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Citral: antifungal activity and mode of action, against *Cladosporium oxysporum*

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ABSTRACT

Dematiaceous fungi are a heterogeneous group of fungi with dark colonies and pigmented fungal elements. The spectrum of diseases associated with dematiaceous fungi ranges from superficial skin and soft tissue infections to disseminated sepsis with high mortality. Therefore, it is necessary to study molecules with an antifungal action against these fungis. Attention has been drawn to the antimicrobial activity of aromatic compounds because of their promising biological properties. Citral is a monoterpene with known pharmacological properties, including antimicrobial action. Therefore, we investigated the antifungal activity of citral against strains of *C. oxysporum*, which involved determining its minimum inhibitory concentration (MIC), minimum fungicidal concentration (MFC) and effects on mycelial growth and conidial germination. The effects of citral on the cell wall (sorbitol protect effect) and the cell membrane (citral to ergosterol binding) were investigated. Citral inhibited the growth of 50% of *C. oxysporum* strains employed in this study at an MIC 128µg/mL, as well as mycelial growth and conidia germination. The results of these studies on the mechanisms of action suggested that citral exerts antifungal effects on the cell membrane of *C. oxysporum*. Finally, our studies support the potential use of the citral as an antifungal agent against dematiaceous fungi especially *C. oxysporum*.
Keywords: Mechanism of action; Citral; *Cladosporium oxysporum*

RESUMO

Os fungos dematiáceos são um grupo heterogêneo de fungos com colônias escuras e elementos fúngicos pigmentados. O espectro de doenças associadas a fungos dematiáceos é variável de infecções superficiais da pele e tecidos moles a sepse disseminada com alta mortalidade. Portanto, é necessário o estudo de moléculas com ação antifúngica contra esses fungos. Atenção foi dada à atividade antimicrobiana de compostos aromáticos devido às suas propriedades biológicas promissoras. O citral é um monoterpeno com propriedades farmacológicas conhecidas, incluindo ação antimicrobiana. Dessa forma, foi investigado a atividade antifúngica do citral contra cepas de *C. oxysporum*, que envolveu a determinação de sua concentração inibitória mínima (CIM), concentração fungicida mínima (CFM) e efeitos no crescimento micelial e na germinação dos conídios. Os efeitos do citral na parede celular (efeito protetor com sorbitol) e na membrana celular (ligação do citral ao ergosterol) foram investigados. O citral inibiu o crescimento de 50% das cepas de *C. oxysporum* empregadas neste estudo com CIM 128µg/mL, bem como o crescimento micelial e a germinação dos conídios. Os resultados desses estudos sobre os mecanismos de ação sugeriram que o citral exerce efeitos antifúngicos na membrana celular de *C. oxysporum*. Finalmente, é possível observar que esses estudos apoiam o uso potencial do citral como agente antifúngico contra fungos dematiáceos, especialmente *C. oxysporum*.
Palavras-chave: Mecanismo de ação; Citral; *Cladosporium oxysporum*

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1 INTRODUCTION

Dematiaceous fungi (black fungi) are a heterogeneous group of fungi present in diverse environments worldwide. Many species in this group are known to cause allergic reactions and potentially fatal diseases in humans and animals, especially in tropical and subtropical climates (YEW *et al*., 2016). *Cladosporium* is mainly known as a ubiquitous environmental saprobic fungus or plant endophyte, and to date, just a few species have been documented as etiologic agents in vertebrate hosts, including humans. They are among the most important allergenic fungi linked to allergic rhinitis and respiratory arrest in asthmatic patients (BLACK *et al*., 2000; SELLART-ALTISENT *et al*., 2007). Some species are described as a cause of opportunistic phaeohyphomycosis, including subcutaneous and deep infections in humans and animals (DE HOOG *et al.*, 2011; SANDOVAL-DENIS *et al.*, 2015).

Cladosporium oxysporum is a common saprophyte frequently grows on various substrat (BARNETT and HUNTER, 1972). This species is often found as culture contaminants (ZHENG *et al*., 2014) and cited in cases of cutaneous and subcutaneous disease (ROMANO *et al*., 1999; GUGNANI *et al*., 2006). The increased incidence of these fungal infections, especially dangerous hospital-acquired infections and infections in immunocompromised patients, has accentuated the need for new antifungal treatments (GEORGE and SELITRENNIKOFF, 2006). There exists a clear demand for additional antifungals with therapeutic potential. In this context, attention has focused on the antifungal activity of aromatic plants and their constituents due to their potential biological properties (MICELI *et al*., 2011).

Studies of plant species have been conducted to evaluate the characteristics of natural drug products, including their sustainability, affordability, and antimicrobial activity (NEGRI *et al*., 2014). Citral ($C_{10}H_{16}O$) is one of the most common flavor compounds found in citrus oils, which has been already widely used in foods and beverages (e.g., soft drinks and desserts) (CHOI *et al*., 2010). Citral is a monoterpenoid aldehyde (HYLDGAARD *et al*., 2010) often present in the form of stereoisomers geranial and neral (BENVENUTI *et al*., 2011) that has been identified in the leaves and fruits of several plant species including myrtle trees, basil, lemon, lime, lemongrass, orange, and bergamot (HYLDGAARD *et al*., 2010; FISHER and PHILLIPS, 2006). A number of experimental and clinical studies have shown that citral has favorable anti-inflammatory (ORTIZ *et al*., 2010), antitumoral (XIA *et al*., 2013) effects, and there is increasing evidence that citral acts as a fungicidal and bactericidal agent (LEITE *et al*., 2014; SHI *et al*., 2016).

Although citral has been reported to be effective against a variety of microbial species, there have been no reports on its antimicrobial activity against *C. oxysporum* and possible mode of action. To fill this gap, the aim of the present study was to determine antifungal effect and mode of action of citral against *C. oxysporum*.

2 MATERIAL AND METHOD

2.1 Microorganisms

Cladosporium oxysporum (URM 5234, URM 6056 and URM 5412) strains used in the antifungal assay were obtained from the Mycology Department fungal collection (URM), Biological Sciences Center, Federal University of Pernambuco, Brazil. The samples were maintained on Sabouraud Dextrose Agar (SDA) (DIFCO®) at room temperature (28°C) and under refrigeration (4°C). Stock inoculations (suspensions) of *C. oxysporum* were prepared from 7-14 day old sabouraud dextrose agar (Difco Lab., USA); the cultures were grown at room temperature. Fungal colonies were covered with 5mL of sterile saline solution (0.9%), the surface was gently agitated with vortexes, and fungal elements in saline solution were transferred to sterile tubes. Inoculator was standardized at 0.5 tube of McFarland scale (10^6 CFU/mL). The final concentration confirmation was done by counting the microorganisms in a Neubauer chamber (CLEELAND and SQUIRES, 1991; HADACEK and GREGER, 2000; SAHIN *et al*., 2004).

2.2 Chemicals

The product tested was the monoterpene citral, obtained from Sigma Aldrich, Brazil. Amphotericin B and voriconazole were obtained from Sigma Aldrich, Brazil. The monoterpene was dissolved in tween 80 (2%) and DMSO (dimethylsulfoxide acid). The antifungal standards were dissolved in DMSO, and sterile distilled water to obtain solutions of 2048µg/mL for each antifungal. The concentration of DMSO did not exceed 0.5% in the assays.

2.3 Culture Media

To test the biological activity of the products, Sabouraud dextrose agar (SDA) purchased from Difco Laboratories (Detroit, MI, USA), and RPMI-1640-L-glutamine (without sodium bicarbonate) (Sigma-Aldrich, Sao Paulo, SP, Brazil) culture media were used. Both were prepared and used according to the manufacturers' instructions.

2.4 Determination of Minimum Inhibitory Concentration (MIC) and Minimum Fungicidal Concentration (MFC)

Broth microdilution assays were used to determine the MICs of monoterpene citral, amphotericin B, and voriconazole against *C. oxysporum* (URM 5234, URM 6056 and URM 5412). RPMI-1640 was added to all the wells of 96-well plates. Two-fold serial dilutions of the agents were prepared to obtain concentrations varying between 4µg/mL and 1024µg/mL. Finally, 10µL aliquots of the inoculate suspension were added to the wells, and the plates were incubated at 28°C for 5 days. Negative controls (without drugs) were used to confirm conidia viability, and sensitivity controls (for DMSO and tween 80) were also included in the studies. At 5 days, there were visual observations of fungal growth. The MIC was defined as the lowest concentration capable of visually inhibiting fungal growth by 100%. The results were expressed as the arithmetic mean of three experiments (CLEELAND and SQUIRES, 1991; HADACEK and GREGER, 2000).

The MFC was determined by microdilution method to verify if the inhibition was reversible or permanent (DENNING *et al*., 2004; RASOOLI and ABYANEH, 2004). Aliquots of 20µL (from the wells that did not show growth in the MIC procedure) were transferred to 96-well plates previously prepared with 100µL of RPMI-1640. The plates were aseptically sealed followed by mixing on a plate shaker (300rpm) for 30 seconds, incubated at 28ºC and read at 5 days of incubation. Tests were performed in duplicate and the geometric mean values were calculated. MFC was defined as the lowest citral concentration in which no visible growth occurred when subcultured on the 96-well plates containing broth without antifungal products.

After determination of the MIC and MFC we selected 1 strain (*C. oxysporum* URM 5234), to continue the citral antifungal activity study.

2.5 Effects on Mycelia Growth

Analyses of the interferences of citral, voriconazole, and amphotericin B on *C. oxysporum* URM 5234 mycelia growth were determined using poisoned substrate technique (dilution in solid medium), by daily measuring of radial mycelial growth on SDA, by adding products in an amount adjusted to provide final concentrations similar to the MIC, MIC × 2 and MIC × 4 previously found. For this, 2mm plugs taken from a 10-day-old mold culture cultivated on SDA slants at 28ºC were placed at the center of the sterile SDA Petri dishes containing the test drugs. At different intervals (0, 2, 4, 6, 8, 10, 12, and 14 days) of incubation at 28ºC, the mold's radial mycelial growth was measured (mm) with calipers. The controls in this assay revealed the mold's radial growth on SDA without adding drugs. Each assay was performed twice and the results were expressed as the average of the two repetitions (ADAN *et al*., 1998; THYÁGARA and HOSONO, 1996; DAFERERA *et al*., 2003).

2.6 Conidial Germination Assay

Citral, voriconazole and amphotericin B were tested to evaluate effects on the germination of *C. oxysporum* URM 5234 fungal conidia. Flasks containing MIC, MIC × 2 and MIC × 4 of citral, voriconazole, amphotericin and a control with distilled water were used. In sterile test tubes, 500µL of RPMI-1640 plus citral were evenly mixed with 500µL of fungal conidia suspension and immediately incubated at 28ºC. Samples of the mixture were taken after 48h of incubation for analysis. The whole experiment was performed in duplicate, where the number of conidia was determined in a Neubauer chamber, and the spore germination inhibition percentage at each time point was calculated by comparing the results obtained in the test experiments with the results of the control experiment. The analysis was conducted under an optical microscope (Zeiss Primo Star) (PEREIRA *et al*., 2013; RANA *et al*., 1997).

2.7 Sorbitol Assay Effects

The assay was performed using medium with and without sorbitol (control), to evaluate possible mechanisms involved in the antifungal activity of the test product on the *C. oxysporum* URM 5234 cell wall. The sorbitol was added to the culture medium in a final concentration of 0.8M. The assay was performed by microdilution method in 96-well plates in a "U" (Alamar, Diadema, SP, Brazil) (CLEELAND and SQUIRES, 1991; HADACEK and GREGER, 2000). The plates were sealed aseptically, incubated at 28ºC, and readings were taken at 5 days. Based on the ability of sorbitol to act as a fungal cell wall osmotic protective agent, the higher MIC values observed in the medium with sorbitol added (as compared to the standard medium); suggest the cell wall as a possible target for the product tested (LEITE *et al*., 2014; LIU *et al*., 2007; FROST *et al*., 1995).

The assay was performed in duplicate and expressed as the geometric mean of the results.

2.8 Ergosterol Binding Assay

MIC value determination in the presence of ergosterol. To assess if the product binds to fungal membrane sterols, an experiment was performed according to the method described by Escalante *et al*. 2008, with some modifications. Ergosterol was prepared as described by Leite et al. 2014. The MIC of citral, against *C. oxysporum* URM 5234 was determined by using broth microdilution techniques (CLEELAND and SQUIRES, 1991; HADACEK and GREGER, 2000), in the presence and absence of exogenous ergosterol (Sigma-Aldrich, São Paulo, SP, Brazil) added to the assay medium, in different lines on the same microplate. Briefly, a solution of citral was diluted serially twice with RPMI-1640 (volume = 100µL) containing ergosterol added at a concentration of 400µg/mL. A volume yeast suspension 10µL (0,5 McFarland) was added to each well. The same procedure was realized for amphotericin B, whose interaction with membrane ergosterol is already known, which served as a control drug. The plates were sealed and incubated at 28ºC. The plates were read after 5 days of incubation, and the MIC was determined as the lowest concentration of test agent inhibiting visible growth.

The assay was carried out in duplicate and the geometric mean of the values was calculated. The binding assay reflected the ability of the compound to bind with ergosterol.

2.9 Statistical Analysis

The results are expressed as mean ± S.E. Differences between the means were statistically compared using the Student's t-test. The values were considered significant with $p < 0.05$.

3 RESULTS AND DISCUSSION

According to criteria proposed by Sartoratto *et al*. 2004, citral showed strong antifungal activity against *C. oxysporum* as the MIC values of this monoterpene was lower than 500µg/mL (Table 1). Amphotericin B and voriconazole were used as a positive control because they are commonly used antifungal drugs for the treatment of infections caused by dematiaceous fungi (CHEN *et al*., 2013; KINDO *et al*., 2013). In the literature, citral has been found to be active against yeast and filamentous fungal species (LI *et al*., 2014; ZHOU *et al*., 2014; SOUSA *et al*., 2016). Citral, one of the volatile constituents in plant essential oils, has been demonstrated to have strong antifungal activity against *P. digitatum, P. italicum*, and *G. citri-aurantii* (WURYATMO *et al*., 2014; DROBY *et al*., 2008; WURYATMO *et al*., 2003).

In accordance with the above results, the strain *C. oxysporum* URM 5234 was employed to explore the effects of citral, amphotericin B and voriconazole on mycelial growth, conidial germination, on the cell wall (sorbitol protect effect) and the cell membrane (interaction with ergosterol). The fungi mycelium is (on the whole) the hyphae, and fungal filaments, or segments of filamentous mycelium. Mode of growth can also be an important factor contributing to the virulence of potentially pathogenic fungi, as both biofilm formation and tissue invasion have been shown to contribute to pathogenesis (POWERS-FLETCHER *et al*., 2016). Therefore, some researchers are investigating the products naturals' potential in inhibiting mycelial growth of pathogenic fungi due to their importance in the mycosis development (PEREIRA *et al*., 2011; GUERRA *et al*., 2015).

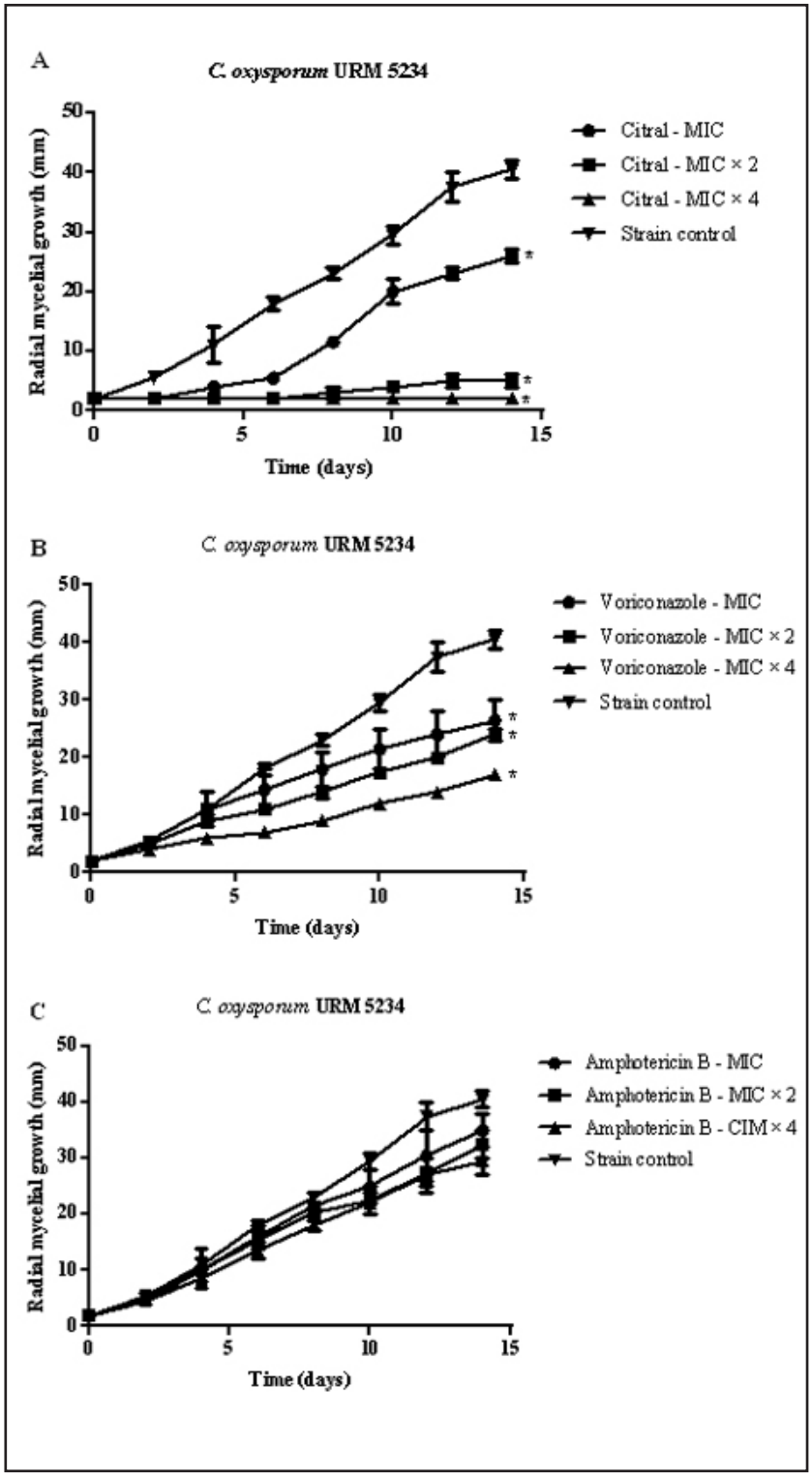
Table 1 - MIC and MFC of citral, amphotericin B and voriconazole against *C. oxysporum*

Microorganisms	Citral (µg/mL)		Amphotericin B (µg/mL)		Voriconazole (µg/mL)		Control strains*
	MIC	MFC	MIC	MFC	MIC	MFC	
C. oxysporum URM 5234	128	256	16	64	16	64	+
C. oxysporum URM 6056	128	256	>1024	ND	>1024	ND	+
C. oxysporum URM 5412	128	256	16	32	8	32	+

Note. * Microorganism growth in RPMI-1640, DMSO (5%), and Tween 80 (2%), without antifungal or monoterpenes. ND- Not determined

The effect of MIC, MIC × 2 and MIC × 4 of the drugs on the mycelial growth was determined by measuring the radial mycelial growth of *C. oxysporum* URM 5234 (Figure 1). As seen in figure 1A, citral all tested concentrations, especially at MIC × 2 and MIC × 4, inhibited the mycelial growth of *C. oxysporum* URM 5234 ($p < 0.05$) as compared with the control (mycelia diameter being 100%). Similar effects were noted with voriconazole in that the drug effectively inhibited the mycelial growth, in all concentrations tested (Figure 1B). Amphotericin B did not show capability to develop a significant inhibitory effect on the mould mycelia growth along 14 days of exposure (Figure 1C). In general, the mould strain when exposed to amphotericin B developed a progressive increasing in their mycelial growth showing a growth profiles similar to the ones found in the control assay.

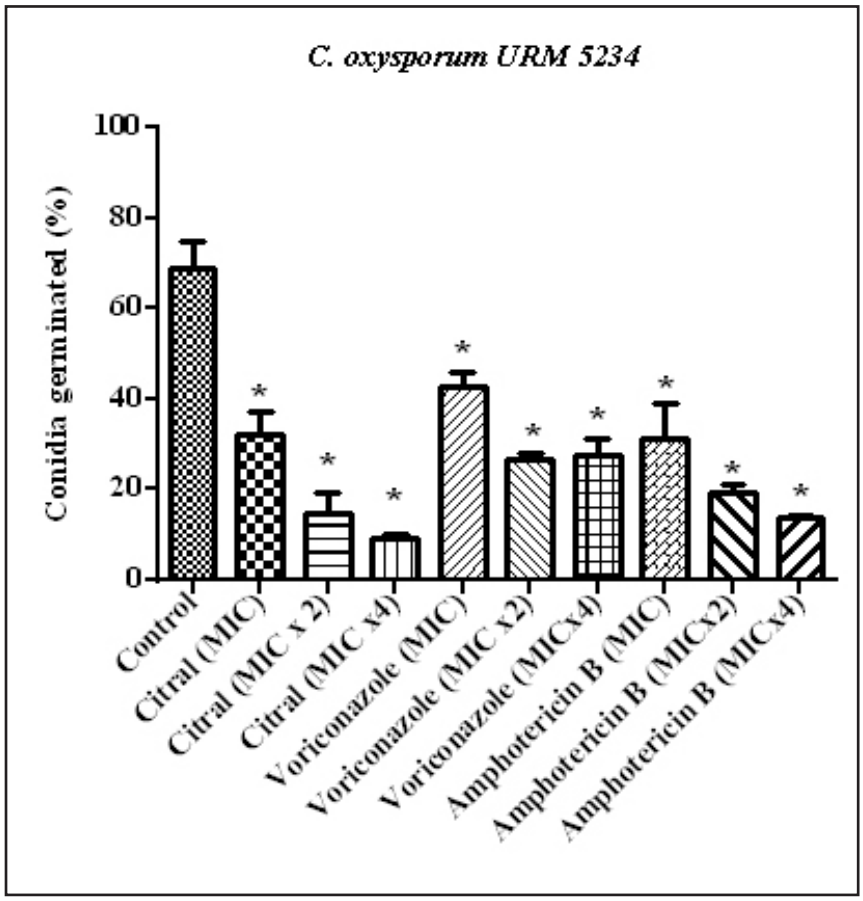
Figure 1 - Radial mycelial growth produced by *C. oxysporum* URM 5234 in the absence (control) and presence citral (A), voriconazole (B) and amphotericin B (C). *$p < 0.05$ compared to control.



In this study, citral it was capable of inhibiting the mycelial growth. In filamentous fungal, hyphal production is important because they penetrate into the deeper layers of the epidermis. This is of particular importance since the outer tissue layers are constantly being lost (BRAND, 2012). These results confirm previously published work; such as that by Ouyang *et al*. 2016 showed that, the citral exhibit its antifungal activity against the mycelial growth of *P. digitatum*. Li *et al*. 2014 showed the effect of citral on *Magnaporthe grisea*, in this study, it was found that mycelial growth was significantly inhibited by citral in a concentration-dependent manner, concerning the efficacy of citral for inhibition of pathogenic fungi growth.

Thus, the study of the germination of conidia has great implications in clinical practice, because it is possible to develop new therapeutic approaches that block the infection at its onset (OSHEROV and MAY, 2001). In this perspective, the effect of the citral on the germination of the conidia of *C. oxysporum* was investigated. The percentages of germinated conidia of *C. oxysporum* URM 5234 are recorded in figure 2. At their MICs, the drugs significantly inhibited conidial germination ($p < 0.05$).

Figure 2 - Percentage of conidial germination of *C. oxysporum* URM 5234 in the absence (control) and presence of citral, voriconazole and amphotericin B. *$p < 0.05$ compared to control.



The asexual spore, or conidium, is critical in the life cycle of many fungi because it is the primary means for dispersion and serves as a `safe house' for the fungal genome in adverse environmental conditions (OSHEROV and MAY, 2001). The study of conidial germination, in addition to being a scientific puzzle of great interest, has far-reaching practical implications. Intensive monoculture and inbreeding have greatly increased the incidence and severity of fungal infections in crops (INGRAM, 1999). Often fatal fungal infections in immunodeficient patients have also increased markedly during the last decade (DENNING, 1998). In almost all cases in both plants and animals, fungal infection is initiated by contact of the host with airborne conidia, which begin the infective process by undergoing conidial germination. By achieving a molecular understanding of this process, it may be possible to develop novel therapeutic approaches that block infection at its outset.

The results showed in this study are agree with those of Neri *et al*. 2006, we reported that citral can inhibited the germination of the conidia of *Penicilium expansum* and Garcia *et al*. 2008 observed that citral inhibited the germination of the conidia of *Colletotrichum musae*, *Colletotrichum gloeosporioides* and *Fusarium subglutinans*. Several targets including cell wall, cell membrane, mitochondrion, and genetic material, have been proposed to account for the antifungal activity of essential oils or their volatile compositions (SHAO, *et al*., 2013; ZHENG *et al*., 2015; PARVEEN *et al*., 2015; RAO *et al*., 2010; YU *et al*., 2010). The fungal cell wall is a dynamic structure

that protects fungal protoplasts from external osmotic shocks and defines fungal morphogenesis. Thus, changes in the organization or functional disruption of the cell wall induced by antifungal agents are involved in fungal death (SARTORATO *et al*., 2004; BOWMAN and FREE, 2006).

The sorbitol protection assay was performed to further explore the mode of action of citral on the integrity of the fungal cell wall. Drugs that act on the cell wall cause lysis of fungal cells in the absence of an osmotic stabilizer (sorbitol), but their growth can continue in the presence of sorbitol (ESCALANTE *et al*., 2008). This effect is detected by increases in the MIC value as observed in medium with sorbitol (0.8M) as compared to the MIC value in medium without sorbitol (standard medium). This assay is known as a broad-spectrum screen that can find not only agents that directly interfere in cell wall synthesis and assembly but also regulatory mechanisms involved in this process, including signal transduction pathways (FROST *et al*., 1995; SVETAZ *et al*., 2007).

The results of the sorbitol protection assay are presented in table 2. The citral MIC remained unchanged, suggesting that the citral does not act by inhibiting fungal cell wall synthesis, but probably by affecting another target in *C. oxysporum* URM 5234. This is the first study to investigate the action of citral on the cell wall of *C. oxysporum* under sorbitol testing, which complicates comparison with other investigations. However, the results confirm earlier studies for other microorganisms; Lima *et al*. 2012 and Leite *et al*. 2014 confirmed that antifungal activity of the citral against *C. albicans* was not reversed in the presence of an osmotic support. Sousa *et al*. 2016 reported that citral does not act on the cell wall of *C. tropicalis.* This would suggest that inhibiting fungal cell wall synthesis or assembly is not altered when the chemical structure of citral is maintained.

Table 2 - MIC values (µg/mL) of drugs in the absence and presence of sorbitol (0.8M) and ergosterol (400µg/mL) against *C. oxysporum* URM 5234

Drugs	Sorbitol		Ergosterol	
	Absence	Presence	Absence	Presence
Citral	128	128	128	2048
Amphotericin B[a]	-	-	16	2048

[a]Positive control. —: not tested.

It is reported that the antimicrobial mechanism of cyclic hydrocarbons, such as citral, is related to its lipophilic character in that they increase the fluidity and permeability of the cell membrane of microorganisms. In fact, these compounds interfere with ion transport, unbalancing osmotic conditions in the membrane and making its associated proteins inefficient. In any case, this can lead to inhibition of microbial growth, and death or cell lysis (DI PASQUA *et al*., 2007). The fungal cell membrane is a dynamic structure composed of a lipid bilayer where enzymes and transport proteins are embedded. Ergosterol is the main sterol component in the plasma membrane of fungi and plays the same role in the fungal membranes that cholesterol does in mammalian cell membranes. Therefore, these two sterols seem to exhibit qualitatively similar properties (BOWMANN and FREE, 2006).

To explore the possible mechanism of interaction of citral with fungal cell membrane, we studied the ability of the compound to form complexes with ergosterol. If the activity of citral were caused by binding to ergosterol, the exogenous ergosterol would prevent the binding to the fungal membrane's ergosterol. Consequently, it would cause an increase in MIC of citral in the presence of exogenous ergosterol with respect to the control experiment (LUND and KUBO, 2000; ESCALANTE *et al*., 2008). Thus, the effect of exogenous ergosterol on the MIC of citral and amphotericin B was determined. Results showed (table 2) that the MIC values of citral against *C. oxysporum* URM 5234 was 2048µg/mL in medium with additional 400µg/mL ergosterol, increased sixteen times in the presence of this sterol, suggesting that the citral act by binding to membrane ergosterol. Regarding amphotericin B, 128 × MIC was observed in the presence of ergosterol. Confirming our results, in others studies citral was found to destroy the membrane permeability and integrity of *P. italicum* and G. citri-aurantii by causing significant losses in total lipids or ergosterol contents (TAO *et al*., 2014; ZHOU *et al*., 2014). According to Rajput and Karuppayil, 2013 the mechanism of anti-Candida activity of citral appears to be associated with damage in the membrane integrity, through inhibition of ergosterol biosynthesis. Recents studies Ouyang *et al*. 2016 suggest that citral could exhibit its antifungal activity against the mycelial growth of *P. digitatum* by disrupting ergosterol biosynthesis.

4 CONCLUSION

From the results presented above, it can be concluded that citral exerts fungicidal effect on *C. oxysporum* strains with MFC = 2 × MIC. In addition, it is possible to observe a significant decrease in conidia formation, directly influencing the fungal filamentation process and consequently the degree of pathogenicity. Finally, it can also be stated that citral exerts its fungicidal effects on *C. oxysporum* from its binding with the ergosterol present in the fungal cell membrane. Thus, our results may serve as a guide for future in vivo studies of clinical use of citral in treating fungi infections by demtiaceous fungi.

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