Antifungal action of *Cinnamomum zeylanicum* Blume essential oil against *Penicillium* spp from environment air of a dry food industry.

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The environment microbiota is a potential contamination source in the food industry, including *Penicillium* spp. By contrast, industry has used chlorinated solutions for the control of microbial species, but the potential antimicrobial and antifungal activity of the essential oil of *Cinnamomum zeylanicum* Blume (OE) has been reported in the literature. Thus, it is justified to study this chemical activity on *Penicillium* strains isolated from the environment of a food industry located in the city of João Pessoa, Paraíba. Tests were conducted to determine the minimum inhibitory concentration (MIC), assess the effect on mycelial growth and spore germination by using the OE and a Vega® chlorine solution (VG), commonly used. MIC values obtained rangeed from 256 to 512µg/mL for both OE and VG. The observed effect on the radial growth was concentration dependent for both products. On the spores germination, OE showed higher effectiveness compared to VG, interfering with conidia germination. The OE is emerging as an excellent alternative to be used in the control of *Penicillium*, once VG needed a higher concentration of application and still not had the same EO effectiveness.

Key words: *Cinnamomum zeylanicum,* essential oil, *Penicillium* spp, environment air, food industry.

INTRODUCTION

Fungi are among the most important pollutants or contaminants in indoor air, being virtually ubiquitous in urban and industrial environments. The development of these fungi may represent an important factor in the emergence of infectious or toxic diseases in animals and plants, including man (Lacz et al. 1998; Burt, 2004). In air environment of Food Industries, the presence of *Penicillium* fungi denotes a possible risk to the ultimate consumers of these products. The fungus is commonly related to food contamination, highlighting the economic losses caused by these contaminants and human health problems from mycotoxin production (Samson et al. 2004). The market offers few sanitizer solutions that have been studied regarding its antimicrobial activity, being mentioned chlorine-based disinfectant solutions, quaternary ammonium compounds, organic acids such as citric acid, lactic acid (Kim, 1999). Moreover, the use of biologically active natural compounds derived from plants such as bactericides and fungicides have been worth noting, with some constituents of these substances proved fungitoxic, as reported in several articles (Okigbo et al. 2006, Araujo et al. 2009; Schwan-Estrada, 2002). One product that has emerged as alternative is the essential oil, highly variable and complex mixture obtained from different plant parts (Aflatuni, 2005). The essential oil extracted from *Cinnamomum zeylanicum* Blume is marketed to cosmetology, cooking and alternative medicine industries due to its analgesic, antiseptic, antispasmodic, aphrodisiac, astringent, insecticide and parasiticide properties (Jayaprakash et al. 2003; Gayoso et al. 2004, Moreira et al. 2007; Trajano et al. 2010). Based on the above, it is important to check the action of essential oil of *Cinnamomum zeylanicum* Blume against wild strains of *Penicillium* spp. collected from

environment air of a food industry and compare its action with the chlorine, usual sanitizer in food industries.

MATERIAL AND METHODS

Products

The essential oil (EO) from leaves of *C. zeylanicum* was obtained from Ferquima Ind. and Com. Ltda (Vargem Grande Paulista, São Paulo, Brazil) and its quality parameters such as appearance, aroma, purity, odor, and density (20 C) and refractive index (20°C) are described in the technical report that accompanies the product. The oil was stored in amber bottle and kept refrigerated at a temperature below 4°C in the Mycology Laboratory. AE Chlorinated Vega® (VG) (batch 2100514-G22), chlorinated alkaline detergent, was obtained in Quimilab - Cleaning Products, and its quality parameters such as physical state, density, odor, pH, water solubility and explosive limits, described in the technical report that accompanies the product. The VG was packaged and stored under the same conditions as the OE.

Products were used in concentrations from 1024 to 256 µg/mL. Products dilutions in different concentrations were prepared at the test time of execution of the tests by dissolving them in Tween 80 q.s. and sterile distilled water to reach the initial concentration of 1024 µg/mL.

Microorganisms

For antifungal activity assays, we selected 14 strains of *Penicillium* spp. obtained from the Laboratory of Mycology collection. Strains were collected from environment air of a dry foods industry (derived from corn and starches), which are codified from *Penicillium* AR1 (PAR1) to *Penicillium* AR14 (PAR14) Stock strains used in the trials were kept in test tubes containing Sabouraud dextrose agar (SDA) tilted under refrigeration (4°C).

Inoculum

In the fungal inoculums preparation, isolates were first cultured on SDA at 28°C for 10 days. Recent fungal colonies were properly covered with 5 mL of sterile saline solution (NaCl 0.85%) and suspensions were made by gentle scraping and agitation with the aid of a “L” bacteriological loop. The suspension was transferred to sterile test tubes.Then, these suspensions were shaken for 2 minutes with aid of a Vortex. After shaking, each suspension had its turbidity visually adjusted and compared to that presented by the barium sulphate suspension in Tube 0.5 of McFarland scale, which corresponds to an inoculum of approximately 10^6 colony forming units/mL (CFU/mL) (Hadacek et al. 2000, Sahin et al. 2004). The inoculums quantification was confirmed by plating 0.01 mL of suspensions on SDA. Plates were incubated at 28°C and daily examined for the colonies counting, confirming the number of CFU/mL.

Antifungal Activity

Minimum Inhibitory Concentration (MIC)

The determination of the essential oil MIC values was performed by the microdilution method (Eloff, 1998), using microtiter plates with 96 wells and "U" bottom (ALAMAR®) (Hadacek et al. 2000, Sahin et al. 2004, Pereira et al. 2011). In each plate hole was added 100 µL of Sabouraud dextrose broth (CSD) doubly concentrated. Subsequently, 100 µL of the products, also doubly concentrated, were dispensed into the wells of the plate’s first line. And concentrations from 1024 µg/mL to 1 µg/mL were obtained through a serial dilution at the ratio of two. 10 µL of fungal species inoculums were added to the cavities. A microorganism control (negative control) was performed in the absence of antifungal products. To verify the solvent interference with emulsion preparation in the results, 100 µL doubly concentrated CSD, 100 µL Tween 80 (10% in sterile distilled water) and 10µL of suspension were added to the control cavities. Plates were sealed and incubated at 28°C for 5 days. MIC for products was defined as the lowest concentration able to visually inhibit fungal growth when compared with control growth. Tests were performed in duplicate and results expressed as geometric mean of results.

Effects on the radial mycelial growth

The test for determining the effects on the radial mycelial growth of PAR7 and PAR12 was performed using the poisoned substrate technique (dilution in solid medium). For this, we initially prepared Petri dishes (Dispo Petri/Interlab®) with 8 mL ASD plus the essential oil or VG at MIC/4, MIC/2, MIC and CIMx2 for each strain individually. Subsequently, a fragment with approximately 2 mm was withdraw from fungal strains grown on SDA at 28°C for 10 days and placed at the center of the plate containing SDA added to products at the aforementioned concentrations. A negative control was also achieved in the absence of any antifungal product. The whole system was incubated at 28-30ºC. At each time interval (0, 2, 4, 6, 8, 10, 12, 14 days), the radial mycelial growth was recorded and the results expressed as mean of two independent experiments in millimeters (mm) (Adam et

Table 1: MIC and MFC values of *C. zeylanicum* essential oil and VG on *Penicillium* spp strains.

Fungi	OE (µg/mL)	VG(µg/mL)	Controls	
	MIC	MIC	Tween 80 (10%)	Microorganisms Control
PAR1	512	512	+	+
PAR2	512	512	+	+
PAR3	256	512	+	+
PAR4	512	512	+	+
PAR5	512	512	+	+
PAR6	256	512	+	+
PAR7	256	512	+	+
PAR8	512	512	+	+
PAR9	512	512	+	+
PAR10	256	512	+	+
PAR11	256	256	+	+
PAR12	512	512	+	+
PAR13	256	256	+	+
PAR14	256	256	+	+

+: Fungal growth in free-antifungal culture medium

al. 1998; Dafera et al. 2003).

Effects on fungal spores germination

In this trial was evaluated the interference of products on the germination of fungal spores of PAR7 and PAR12. Suspensions of these strains were shaken for 2 minutes with aid of a Vortex, remained at rest for 20 minutes and the supernatant containing the conidia was collected. The number of conidia was determined in a Neubauer chamber and adjusted to 10^6 spores/mL. In sterile test tubes, 512 µL of doubly concentrate CSD were added to the essential oil or VG in its respective concentrations of CIM/2, CIM, CIMx2 and CIMx4. Afterwards, 512 µL of fungal conidia suspension were introduced and immediately incubated at 28°C. Samples of this mixture were analyzed after 24 h and the spore germination analysis was conducted using a standard optical microscope Zeiss Primo Star® model. The whole system was carried out twice and the results expressed as arithmetic mean of conidial germination inhibition percentage (Rana et al. 1997, Santiago et al. 2000).

Statistical Analysis

The statistical evaluation of results for the study on the products effects on the mycelial growth and spore germination inhibition percentage was performed to determine statistically significant differences ($p < 0.05$), using the Mann-Whitney test and Fischer test respectively. In all cases, the implementation of statistical analysis was performed using GraphPad Prism version 5.0.

RESULTS

MIC values found to *C. zeylanicum* and VG are shown in Table 1. Overall, the essential oil of *C. zeylanicum* showed similar effectiveness in inhibiting the growth of *Penicillium* spp strains compared to VG, a chlorine product commonly used as industry sanitizing where strains were isolated. All strains used in the tests were sensitive to *C. zeylanicum* essential oil showing variables MIC values ranging from 256 to 512 µg/mL similar to those found for the VG.

For the remaining experiments were used two strains with different sensitivity profiles to essential oils: PAR7 (MIC 256 µg/mL) and PAR12 (MIC 512 µg/mL).

The effect of *C. zeylanicum* essential oil at MIC/4 (64 µg/mL) MIC/2 (128 µg/mL), MIC (256 µg/mL), CIMx2 (512 µg/mL) on the kinetics of radial mycelial growth of PAR7 are shown in Figure 1A and 1B. The results show that the essential oil had effective kinetics inhibition power on the radial growth. From 128 µg/mL, the oil showed significant unlike results ($p < 0.05$) in the last interaction time compared to control. While the VG showed difference ($p < 0.05$) only in the MICx2 (1024 µg/mL) when compared to control in the last interaction time.

Figure 2A-B presents the interference results of the essential oil and VG on the inhibition kinetics of radial mycelial growth for PAR12 strain. The essential oil also had a strong inhibitory effect with significantly different results ($p < 0.05$) compared to the control from the MIC (512 µg/mL) in 12 days. The VG showed significant difference only in the MICx2 (1024 µg/mL) in 12 days. These results confirm the higher resistance profile for PAR12 compared to PAR7 and also show the greatest inhibition power of the *C. zeylanicum* essential oil inb relation to the VG sanitizing.

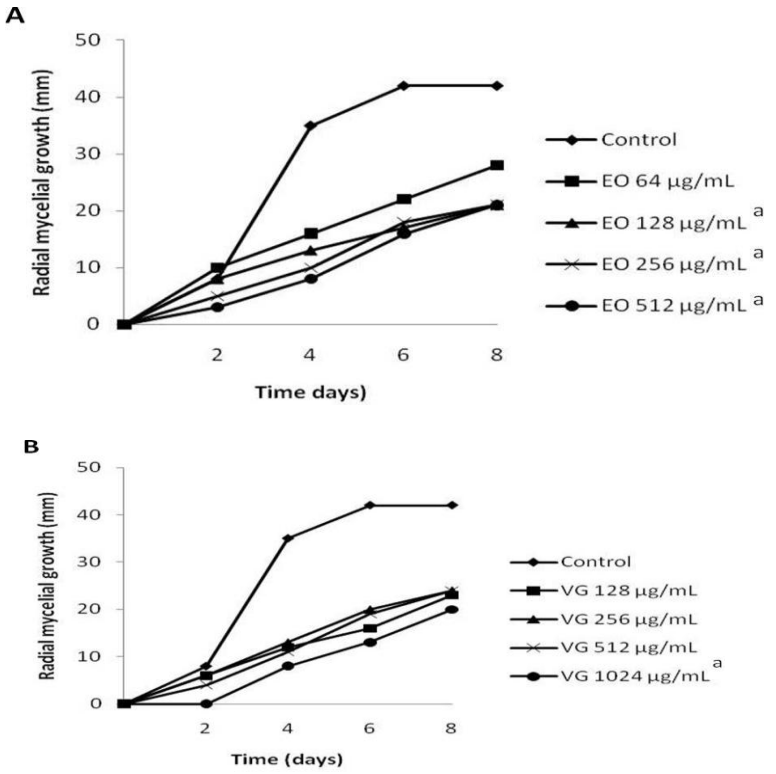

Figure 1. Effects of *C. zeylanicum* essential oil (EO) (A) and VG product (B) in the radial mycelial growth of PAR7. a: p <0.05 compared to control after 12 days of interaction.

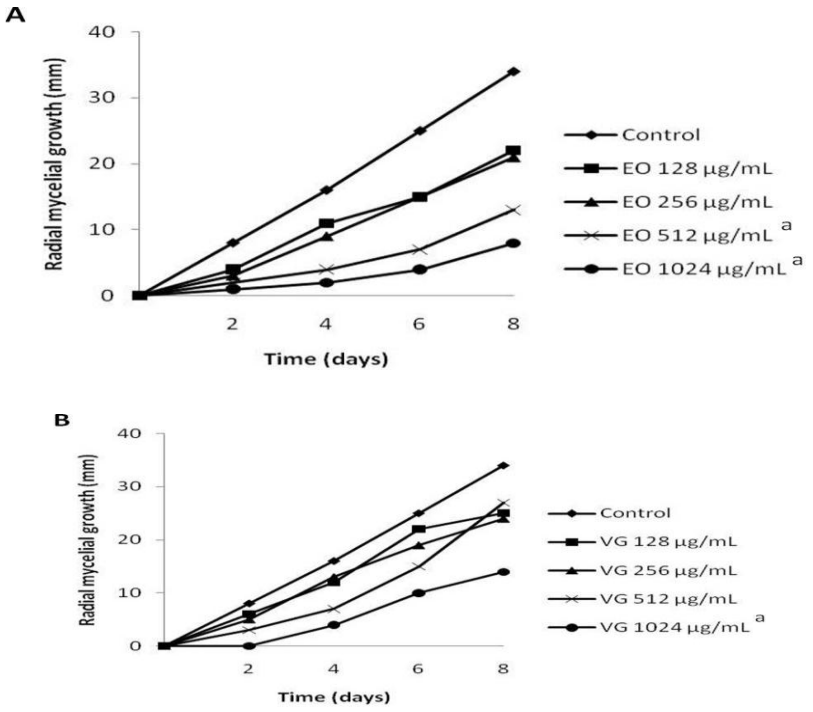

Figure 2. Effects of *C. zeylanicum* essential oil (EO) and Vega® product (VG) in the radial mycelial growth of PAR12. a: p <0.05 compared to control in 12 interaction days.

Table 2: Inhibition percentage of spores' germination for PAR7 and PAR12 after 24h interaction with the *C. zeylanicum* essential oil (EO) and the chlorinated product (VG).

Fungi	OE				VG			
	MIC/4	MIC /2	MIC	MIC x2	MIC /4	MIC /2	MIC	MIC x2
PAR7	66.7	77.7	92.6	94.6	52.7	60.8	81.8	82.2
PAR12	45.4	77.8	90.0	90.7	59.1	81.6	84.6	90.9

The effect of *C. zeylanicum* essential oil and VG on the spores germination of PAR7 and PAR12 are summarized in Table 2. In all cases, there was a gradual increase in the inhibition percentage with the increased concentration of products, showing a concentration-dependent inhibitory effect. Comparing the products, no difference was observed among results for each time separately. However, it is valid to note that the inhibitory concentration values of the essential oil are lower than the respective VG values for PAR7 strain (Table 1). And thus, indicating the greater effectiveness of the essential oil in relation to VG in this case interfering with conidia germination. In the results with PAR12, oil and VG had similar power to inhibit spore germination since there was no difference among results of products for each time separately.

DISCUSSION

The MIC results obtained in this study proved to be equivalent with those reported by Trajano et al (2010), indicating the antibacterial action of this oil against bacteria of interest in food. In our study, there was mycelial growth inhibition using the essential oil, similar to that found by Cvek et al. (2010) who tested the *C. zeylanicum* essential oil against *Peniccilium expasum* strains isolated from apple, resulting in 100% growth inhibition. The activity of *C. zeylanicum* essential oil was compared with the Vega® chlorine product activity - nominated by traditional methods - the use of chlorinated detergents in removing or sanitizing environments, however, the efficiency of chlorine as a fungistatic agent depends on pH and free chlorine concentration (Prusky et al. 2001). Rubin (1983) in his work emphasized mycobactericidal activity of formaldehyde, iodine, iodophor and chlorine compounds. Chlorine, in its various forms, especially salts of hypochlorite, is one of the most successful sanitizers used in food industries. They are efficient and low cost compounds with wide application (Kim, 2004). Results found indicate a decreased efficiency of chlorinated agent in relation to the essential oil, since the MIC was higher for VG against a more resistant strain. To Morato et al. (2009) there is a need for frequent evaluation of disinfectants for measurement of its antimicrobial activity, being important trials to assess adequately the nature and origin of the strain being tested, the inoculum preparation, product's active ingredient and its respective concentration.

Results for the radial growth kinetics of PAR7 and PAR12 with exposure to the essential oil showed a strong inhibiting effect. For exposure to chlorine product, results revealed difference only with the highest concentration and exposure time. In the experiments of Moreira (2007), *C. zeylanicum* oil showed fungicide effect on *Aspergillus flavus, A. fumigatus*, observed by the total inhibition of mycelial growth throughout 14 days of exposure. Rosal et al. (2009) tested the effect of *Salvia officinalis* L. extract at different concentrations on the mycelial growth of *Penicillium* spp., detecting that the higher the extract concentration applied to the culture medium, the more progressively reduced the mycelial growth. In the experiments of Carmo et al. (2008), *C. zeylanicum* essential oil had strong suppression effect on spores' germination of *Aspergillus* species tested. The findings in this study demonstrate inhibition activity of spores' germination related to the concentration, with oil being more effective than the chlorine compound with PAR7 (most sensitive strain) and presenting activity similar to the VG chlorinated compound when using PAR12.

Cinnamaldehyde, linalool, eugenol and 1,8 cineole have been reported as active components present in the essential oil of *Cinnamomum* spp. capable of inhibiting the growth of *Monilia, Botrytis and Mucor* (Goubran et al., 1993). To Ranasinghe et al. (2002), antifungal activity of essential oils can not be easily correlated with any individual component but with a mixture of compounds in these oils.

Given the data found in this work, the essential oil of *Cinnamomum zeylanicum* emerges as an excellent alternative to be used in *Penicillium* control since the commonly used Vega® chlorine product needed a higher application dosage and did not have the same EO effectiveness.

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