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Biological Activity of Lipids Extracted from Two Isolates of *Fusarium oxysporum* (Environmental and Clinical) in *Galleria mellonella*

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Abstract: The species complex *Fusarium oxysporum* (Hypocreales: Nectriaceae) includes plant pathogens of agronomic importance and opportunistic animal and human pathogens. Considering this trans-kingdom capacity, in the present work, we evaluated the virulence of the conidia and the biological activity of lipid extracts from two isolates (environmental and clinical) of *F. oxysporum*. This evaluation was performed on larvae of *Galleria mellonella* model, injecting conidia of both isolates via hemocoel in concentrations of 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/mL. The extracts were characterized by GC and UHPLC-MS and evaluated on *G. mellonella* in concentrations of 1×10^2, 1×10^3 and 1×10^4 µg/mL. Greater mortality was observed with conidia of environmental isolate than with those of clinical isolate, although with both isolates were observed extra cuticular growth of mycelium in dead caterpillars and colonies of *F. oxysporum* were recovered from them. It was also found that the lipid extract obtained with methanol from environmental isolate caused prolonged melanization and larval mortality. The lipidomic characterization showed significant differences between the extracts in the composition of phospholipids and their index of unsaturation, finding a greater number of phospholipid species and a higher unsaturation index for the clinical isolate than for the environmental isolate. Co-stimulus of methanol lipid extract and conidia of the environmental isolate was also evaluated and mortality of the whole larvae was obtained, which suggests a synergic effect between lipids and conidia.

Keywords: Pathogenesis, Trans-kingdom Pathogen, Virulence, Lipidomic

1. Introduction

Fusarium oxysporum (Sordariomycetes: Hypocreales: Nectriaceae) is a species complex that includes primarily plant pathogens that cause millionaire loses in the food industry worldwide. [1] These species also have the enzymatic capacity to synthesize a wide variety of secondary metabolites as mycotoxins that are associated with fungal pathogenesis and food contamination. [1-2] *F. oxysporum* complex also includes human opportunistic pathogens that affect immunocompetent and immunocompromised persons (HIV positives, transplanted and neutropenic patients, etc.). [3] The most common diseases caused by this pathogen are superficial mycoses such as onychomycosis (32.8% of mold onychomycosis in Colombia) [4] subcutaneous mycosis and systemic mycosis.

In the fungal cellular membrane, there are lipid rafts rich in ergosterol and sphingolipids that could function as signaling and adhesion mediators in the host tissues). [5] Glucosylceramides (GlcCer) are the more abundant

sphingolipids in fungi and could participate in the fungal pathogenesis. *Cryptococcus neoformans* mutated isolates lacking GlcCer are incapable of growing up *in vitro* at neutral or alkaline pH and 5% CO_2, conditions mimicking the alveolar space and bloodstream environments. [6] Other investigations show that if mice are immunized with an antibody against GlcCer, it increases the survival of mice infected with *C. neoformans.* [7] Another study demonstrated the importance of these molecules in the virulence of *C. neoformans* since a mutation in C9 sphingolipid methyltransferase gene (STM1) responsible for the methylation of carbon 9 (C9) in the sphingosine skeleton of the GlcCer, results in a loss of more than 80% of its virulence when compared with wild-type isolates. [8] It has also been observed that the glycosilinositol-phosphorylceraramides induce an immune response with the production of specific antibodies in patients with paracoccidioidomycosis. [9] Although these investigations show that lipids are important macromolecules for fungal growth and virulence, the lipid composition and their role in *F. oxysporum* pathogenesis are poorly understood.

To study the trans-kingdom capacity of *F. oxysporum* and the role of the lipids in its pathogenesis, we evaluate the virulence of conidia, the chemical composition and the biological activity of lipids extracted from two *F. oxysporum* isolates (environmental and clinical) in the *G. mellonella* invertebrate model.

2. Materials

2.1. *Fusarium oxysporum* Isolates Identification and Culture

The environmental isolate was obtained from decomposing plant material (UDEAGIEM-15H07, Grupo Interdisciplinario de Estudios Moleculares (GIEM), University of Antioquia) and the clinical isolate was obtained from a patient with onychomycosis (09155, Grupo de Micología Médica, University of Antioquia). The isolates were classified using taxonomical keys and molecular markers such as ITS1, ITS4 and calmodulin gene by Centro Nacional de Secuenciación Genómica (CNSG), University of Antioquia, through Sanger capillary sequencing. The isolates were grown in potato dextrose agar (PDA) (BD Difco™) with oxytetracycline (Mk® Tecnoquímicas S. A.) during 14 days at room temperature. The working strains were maintained in PDA at 4°C and the isolates were conserved in glycerol 20% at -20°C.

2.2. Maintenance of *Galleria mellonella*

G. mellonella colony was maintained in darkness in acrylic containers and fed with an artificial diet (5.54% dry yeast, 22.17% wheat bran, 11.09% yellow corn flour, 11.09% powdered milk, 5.54% wheat germ, 17.65% honey, 22.70% glycerol, 3.88% beeswax and 0.033% formaldehyde solution). [10] Caterpillars were harvested for treatments with the best quality control (color, size, and vigor) and body weight between 200 and 300 mg.

2.3. *Fusarium oxysporum* Conidia Preparation

Conidia were obtained of 14-days *F. oxysporum* cultures using Tween 20 as surfactant. Each culture was added 4mL of Tween 80 (0.1%) and scraped with a scalpel to separate the conidia. This solution was filtered through sterile gauze and filter paper, recovered in a falcon tube and its volume adjusted up to 10 mL with Tween 20 (0.1%). The concentration of conidia per milliliter was estimated by Neubauer- improved chamber counting (Boeco Germany) and adjusted to 1×10^4-1×10^7 conidia/mL

2.4. Lipids Extraction

Mycelia of 14-days *F. oxysporum* cultures were placed in pure hexane for 15 days. Then lipids were sequentially extracted by the Soxhlet system using solvents of increasing polarity (hexane, dichloromethane, and methanol (JT Baker)), for 2 hours per solvent. The extracts were concentrated by rota-evaporation and dried with gaseous nitrogen. The extracts emulsions were formulated with Tween 20 and sterile water using a three-way key and adjusted to 1×10^2-1×10^4 μg/mL

2.5. Derivatization and Preparation of Fatty Acid Methyl Esters (FAMEs)

The different extracts were treated with 4ml of 0.5 N Sodium hydroxide (NaOH) in methanol and placed in a water bath at 90 °C under a reflux system for 7 min. Thereafter, 20% boron trifluoride (BF_3) in methanol was added and maintained under a reflux system for 2 min. Then the samples were mixed with n-heptane and left to reflux for one additional min. The samples were removed from the water bath and allowed to stand for 5 min. Then of a saturated solution of NaCl was added to improve the separation of the organic phases. The organic phases were then collected into Eppendorf tubes with anhydrous sodium sulfate (Na_2SO_4) to dry the samples. The samples were centrifuged and a fraction was collected from the supernatant into auto-sampler glass-crimp cap vials (Agilent, Santa Clara, CA, USA) to undergo gas chromatography.

2.6. Gas Chromatography (GC)

The internal standards mixture of FAMEs in n-heptane (laureate, myristate, palmitate, stearate, oleate, linoleate, linolenate, arachidonate, and docosahexaenoate) were run before each assay to estimate the retention times, followed by the different extracts that were run independently in the Agilent 6890N gas chromatograph with flame ionization detector (FID, Santa Clara, CA, USA). The chromatographic running was performed in a FAME column (FAME model JIWDB23, Agilent) with hydrogen gas. The data were analyzed with Agilent ChemStation software using the areas under the curve of each analyte.

2.7. Lipids Chemical Characterization

The qualitative characterization of *F. oxysporum* lipids extracts was performed by mono and two-dimensional thin-layer chromatography (TLC) in silica gel 60 F254 (Merck®). The spots were visualized by short and long length UV light and vanillin. The fractions were also characterized by ultra-high performance liquid chromatography-mass spectrometry (UHPLC-MS) with reverse-phase liquid chromatography-mass spectrometry (LC-MS / MS) using a capillary column Atlantis dC18 300 mm × 15 cm (Waters Corp.) Column temperature 25°C, Injection volume: 20 µL flow 1.0 mL / min, Wavelength 250 nm and mobile phase: acetonitrile-water with gradient programmed for 10 minutes at 35:65, 35:65 until 80:20 in 5 minutes, 80:20 for 7 min, 80:20 to 90:10 in 13 min, to 90:10 for 5 min.

2.8. Biological Assays

G. mellonella caterpillars in the sixth instar were injected with 20 µL into the hemocoel, via the last proleg with 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/mL from both *F. oxysporum* isolates using Tween 80 as control. To evaluate the biological activity: 1) the lipids extracted were injected at 1×10^2, 1×10^3 and 1×10^4 µg/mL using Tween 20 as control and 2) co-stimulus with conidia at sub-lethal concentrations (when approximately 80–100% of the caterpillars survived) and lipids. Before the injection, the area was disinfected with an alcohol solution (70%). A 1 mL disposable syringe was used with a 31G×8.0 mm needle (BD Ultra-Fine, Becton Dickinson). Caterpillars were stored in the dark and incubated at 28°C in Petri dishes and the mortality was evaluated at 48 hours post-injection based on the cuticle melanization and the absence of movement in response to stimulation. All the assays were performed by triplicate with groups of 10 caterpillars. Data were analyzed with statistical software R v3.4.2 using nonparametric tests of Kruskal-Wallis and pairwise comparison t-test. Graphics were made in OriginPro 8 SR0 v8.0724 (B724).

2.9. *Fusarium oxysporum* Isolation from *Galleria mellonella* Caterpillars

Caterpillars injected with conidia and co-stimulus (conidia and lipids), at 48 hours post-injection were divided into two groups to determine the best isolation method: the first group was anesthetized with cold (4°C) for 5 minutes and then macerated in a Falcon tube containing PBS. The second group was only anesthetized with cold (4°C) for 5 minutes. Caterpillars of both groups were put in PDA medium (BD Difco™) for 14 days to recover *F. oxysporum* colonies.

2.10. Scanning Electron Microscope (SEM)

Phenom Scanning electron microscope. Optical zoom: 20x - 135x. SEM zoom 80x - 130000x. Sample size 32 nm. Acceleration voltage: 5 kV - 10 kV.

3. Results

3.1. Conidia Virulence of *Fusarium oxysporum* Isolates in *Galleria mellonella*

Progressive melanization was observed in caterpillars injected with conidia at the concentrations studied for 48h. We also observed extra cuticular growth of mycelium in dead caterpillars injected with the maximum concentration of conidia of both isolates and it was possible to recover *F. oxysporum* colonies from macerated and non-macerated caterpillars (Figure 1). The caterpillars average mortality at 48 hours post-injection with 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/mL of the environmental isolate was 30%, 53.3%, 93.3% and 100% respectively. The nonparametric tests of Kruskal-Wallis and pairwise comparison t-test with 95% confidence show statistical differences between medians of mortalities of all conidia concentration compared to control with Tween 20. The average mortality of the caterpillars at 48 hours post-injection with 1×10^4, 1×10^5, 1×10^6 and 1×10^7 conidia/mL of the clinical isolate was 6.7%, 16.7%, 23.3%, and 76.7%. Only the mortality with the treatment of 10^7 conidia/mL, had a statistically significant difference (p-value: 1.2×10^{-7}) compared to the control with Tween 20 (Figure 2). Finally, the mortalities obtained by concentration of conidia between isolates were compared and statistically significant differences were found in of 1×10^5 and 1×10^6 conidia/mL treatments (p-values: 0.00274 and 5.0×10^{-7}, respectively) (Figure 2). These results confirm the significative differences in conidia virulence between the isolates of *F. oxysporum,* where the environmental isolate was more virulent than the clinical isolate.

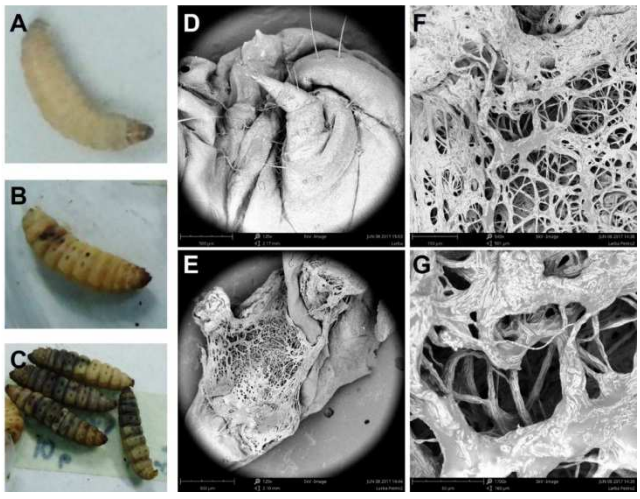

Figure 1. *Effect of F. oxysporum isolates in G. mellonella. (A–C) Progressive melanization of G. mellonella larvae after inoculation with 1×10^4 conidia/mL of F. oxysporum and incubation at 28°C. (D–E) Colonization of a larva killed by F. oxysporum for SEM microscopy at 125x. (F–G) SEM microscopy extra cuticular growth of F. oxysporum and larvae tissue degradation at 540x and 1700x.*

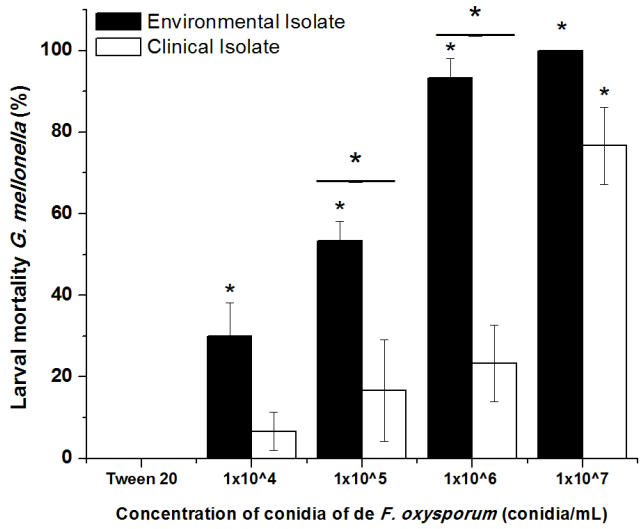

Figure 2. *Virulence of F. oxysporum isolates in G. mellonella. All the conidia concentrations caused caterpillar mortality, but the environmental isolate conidia were more virulent than the clinical conidia, at 48 hpi (* p-value<0.05).*

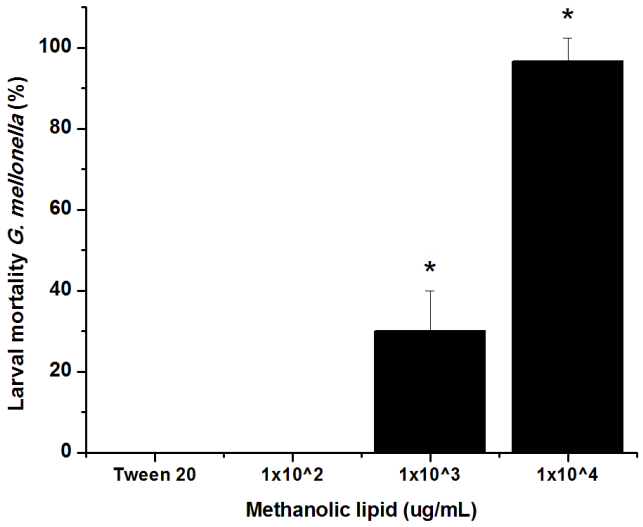

Figure 3. *Virulence of methanolic lipids extracted from F. oxysporum environmental isolate in G. mellonella. Caterpillar mortality was found with 1×10^3 and 1×10^4 µg/mL of the extract (* p-value<0.05).*

3.2. Lipids Virulence of *Fusarium oxysporum* Isolates in *Galleria mellonella*

The lipid extracts were colorless or yellowish for all solvents except for the methanol extract from environmental isolate that was light violet, which is the color of the cultures in PDA. It was observed that the hexane and dichloromethane extracts of both isolates and the methanol extract of the clinical isolate, did not cause melanization, abnormal behavior or death in the caterpillars at 48h post-injection. Caterpillars injected with the methanol extract of the environmental isolate showed early melanization since the first hour of injection and lasted until 48 hours. The average mortality of the caterpillar injected with 1×10^2, 1×10^3 and 1×10^4 µg/mL of methanolic lipid extract of the environmental isolate was 0%, 30%, and 96.7%, respectively. The non-parametric tests of Kruskal-Wallis and pairwise comparison t-test with 95% confidence, show statistically significant differences (p-value <0.05) between mortality medians of caterpillars injected with 1×10^3 and 1×10^4 µg/mL, compared to the control with Tween 20 (Figure 3). These results show a significant difference in the lipid virulence between the environmental and clinical isolate of *F. oxysporum.*

3.3. GC Analysis of Fatty Acids of Methanolic Extract of *Fusarium oxysporum* Isolates

In methanolic extracts from *F. oxysporum* isolates were found 6 fatty acids: Lauric (C12:0), Myristic (C14:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2). Lauric and myristic were only found in the clinical isolate extract in 0.24 ± 0.1% and 0.30 ± 0.1% respectively. The concentration of palmitic, stearic, oleic and linoleic were 21.01 ± 0.13%, 21.00 ± 0.38%, 48.72 ± 0.46% and 2.53 ± 0.13%, respectively for the clinical isolate and for the environmental isolate were 36.1 ± 1.1%, 24.8 ± 0.7%, 30.9 ± 2.7%, 8.2 ± 0.2%, respectively (Figure 4). These results show a significant difference in the fatty acid composition of the *F. oxysporum* isolates, presenting an unsaturation index of 1.20 for the clinic isolate compared to the environmental isolate of 0.642. These differences between the unsaturation index suggest that the *F. oxysporum* environmental isolate was able to change the relationship between saturated and unsaturated fatty acids on the cell wall, to adapt to the body temperature of the host.

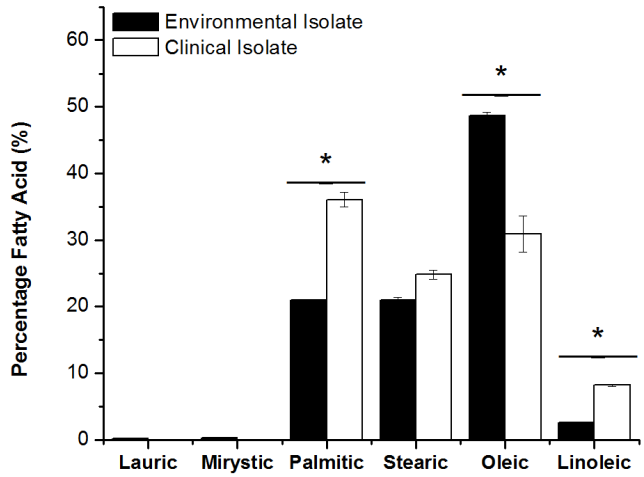

Figure 4. *Fatty acids composition of methanolic lipids extracted from F. oxysporum isolates. The isolates had different percentages of fatty acids. The environmental isolate had greater percentages of palmitic acid and linoleic acid while the clinical isolate had a greater percentage of oleic and linoleic acids (* p-value<0.05).*

3.4. UHPLC-MS Analysis of Phospholipids

The difference between methanol extracts from environmental and clinical isolates of *F. oxysporum* was also observed by liquid chromatography of high efficiency-mass spectrometry (UHPLC-MS). For the clinical isolate, were found 13 phospholipids species, being 2 phosphatidylethanolamines (PE), 4 phosphatidylcholines (PC), 4 phosphatidic acid (PA), 2 phosphatidylglycerol (PG),

one phosphatidylinositol (PI), and one monoacylglycerol (MG), with a distinct fatty acid chain (Table 1). For the environmental isolate only were identified 2 phospholipid species, being one PE and one PC. The compounds found are not shared between isolates. These results confirm that there are significative differences in the composition of methanolic extracts from both isolates of *F. oxysporum*.

Table 1. *Composition of major phospholipids identified by UHPLC-MS positive –ion from the clinical and environmental isolate of F. oxysporum.*

Observed species					
Clinical isolate		**Environmental isolate**			
Ion species	**m/z**	**Ion species**	**m/z**	**Proposed composition**	**Predicted mass (Da)**
Phosphatidylethanolamine (PE)					
M	667.372	ND	ND	C16:0/C14:0	663.918
ND	ND	M	474.314	C18:2	476.571
M	463.320	ND	ND	C16:0	453.557
Phosphatidylcholine (PC)					
$[M+Na]^+$	792.383	$[M+Na]^+$	792.383	C16:0/C18:0	785.097
M	701.493	ND	ND	C12:0/C18:2	701.967
$[M+Na]^+$	541.449	ND	ND	C18:2	542.650
m	519.070	ND	ND	C18:0	523.962
Phosphatidic acid (PA)					
$[M+Na]^+$	733.284	ND	ND	C18:1/C18:1	721.953
M	703.414	ND	ND	C18:0/C18:0	704.003
$[M+Na]^+$	460.320	ND	ND	C18:0	460.526
Phosphatidylglycerol (PG)					
$[M+Na]^+$	541.449	ND	ND	C18:2	542.650
$[M+Na]^+$	473.124	ND	ND	C14:0	478.495
Phosphatidylinositol (PI)					
$[M+Na]^+$	615.497	ND	ND	C18:2	618.633
Monoacylglycerol (MG)					
M	346.312	ND	ND	C18:2	354.531

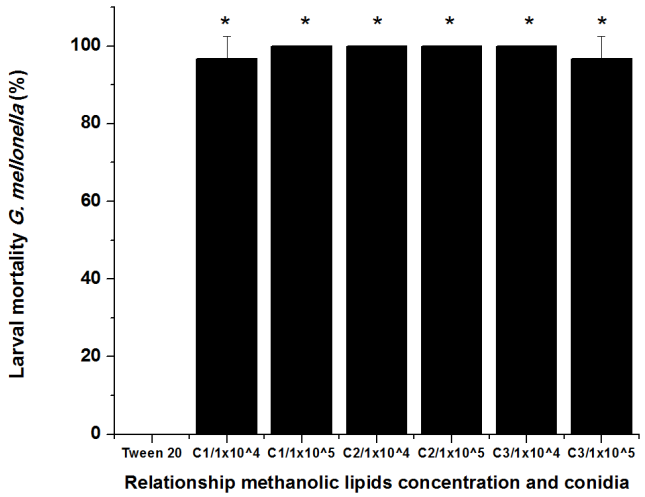

Figure 5. *Co-stimulus with conidia and methanolic lipids extract from the environmental isolate of F. oxysporum in G. mellonella. The caterpillar mortality increased (100% approximately) with the co-stimulus of conidia and lipids compared to the individual treatments (* p-value<0.05).*

3.5. Co-Stimulus of Conidia and Lipids

Sub-lethal concentrations of conidia (1×10^4 and 1×10^5 conidia/mL) of the environmental isolate was chosen and was injected with the methanol lipids extract from the same isolate. The combined inoculation of lipids and conidia resulted in an increase in the mortality of the caterpillars injected (approximately 100%) compared with the mortalities obtained with individual treatments of conidia (30% and 53.3% respectively) and lipids (0%, 30% y 96.7% respectively), and the mortalities were significantly different from the control with Tween 20 / Tween 80 (p-value <0.05) (Figure 4). These results suggest a synergic effect of conidia and methanolic lipids from the environmental isolate of *F. oxysporum*, in the infection of *G. mellonella* caterpillars.

4. Discussion

The species complex *Fusarium oxysporum* (Sordariomycetes: Hypocreales: Nectriaceae) includes plant pathogens of agronomic importance and opportunistic animal and human pathogens. The number of infections in humans is increasing worldwide due not only to the development of better detection methods but also by the growing number of susceptible and immunocompromised patients. [11] Raad et al, 2002 show that the most likely source of fusariosis in patients of the M. D. Anderson Cancer Center, Houston, Texas, was the outside environment, [12] which highlights the trans-kingdom ability of this microorganism like our findings where both isolates, regardless of the origin (as environment saprophyte or as onychomycosis agent), were able to infect *Galleria mellonella* caterpillars, causing melanization, tissue degradation and extra cuticular mycelial growth in dead caterpillars (Figure 1).

Multiple studies have been done to characterize environmental and clinical isolates of pathogenic fungi as *Aspergillus fumigatus* and *Sporothix schenkii*, finding great genetic diversity among them. [13-14] Studies in different species of the genus *Fusarium* found differences in antifungal susceptibility between environmental and clinical isolates [15] and this difference in susceptibility is found even in isolates

from the same type (isolates from onychomycosis patients) from *Fusarium* spp. [16] Our study shows significant differences in the virulence of conidia between environmental and clinical isolates of *F. oxysporum*, using *G. mellonella* as an invertebrate host (Figure 2). The environmental isolate caused greater caterpillar mortality in all concentrations evaluated, respect to the control. On the other hand, clinical isolate caused the same symptomatology only with the greatest conidia concentration evaluated. These findings suggest that *F. oxysporum* isolates have different pathogenicity mechanisms in the interaction with *G. mellonella* as a host. The environmental isolate could be producing toxic metabolites to the caterpillars since this species can synthesize a wide variety of mycotoxins like fumonisins and trichothecenes, which are recognized as virulence factors in plant infections and serve to compete with other microorganisms in the soils. [2] Fumonisins are inhibitors of ceramide synthase and cause an accumulation of compounds from the pathway that is toxic for the cell [17] and trichothecenes inhibit mitochondrial translation and affect the integrity of the membrane of this organelle, causing cell death by apoptosis.[18] Similar studies with *Beauveria bassiana* in *G. mellonella*, also show that the ability of the microorganism to kill the host depends on the active development of mycelium and the production of melanizing-toxic macromolecules. [19] On the other hand, clinical isolate could be producing many lytic enzymes as a mechanism of pathogenicity since it was recovered as an onychomycosis agent, degrading the keratin of the patient nail. This mechanism could explain that only the maximum conidia concentration caused caterpillar mortality because to synthesize this great number of lytic enzymes, the fungus had to grow actively within the caterpillar, colonizing and degrading, to finally come out and grow in the cuticle of the integument.

The difference between the two isolates was also found in lipid extracts treatment since only the methanol extract of the environmental isolate caused melanization and death in the caterpillars (Figure 3). Also, in the methanol extract from the environmental isolate was observed a light purple pigment that was not found for the clinical isolate (Data not shown). Since only the methanol extract caused death in caterpillars and was the only one with pigment, this could act as an immune modulator in *G. mellonella* causing melanization, as was concluded by Liu and Nizet, 2006 that point out that the cytotoxic effect can also be mediated by pigments that induce inflammatory responses in the host, such as those produced by *Cryptococcus neoformans* and *Aspergillus* spp [20].

The fatty acid composition of methanolic extracts from the isolates shows more compounds for the clinical isolate and a greater percentage of palmitic acid and linolenic acid, however, the percentage of oleic acid was greater for the environmental isolate (Figure 4). Characterization by UHPLC-MS shows differences in the phospholipid composition and the unsaturation index between isolates, finding a greater number of phospholipid species and major unsaturation index for the clinical isolate than for the environmental one (Table 1). These differences in the unsaturation index suggest that the clinical isolate can change the relationship between saturated and unsaturated fatty acids on the cell wall, to adapt to the body temperature of the host, what could signify that the original environment where the isolates were isolated has greater effects on cell wall lipids composition. Fatty acids and phospholipids found in the extracts of *F. oxysporum* are major components of fungal cell wall lipids as found in human dimorphic pathogens *Histoplasma capsulatum* and *Paracoccidiodes brasiliensis* [21-22]. The phosphatidic acid found in the extracts is very interesting since this could act as a second messenger in signaling pathways in eukaryotic cells and it has been identified as a mechanism of defensins in tomato interacting with the plant pathogen *Cladosporium fulvum*. [23] Similar to phosphatidic acid, phosphatidylinositol found could act as a signaling second messenger and its translocation across the membrane by an inositol transporter protein (Pdr16p) could mediate azole resistance in *Saccharomyces cerevisiae*. [24]

Finally, co-stimulus of methanol lipid extract with conidia sub-lethal concentrations of environmental isolate show a synergistic effect that resulted in the death of all the caterpillars injected (Figure 5). This finding suggests an immune-modulator effect of lipids and perhaps the pigment in the methanol extract that triggers a melanizing response in *G. mellonella* caterpillars.

5. Conclusions

Environmental and clinical isolates of *Fusarium oxysporum* were evaluated using *Galleria mellonella* as an invertebrate host. Conidia and lipids extracted from the isolates were injected in larvae and mortality was evaluated. Our results show significative differences between environmental and clinical isolates. The environmental isolate caused greater larval mortality than the clinical isolate and its methanol extract had melanizing and mortal effects in larvae. Characterization of methanol extracts from both isolates shows differences in fatty acid composition, phospholipid species, and unsaturation index, suggesting that the temperature of the environment from they were isolated (plant decomposing material for the environmental isolate or a patient with onychomycosis for the clinical isolate) has greater effects on cell wall lipid composition. Co-stimulus between conidia and lipids from environmental isolate caused greater mortality in caterpillars what suggests a possible synergic effect on *Galleria mellonella* infection.

Conflict of Interest

All authors declared that there is no conflict of interest.

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