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Combined effect of chitosan and water activity on growth and fumonisin production by *Fusarium verticillioides* and *Fusarium proliferatum* on maize-based media

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ABSTRACT

The objectives of the present study were to determine the *in vitro* efficacy of chitosan (0.5, 1.0, 2.0 and 3.0 mg/mL) under different water availabilities (0.995, 0.99, 0.98, 0.96 and 0.93) at 25 °C on lag phase, growth rate and fumonisin production by isolates of *Fusarium verticillioides* and *Fusarium proliferatum*. The presence of chitosan affected growth and fumonisin production, and this effect was dependent on the dose and a_W treatment used. The presence of chitosan increased the lag phase, and reduced the growth rate of both *Fusarium* species significantly at all concentrations used, especially at 0.93 a_W. Also, significant reduction of fumonisin production was observed in both *Fusarium* species at all conditions assayed. The present study has shown the combined effects of chitosan and a_W on growth and fumonisin production by the two most important *Fusarium* species present on maize. Low molecular weight (Mw) chitosan with more than 70% of degree of deacetylation (DD) at 0.5 mg/mL was able to significantly reduce growth rate and fumonisin production on maize-based media, with maximum levels of reduction in both parameters obtained at the highest doses used. As fumonisins are unavoidable contaminants in food and feed chains, their presence needs to be reduced to minimize their effects on human and animal health and to diminish the annual market loss through rejected maize. In this scenario post-harvest use of chitosan could be an important alternative treatment.

1. Introduction

Fumonisins are polyketide mycotoxins produced by several *Fusarium* species, especially *Fusarium verticillioides* and *F. proliferatum*, both of which are common pathogens of maize (*Zea mays*) worldwide (Marasas, 2001). The major naturally occurring fumonisin analogues in maize and maize-based products intended for human consumption are fumonisin B_1 (FB_1), B_2 (FB_2) and B_3 (FB_3) (Shephard et al., 1996). The contamination of maize with fumonisins is of concern as these mycotoxins cause various animal diseases and occur in maize and maize-based products intended for human consumption (Shephard et al., 1996). Dietary exposure to fumonisins causes adverse effects in farm and laboratory animals. These toxins have been associated with leukoencephalomalacia in horses (Ross et al., 1992), pulmonary edema syndrome in pigs (Harrison et al., 1990), liver and kidney toxicity in rats (Voss et al., 1988) and apoptosis in many types of cells (Jones et al., 2001). Epidemiological studies have shown some evidence between the intake of fumonisins and oesophageal cancer in Africa, Brazil, China and Italy. Also fumonisin B_1 reduces the folate uptake in cell lines, and fumonisin intake has been implicated as a cause of neural tube defects (Marasas et al., 2004). Based on current data, the International Agency for Research on Cancer has classified FB_1 as possibly carcinogenic to humans (group 2B carcinogen) (IARC, 2002).

Previous reports of the mycotoxin situation in South America encompassing the periods 1995–2000 and 1999–2010 showed that fumonisins appeared to be a major problem in maize and maize products (Garrido et al., 2012; Rodriguez Amaya and Sabino, 2002). Since fumonisins are unavoidable contaminants in food and feed chains, their presence needs to be minimized in order to reduce their effects on human and animal health and to diminish the annual market loss through rejected maize. *Fusarium* species are considered as field fungi and fumonisin contamination in maize occurs mainly pre-harvest, although it has been reported that fumonisin production can occur post-harvest under inadequate storage conditions (Chulze, 2010; Marin et al., 2004). Different strategies have been developed to diminish fumonisin contamination. Adequate drying after harvest is the most effective way to reduce fumonisin contamination, but this is not possible in many cases. Post-harvest treatment with antimicrobials agents

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can cause problems such as ambient pollution, chemical residues in food and feed, and the development of chemical resistance fungi species. Due to these problems there is an increased interest on the study and utilization of antifungal compounds obtained from natural sources to replace synthetic fungicides. Chitosan is a possible option, because it is a biodegradable high molecular weight cationic polysaccharide that can be easily obtained by partial alkaline *N*-deacetylation of chitin, which is the most abundant polysaccharide found in nature after cellulose (Chung et al., 2003). Chitosan is a lineal copolymer of β(1–4) 2-acetamido-2deoxy-β-D-glucopyranose and 2-amino-2-deoxy-β-D-glucopyranose. Its biological activities are attributable to several properties including deacetylation degree and molecular mass concentration (Ziani et al., 2009). Due to its unique biological characteristics, including biodegradability and non-toxicity, many applications for chitosan have been found in foods, pharmaceuticals, textiles, water treatment, cosmetic industries and agriculture (Kong et al., 2010).

There are several reports regarding the antifungal activity of chitosan against plant pathogenic fungi, and inhibition of different stages of growth such as mycelia growth, sporulation, germination and spore viability have been observed, as well as inhibition of production of fungal virulence factors (Bautista-Baños et al., 2003, 2004; El Ghaouth et al., 1992; Falcón-Rodriguez et al., 2012; Ziani et al., 2009). Chitosan's antifungal activity depends on its molecular weight, acetylating grade, pH of chitosan solution, and on the target organism (Xu et al., 2007). Recent studies have shown that chitosan is not only effective in stopping the pathogen growth, but it also induces marked morphological changes, structural alterations and molecular disorganization of the fungal cells. The positive charge of the chitosan is due to the protonization of its functional amino group. This group reacts with the negatively charged cell walls of macromolecules, causing a dramatic increase in the permeability of the cell membrane, causing disruptions that lead to cell death (Ziani et al., 2009).

In vitro analysis has demonstrated that chitosan has a fungistatic activity against *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Alternaria alternata*, *Colletotrichum gloeosporioides*, *Monilinia fructicola*, *Penicillium digitatum*, *Rhizopus stolonifer* and *Aspergillus niger* (Hernández-Lauzardo et al., 2008; Martínez-Camacho et al., 2010). To date, only a few studies have reported the effect of chitosan on the growth and mycotoxin (aflatoxin) production by *Aspergillus flavus* and *Aspergillus parasiticus* (Cota-Arriola et al., 2011; Cuero et al., 1991). These experiments were carried out in nutrient rich liquid media and grain without any consideration of the effect of key environmental factors, particularly water availability (a_W) and temperature.

Antioxidants or essential oils under different conditions of a_W, and temperature and controlled atmospheres have been evaluated as possible post-harvest strategies for the reduction of growth of *Fusarium* species and fumonisin production in stored maize (Chulze, 2010), but there is no research about the use of chitosan as a possible strategy. Thus, the objectives of the present investigation were to determine the in vitro efficacy of chitosan under different water availabilities at 25 °C on (i) lag phase, (ii) growth rate and (iii) fumonisin production by isolates of *F. verticillioides* and *F. proliferatum* isolated from Argentinean maize.

2. Material and methods

2.1. Chitosan solution

The chitosan used was low-viscous chitosan (Fluka 50494; LVC; viscosity: ≤200 mPa s), obtained from Sigma-Aldrich Co. A stock solution was prepared by dissolving 10 g/L chitosan in 1% acetic acid (AcH) and stirring for 24 h at 28 °C. The pH of the chitosan solution was adjusted to 5.6 using NaOH. This solution was maintained at 4 °C and brought to ambient temperature before use.

2.2. Chitosan characterization

The viscosity–average molecular weight (*Mv*) of chitosan was determined using the intrinsic viscometric method using the Mark–Houwink–Sakurada equation (Knaul et al., 1998).

The percentage of the chitosan amino groups (degree of deacetylation, DD) was determined using an infrared spectroscopy (Bruker Tensor 27) analysis, applying the following equation:

$$DD(\%) = 97.67 - 26.486 \times (A_{1655}/A_{3450})$$

where A_{1655} is the absorbance at 1655/cm of the amide I band and A_{3450} is the absorbance at 3450/cm of the hydroxyl band (Cota-Arriola et al., 2011).

2.3. Fungal strains

F. verticillioides (M7075) and *F. proliferatum* (RC 2080) strains were used. Both strains were isolated from maize in Argentina (Etcheverry et al., 2002; Reynoso et al., 2004). These strains have been characterized using a polyphasic approach: morphologically, amplified fragment length polymorphisms (AFLP) and fumonisin production. The strains are deposited at the Department of Microbiology and Immunology, Universidad Nacional de Rio Cuarto culture collection (RC). Cultures are maintained in 15% glycerol at −80 °C.

2.4. Medium

Maize was finely milled with a Romer mill (Romer Labs GmbH, Tulln, Austria). Mixtures of 2% (*w*/*v*) milled maize in water were prepared and 2% (*w*/*v*) agar (technical agar No. 2, Oxoid) added. The a_W of the basic medium was adjusted to 0.995, 0.99, 0.98, 0.96 and 0.93, by the addition of different amounts of glycerol (Dallyn and Fox, 1980). From the chitosan stock solution different aliquots were taken in order to produce different concentrations (0.5, 1, 2 and 3 mg/mL) and added to the medium before sterilization. The media were autoclaved at 120 °C for 20 min. Flasks of molten media were thoroughly shaken prior to pouring into 9 cm sterile Petri dishes. The final pH of the medium containing chitosan ranged from 5.5 to 6, in order to ensure that all the chitosan amino groups were positively charged (Kong et al., 2010; Liu et al., 2004).The a_W and pH of representative samples of media were checked with an Aqualab Series 3 (Decagon devices, Inc., WA, USA) and a pH meter (Orion 250A, Boston), respectively. Control plates were prepared and measured at the end of the experiment in order to detect any significant deviation of both parameters.

In order to discount fungal inhibition due to acetic acid in the chitosan solution a maize based-medium without chitosan (control AcH) was prepared for each a_W level used. Those AcH control plates were prepared as described above, adding the same volume of 1% AcH used to prepare 3 mg/mL of chitosan amended media.

A preliminary experiment was performed to choose the range of concentration of chitosan (1, 3, 5 and 10 mg/mL) to be added to obtain a dose–response curve. As fungal growth was completely inhibited in 5 and 10 mg/mL chitosan solutions, solutions below 5 mg/mL were used in the following experiments.

2.5. Inoculation, incubation and growth assessment

All media, with and without chitosan, were inoculated centrally with a 4 mm diameter agar disk taken from the margin of a 7-day-old colony of each isolate grown on synthetic nutrient agar (SNA) (Gerlach and Nirenberg, 1982) at 25 °C. The disks were transferred face down onto the center of each plate. Inoculated Petri plates of the same a_W were