



A chloroacetamide derivative as a potent candidate for fusariosis treatment

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Abstract

Fusariosis has presented a significant increase in their incidence in the last years. This epidemiological panorama probably is due to the increasing profile of refractory susceptibility of *Fusarium* spp. to available drugs, especially in immunocompromised individuals. Thus, the development of new compounds with effectiveness on these organisms is a necessity. This study evaluated the antifungal potential of a chloroacetamide derivative (4-BFCA) against resistant *Fusarium* strains. As a result, the compound was effective against all strains (MIC range 12.5–50 µg/mL). The time kill assay demonstrated that 4-BFCA presents a concentration-dependent fungicidal action. Although its action mechanism has not yet been elucidated, it was possible to observe its efficacy through damages and alterations provoked along the hyphae of *Fusarium* spp. 4-BFCA maintained a high survival rate of *Tenebrio molitor* larvae, suggesting that it does not cause acute systemic toxicity on this host at the concentration evaluated. In addition, 4-BFCA was 83.33% effective in combating a fungal infection in vivo on the chorioallantoid membrane of embryonated eggs. Our results are very promising and arouse interest to investigate the action of 4-BFCA on *Fusarium* strains since it acts as a possible candidate for the development of new therapies for the treatment of fusariosis.

Keywords *Fusarium* · Antifungal resistance · Chloroacetamide derivatives · Antifungal therapy

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Introduction

Fusarium spp. are organisms saprophytic and ubiquitous considered opportunistic pathogens, capable to cause infections in a human known as fusariosis [1–3]. In recent years, the incidences of these infections have increased considerably, since these fungi represent the second most common cause of infections due to filamentous fungi in humans, after only *Aspergillus* spp. [4–9]. Fusariosis can be localized, with clinical manifestations in the skin, nails (onychomycosis), and eyes (keratitis or endophthalmitis)—mainly related to healthy individuals—or invasive and disseminated—mainly reported in immunocompromised individuals [10].

Among the main species related to fusariosis, *Fusarium solani* appears as most prevalent, followed by *F. oxysporum* and *F. verticillioides* [11]. Cases of multidrug resistance are the main problem related to *Fusarium* species, since fusariosis is usually refractory to the antifungal therapy available, especially in the cases related to immunocompromised patients [7, 12]. Due to this, in Brazil, the options for the treatment of fusariosis are restricted to amphotericin B,

natamicin, and voriconazole [7, 12]. However, there is no consensus for establishing the best antifungal therapy, since there are variations of susceptibility among species that also depend on the related clinical manifestation [6, 8, 13].

When compared to advances in the development of new antibacterial agents, studies related to the discovery of new antifungal agents have disadvantages [14, 15]. Thus, the prospection of new therapeutic options capable of overcoming the cases of multi-resistance in *Fusarium* spp. is essential. In this scenario, chloroacetamides are used widely globally as herbicides for the control of weeds mainly in corn, soybean, and cotton crops [16, 17]. In addition to the action on plants, studies have shown that these agents and their derivatives are biologically active. Thus, can be mentioned the presence of antileishmania action, antibacterial and antifungal [16, 18, 19]. In addition, according to Machado [20], chloroacetamide derivatives, such as *N*-4-bromophenyl-2-chloroacetamide (4-BFCA), showed anti-*Candida* activity, efficacy to inhibit the growth of these yeasts, and fungicide action against dermatophyte. However, no studies are evaluating the antifungal potential of these derivatives against *Fusarium* spp.

Thus, this study evaluated the antifungal potential and toxicological parameters of 4-BFCA against multi-resistant *Fusarium* strains.

Material and methods

Preparation of compound

The *N*-(4-bromophenyl)-2-chloroacetamide (4-BFCA—MW: 248.5 g/mol) (Fig. 1) was synthesized by Lavorato et al. [21]. The compound was fully characterized by its melting points and ¹R, ¹H, and ¹³C through nuclear magnetic resonance spectroscopy (NMR) spectra and its demonstrated melting point consistent with a previous report [22, 23].

For biological assays, 4-BFCA was diluted in 2% DMSO (Sigma-Aldrich, St. Louis, Missouri, USA) and the remaining volume was completed with RPMI 1640 (Gibco, Grand Island, NY, USA) to obtain the required concentrations for each biological assay. The antifungal amphotericin B (AMB—Cristália, São Paulo, Brazil) was prepared

according to CLSI [24]. Thus, an AMB stock solution was initially diluted in 2% DMSO and the remaining volume supplemented with RPMI medium to obtain a solution at 128 µg/mL, which was used as a control drug for the test.

Fungal strains

In this study, a total of 16 *Fusarium* spp. strains were evaluated: *F. falciforme* (F 9, F 20, and HCF 19), *F. keratoplasticum* (F 21, HCF 17, and HCF 26), *F. oxysporum* (F 24, F 35, HCF 22, and HCF 46), *F. proliferatum* (F2), and *F. solani* (ATCC 16,031, F 28, F 33, F 34, and HCF 41). All strains are from the mycology collection of yeasts of the Federal University of Rio Grande do Sul (Porto Alegre, Rio Grande do Sul, Brazil). *Fusarium* spp. strains were first identified through DNA sequence analysis, which were compared to Basic Local Alignment Search Tool (BLAST) for these sequences in the Database Multilocus Sequence Typing (MLST) for *Fusarium* genus. As a control, the standard ATCC 36,031 strain obtained from ATCC (American Type Culture Collection, Manassas, VA, USA) was used.

Antifungal susceptibility testing

The broth microdilution assay was performed to determine the minimum inhibitory concentration (MIC) of 4-BFCA against multi-resistant *Fusarium* strains. The compound was prepared at 100 µg/mL, with concentrations ranging between 0.09 and 50 µg/mL. AMB solution at 128 µg/mL was used as control. A suspension containing each fungal inoculum in saline solution (NaCl 0.85%) was prepared. The suspensions were homogenized in a vortex and after 5 min the supernatant containing the fungal conidia was removed and transferred to a new sterile tube. Then, conidial density was read in a spectrophotometer at 530 nm, and a 1:50 dilution of the fungal inoculum was performed to obtain a concentration of approximately $0.5\text{--}4 \times 10^4$ UFC/mL. The microplates were incubated at 32 °C for 48 h, without shaking. The minimum inhibitory concentration (MIC) was determined as the lowest concentration of 4-BFCA capable of inhibiting 100% of fungal growth visually. The methodology followed the one proposed by document M38-A2 [24].

Time kill assay

The 4-BFCA solutions at MIC, MICx2, MICx4, and MICx8 concentrations were prepared in RPMI 1640 and evaluated against *F. solani* (ATCC 31,036) strain. A fungal inoculum in sterile saline solution (NaCl 0.85%) was prepared and the suspension reading was performed in a spectrophotometer at 530 nm. An aliquot of this fungal suspension was added in each 4-BFCA solution to obtain approximately $0.5\text{--}4 \times 10^4$ UFC/mL. As a control, solutions containing only fungal

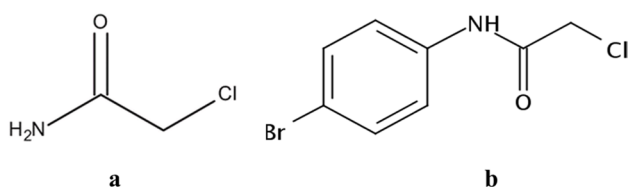


Fig. 1 Chemical structure of the chloroacetamide (a) and of *N*-(4-bromophenyl)-2-chloroacetamide (4-BFCA) (b)

inoculum in RPMI 1640 were used. All 4-BFCA solutions were made in triplicate. The determination of kinetics curves was performed in the times 0, 3, 6, 12, 24, and 48 h at 32 °C without shaking. During each time, an aliquot of 100 µL of each sample and control was removed and diluted to 10^{-3} to allow counting of the *Fusarium* colonies. Then, 20 µL of each sample were plated on sabouraud dextrose agar (SDA) using a Drigalski spreader through spreading-plate method, making it possible to reliably count the filamentous colonies. The plates were incubated at 32 °C at 48 h. After growth, colony count was performed, and the results were expressed by plotting mean \log_{10} CFU/mL. The presence of fungicidal action was defined when a decrease of $\geq 99.9\%$ in the \log_{10} CFU/mL was observed. When the reduction in the \log_{10} CFU/mL was $< 99.9\%$, the 4-BFCA action is considered fungistatic [25].

Scanning electron microscopy

After determination of MIC values for 4-BFCA and AMB against *F. solani* (ATCC 31,036) strain, an aliquot at MIC/2 concentration of both samples was collected for scanning electron microscopy (SEM). Firstly, the samples were prepared through several steps, including washing, fixing, and centrifugation [26]. Thus, pellets containing the samples were obtained and adhered to coverslips previously treated with poly-L-lysine for 1 h. Then, the coverslips were dehydrated in a series of acetone solutions: 30, 50, 70, 95 (5 min), and 100% (10 min) (Dinâmica, Diadema, São Paulo, Brazil). Lastly, the samples were subject to critical point drying (MS CPD 300, Leica), coated with gold and palladium for SEM viewing (Carl Zeiss EVO® MA10—Carl, Oberkochen, Germany). A current of 10 kV was used.

Acute toxicity tests in mealworms

Tenebrio molitor larvae (Coleoptera: Tenebrionidae) were anesthetized by cooling (2 °C) during 2 min. Then, 50 µL of 4-BFCA solution at 100 µg/mL was applied in each larva with support of a micro syringe into the hemocoel, at the second or third visible sternite above the legs, in the ventral portion. This was the maximum volume that did not cause larvae death at 48 h, and thus used in this assay. The equivalent dose was calculated as 5 µg/200 mg; so, this dose is below of the recommended limit of chloroacetamides for most sensitive mammals. The larvae were incubated at 37 °C in Petri dishes containing a rearing diet. The number of dead larvae was recorded at 4 h intervals during 48 h. The assay was performed in triplicate with groups of seven larvae, with a total of twenty-one larvae per treatment. The methodology followed the one proposed by De Souza et al. and Usui et al. [27, 28].

Fungal infection model on the chorioallantoic membrane (CAM)

White fertile eggs fresh Lohmann (Lohmann selected Leghorn, LSL) for this infection model were used. Firstly, optimal conditions of incubation temperature (38 to 39 °C) and humidity (55 to 60%) were established for the eggs' maintenance during 12 days. In parallel, the *F. solani* ATCC 31,036 strain was grown on SDA for 48 h at 32 °C. Thus, a fungal inoculum suspension was prepared in 5 mL of 0.85% sterile saline solution, according to M38-A2 protocol [24]. The resulting suspension was vortexed, and the fungal cell density was adjusted in a spectrophotometer at 530 nm wavelength. Thus, a final suspension of the ATCC strain was prepared at 1:100 dilution by counting Neubauer chamber resulting in approximately $1-4 \times 10^3$ cells/mL [29]. From the fourth day of incubation onward, the eggs were turned four times a day until they were infected with 100 µL of the fungal inoculum, the holes were sealed with paraffin, and embryo viability was checked daily. In brief, on developmental day 8, 100 µL of 4-BFCA at 100 µg/mL was added in each egg (negative control—0.85% sterile saline solution). On day 12, the eggs were opened again and 100 µL of the 4-BFCA solution and of the negative control was removed, and spread with Drigalski strap on Petri dishes containing SDA. Thus, the plates were incubated at 30 °C for 72 h [30, 31].

Statistical analysis

Statistical analysis was performed for CAM test, through non-parametric Student's *t*-test, and two-way ANOVA followed by Dunnett's test to assess the fungicidal effect of 4-BFCA at different concentrations when compared to the control (untreated control). Both analyses consider $P < 0.05$ significant (*), and GraphPad Prism 7.0 was used. The experiments were performed in triplicate.

Results

The 4-BFCA was effective against all *Fusarium* spp. strains evaluated, presenting a similar susceptibility profile among species, with MIC values ranging from 12.5 to 50 µg/mL. The *F. keratoplasticum* specie was the most sensitive to 4-BFCA, since this had the lowest MIC value (12.5 µg/mL). The strains HCF 17, HCF 22, and HCF 41 showed low susceptibility to AMB (MIC > 16 µg/mL) (Table 1).

The evaluation of time kill kinetics indicates that the 4-BFCA has fungicidal action (reduction $\geq 99.9\%$ in the \log_{10} CFU/mL) against *Fusarium* spp. (Fig. 2). As a result, this action is dependent on the 4-BFCA concentration and elapsed time. At higher concentrations, such as MICX2,

Table 1 Minimum inhibitory concentration (MIC $\mu\text{g/mL}$) values refer to 4-BFCA and amphotericin B (AMB) against *Fusarium* spp. strains

<i>Fusarium</i> spp.	Strains	4-BFCA	AMB
<i>F. falciforme</i> (n=3)	F 9	25	4
	F 20	25	4
	HCF 19	50	8
<i>F. keratoplasticum</i> (n=3)	F 21	12.5	8
	HCF 17	25	> 16
	HCF 26	50	4
<i>F. oxysporum</i> (n=4)	F 24	25	2
	F 35	25	4
	HCF 22	50	> 16
	HCF 46	50	8
<i>F. proliferatum</i>	F2	25	4
<i>F. solani</i> (n=5)	ATCC 36,031	25	4
	F 28	25	8
	F 33	50	2
	F 34	25	8
	HCF 41	25	> 16
MIC range	-	12.5–50	2–> 16
Geometric mean	-	31.04	4.45

Resistant strains at the highest concentration tested for 4-BFCA (MIC > 50 $\mu\text{g/mL}$) and amphotericin B (AMB—MIC > 16 $\mu\text{g/mL}$)

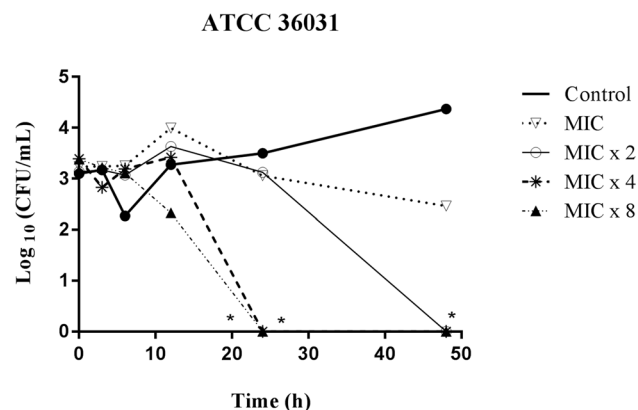


Fig. 2 Log plots of killing kinetics for 4-BFCA against *F. solani* (ATCC 31,036) strain at MIC, MICx2, MICx4, and MICx8 concentrations and control untreated. The concentrations ranged from 25 to 200 $\mu\text{g/mL}$ (MIC = 25 $\mu\text{g/mL}$). The asterisks (*) indicate statistical differences when compared to the untreated control (* $P < 0.05$)

MICx4, and MICx8, the fungicidal action starts from 24 h with significant reduction of the *Fusarium* cells (* $P < 0.05$) when compared to the untreated control. At MIC, the 4-BFCA is not able to promote a cell kill $\geq 99.9\%$ in the \log_{10} CFU/mL, not showing a significant reduction when compared to the control, acting fungistatically on *Fusarium* strain.

The antifungal effect of the 4-BFCA on *F. solani* ATCC 31,036 cells can be revealed through microphotographs of SEM. For untreated cells, the qualitative presence of the fungal mycelium formed by a large amount of branched

and elongated hyphae can be observed (Fig. 3a and d). On the other hand, 4-BFCA appears to reduce the presence of *Fusarium* hyphae when the microphotographs are compared to the untreated control. (Fig. 3b and e).

The Kaplan–Meier plot showed that all *T. molitor* larvae survived the first 24 h of exposure to 4-BFCA tested and the PBS control. From 24 h, the survival rate remained at 71.4% up to 48 h, for both treatments. There was no significant difference among the groups ($p > 0.5$), confirming that the 4-BFCA does not present toxicity to larvae (Fig. 4). In parallel, the results of the CAM model infection showed eggs with fungal growth in which embryos were dead and eggs without fungal growth in which embryos were still alive at the time of hatching (Table 2). The eggs treated with 4-BFCA solution at 100 $\mu\text{g/mL}$ showed the absence of fungal growth, while the untreated eggs in the *F. solani* growth were visualized. The 4-BFCA was 83.33% effective in combating the fungal infection in vivo (Fig. 5).

Discussion

The antimicrobial efficacy of chloroacetamides has been proven by the use of 2-chloroacetamide compound as a preservative in hygiene and cosmetic products for human use. This substance is authorized by Food and Drug Administration (FDA) as a preservative in these products, since it has no dermal toxicity, irritation, and ocular sensitivity at low concentrations, as demonstrated in an in vivo study conducted with mammalian animals [32, 33]. In Brazil, the Agência Nacional de Vigilância Sanitária (ANVISA) determines that cosmetics and hygiene products for human use contain a maximum of 0.3% of this preservative, suggesting its safety for topical use at low concentrations [34]. Despite preservative action of chloroacetamides, studies evaluating the antifungal action of this class are scarce. The findings show only information about the susceptibility profile of a *Candida* species, dermatophytes, *A. niger*, and *F. oxysporum* strain against these derivatives [16, 19, 20]. In this study, we suggest that the anti-*Fusarium* action presented by 4-BFCA is probably due to its lipophilic characteristics.

According to Lavorato et al. [21], studies of quantitative structure–activity relationship (QSAR) had shown a high value of the partition coefficient (LogP 2.52) for 4-BFCA, relating their liposolubility to its antileishmanial action, since the higher the LogP of a compound, the greater its lipophilic character. In this context, the lipophilicity can facilitate the penetration of 4-BFCA through the *Fusarium* cell membrane, leading to fungal death. Thus, we suggest that the 4-BFCA anti-*Fusarium* activity is also related to its physicochemical characteristics, such as its liposolubility. In addition, according to Machado et al. [20] the 4-BFCA presented strong anti-dermatophytic and

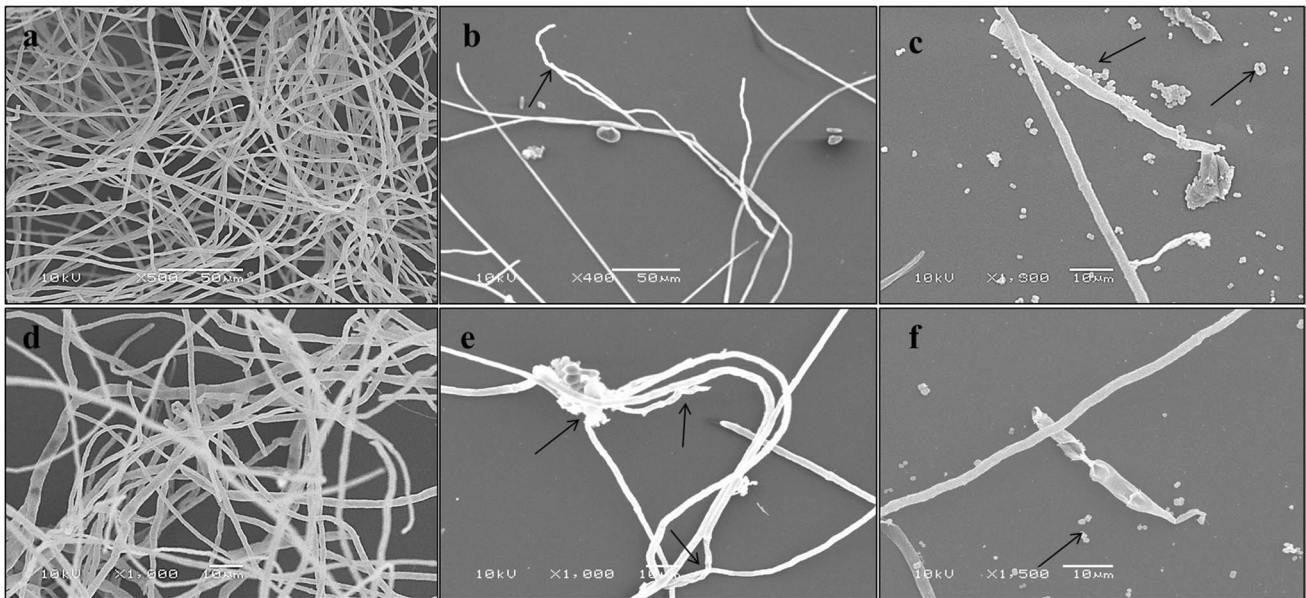


Fig. 3 Microphotographs of SEM analysis of *F. solani* (ATCC 36,031) strain in the presence of 4-BFCA at MIC/2 concentration, amphotericin B (AMB). Untreated cells were used as a control: **a** and **d** ATCC 36,031—untreated cells at different magnifications; **b** and **e** ATCC 36,031—cells treated with 4-BFCA at different magnifications; **c** and **f** ATCC 36,031—cells treated with AMB at different magnifications. The changes provoked on *F. solani* cell structure after treatment with 4-BFCA and AMB are indicated by the arrows. Currently used: 10 kV

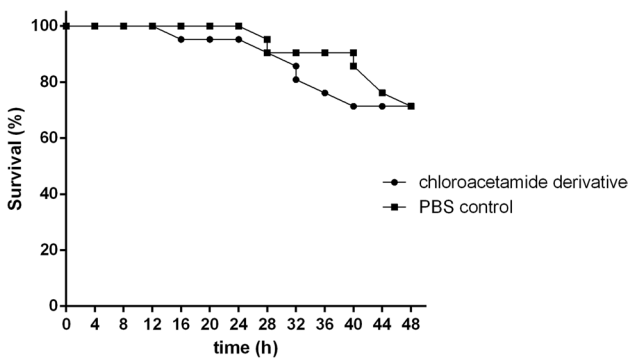


Fig. 4 Kaplan–Meier plots showing survival curve of mealworms after of exposition to 4-BFCA at 100 µg/mL (■) and phosphate buffer solution (PBS) (●)

Table 2 Chorioallantoic membrane (CAM) infection model by *F. solani* showing the antifungal activity of 4-BFCA at 100 µg/mL on eggs and embryos tested

Strain	Eggs (n=12)		Embryos (n=12)		4-BFCA efficiency
	With fungal growth	Without fungal growth	Deaths	Lives	
<i>F. solani</i> ATCC 31,036	2	10	1	11	83.33%

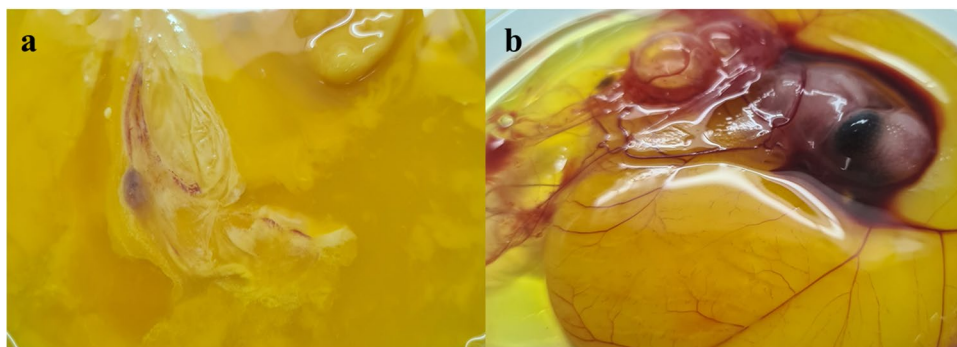
Total number of eggs and embryos tested: n=12. *F. solani* ATCC 31,036 inoculum at 1–4 × 10³ CFU/mL

anti-*Candida* action (MIC range 6.25–50 µg/mL), highlighting the greater effectiveness of this compound against filamentous fungi. The presence of bromine at the *para* position of the phenyl ring has improved the activity of the phenylchloroacetamide derivatives. This is in agreement with the data observed for the antifungals used in clinical therapy that have halogens in their structure, such as fluorine-fluconazole [35] and chloro-clotrimazole [36]. These substituents present characteristic that apparently enhances the potency of these drugs against fungal cells.

Results of analysis of time kill curves indicate that at concentrations higher than MIC, the 4-BFCA provoked a dose-dependent fungicidal action on *Fusarium* strain. According to Machado et al. [20], 4-BFCA showed only a fungistatic effect on *Candida* strains. However, on dermatophytes, a fungicidal action at concentrations higher than MIC and dependent on the elapsed time was also observed, similar to the behavior against *Fusarium*. Both fungistatic and fungicidal behavior also occurs in the action of commercial antifungal, such as fluconazole, amphotericin B, terbinafine, and echinocandins, since the fungicidal effect of the same will depend on their concentration [35–37]. Our findings are very promising, since the presence of fungicidal action is one of the essential characteristics for choosing a new antifungal candidate. Although the use of fungistatic antifungals can be considered, they only reduce the growth and reproduction of fungi, keeping the residual

antifungal candidate. Although the use of fungistatic antifungals can be considered, they only reduce the growth and reproduction of fungi, keeping the residual

Fig. 5 Macroscopic changes in infected embryonated eggs. Eggs were infected on developmental day 4. **a** Control, no treatment (embryo dead). **b** Treatment with 4-BFCA at 100 µg/mL (embryo live)



cells that can become resistant from successive expositions to these agents, causing therapeutic failures [37].

The 4-BFCA causes extravasation of the intracellular material and changes in the *F. solani* cells (Fig. 3b and e). This same behavior was observed by Machado et al. [20] when 4-BFCA was evaluated against *Candida* species and dermatophytes. However, against dermatophytes, as well as against *Fusarium* spp., 4-BFCA effect is more pronounced, which suggests a greater efficacy of this compound against filamentous fungi when compared to yeasts. AMB action on *F. solani* cells (Fig. 3c and f) demonstrates the presence of numerous *F. solani* conidia. Although further studies are needed, we suggest that the antifungal mechanism of 4-BFCA on *Fusarium* is similar to its anti-dermatophytic effect, as observed by Machado et al. [20].

Alternative models to the use of mammalian animals have been widely applied as screening tests for evaluation of acute systemic toxicity and efficacy of new compounds. Larvae of *T. molitor* appear as a new model of promising invertebrate host for evaluation of this parameter, avoiding the use of vertebrate animals in preliminary tests [27, 28]. Likewise, the in vivo infection model using the chorioallantoic membrane is also effective for evaluating the antifungal efficacy of new compounds. In this study, embryonic death in the treated eggs may be related to infection by *F. solani* strain. According to Jacobsen et al. [30], the fungal infection dose, $1\text{--}4 \times 10^3$ CFU/mL, is considered to be low. Thus, the infection would not be assigned to the dominant as one cause of the non-viability of the embryos. Our results are promising, although in vivo studies in mammalian models are needed to confirm these data.

Conclusion

The 4-BFCA was effective against multi-resistant *Fusarium* strains, showing a dose-dependent fungicidal action and able to damage the structure of fungal cells. In addition, the compound suggests safety for possible systemic use, although in vivo studies with mammalian animal models are needed. These findings are very promising, since this compound has

a high potential as a candidate for the development of a new therapy for the treatment of fusariosis.

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Author contribution Gabriella da Rosa Monte Machado participated in the conceptualization, methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, and writing—review and editing of the text. Stefânia Neiva Lavorato participated in the methodology, validation, formal analysis, and data investigation. William Lopes and Mário Teixeira participated in the methodology, data curation, and formal analysis. The professors Marilene Henning Vainstein, Ricardo José Alves, Saulo Fernandes de Andrade, and Alexandre Meneghello Fuentefria participated in the visualization, supervision, project administration, and acquisition of the financial support.

Declarations

Conflict of interest The authors declare no competing interests.

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