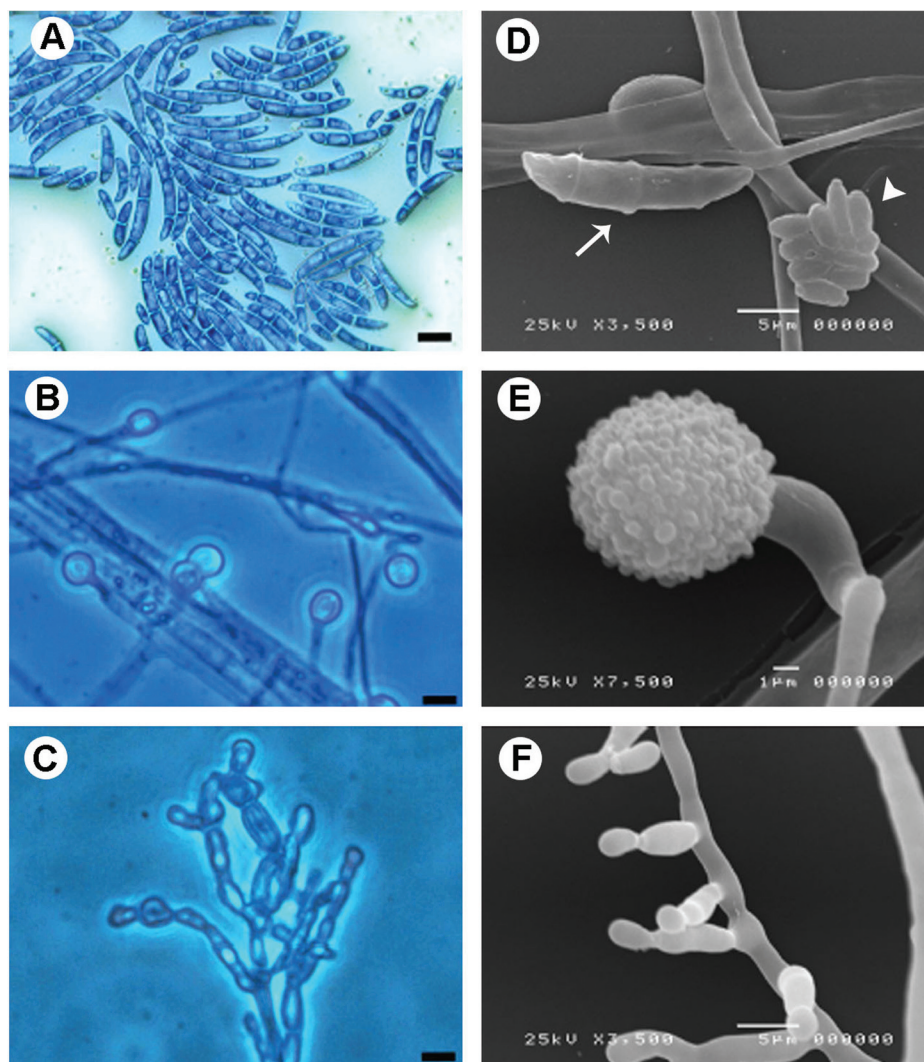


Figure 1. Light (A-C) and scanning electron microscopy micrographs (D-F) of *Fusarium* spp reproductive structures. *Fusarium oxysporum* (A) and *F. solani* species complex (D, white arrow) macroconidia. *F. solani* species complex microconidia can also be observed in D (white arrowhead). *F. oxysporum* (B) and *F. solani* (E) species complex chlamydospores. *F. oxysporum* (F) and *F. solani* (C) species complex phialides. Scale bars: 20 µm (A-C), 5 µm (D and F) and 1 µm (E).

multicelled macroconidia with 3 or 4 septa (Figure 1D, white arrow), microconidia (Figure 1D, white arrowhead), lengthy phialides (Figure 1C) and abundant chlamydoconidia (Figure 1E). The two FOSC isolates (both recovered from the intensive care bathroom) presented curved and multicelled macroconidia with 1-4 septa (Figure 1A), chlamydoconidia (Figure 1B) and short phialides (Figure 1F).

Although the clinical manifestations and outcome of fusariosis depend largely on the immune status of the host, the virulence attributes expressed by the fungus enhance the infective process (1). The fungal adhesion to host tissue is an extremely important step for establishing the infection and tissue invasion, by digesting and destroying cell membranes and/or extracellular matrix components (10). The activity of hydrolytic enzymes, including proteases, lipases (phospholipases and esterases) and hemolysins, is associated with adhesion, cell damage, tissue invasion, and blood dissemination in several infections caused by bacteria and fungi (10).

Herein, we evaluated the extracellular esterase (Figure 2B) and phospholipase (Figure 2C) production by the 16 *Fusarium* isolates cultured on modified Sabouraud

medium for 15 and 30 days. These assays, which detect phospholipase and esterase activities by measurement of the zone precipitation around colonies, have been largely employed to study enzyme production by various bacteria and fungi (8,11,12). In the present study, all isolates were able to secrete esterase and phospholipase enzymes (Figure 2A-C and Table 1). The phospholipase and esterase activities were time-dependent, increasing with culture growth time (Table 1). After 30 days of culture, most of the isolates showed moderate phospholipase and/or esterase activity. Interestingly, we observed that three *Fusarium* isolates from the hospital environment (IA-1, IA-2, and IA-3) presented the highest esterase activity (lowest Pz values of 0.3-0.37) (Table 1 and Figure 2E), whereas the isolates scraped from the interdigital spaces presented the highest phospholipase activities (Table 1). It is important to remember that low Pz values mean high phospholipase or esterase production and, conversely, high Pz values indicate low enzymatic production.

Additionally, we observed a negative correlation ($r = -0.43$, $P < 0.05$, Pearson test) between the two lipases studied (phospholipases and esterases; Figure 2D), indicating

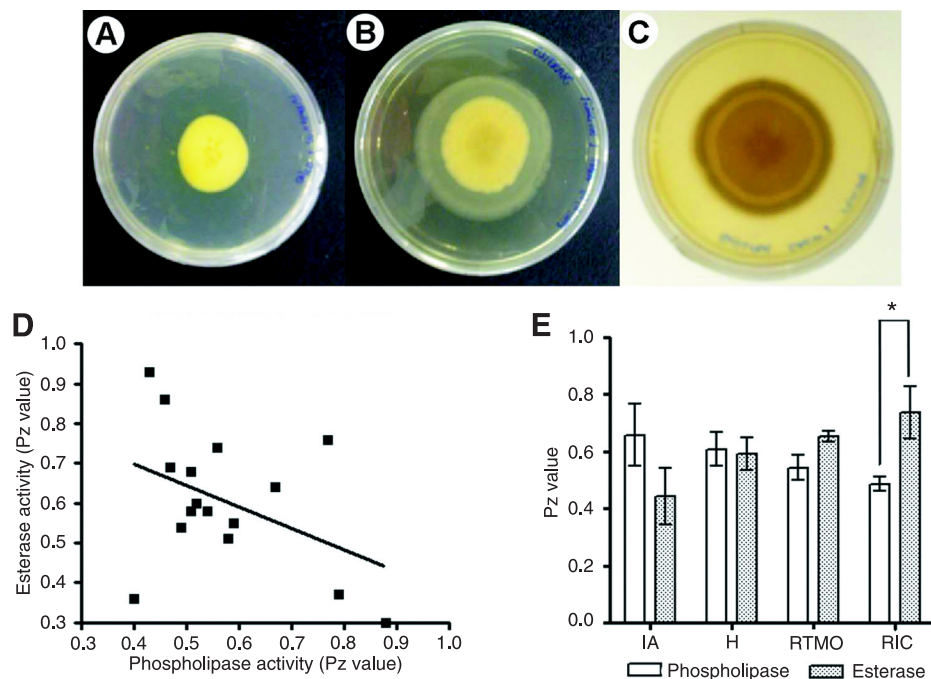


Figure 2. Phospholipase and esterase activities of *Fusarium* spp isolates. A, *Fusarium* sp grown on Sabouraud dextrose agar (SDA, control); B, *Fusarium* sp grown on SDA supplemented with 0.1% Tween 80 - esterase production; C, *Fusarium* sp on SDA supplemented with 2% egg yolk - phospholipase production. D, Negative correlation between phospholipase and esterase Pz values extracted from Table 1 ($r = -0.43$, $P < 0.05$, Pearson test); E, phospholipase and esterase activities by the following fungal isolate groups: hospital environment (IA), blood cultures (H) from immunocompromised patients, foot interdigital space scrapings from immunocompromised patients (RTMO), and foot interdigital space scrapings from immunocompetent patients (RIC). * $P < 0.05$ (Student *t*-test).

that *Fusarium* isolates with high phospholipase activity have a low esterase production and vice versa. The production of enzymes was also analyzed according to fungal group (IA, H, RTMO, and RIC) and compared between them (Figure 2E). We observed that *Fusarium* isolates from clinical material produced more phospholipase, an enzyme that degrades complex lipids such as phospholipids present in tissues. In contrast, environmental strains produced more esterase, allowing them to degrade simple lipids (monoacylphospholipids) that can be present on hospital surfaces and/or in water, and are important to fungal nutrition. In summary, the phospholipase production profile of the different fungal groups was characterized as follow: IA < H < RTMO < RIC, whereas the esterase production was inversely proportional in the same fungal groups: IA > H > RTMO > RIC. Moreover, the isolates scraped from the foot interdigital space of immunocompetent patients (RIC) showed a significant difference between phospholipase and esterase production ($P < 0.05$, Student *t*-test; Figure 2E). Probably, these environmental strains may require an enzymatic system to degrade simple lipids such as monoacylphospholipids - esterase substrate - present in the environment. Meanwhile, further studies analyzing more *Fusarium* isolates from environmental and clinical material should be performed to confirm these hypotheses.

Phospholipases are ubiquitous enzymes with hydrolytic characteristics that attack phospholipids, mainly glycerophospholipids, common to all cell membranes, and involved in diverse processes such as membrane homeostasis, nutrient acquisition and generation of bioactive molecules. Some phospholipases play an important role in fungal pathogenesis and virulence (13). Ibrahim et al. (14) demonstrated that *Candida albicans* isolates with increased phospholipase activity were associated with a higher virulence profile in murine models of disseminated candidiasis. Ghannoum (6) showed that a phospholipase B1 knockout *Candida* strain produced fewer enzymes and was less virulent compared to a wild-type strain. In addition, phospholipase B produced by *Paracoccidioides brasiliensis* is also involved in the process of adhesion and internalization of yeast cells by MH-S cells and may enhance their virulence and the subsequent down-regulation of macrophage activation (15).

In contrast to phospholipase, few studies have shown esterase production by fungi. Kumar et al. (11) described different *Candida* species isolated from immunocompromised patients, such as *Candida albicans*, *C. tropicalis*, *C. lipolytica*, and *C. inconspicua*, which produced esterase,

Table 1. Phospholipase and esterase activities from 16 *Fusarium* spp isolated from hospital environment (IA), immunocompromised patients' blood cultures (H), foot interdigital space scrapings from immunocompromised patients (RTMO), and foot interdigital space scrapings from immunocompetent patients (RIC).

Isolates	Species	Pz values			
		Phospholipase activity		Esterase activity	
		15 days	30 days	15 days	30 days
IA-1	FOSC	0.92 (+)	0.88 (+)	0.77 (+)	0.30 (+++)
IA-2	FSSC	0.92 (+)	0.40 (++)	0.86 (+)	0.36 (+++)
IA-3	FOSC	0.90 (+)	0.79 (+)	0.81 (+)	0.37 (+++)
IA-4	FSSC	0.76 (+)	0.56 (++)	0.85 (+)	0.74 (+)
H-1	FSSC	0.87 (+)	0.77 (+)	0.66 (++)	0.76 (+)
H-2	FSSC	0.83 (+)	0.58 (++)	0.76 (+)	0.51 (++)
H-3	FSSC	0.89 (+)	0.49 (++)	0.83 (+)	0.54 (++)
H-4	FSSC	0.91 (+)	0.59 (++)	0.81 (+)	0.55 (++)
RTMO-1	FSSC	0.84 (+)	0.47 (++)	0.91 (+)	0.69 (++)
RTMO-2	FSSC	0.94 (+)	0.67 (++)	0.88 (+)	0.64 (++)
RTMO-3	FSSC	0.98 (+)	0.51 (++)	0.89 (+)	0.68 (++)
RTMO-4	FSSC	0.96 (+)	0.52 (++)	0.69 (++)	0.60 (++)
RIC-1	FSSC	0.83 (+)	0.46 (++)	nd	0.86 (+)
RIC-2	FSSC	0.84 (+)	0.43 (++)	0.80 (+)	0.93 (+)
RIC-3	FSSC	0.93 (+)	0.51 (++)	0.94 (+)	0.58 (++)
RIC-4	FSSC	0.86 (+)	0.54 (++)	0.82 (+)	0.58 (++)

The isolates were incubated for 15 and 30 days on modified Sabouraud dextrose agar. The Pz value was scored into four categories: high Pz values of 1.0 were scored as negative enzymatic production, Pz values between 0.999 and 0.700 as low enzymatic production (+), Pz values between 0.699 and 0.400 as moderate enzymatic production (++), and Pz values between 0.399 and 0.100 as high enzymatic production (+++). FOSC = *Fusarium oxysporum* species complex; FSSC = *Fusarium solani* species complex; nd = not determined.

and the increase in the level of *C. albicans* esterase activity was correlated with fungal growth (16). More recently, Palmeira et al. (12) reported that high levels of esterase activity in the fungus *Fonsecaea pedrosoi* were related to adherence to mammalian cells. Additionally, *Malassezia furfur*, an important etiologic agent of mycosis on the stratum corneum of human skin, produces high levels of extracellular esterase activity (17). Independently of the source of fungal isolation, all the strains analyzed in the present study showed resistance to amphotericin B, fluconazole and itraconazole in an *in vitro* antifungal susceptibility assay (data not shown).

Invasive fungal infections are serious and escalate health problems. They are associated with high morbidity and mortality (18). Current therapies are limited in safety and/or efficacy and resistant fungal pathogens are an emerging problem. Of the antifungal drugs marketed for systemic use, all, except echinocandins (which target the cell wall synthesis enzyme, β -1,3 glucan synthase) act on the fungal membrane lipid, ergosterol, or its biosynthetic pathways (19). Thus, new drugs acting on novel targets

are needed. Virulence factors, like multifunctional enzyme phospholipase B1, are an example of potential targets for drug discovery (18).

Although some studies have shown the importance of *Fusarium* enzymatic hydrolysis in the production processes of dairy foods such as milk modification (20), our study is the first one to correlate the expression of the preferential hydrolytic enzyme fungal habitat. In this respect, the data obtained in the present study open several possibilities to study phospholipase and esterase activities by *Fusarium*

isolates, such as purification for better biochemical characterization of their activities, and their importance for biotechnology and during fungal-host interaction. In addition, these lipases may be considered to be an interesting target for antifungal drug discovery.

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