Inorganic compounds at regular and nanoparticle size and their anti-toxigenic fungi activity

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Abstract

Zinc-compounds are inexpensive, stable and can present strong antibacterial activity; however, there are only a few studies that report its antifungal activity. Their application advantage is that Zn-compounds are utilized often as dietary supplements and some of them (zinc acetate, oxide, carbonate and sulphate) are considered as GRAS (generally recognized as safe) and authorized for the fortification of foods by the Food and Drug Administration (US/FDA). Therefore, the aim of this study was to evaluate the antifungal properties of Zinc compounds at regular and nanoparticles (NPs) size against toxigenic strains of *Fusarium verticillioides*, *Aspergillus flavus* and *Penicillium citrinum* by the Fraternale and Kirby-Bauer methods. In addition, it was verified their activity on fungi hyphae morphological alterations, mortality and production of reactive oxygen species (ROS) by scanning electron, optical and fluorescence microscopies. The Groups were Zn treated: with $ZnSO_{4\ (regular)}$ & $ZnO_{(NPsize)}$ (conc: 25/50/100 mM) and a Control. They were added to potato dextrose agar medium, followed by mycelia material inoculation. *P. citrinum* was the fungi more sensible to treatments, which was completely inhibited by $ZnSO_{4(regular)}$ and $ZnO_{(NPsize)}$ in the concentration of 100 mM. At that concentration, *F.verticillioides* and *A. flavus* also were significantly reduced by both Zn-compounds. After ZnO-NPs treatment, *F. verticillioides* and *A. flavus* were reduced by 70.1 and 23.68%. For $ZnSO_4$ treatment, *F. verticillioides* was totally reduced, while *A. flavus* reduced 61.54%. Regarding fungi growth inhibition by the Kirby-Bauer method, the highest inhibition diameter zone was observed with the $ZnSO_4$ treatment, against all fungi strains (*P. citrinum*, *F. verticillioides* and *A. flavus*) in decreasing in order: 51, 36 and 28 mm (Control: no inhibition zone). The morphological alterations occurred in the Zn-treated fungi were hyphae ruptures and deformations, which led to cell death and ROS production. These compounds showed strong antifungal activity at low concentrations, implying that have potential for industrial application, either because they are considered essential elements for the human body and non-toxic in adequate amounts.

Keywords: Zn-compounds, toxigenic fungi, hyphae, maize grains, food safety

1. Introduction

Fungi are responsible for deterioration of raw and processed grain when exposed to optimal environment conditions such as high temperature and humidity. Maize (*Zea mays* L.) is quite prone to fungal attack and mycotoxin contamination. Some species of toxigenic fungi can cause loss of germination, discoloration and reduction of nutritional values. *Fusarium verticillioides* and *F. proliferatum*, well known as field producers of fumonisins (FBs), is often associated with economic loss in maize (Waskiewicz et al., 2012). The presence of FBs in food is reported in Brazil (Bittencourt et al., 2005; Moreno et al., 2009; Queiroz et al., 2012) and worldwide (Wang et al., 2008; Van der Westhuizen et al., 2010; Garrido et al., 2012).

Aspergillus and *Penicillium* sp. are found in stored grains, reducing their quality (Scussel et al., 2011). Moreover, toxigenic species of *Aspergillus* sp. can produce AFLs that are teratogenic, mutagenic and hepatotoxic (Benett and Klich, 2003) and among the AFLs, AFB_1 presents the highest toxic potential and is classified by the International Agency for Research on Cancer (IARC) as a Group 1 carcinogen (IARC, 1993). *Penicillium* sp. can produce citrinin (CTR) causing depression, glycosuria, proteinuria and renal lesions (Kumar et al., 2007). The occurrence of those mycotoxins can be found in a wide variety of important agricultural commodities, including maize (Soleimany et al., 2012; Vrabcheva et al., 2000).

These fungi can be controlled by organic / synthetic fungicide application in the field or in storage. However, this treatment has several disadvantages due to high toxicity to mammals and to residuals which may remain in the food (Barlow, 1985; Boobis et al., 2008). For these reasons, the interest in inorganic compounds, such as zinc (Zn) is increasing (Seven et al., 2004; Zhang et al., 2007), as they are non-toxic in appropriate amounts and can present strong antimicrobial activity at low concentrations (Burguera-Pascu et al., 2007; Guangjian et al., 2012; Kumar et al., 2013). Moreover, they are essential elements for the human body (Prasad 1995) and also can be utilized as dietary supplements.

In recent years, Zinc oxide nanoparticles (ZnO-NPs), also have received special attention due to their interesting physical chemical properties and biological application potential as antimicrobial agents (Hanely et al., 2009; Ostrovsky et al., 2009). Despite its advantages, a clear understanding of the possible health effects of nanoparticles is still unavailable, resulting in a limitation to its widespread use, especially in the area of food security (FDA, 2012).Zinc-compounds are inexpensive, stable and can present strong antibacterial activity (Zhang et al., 2007), however, there are only a few studies that report its antifungal activity (He et al., 2011). Their application advantage, apart from being often utilized as dietary supplements, some of them (zinc acetate, oxide, carbonate and sulphate) are considered as generally recognized as safe (GRAS) and authorized for the fortification of foods by the Food and Drug Administration (FDA, 2012; ODS, 2011).

This study evaluated antifungal properties of Zinc compounds at regular and nanoparticles (NPs) sizes against toxigenic strains of *F. verticillioides*, *A. flavus* and *P. citrinum* (found frequently in maize grains) by the Fraternale and Kirby-Bauer methods. Activity on fungi hyphae morphological alterations, mortality and production of reactive oxygen species (ROS) was verified by scanning electron, optical and fluorescence microscopies.

2. Materials and Methods

2.1. Fungi strain

F. verticillioides, *P. citrinum* and *A. flavus* were obtained from the Food Mycology Laboratory of Mycotoxicology and Food Contaminants (LABMICO) culture collection at the Federal University of Santa Catarina, Florianopolis, SC, Brazil. *Sample*: maize grain (15 kg).

2.2. Culture media and chemicals

Culture media: potato dextrose agar (PDA) and peptone bacteriology media were purchased from Himedia (Curitiba, Parana, Brazil). *Chemicals:* $ZnSO_4$, ZnO and Evans blue dye obtained from Sigma Aldrich Chemicals (St. Louis, MO, USA); chloramphenicol from Vetec (Duque de Caxias, RJ, Brazil) and tween 80 from Synth (Diadema, SP, Brazil). Water was obtained from a Milli-Q system 18.2 MΩ/cm.

2.3. Instruments

X-ray diffraction (XRD) system, model Cade-4, Enraf (Nonius-Eugene, OR, USA); field emission transmission electron microscope (TEM), model JEM-2100, Jeol (Peabody, MA, USA); light microscope (LM), CH-Bl45-2, Olympus (Shinjuku, Tokyo, Japan); scanning electron microscopy (SEM), Jeol (Peabody, MA, USA); confocal optical microscope (COM), model DMI6000B, Leica (Sao Paulo, SP, Brazil); autoclave, Phoenix (Araraquara, SP, Brazil); microwave oven, Philco (Sao Paulo, SP, Brazil); laminar flow cabinet, Veco (Campinas, SP, Brazil); fume cabinet, Quimis (Diadema, SP, Brazil); rotary shaker, Marconi (Piracicaba, SP, Brazil); microbiological incubator, Quimis (Diadema, SP, Brazil) and ultraviolet cabine, Dist (Florianopolis, SC, Brasil).

2.4. Preparation of Zinc-compounds

The ZnO-NPs were synthesised according to Sharma et al. (2011) and characterised by XRD and TEM. The concentrations of 25, 50 and 100 mM were prepared for ZnO-NPs and $ZnSO_4$ by diluting each compound in water.

2.5. Antifungal activities

The antifungal activity was performed according to the Fraternale et al. (2003) method. Different concentrations (50 and 100 mM) of ZnO-NPs and $ZnSO_4$ were added to autoclaved potato dextrose agar (PDA) medium into the Petri dishes, keeping one as a control (PDA without Zn-treatment). A disc (6 mm) of mycelia material, taken from the edge of 7-day-old fungal cultures, was placed in the centre of each Petri dish containing the PDA culture medium Zn-treated and incubated at 25°C for 8 days. The efficiency of Zn-compounds treatment was evaluated until the 8th day after incubation by measuring the fungi colonies diameters in millimeters.

A second methodology (Kirby-Bauer method) was performed to check the antifungal activity of the compounds, especially $ZnSO_4$. This method was evaluated by measuring the diameter of inhibition zone of according to Jorgensen and Turnidge (2007) with modifications. The fungal cultures were diluted separately with a saline solution (NaCl 0.9%) up to obtain a fungal cell density around 10^8 CFU/mL and an aliquot of this suspension was spread on the PDA media of each plate and distributed homogeneously. Wells were performed with 6 mm in diameter in the culture medium and were subsequently distributed aseptically inside wells, in triplicate, 60µL of Zn-compounds (100 mM), maintaining a well only with water for Control. All plates were incubated at 27°C for 5 days. After incubation the presence of fungi growth inhibition zone around were observed and their diameter in millimeters was measured.

2.6. Hyphae morphological alterations, mortality determination and ROS production

2.6.1. Morphological alterations

The treated fungi mycelia sections were collected, fixed with formaldehyde, washed with phosphate buffer solution and dehydrated with alcohol solution (30, 60, 80, 90 and 100%, maintaining the mycelia at 100%) and then submitted to critical point drying according to Bray (2000). After, the fungi mycelia were prepared for SEM analysis, submitted again to vacuum, and the cells were visualised, identified at different magnifications and registered by micrographies (taken at a voltage of 0.5–30 kV).

2.6.2. Mortality determination

Cell death was verified with Evans blue dye that has the ability to penetrate and remain in nonviable cells. The fungal spores were incubated in saboraud liquid medium for 24 h at 28°C for hyphae formation and later transferred to this same medium containing the Zn-compounds

for another incubation of 24 h at 28°C. The hyphae were centrifuged, soaked in 0.05% Evans blue solution and left for 5 min. Finally, the hyphae were washed three times with 1x PBS to remove the excess of dye. The resultant sediment was verified in OM at x400 magnification. The mortality was observed by coloration of blue hyphae (Semighini and Harris, 2010).

2.6.3. ROS production

For the evaluation of the ROS levels in the Zn-treated fungi hyphae, we utilised a 2,7-dichlorofluorescein diacetate (H_2DCFDA) probe that reacts with ROS and becomes fluorescent, thus being possible to evaluate the oxidative stress inside fungi cell structures. The fungal spores were incubated in saboraud liquid medium for 24 h at 28°C, for hyphae formation. After, the hyphae were incubated in this same medium containing Zn-compounds for 24 h at 28°C. The fungi suspension was centrifuged and treated with 40 µM H_2DCFDA for 30 min at 28°C in the dark. Finally, the sediments were washed three times with 1x PBS and analysed by COM x300 magnification. The ROS production was observed by coloration of green fluorescent hyphae (Liu et al., 2010).

2.7. Statistical analysis

The data of antifungal activities were analysed by analysis of variance (ANOVA) followed by Bonferroni post-test. All analyses were expressed as mean±SD, and the *p* values <0.05 were considered statistically significant.

3. Results and Discussion

ZnO-NPs were synthesized and characterized by the microwave method (Sharma et al. 2011) and resulted in particle size of 30 nm mean diameter. Figure 1 shows the XRD pattern of the synthesized ZnO-NPs. All the diffraction peaks are in good agreement with those of hexagonal wurtzite structure of ZnO. The peaks of sharp intensity between 30º and 40º theta scale can be indexed to the wurtzite ZnO with high crystallinity (Guo et al. 2005).

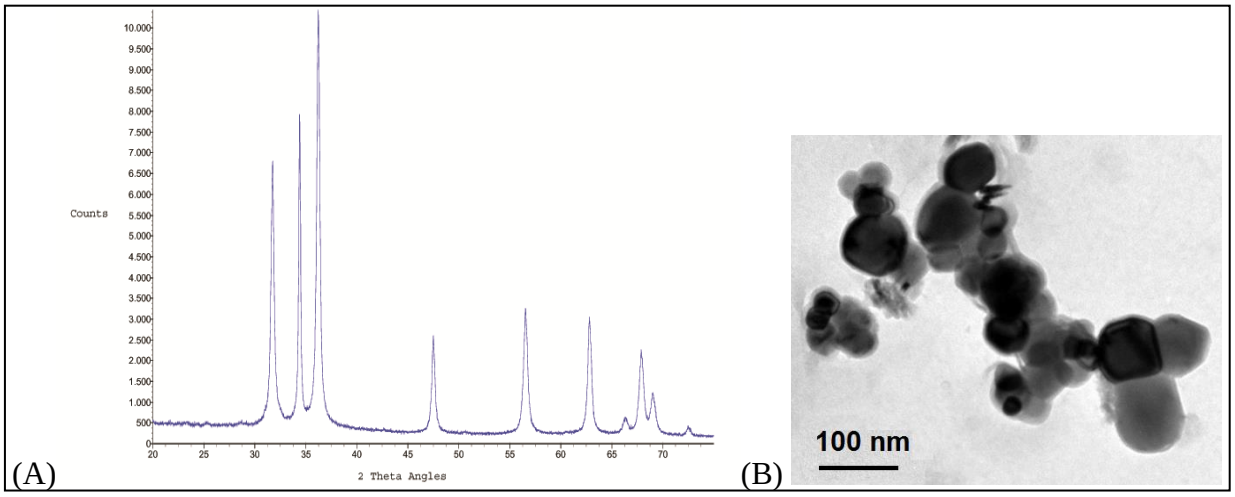

Figure 1 Characterization of ZnO-NPs: (A) X-ray diffraction plot and (B) TEM imaging showing mean diameter of 30 nm size.

Zn-compounds showed significant results against fungi toxigenic contaminants of the maize grains. *P. citrinum* was the fungi most susceptible to treatments, which was completely inhibited by $ZnSO_{4(regular)}$ and $ZnO_{(NPsize)}$ in the concentration of 100 mM. On the other hand, at that same concentration, *F.verticillioides* and *A. flavus* also were significantly reduced by both Zn-compounds. After ZnO-NPs treatment, *F. verticillioides* and *A. flavus* were reduced in 70.1 and 23.68% (Figure 2). For $ZnSO_4$ treatment, *F. verticillioides* was totally reduced,

while *A. flavus* reduced 61.54% (Figure 3). Others studies already were performed previously in our laboratory showing the efficacy of ZnO and others Zn compounds, reducing significantly the *F. graminearum* colonies growth (Savi et al., 2013).

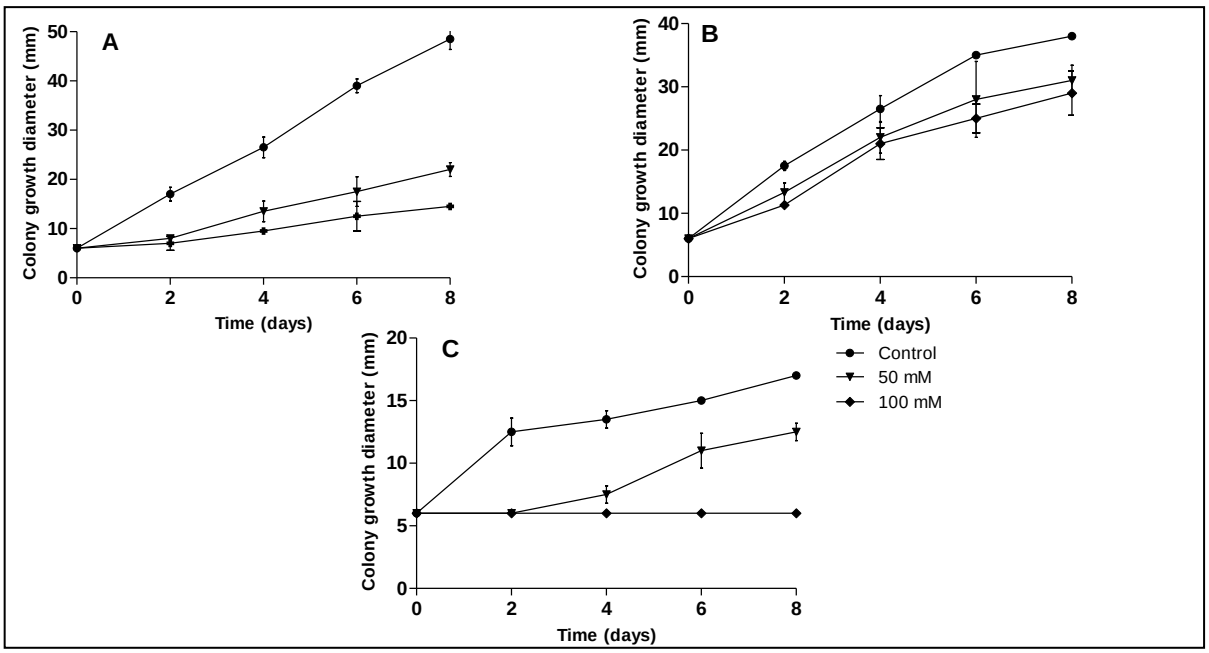

Figure 2 Antifungal activities of ZnO-NPs against (A) *F. verticillioides*, (B) *A. flavus* and (C) *P. citrinum* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal colony - each point represents an average of triplicate measurements).

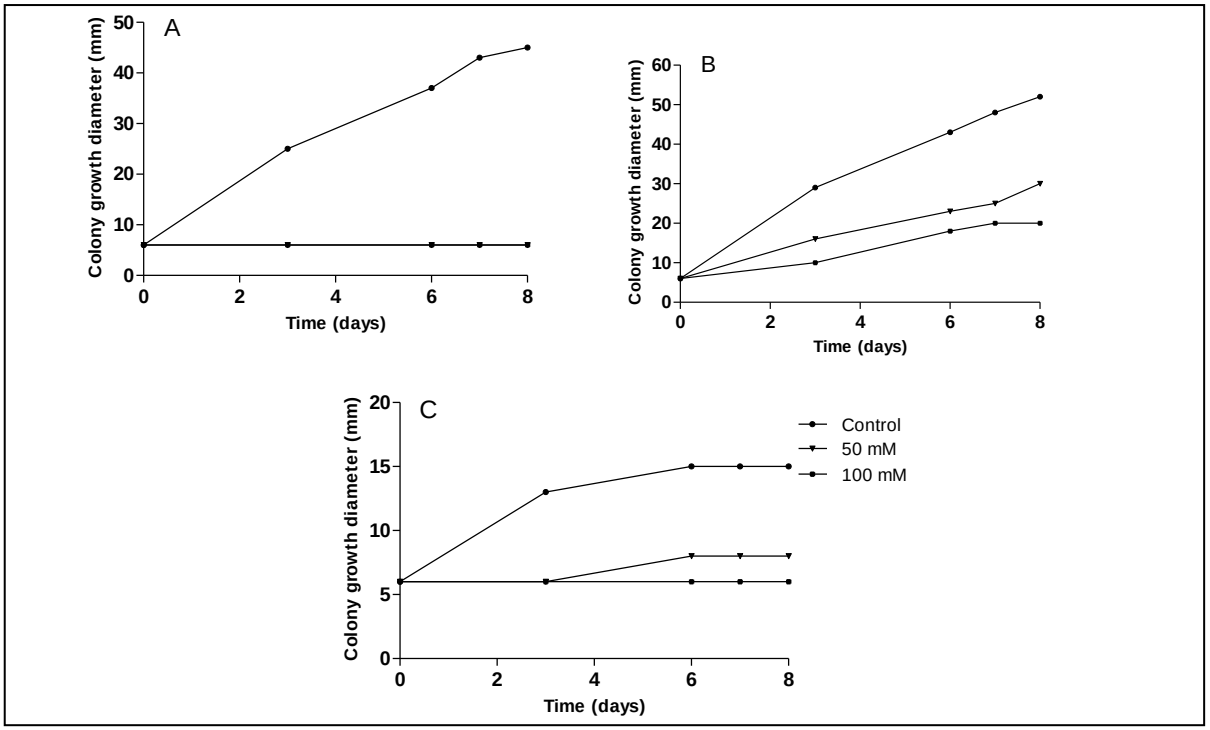

Figure 3 Antifungal activities of $ZnSO_4$ against (A) *F. verticillioides*, (B) *A. flavus* and (C) *P. citrinum* on PDA at different concentrations (data are shown as average values and standard deviation of diameter fungal - each point represents an average of triplicate measurements).

Regarding fungi growth inhibition by the Kirby-Bauer method, the highest inhibition zone was observed with the $ZnSO_4$ treatment, against all fungi strains (*P. citrinum*, *F. verticillioides* and *A. flavus*) in decreasing in order: 51, 36 and 28 mm (Control: no inhibition zone) (Figure 4). This second methodology used for evaluate the antifungal activity of Zn-compounds was highly sensible and proved to be efficient against *P. citrinum* (strong inhibition zone) that presented higher growth reduction as verified in the previous method.

Recent studies have showed antimicrobial activity of Zn-compounds similar to ours results. Sharma et al. (2011) found strong antifungal activity of ZnO-NPs synthetized by microwave method against plant fungus *Pythium debarynum* in the concentration of 10 mM. He et al. (2011) showed that 12 mM of ZnO-NPs were sufficient to completely inhibit the growth of *Botrytis cinerea* and *P. expansum*. Antibacterial properties of $ZnSO_4$ also were studied, showing that their addition to glass-ionomer-based cements led to significant inhibition of *S. mutans* growth (Osinaga et al., 2003).

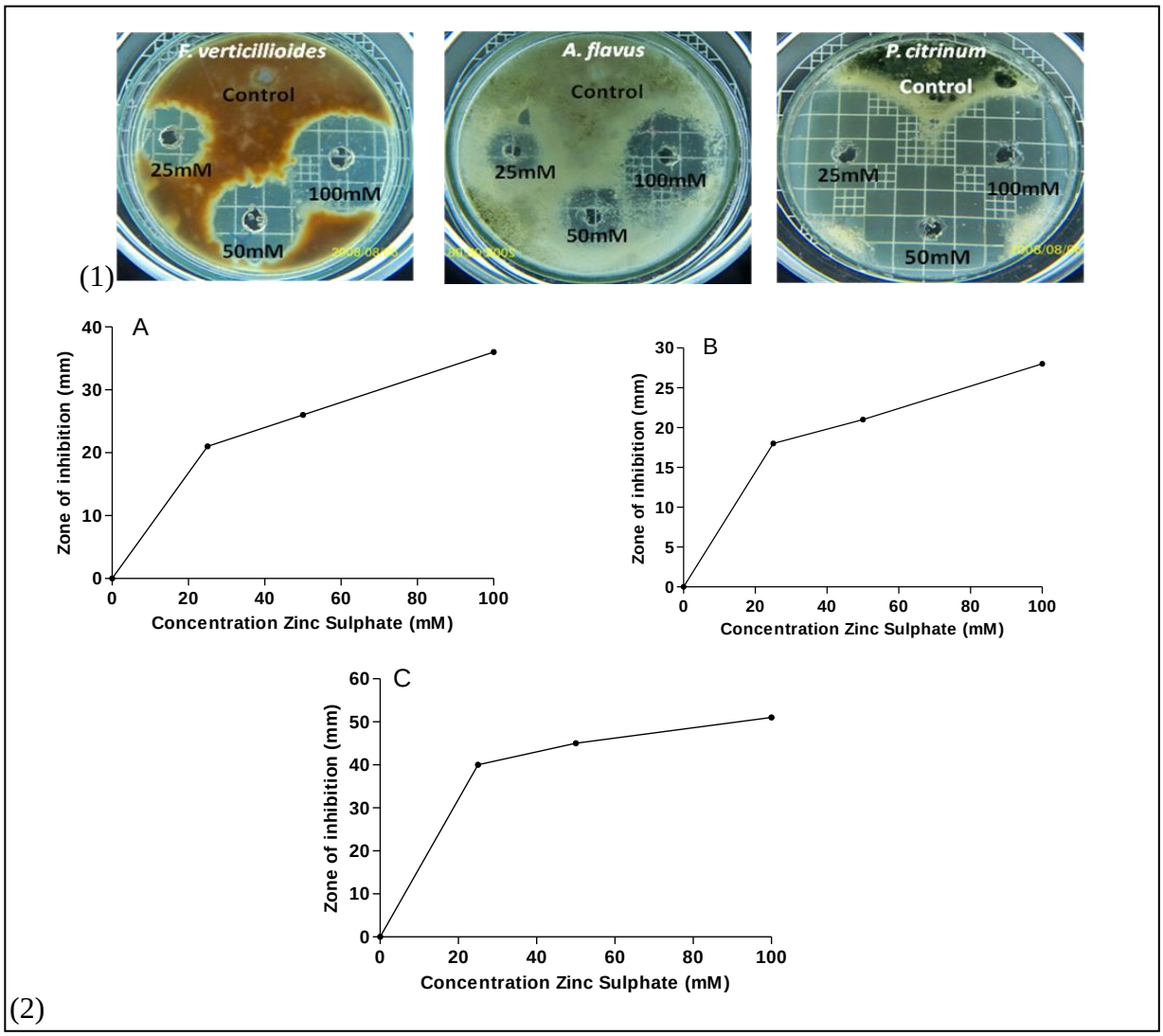

Figure 4 Antifungal activities of $ZnSO_4$ by *Kirby-Bauer method*: (1) culture PDA media fungi inhibition of (A) *F. verticillioides*, (B) *A. flavus* and (C) *P. citrinum* at different concentrations. (2) Inhibition zone versus concentration (data are shown as average values of inhibition zone diameter).

The hyphae morphological alterations that occurred after Zn-treated fungi were ruptures and deformations, observed by SEM analysis (Figure 5A). Changes in the structure of the fungus were also observed using ZnO-NPs against *P. expansum* and *B. cinerea* (He et al., 2011). SEM analized also can confirm changes and rupture of the fungal cell membrane in *F. verticillioides, A. flavus* and *P. citrinum* due to gold NPs presence in culture medium (Savi et al., 2012). In our study, it was observed the development of a very strong intensity Evans blue staining in all the Zn-compounds hyphae treated indicating that they inflicted high damage to those fungi (Figure 5B).

ROS has important roles in cell signaling and homeostasis, however under stress conditions, their production can be greatly increased. To evaluate if fungi cell death occurred due to the increase of intracellular ROS production, we utilized H_2DCFDA as a specific proof of general oxidative stress. H_2DCFDA is permeable to cell membrane and undergoes intracellular conversion by nonspecific esterases to form nonfluorescence 2,7 dichlorofluorescein (DCFH). DCFH oxidizes in the presence of ROS to form 2,7 dichlorofluorescein (DCF), which emits a high green fluorescence (Cathcart et al., 1983). Our results pointed out to an increase of ROS production in the fungi hyphae treated with Zn-compounds, which were observed a stronger fluorescence intensity (Figure 5C).

The morphological alterations that occurred in the Zn-treated fungi can lead to cell death and ROS production, reducing the colony growth of the *F. verticillioides*, *A. flavus* and *P. citrinum*, species frequently found in maize grains.

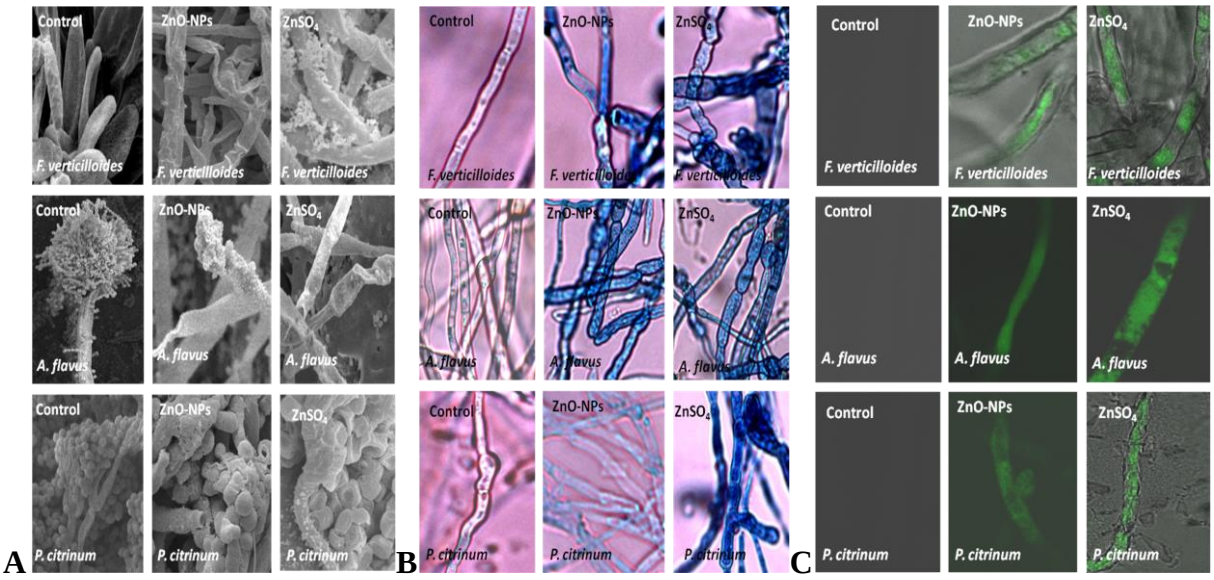

Figure 5 Effect of Zn-treatments on *F. verticillioides, A. flavus* and *P. citrinum* showing hyphae: (A) alterations by scanning electron microscopy; (B) mortality after Evans blue staining, by light microscopy; (C) fluorescence with ROS production after H_2DCFDA reaction, by confocal optical microscopy [Treatment *Groups:* Control (no Zn treated); ZnO-NPs and $ZnSO_4$ - concentration of 100 mM].

4. Conclusions

The mechanism of Zn-compounds expressed as the fungi hyphae alterations is related to the antifungal activities of the treatments utilized in this study. Their strong antifungal activity were observed at low concentrations, implying that they have potential for industrial

application, either because they are considered (a) essential elements for the human body and (b) non-toxic in adequate & effective amounts.

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