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Combined analytical and microbiological tools to study the effect on *Aspergillus flavus* of cinnamon essential oil contained in food packaging

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ABSTRACT

Cinnamon essential oil has been used for centuries to protect food from microbiological infection, and in the last ten years cinnamon essential oil is also incorporated into food packaging materials as antimicrobial agent. However, very little is known about the real effect that it has on the microorganism cells. This study combines analytical and microbiological tools to elucidate cell damage produced on *Aspergillus flavus*. First, antifungal activity of cinnamon essential oil was evaluated at 10^3,10^4, 10^5 and 10^6 CFU/mL. Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) were determined by macrodilution in direct contact with the mold. A strong activity was obtained, with a MIC of 0.05–0.1 mg/mL, and a MFC of 0.05–0.2 mg/mL, both ranges depending on the initial fungal suspensions.

Polyethylene terephthalate films containing cinnamon essential oil were tested in vapor phase, without direct contact with the mold. Active PET started showing activity at 2% CIN EO load and produced total inhibition at 4% CIN EO. SEM and FTIR were used to study the cell damage on the mold exposed to the cinnamon essential oil. Evident damage and a strong decrease in sporulation were found by SEM, while biochemical changes in conidia could be suggested from the FTIR spectra analysis. Two deposition techniques were used to prepare the samples for FTIR. The results obtained are shown and discussed.

1. Introduction

In the last ten years many studies have been published about the development of active packaging materials, most of them focused on food applications (Becerril, Gomez-Lus, Goni, Lopez, & Nerin, 2007; Goni et al., 2009; Lopez, Sanchez, Batlle, & Nerin, 2005; Lopez, Sanchez, Batlle, & Nerin, 2007a; Nielsen & Rios, 2000). This is an area of great interest for both industry and academia, as the introduction of protective agents in the packaging materials can be used to protect the food without direct addition of new chemicals. The current trend of having more natural and ecologically produced foodstuffs, while simultaneously requiring longer shelf life, is a challenge the food industry has to face. One key point in this research is the selection of the active agents, i.e. the protective substances to be incorporated into the packaging materials (Coma, 2008). Natural extracts, such as essential oils (EO) and their constituents, are categorized as flavorings in Europe (European Decision 2002/113/EC of January 23rd 2002, notified under document number C(2002) 88). In addition, essential oils and their constituents are categorized as GRAS (Generally Recognized as Safe) by the US Food and Drug Administration. For this reason, essential oils (EOs) have been often proposed and used as antimicrobial, antifungal and antioxidant agents, in general with good results (Gutierrez, Batlle, Sanchez, & Nerin, 2010; Gutierrez, Escudero, Batlle, & Nerin, 2009; Gutierrez, Sanchez, Batlle, & Nerin, 2009; Rodriguez, Batlle, & Nerin, 2007; Rodriguez-Lafuente, Nerin de la Puerta, & Batlle, 2009). Among them, cinnamon EO has demonstrated a strong antimicrobial activity, although most of the studies show the behavior versus bacteria and only few reports are dedicated to molds. It is not possible to compare antifungal and antibacterial activity (Ghannoum & Rice, 1999). Furthermore, antifungal activity is studied and reported using widely variable methodology, as there are no standard protocols to test EO on fungi (Holley & Patel, 2005; Tullio et al., 2007). Molds are difficult to inhibit due to their complex structure. They reproduce through spores, from which they grow in the form of multicellular filaments called hyphae. The interconnected network of hyphae forms the mycelium, and supports the fertile conidiophore containing the spores that can be propagated. There are thousands of known species of molds, some of which are

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opportunistic pathogens which can cause serious diseases, even with lethal effects (Fischer & Dott, 2003; Samson, Hoekstra, & Frisvad, 2004; Samson, Houbraken, Thrane, Frisvad, & Andersen, 2010).

One of the most common mold genera is *Aspergillus*, which is a pathogen for plants, animals, and humans that produces aflatoxin, a potent carcinogen (Fischer & Dott, 2003) that can also cause acute or chronic intoxication upon ingestion of contaminated food (Riba et al., 2010). This highlights the importance of inhibiting this particular mold in foodstuffs.

It is generally accepted that the release of the active substance depends on the underlying substrate (plastic film, paper, cardboard…), as happens in most of the cases with bacteria. However, the metabolism of molds is very different and the influence of the substrate and formulation on the release kinetics should be studied.

Mold inhibition is mediated by the biochemical interaction between the antifungal substance and the microorganism. However, this interaction has not been studied in depth yet, and only macroscopic effects have been reported. Thus it is not clear whether the mycelium or the spores are affected, and whether this effect is concentrated in the membrane or inside the cell.

For the purpose of active packaging material design, it is not only necessary to demonstrate the antifungal activity, but also to establish which compounds are responsible for those properties, and what the optimal concentration is to obtain maximum inhibition. On the other hand, in order to study the mechanism of action of the essential oil, few analytical techniques can be applied as most of them are destructive for the cells. In the last decade, high resolution FTIR (HR-FTIR) has become a powerful technique to identify cell biochemical composition (Jilkine, Gough, Julian, & Kaminskyj, 2008). Although the spatial resolution is limited, the non-destructive character and the selectivity of the specific bands associated with selected functional groups, allow studying changes produced by the exposure of the microorganism to the active packaging materials. Cellular changes can also be studied with high spatial resolution by scanning electron microscopy (SEM). Although this technique does not provide the key chemical selectivity needed to detect subtle changes in the sample, it is a powerful complement to observe clearly the integrity loss of the cell wall.

The two main objectives of this work are: i) to analyze the influence of the substrate on the antifungal activity versus *Aspergillus flavus* of several packaging materials containing cinnamon EO as active agent and ii) to provide data to elucidate the mode of action of this substance. Microbiological performance of the mold is analyzed in the presence of the active agent in pure form and as released from the packaging materials (PET and paper). Cell damage is assessed in the presence of cinnamon EO released from the active packaging materials.

2. Materials and methods

2.1. Microorganism and culture media

Strain of *A. flavus* CECT 2949 (Colección Española de Cultivos Tipo) was provided by the Department of Microbiology of the University of Valencia (Spain). For the culture media, Czapek (CZP) as solid media and Yeast Extract Broth (YEB) as liquid media were employed, all supplied by Scharlau (Spain). As diluents, distilled water with 0.1% Tween 80 and physiological solution (NaCl 0.9%) were used, both provided by Panreac (Spain).

2.2. Reagents

Cinnamon essential oil (CIN EO) from the bark, fortified with cinnamaldehyde (*cinnamon zeylanicum,* CAS 8015-91-6) and with a final concentration of cinamaldehyde of 900 mg/g was supplied by Argolide (Spain). Cinnamon EO from the leaves usually contains eugenol as major component and little amount of cinamaldehyde. The efficiency of this latter EO as antimicrobial agent has been demonstrated to be much lower than that coming from the bark. The CIN EO was fortified by the company Argolide, who certifies the final concentration of cinnamaldehyde. Minor components such as b-caryophyllene, linalool and safrol were also present but their influence was very low, according to the previous studies mentioned above. Ethanol without stabilizer, HPLC grade, was supplied by Scharlau (Spain). Appropriate dilutions of the pure CIN EO in ethanol were used for some experiments. Glutaraldehyde, sodium cacodilate and sacarose, all provided by Panreac (Spain) were used as dehydration reagents.

2.3. Active PET

Active PET containing CIN EO was provided by Artibal (Sabiñánigo, Spain), and consisted of a 25 μm thick layer of PET coated with an organic solvent base formulation containing the essential oil. The grammage of the coating was between 2.0 and 2.5 g/m^2. Active PET layers with 2, 4, 6 and 8% CIN EO amounts were tested, where each percentual point amounted to 0.0355 g/m^2.

2.4. Minimal Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

A. flavus inocula of 10^3, 10^4, 10^5 and 10^6 CFU/mL were prepared in NaCl 0.9% and confirmed by plate counting. MIC values were obtained by macrodilution in Yeast Extract Broth (YEB) test tubes (Manso, Nerín, & Gómez-Lus, 2010). Similar methodology has been employed by other authors in the antifungal determination of the EOs (Mitchell, Stamford, de Souza, Lima, & Carmo, 2010; Rasooli & Owlia, 2005; Rasooli, Rezaei, & Allameh, 2006) Samples were prepared as follows: first, serial dilutions of CIN EO in ethanol were prepared in the range from 160 mg/mL to 2.5 mg/mL. Tubes containing 1.78 mL of YEB were inoculated with 200 μL of fungal suspension and 20 μL of CIN EO dilution, so that the final EO concentration in the sample tubes was diluted by a factor 100. Controls with 20 μL of ethanol were added to the test. The same procedure was employed for the 4 fungal concentrations. The samples were incubated for 48 h at 25 °C under continuous shaking, except those with the lowest fungal concentration, which were incubated for 72 h to ensure complete mold growth. To assure no changes versus time and confirm final MIC concentrations, test tubes were kept in the incubator at 25 °C for a total of 5 days.

After incubation, the lowest EO concentration with non-growth was named the MIC. Evident development of a mycelium mass was observed in the glass tubes in the case of non inhibition. Below the MIC, subinhibitory concentration was determined as that with reduced growth compared to the control.

To determine the minimal fungicidal concentration, 100 μL of the non-growth suspensions were seeded with a sterile Drigalsky spatel onto Petri dishes containing 15 mL of CZP. After 5 days of incubation at 25 °C, the MFC was determined as the lowest concentration where no colony had developed.

The MIC and the MFC were both determined three times by duplicate.

2.5. Antifungal characterization of active PET

A. flavus was incubated on CZP for 7 days at 25 °C. Suspensions of 10^4, 10^5 and 10^6 CFU/mL were prepared as in the previous assay, and 100 μL of each population were seeded with a sterile Drigalsky spatel onto CZP Petri dishes. PET films were evaluated in vapor