Glucosylceramides in *Colletotrichum gloeosporioides* are involved in the differentiation of conidia into mycelial cells

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Abstract Glucosylceramides (GlcCer) were extracted from the plant pathogen *Colletotrichum gloeosporioides* and purified by several chromatographic steps. By using electrospray ionization mass spectrometry and nuclear magnetic resonance, GlcCer from *C. gloeosporioides* were identified as *N*-2′-hydroxyoctadecanoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and *N*-2′-hydroxyoctadecenoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. Monoclonal antibodies against these structures were produced and used as tools for the evaluation of the role of GlcCer in the morphological transition of *C. gloeosporioides*. In the presence of antibodies to GlcCer, the differentiation of conidia into mycelia was blocked. Since GlcCer is present in several plant pathogens, the inhibitory activity of external ligands recognizing these structures may be applicable in other models of fungal infections.

Key words: Glucosylceramide; Monoclonal antibody to GlcCer; *Colletotrichum gloeosporioides*

1. Introduction

Colletotrichum is a large genus of ascomycete fungi that comprises several plant pathogens. Plant diseases caused by *Colletotrichum* species include anthracnose or blight on a wide range of important crop and ornamental plants [1,2]. The control of anthracnose has become increasingly complex because of the recent appearance of isolates resistant to chemical fungicides [1]. In this context, the search for new strategies to inhibit the colonization of plant hosts by *Colletotrichum* species is imperative. The need of new antifungal agents effective against members of the genus *Colletotrichum* is supported by their recent identification as the causing agents of phaeohyphomycosis in human hosts [3].

The infection by *Colletotrichum* species includes stages of spore adhesion and germination, formation of germ tube and appressorium, and development of biotrophic and necrotrophic hyphae [2]. Therefore, the understanding of the process of differentiation by molecular, biochemical, and immunological approaches may reveal the existence of new targets to be used in the inhibition of fungal morphogenesis and, consequently, establishment of infection.

Fungal cerebrosides are apparently involved with morphogenesis in several human and plant pathogens [4]. They are composed of a sugar unit, usually glucose or galactose, bound to a hydrophobic ceramide, containing the conserved sphingobase 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyhexadecanoic acids [4–14]. For that reason, they are also called glycosylceramides or ceramide monohexosides. The involvement of glycosylceramides with fungal differentiation was first reported by Kawai and coworkers [15,16], who demonstrated that these molecules had fruiting-inducing activity in bioassays with *Schizophyllum commune*. The intact 9-methyl-4,8-sphingadienine but not the β-glucopyranosyl residue was essential for this activity. The involvement of cerebrosides in fungal growth was further confirmed by experiments using a family of compounds known to inhibit GlcCer synthase in mammals. Two analogs, D-threo-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (P4) and D-threo-3P,4P-ethylenedioxy-P4, strongly inhibited germination and hyphal growth of *Aspergillus nidulans* and *Aspergillus fumigatus* [17].

Antibodies to cerebrosides have also been recently used as inhibitory tools of the morphological transitions of fungal species. We have demonstrated that the binding of polyclonal anti-GlcCer antibodies to the fungal cell surface inhibits bud formation in *Cryptococcus neoformans* [11], and germ tube formation in *Pseudallescheria boydii* and *Candida albicans* [10]. In the present work, we report on the characterization of cerebrosides in *Colletotrichum gloeosporioides*. They are glucosylceramides (GlcCer) containing 9-methyl-4,8-sphingadienine in amidic linkage to 2-hydroxyoctadecanoic or 2-hydroxyoctadecenoic acids, as demonstrated by electrospray ionization mass spectrometry (ESI-MS) and nuclear magnetic resonance (NMR). Polyclonal and monoclonal antibodies to GlcCer were generated and used in differentiation assays of *C. gloeosporioides*, demonstrating that binding of anti-GlcCer antibodies to the fungal surface results in the inhibition of the morphological transition of conidia to mycelia. These results suggest that external ligands recognizing GlcCer can inhibit fungal morphogenesis, which could be also applicable in plant pathogens expressing similar GlcCer [6,8,18–20].

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Abbreviations: GlcCer, glucosylceramides; ESI-MS, electrospray ionization mass spectrometry; NMR, nuclear magnetic resonance; GC, gas chromatography

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2. Materials and methods

2.1. Microorganism

C. gloeosporioides was kindly supplied by Prof. Rosalie R.R. Coelho from the Soil Laboratory, Departamento de Microbiologia Geral, Instituto de Microbiologia, UFRJ, Rio de Janeiro, RJ, Brazil. Mycelial cells were cultivated at room temperature in Sabouraud's modified liquid medium, containing (g/l) 20 glucose, 10 peptone and 5 yeast extract. After 7 days of growth, fungal cells were obtained by filtration, washed three times with distilled water and stored at −20°C. Conidial forms of *C. gloeosporioides* were grown on malt agar plates containing (g/l) 4 glucose, 4.5 yeast extract, 10 malt extract, 20 agar, at room temperature for 7 days. After this period, a preparation of conidial cells was obtained by washing plate surface with phosphate-buffered saline (PBS). Cells were then washed three times with PBS and counted in a Neubauer chamber.

2.2. Extraction and purification of GlcCer

C. gloeosporioides cells were successively extracted at 28°C with chloroform/methanol 2:1 and 1:2 (v/v). Extracts were combined, dried and the crude lipid extract was partitioned according to Folch and coworkers [21]. The lipids recovered from the Folch lower phase were fractionated on a silica gel column eluted with chloroform, acetone and then methanol. The glycolipid fraction eluted with acetone was purified by further silica gel column chromatography. This column was eluted sequentially with chloroform/methanol (95:5, 9:1, 8:2, 7:3 and 1:1, v/v) and finally methanol. Fractions of 5 ml were collected and analyzed by high-performance thin layer chromatography (HPTLC), developed with chloroform/methanol/water 65:25:4 (v/v). The spots were visualized with iodine and by spraying with orcinol/H_2SO_4. The chloroform/methanol 7:3 (v/v) fraction was further purified by chromatography on Iatrobeads RS 2060 (Macherey &Nagel, Düren, Germany), using the same elution system, yielding a partially purified glycosphingolipid fraction. Final purification was attained after passage through a second Iatrobeads column eluted with chloroform/methanol mixtures (9:1, 85:15, 8:2, 75:25, and 1:1, v/v) and finally methanol. The 8:2 chloroform/methanol fraction contained the finally purified glycolipid, as visualized by HPTLC.

2.3. Sugar analysis

Glycosphingolipids were hydrolyzed with 3 M trifluoroacetic acid at 100°C for 3 h, and the resulting monosaccharides were characterized by TLC and quantified by gas chromatography (GC) as alditol-acetate derivatives [22] using an OV–225 fused silica capillary column (30 m×0.25 mm internal diameter), with temperature programmed from 50 to 220°C at 50°C/min.

2.4. ESI-MS analysis

Glycolipids were analyzed by ESI-MS using an LCQ-Duo ion trap instrument (Finnigan Thermoquest, San Jose, CA, USA). Samples were diluted in chloroform:methanol (1:1, v/v), containing either 10 mM ammonium acetate, 1% ammonium hydroxide or 10 mM lithium iodide, and introduced into ESI-MS at 5–10 μl/min flow rate, with the assistance of an infusion micropump (Harvard Apparatus, Cambridge, MA, USA). Alternatively, samples were delivered (at 10–20 μl/min) to the ion source through the mass spectrometer-embedded valve loop using a solvent delivery system, pressurized with 9–10 psi N_2. Analyses were carried out at both positive (ESI+) and negative (ESI−) ion modes. Source and capillary voltages were 4.5 kV and 3 V (ESI+), or −4.5 kV and −39 V (ESI−). Capillary temperature was kept at 200°C for both ion modes. Spectra were collected at 200–2000 *m/z* range. Source-induced dissociation (SID) was obtained at 25 V. Ion trap collision-induced dissociation (tandem ESI-MS/MS or ESI-MS/MS) experiments were carried out at 20–40% (1–2 eV) energy. All spectra were processed using the Xcalibur software (Finnigan Thermoquest).

2.5. ^{1}H-NMR

A sample of GlcCer was dissolved in 0.5 ml $CDCl_3$–CD_3OD (2:1 v/v) (99.9%; Merck, Darmstadt, Germany). The ^{1}H-NMR spectrum was acquired at 400 MHz using a Bruker Advance 400 spectrometer, at a probe temperature of 303 K and a sweep width of 6127 Hz with 64K points.

2.6. Monoclonal antibodies to GlcCer

A purified GlcCer fraction (50 μg) was resolved by TLC on a silica plate. The purified cerebroside was scraped from the plate and, together with the silica, was suspended in phosphate buffer (pH 7.2), followed by sonication and emulsification with complete Freund's adjuvant. The final mixture (200 μl) containing silica and 50 μg of the purified GlcCer was then used to immunize 6 weeks old BALB/c mice intraperitoneally. After 4 weeks, this procedure was repeated, using incomplete Freund's adjuvant. One week later, a last intraperitoneal injection was made, containing no adjuvant. 3 days before fusion, the animals were boosted with an intrasplenic injection of 50 μg of silica-adsorbed GlcCer.

Animals were killed and their spleens were removed and rinsed. For cellular fusion, the myeloma mouse cell line Sp2/0 was then mixed in a ratio of 1:5 with spleen cells. While gently shaking the cell mixture, 1 ml of a solution of polyethylene glycol (PEG 3000) was slowly added drop by drop. The cell mixture was diluted in 10 ml of RPMI-1640 medium, followed by settling for 15 min and centrifugation for 10 min (4°C, 200×*g*). The cells were resuspended in RPMI-HAT medium supplemented with bovine fetal serum (10%) and plated onto the wells of a flat-bottomed polystyrene microtiter plate. 10 days after the fusion, hybridomas producing antibodies to GlcCer were screened by enzyme-linked immunosorbent assay (ELISA) [11]. The hybridoma culture whose supernatant had produced the highest level of antibodies to GlcCer was expanded and cloned by limiting dilution, over a feeder layer of macrophages, in a 96-well microtiter plate. The feeder layer was obtained by washing the peritoneal cavity of non-stimulated BALB/c mice with 3 ml of cold RPMI. Peritoneal cells were resuspended in cold RPMI-HAT supplemented with bovine fetal serum and distributed over microtiter plates (5×10^3 cells per well). For preparation of antibodies in higher concentrations, antibody-producing cells were injected into the peritoneal cavity of BALB/c mice. Antibodies to GlcCer were purified by protein G affinity chromatography from ascitic fluids and isotyped as IgG2b using the Sigma ISO/2 kit.

2.7. Polyclonal antibodies to GlcCer

Polyclonal anti-GlcCer antibodies were obtained from serum of a rabbit immunized with *P. boydii*, an opportunistic fungus that also expresses cerebrosides similar to those described in this work [10]. Antibodies were purified by incubation of serum with immobilized GlcCer followed by acid elution and immediate neutralization, as previously described [10,11]. As expected, purified antibodies cross-reacted with the purified GlcCer from *C. gloeosporioides*, as inferred from ELISA and immunofluorescence reactions (data not shown). To exclude the possibility of unspecific recognition of fungal structures by antibodies to GlcCer, their reactivity with protein and lipid extracts from fungal cells was also evaluated. As demonstrated by immunostaining and Western blot analyses, GlcCer from *C. gloeosporioides* were the only structures recognized by purified antibodies (not shown).

2.8. Immunofluorescence

C. gloeosporioides conidia and mycelia were fixed in 4% paraformaldehyde cacodylate buffer (0.1 M, pH 7.2) for 1 h at room temperature. Systems were rinsed in PBS and then in 1% bovine serum albumin (BSA) in PBS for 1 h for further incubation with monoclonal antibody to GlcCer (10 μg/ml) and fluorescein isothiocyanate (FITC)-labeled anti-mouse IgG (at 1:100 dilution). Control cells, which were incubated with the fluorescein conjugate but not with antibodies to GlcCer, were also prepared. Fluorescent reactions were observed in a Zeiss epifluorescence microscope (Axioplan 2).

2.9. Fungal differentiation

10^6 conidia were suspended in 1 ml of a minimal medium (30 mM glycerol, 10 mM $MgSO_4$, 29.4 mM KH_2PO_4, 13 mM glycine, and 3 mM thiamine). This suspension was added to the wells of a 24-well microtiter plate and supplemented with polyclonal or monoclonal antibodies to GlcCer (25 μg/ml). After 8, 12, 18 or 24 h of incubation at 37°C, glutaraldehyde was added to a final concentration of 2.5%. Fungal differentiation into mycelial forms was then assessed in an inverted microscope. Control systems were performed identically, except for the replacement of antibodies to GlcCer by irrelevant immunoglobulins (with no specific affinity for any fungal antigen) of the same organism and isotype. All the systems were performed in tripli-

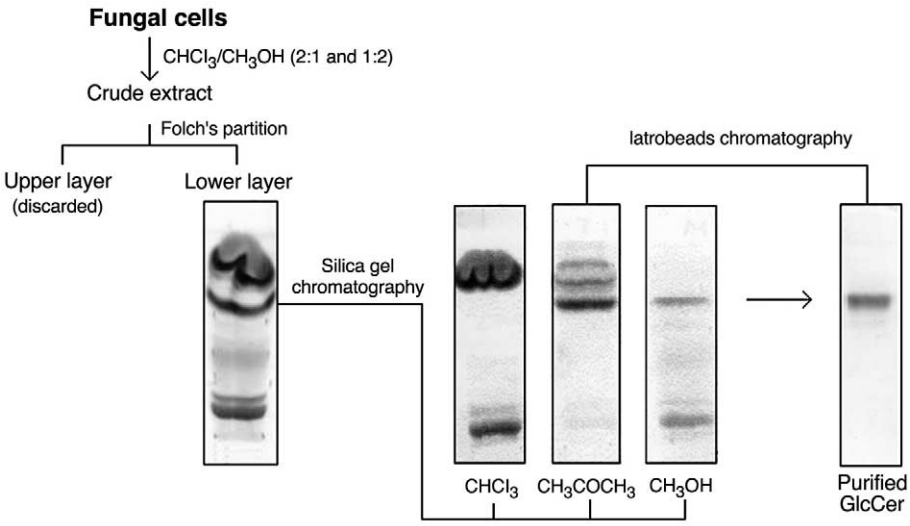


Fig. 1. Purification of GlcCer from *C. gloeosporioides*. Purified or partially purified fractions are visualized by reaction with orcinol-H_2SO_4 after separation by HPTLC. For details of the steps of purification, see Section 2.

cate sets and representative images of these experiments are shown in Section 3.

3. Results

3.1. C. gloeosporioides *expresses conserved GlcCer*

The purification steps of GlcCer from *C. gloeosporioides* are shown in Fig. 1. After extraction with mixtures of chloroform and methanol followed by different steps of chromatographic separation, an orcinol-reactive band was detected by HPTLC, with a chromatographic mobility corresponding to a standard GlcCer from bovine brain.

The GlcCer-enriched fraction was analyzed by positive ion mode ESI-MS, showing a cluster of singly-charged ions with *m/z* ranging from 700 to 860. Two major ion species were observed at *m/z* 754 and 756, as well as their corresponding fragments at *m/z* 736 and 738, generated after loss of water (Fig. 2A). When submitted to ESI-MS/MS, these species gave rise to major daughter ions at *m/z* 592 and 594, respectively, 162 mass units shorter than the parent ions, which most likely corresponds to the loss of a hexose residue (Fig. 2B and D). These daughter ions were tentatively assigned to the ceramide moiety of the GlcCer species. Cleavage of the amide linkage [23,24] is responsible for the low mass ion at *m/z* 294 and its dehydration product at *m/z* 276, which are diagnostic of a C19 sphingadienine (Fig. 2C and E). The ceramide ion species at *m/z* 594 and 592 could be assigned as *N*-2′-hydroxyoctadecanoyl-9-methyl-4,8-sphingadienine and *N*-2′-hydroxyoctadecenoyl-9-methyl-4,8-sphingadienine, respectively.

The constituent monosaccharide was liberated by hydrolysis and identified by TLC and GC-MS as glucose (not shown). In the ^{1}H-NMR spectrum of native GlcCer, protons corresponding to a β-glucopyranosyl residue were recognized by their characteristic chemical shifts (Figs. 3 and 4). The spectrum displayed one sharp doublet at δ 4.27 ppm with a coupling constant ($J_{1,2}$) of 7.85 Hz (Fig. 3), due to the protons bound to anomeric carbon C-1. The remaining ring-bound protons observed in the spectrum were in agreement with the β-glucopyranoside structure. The long-chain base was consistent with a 9-methyl-4,8-sphingadienine structure. A single vinyl resonance (5.11 ppm) for the Δ^8 unsaturation when C-9 is substituted with a methyl group (1.58 ppm) is characteristic of cerebrosides from fungi [6,11,12] and some marine organisms [25]. The resonance at 5.45 ppm was assigned as H-4 of the 9-methyl-4,8-sphingadienine, characteristic of vinyl resonance for the Δ^4 unsaturation which is found in many glycosphingolipids present in fungi [4] as well as in mammals [26].

3.2. Immunofluorescence

Polyclonal and monoclonal antibodies to GlcCer were prepared and incubated with different forms of *C. gloeosporioides*. The binding of these antibodies to the fungal surface was then analyzed by fluorescence microscopy. As shown in Fig. 5, mycelial forms of *C. gloeosporioides* were strongly reactive with monoclonal antibodies to GlcCer. Surprisingly, fungal septa were not recognized by antibodies. Conidial forms also presented a diffuse profile of reactivity with the antibodies, but were less reactive than mycelia. The use of polyclonal antibodies in these experiments produced similar results (data not shown). Control cells, which were incubated with the fluorescein conjugate but not with antibodies to GlcCer, presented insignificant fluorescent reactions (not shown).

3.3. Antibodies to GlcCer inhibit the differentiation of conidia to mycelium in C. gloeosporioides

Several previous reports indicated the existence of a link between cerebrosides in fungi and morphological transitions or fungal growth [11,12,15–17,27]. In this context, we used polyclonal and monoclonal antibodies to GlcCer as tools to evaluate the relevance of glycosphingolipids as targets to inhibit the differentiation of *C. gloeosporioides*. Conidia were incubated in a chemically defined medium supplemented with antibodies to GlcCer and the process of differentiation into mycelia was evaluated after different intervals. In control systems, which were performed in the absence of antibodies (not shown) or in the presence of irrelevant immunoglobulins (Fig. 6), conversion of conidia into mycelia was first detected after 12 h of incubation at 37°C (Fig. 6B). This morphological transition progressively proceeded and reached a maximum

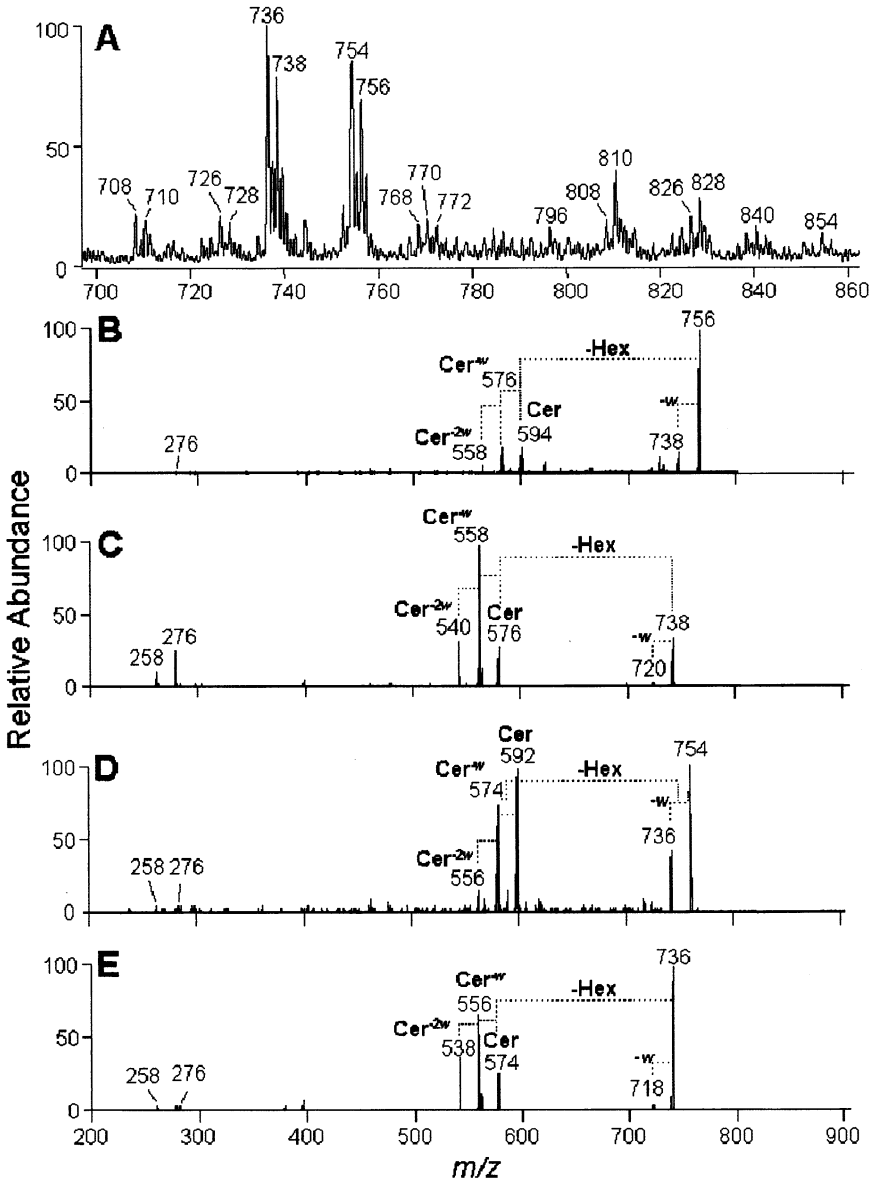


Fig. 2. ESI-MS (positive ion mode) analysis of the GlcCer species of *C. gloeosporioides*. A: MS1 spectrum. B–E: ESI-MS2 of the ion species *m/z* 756, 738, 754 and 736, observed in A. Cer, ceramide; Hex, hexose; w, water.

intensity after 24 h. When the experiment was performed in the presence of the anti-GlcCer monoclonal antibody, the morphological transition of conidial forms into mycelia was delayed; mycelial cells were detected only after 24 h of incubation (Fig. 6E, F). Similar results were observed with polyclonal antibodies to GlcCer but, in this case, the inhibition of differentiation was even more efficient, since no mycelial forms of *C. gloeosporioides* were detected after 24 h of incubation (Fig. 6I–L).

4. Discussion

Species from the genus *Colletotrichum* have been used for many years in studies concerning fungal differentiation and fungal–plant interactions [2]. *Colletotrichum* species develop a series of specialized infection structures including germ tubes, appressoria, intracellular hyphae, and secondary necrotrophic hyphae. Blocking the morphological transition therefore appears to be an interesting alternative to prevent the infection by the plant pathogen *C. gloeosporioides*.

In the present work, GlcCer were extracted from *C. gloeosporioides* cells and characterized as *N*-2′-hydroxyoctadecanoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine and *N*-2′-hydroxyoctadecenoyl-1-β-D-glucopyranosyl-9-methyl-4,8-sphingadienine. Monoclonal antibodies against the GlcCer, which is a very conserved structure in different fungal species, were produced and used as the tools for the evaluation of the role of GlcCer in the differentiation of *C. gloeosporioides*. Our results demonstrated for the first time that molecules recognizing fungal cerebrosides interfere with the process of differentiation of a plant pathogen.

GlcCer have been precisely characterized in a number of fungal species [4–14]. For many years, these molecules were thought to play exclusively structural roles in cell membranes [28], but several results from recent reports argue against this hypothesis [11,12,17,27]. Synthesis of GlcCer by fungi has

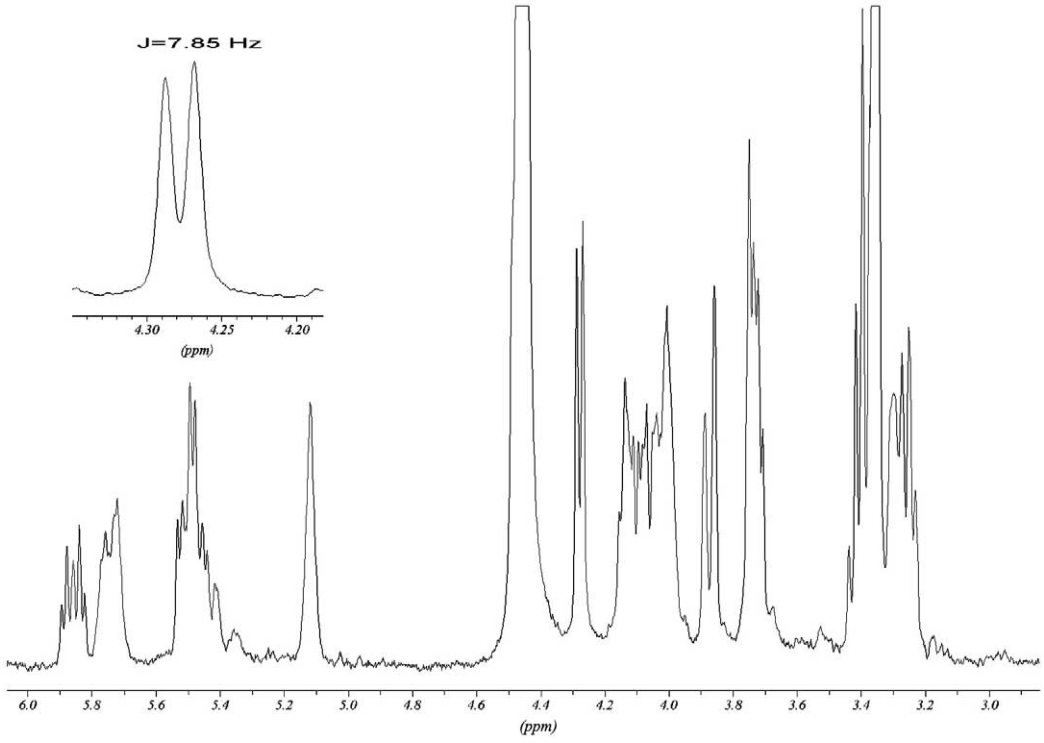
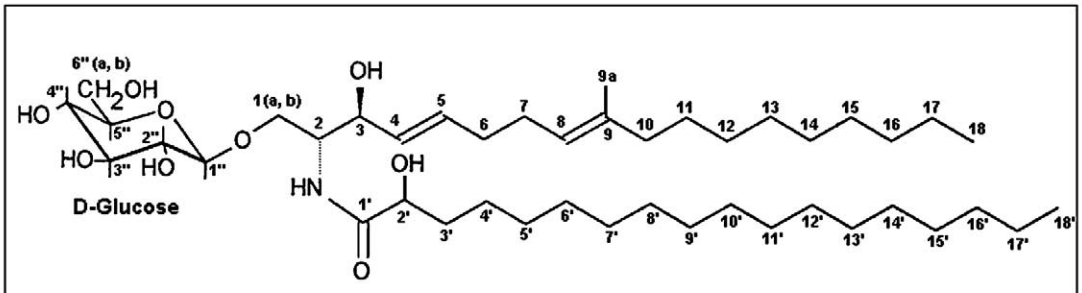


Fig. 3. ^{1}H-NMR spectrum of GlcCer from *C. gloeosporioides*. Inset: Anomeric region (H-1) and coupling constant ($J_{1,2}$ 7.85 Hz).



Glucose		Long-chain base		Fatty acid	
Carbon	δ-^{1}H	Carbon	δ-^{1}H	Carbon	δ-^{1}H
1''	4.27	1 a	3.74	2'	4.46
2''	3,25	1 b	4.06	3'	3.60
3''	3.39	2	4.00	4'	5.84
4''	3.27	3	4.13	5'	2.12
5''	3.29	4	5.45	Aliphatic-CH_3	0.88-2.05
6''a	3.72	5	5.72		
6''b	3.88	6	2.05		
		7	2.05		
		8	5.11		
		9 a	1.58		

Fig. 4. ^{1}H chemical shifts (ppm) for the major GlcCer from *C. gloeosporioides* and its corresponding structure.

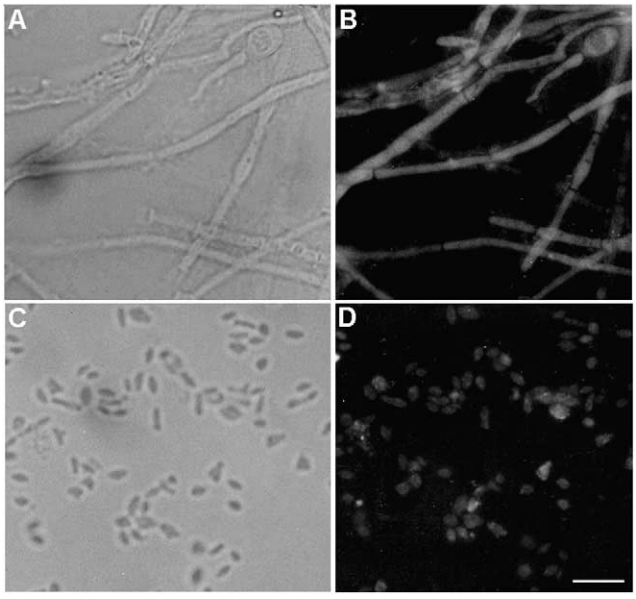


Fig. 5. Immunofluorescence analysis of *C. gloeosporioides* mycelia (A, B) and conidia (C, D) incubated with anti-GlcCer monoclonal antibody. A and C show fungal cells under contrast phase microscopy, while fluorescent images are shown in B and D. Control cells, which had not been incubated with antibodies to GlcCer, did not present any significant fluorescent reaction (shown). Scale bar: 10 μm.

emerged as an interesting target for the action of substances with antifungal properties. For instance, Levery and coworkers [17] reported on the block of germination and hyphal growth of *A. nidulans* and *A. fumigatus* by D-threo-1-phenyl-2-palmitoyl-3-pyrrolidinopropanol (P4) and D-threo-3P,4P-ethylenedioxy-P4, two inhibitors of GlcCer synthase. This enzyme, which is encoded by *GCS* genes, catalyzes the final step in the biosynthesis of GlcCer [29].

Binding of external ligands to GlcCer at the fungal surface also results in the inhibition of fungal growth. In a very recent study, Thevissen and coworkers [27] demonstrated that *gcs* deletion mutants of both *C. albicans* and *Pichia pastoris* are resistant against RsAFP2, an antifungal peptide isolated from radish seed. These observations confirmed previous results demonstrating that the replication of *Saccharomyces cerevisiae*, which does not synthesize GlcCer, is not affected by RsAFP2 [27], which strongly suggests a role of GlcCer in RsAFP2-mediated growth inhibition. Accordingly, GlcCer were identified as the molecular targets for RsAFP2 [27]. In addition, the interaction of RsAFP2 with fungal GlcCer was found to be selective since RsAFP2 did not interact with human or plant GlcCer nor with plant monogalactosyldiacylglycerols. Similarly to RsAFP2, heliomicin, a defensin-like peptide from the insect *Heliothis virescens*, is active on *C. albicans* and *P. pastoris* parental strains, but displays no activity on the *gcs* deletion mutants of both yeast species. Furthermore, heliomicin interacts with GlcCer isolated from *P. pastoris* and soybean but not with human GlcCer [27].

Antibodies to GlcCer have also been used to target glycosphingolipids in fungal cells, producing inhibitory effects [10,11]. They were identified as inhibitors of the morphological transition of *P. boydii*, although they did not influence mycelial growth [10]. Differentiation into mycelial forms is also recognized as a crucial event in tissue invasion by *C. albicans* [30], a fungus that synthesizes GlcCer [9]. As with *P. boydii*, anti-GlcCer antibodies inhibited germ tube formation in *C. albicans* [10]. In addition, they arrested the growth of the human pathogen *C. neoformans* [11]. In these reports, polyclonal antibodies were purified from sera of infected individuals and added to experimental systems of fungal growth or differentiation. In the present work, we aimed to evaluate the effects of a GlcCer-binding structure on the differentiation of the plant pathogen *C. gloeosporioides*. In this context, a monoclonal antibody to GlcCer was produced and used as the GlcCer-binding tool in our experimental model.

From immunofluorescence experiments using this antibody, it can be concluded that the distribution of GlcCer in *C. gloeosporioides* is different from those described in other

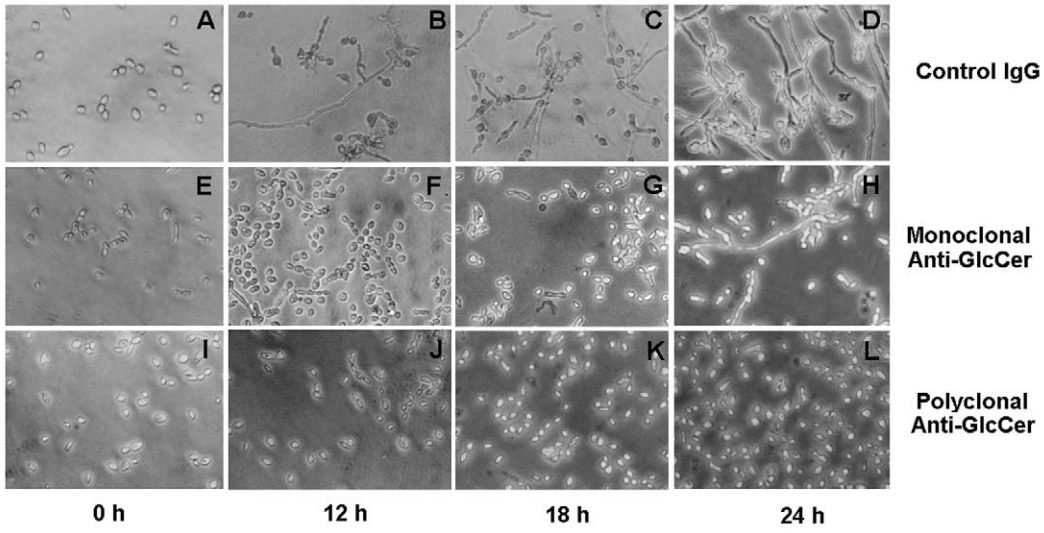


Fig. 6. Antibodies to GlcCer inhibit the differentiation of *C. gloeosporioides* conidia to mycelia. Conidial cells were incubated with control immunoglobulins (A–D), anti-GlcCer monoclonal antibody (E–H) or polyclonal antibodies to GlcCer (I–L) for varying periods. Formation of mycelium was assessed in an inverted microscope.

pathogens [10,11,31], in which this glycosphingolipid was mainly accumulated into the sites of cell division. Points of transport of presumed GlcCer-containing vesicles from the plasma membrane to the cell wall were also suggested in other species [11]. However, the reaction of *C. gloeosporioides* with the anti-GlcCer monoclonal antibody revealed that, in this plant pathogen, GlcCer is uniformly distributed over the fungal surface. In agreement with a singular surface distribution of GlcCer in *C. gloeosporioides*, fungal septa were not recognized by antibodies. Conidial forms also presented a diffuse profile of reactivity with the antibodies, but were less reactive than mycelia. The lower reactivity of the conidial surface with antibodies to GlcCer could be explained by (i) a lesser expression of GlcCer at the cell wall of conidia, (ii) the expression of structurally distinct surface GlcCer in conidial cells, or (iii) a different wall assembly in conidia, in which conserved GlcCer would be less accessible to antibodies. These hypotheses are currently under investigation in our laboratory, through the search of GlcCer in purified cell walls from *C. gloeosporioides* mycelia and conidia. The isolation of GlcCer from *C. gloeosporioides* cell walls will therefore allow the accomplishment of quantitative tests followed by structural and immunological analyses.

As described for *P. boydii* and *C. albicans* [10], polyclonal anti-GlcCer antibodies purified from sera of infected individuals inhibited the differentiation of *C. gloeosporioides* with a striking efficiency. The monoclonal antibody was also effective, although to a lesser extent. This could be explained by the absence of highly inhibitory classes (or isotypes) of immunoglobulins putatively present only at the polyclonal preparation. In this regard, it is our intent to prepare additional monoclonal antibodies to GlcCer of different classes, which could present variable antifungal activity. Nevertheless, it is now clear that external ligands recognizing GlcCer at the fungal surface directly interfere with the morphological transition of human and plant pathogens. The identification of selective probes with inhibitory activity against fungal pathogens may therefore reveal alternative strategies for the control of human and plant infections caused by fungi. In this context, experimental models to construct transgenic plants expressing antifungal polypeptides have been recently proposed. For instance, transgenic Arabidopsis plants expressing a chimeric protein containing sequences of two different antimicrobial peptides, including the above-cited defensin RsAFP2, were recently developed [32]. In this plant, the chimeric polyprotein was found to be cleaved and the individual peptides were functionally secreted into the extracellular space, showing antifungal activity. In this regard, it would be also interesting to identify the specific sequences of antibodies to GlcCer responsible for their antifungal action, which could allow the development of alternative models of transgenic plants less susceptible to fungal infections.

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