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ORIGINAL ARTICLE

Ultraviolet radiation and generally recognized as safe (GRAS) preservatives for inactivation of *Aspergillus niger in vitro* and corn dough

Radiação ultravioleta e conservantes GRAS na inativação de *Aspergillus niger in vitro* e em massa de milho

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Abstract

Corn is the main cereal produced in the world, it is also used for direct human consumption and for the production of various food products; however, it is prone to being contaminated by fungi, especially by mycotoxin producers. *Aspergillus* spp. is a contaminant fungus related to postharvest of stored grains, especially in corn. This study evaluated the effect of Ultraviolet radiation (UV) and Generally Recognized As Safe (GRAS) treatments on the inhibition of mycelial growth of *A. niger* (GIBI_00056) *in vitro* and in corn (*Zea mays* L.) dough. For the *in vitro* study, UV radiation and solutions of citric acid, potassium sorbate, sodium bicarbonate, sodium benzoate, and ascorbic acid were used, which were added to PDA agar in which *A. niger* was inoculated and evaluated at 24, 48, and 72 h. Subsequently, the best treatment was selected and applied in different concentrations in an *A. niger* inoculated corn dough, thus evaluating the incidence of contamination at 24, 48, and 72 h. The sodium bicarbonate and sodium benzoate solutions had the best effect on the inhibition of *A. niger in vitro* compared to the control, whereas the other treatments evaluated did not show differences in the mycelial inhibition. In the corn dough inoculated with *A. niger*, the effect of sodium bicarbonate depended on the concentration used; the lowest incidence of contamination of the microorganism at 72 h was 0% with 1.8 and 2.7% (w/w) of sodium bicarbonate, whereas the highest was for the control with 100% incidence. The potential of sodium bicarbonate to inactivate *A. niger* growth *in vitro* and corn dough was observed.

Keywords: Fungal inhibition; Carbonate ion; Maize; Food-grade solutions; Reduction; Arepas.

Resumo

O milho é o principal cereal produzido no mundo e é utilizado para o consumo humano direto e para a produção de diversos produtos alimentícios. No entanto, está sujeito a ser contaminado por fungos, especialmente por produtores de micotoxinas. *Aspergillus* spp. É um fungo contaminante relacionado à pós-colheita de grãos armazenados, principalmente nos grãos de milho. Este estudo avaliou o efeito da radiação ultravioleta (UV) e de tratamentos geralmente reconhecidos como seguros (GRAS) na inibição do crescimento micelial de *Aspergillus niger* (GIBI_00056) *in vitro* e em massa de milho (*Zea mays*). Para o estudo *in vitro*, foram utilizadas a radiação UV e as soluções de ácido cítrico, sorbato de potássio, bicarbonato de sódio, benzoato de sódio e ácido ascórbico, que foram adicionadas ao meio de cultura BDA, no qual *A. niger* foi inoculado sendo avaliado nos períodos de 24, 48 e 72 h. Posteriormente, o melhor tratamento foi selecionado e aplicado em diferentes concentrações em uma massa de milho inoculada com *A. niger*, avaliando a incidência de contaminação nos períodos de 24, 48 e 72 h. As soluções de bicarbonato de sódio e benzoato de sódio tiveram o melhor efeito na inibição de *A. niger in vitro*, em relação ao controle, enquanto os demais tratamentos avaliados não apresentaram diferenças na inibição micelial. Na massa de milho inoculada com *A. niger*, o efeito do bicarbonato de sódio dependeu da concentração utilizada: a menor incidência de contaminação do microrganismo em 72 h foi de 0% com 1,8 e 2,7% (p/p) de bicarbonato de sódio, enquanto a maior foi para o controle, com 100% de incidência. Foi observado o potencial do bicarbonato de sódio para inativar o crescimento de *A. niger in vitro* e na massa de milho.

Palavras-chave: Inibição fúngica; Íon carbonato; Milho; Soluções de qualidade alimentar; Redução; Arepas.

Highlights

- Sodium bicarbonate and sodium benzoate had an effect on the inhibition of *A.niger in vitro*
- Sodium bicarbonate had a potential to control *A. niger* in a corn dough
- Concentration was an important factor in the inhibition of *A. niger*

1 Introduction

Maize or corn (*Zea mays* L.) is the cereal with the highest production worldwide, with a world production estimated at 1124 million tons (2019/2020) since the main consumers are countries like Mexico, Colombia, Perú, Iran, Saudi Arabia, China, Japan, Korea, and European Union (EU), which is mainly intended for food, feed, industrial, and ethanol production (García-Lara & Serna-Saldivar, 2019; International Grains Council, 2021). The consumption of corn in countries such as Mexico and Colombia is very important due to its cultural and nutritional value (carbohydrates, proteins, and lipids), thus being the main raw material for ethnic products such as “tortillas”, “arepas”, “tamal”, and “empanadas”, which are made with corn dough (Fideicomisos Instituidos en Relación con la Agricultura, 2016; Hernández Montoya et al., 2019b). However, studies have evidenced high fungal contamination in this type of product, impacting the quality of the final product and the shelf-life (Hernández Montoya et al., 2019a, 2019b).

Aspergillus spp. is a common contaminant of corn, which grows at an optimum temperature of 25 °C, relative humidity of 95%; furthermore, some species can produce toxic metabolites such as aflatoxins and ochratoxins (Karami-Osboo et al., 2012; Serrano-Coll & Cardona-Castro, 2015). It usually grows and contaminates cereals like corn, rice, wheat, and sorghum. *Aspergillus* spp. grows and contaminates before harvest, during harvest, transportation, storage, and processing, where humidity and high temperatures are favorable for fungal growth (Karami-Osboo et al., 2012; Londoño-Cifuentes & Martínez-Miranda, 2017).

To avoid contamination of food with fungi and the production of mycotoxins, it is necessary the implementation of Good Agricultural Practices (GAP), Good Handling Practices (GHP), Good

Manufacturing Practices (GMP), and the control of variables in storage such as humidity (less than 12%), water activity in food (less than 0.7), and temperature (less than 22 °C) (Martínez-Miranda et al., 2013). Another way to avoid the proliferation of this type of microorganism is by applying Generally Recognized As Safe (GRAS) preservatives, such as benzoates, sodium bicarbonate, propionates, and sorbates; which according to the general rule for food additives, these substances are allowed in some foods in a concentration limit for each one (Food and Agriculture Organization, 2018; U.S. Food and Drug Administration, 2020).

Some studies have shown the potential of some physicochemical treatments to control *Aspergillus* spp. in food. Thanaboripat et al. (2012) proposed the use of chemical inhibitors (ammonium carbonate and sodium bisulfite at concentrations between 1% and 5%) as a strategy for the elimination, detoxification, or reduction of aflatoxins, suppressing the germination of fungi spores, as well as the development of *A. flavus*, *A. parasiticus*, *A. nomius*, *A. tamarii*, *A. bombycis* and *A. pseudotamarii* in corn. The results showed that sodium chloride, ammonium carbonate, and sodium bisulfite inhibited fungal growth in corn for 28 days. Ratnayake et al. (2009) evaluated the effect of substances sodium bicarbonate, calcium chloride, sodium benzoate, and citric acid, using concentrations between 1% and 5% in the inhibition of *A. niger in vitro* in wood apple, finding that the bicarbonate of sodium inhibited the growth and germination of conidia of *A. niger*; seeing that it also extended the shelf life 28 days. Samapundo et al. (2010) evaluated substances such as sodium, calcium, magnesium, potassium chloride, and magnesium sulfate in the inhibition of *Penicillium roqueforti* and *A. niger* in bread samples, finding that sodium chloride and magnesium chloride presented the best effect for inhibiting the growth of microorganisms.

Physical methods such as ultraviolet (UV) radiation have also been used to control *Aspergillus* spp. Pulsed UV light was used to inactivate spores of *A. niger* in corn meal with a 4 $\log_{10}$ reduction in the number of viable spores (Jun et al., 2003). The effect of UV radiation on the survival and susceptibility of some species of fungi such as *A. niger*, isolated from the indoor air of agricultural work areas, has also been evaluated, finding that the survival of conidia is inversely proportional to the exposure time, to UVC radiation, since between 77% and 88.5% of the conidia were eliminated within six hours after exposure (Abdel Hameed et al., 2013). The mechanism of inactivation of the microorganism with this method has been attributed to an irreversible change in DNA (Moreau et al., 2013).

As aforementioned, it was hypothesized that the use of physicochemical treatments such as preservatives and UV radiation could allow the inhibition of the mycelial growth of *A. niger in vitro* and corn dough, thus impacting product shelf-life and consumer safety.

2 Materials and methods

2.1 Reactivation of the fungal strain

The strain GIBI_00056 of *A. niger* used in this study was obtained from the Microorganisms Collection of the *Universidad Católica de Manizales*. This isolate was preserved in 15% glycerol at -80 °C. The strain was inoculated on Potato Dextrose Agar (PDA Agar, Scharlau®) and incubated (Binder, Model Red Line RI 115®) at 22 °C for 5 days for the reactivation. Subsequently, the pure fungal isolate was verified by observing the macroscopic characteristics, and a lactophenol blue solution staining was performed to observe the microscopic characteristics.

2.2 Treatments

The solutions were prepared with GRAS preservatives, citric acid, potassium sorbate, sodium bicarbonate, sodium benzoate, and ascorbic acid, which were added to the PDA agar at a temperature between 45 and 50 °C (preventing it from solidifying) in the concentrations mentioned in Table 1. The concentrations were selected

following the recommendations for food additives for human consumption (Food and Agriculture Organization, 2018). The UV treatment was carried out by exposing a fragment of the fungus to radiation for 10 min, later inoculated on the PDA agar according to the methodology of Castro-Ríos et al. (2021).

2.3 Evaluation of the growth inhibition of *Aspergillus niger in vitro*

A portion of the mycelium of *A. niger* was taken with a stylet and was inoculated by a central puncture in the PDA Agar with the treatment of interest (Table 1); in addition, they were incubated at 22 °C, for 72 h (Castro-Ríos et al., 2021). The evaluations were carried out by measuring the mycelial radial growth of the central colony in millimeters at 24, 48, and 72 h. When satellite colonies were present, these were not measured; a simple macroscopic description of them was made, and their presence was reported.

2.4 Evaluation of the inhibition of mycelial growth of *Aspergillus niger* in a corn dough

According to results with all the treatments, sodium bicarbonate was selected to evaluate *A. niger* inhibition in corn dough. For this, a solution with the microorganism was prepared at a concentration of 2.8 x 10^6 spores/mL; the quantification was carried out in a Neubauer chamber, with the Calibra software (Empresa Brasileira de Pesquisa Agropecuária, 2010).

For the elaboration of the corn dough, commercial cornflour (Colombia) was used, and it was mixed with water (350 mL) to form a homogeneous dough; this was divided into four parts, and sodium bicarbonate was added in the concentrations 1%, 1.8% and 2.7% (w/w), the control mass did not have sodium bicarbonate. All samples were inoculated with 1 mL of the solution prepared with the microorganism for every 50 g of dough (Russo et al., 2017). Five samples per treatment of 10 g were deposited in a humidity chamber (Petri dishes with wet absorbent paper), and subsequently incubated for 72 h at 25 °C.

Aspergillus niger growth in the corn dough was evaluated with the percentage of contamination incidence. Each experimental unit (Petri dish with 10 gr of the sample) was verified with a stereomicroscope (Leica EZ4) at 24, 48, and 72 h. The calculation of the incidence was carried out according to the Equation 1:

$$Incidence(\%) = \frac{Number\ of\ contaminated\ samples}{Number\ of\ total\ samples} * 100 \quad (1)$$

2.5 Statistical analysis

The data of the inhibition of *A. niger in vitro* and the contamination incidence in corn dough was analyzed using descriptive statistics and Analysis of Variance (ANOVA), where the effects of the physicochemical treatments were compared, determining if there were statistical differences between the control and the treatments. Significant differences were determined using the Tukey's test with a significance level of $p < 0.05$. The experimental unit was each Petri dish, and the response variable was mycelial growth in mm/day (*in vitro* analysis) and the percentage of incidence in the corn dough. It was conducted five replicates per treatment, in two experiments at different times. The statistical analyses were performed with Jamovi software version 1.2 (Jamovi, 2020).

Table 1. Concentrations / Exposure time of physicochemical methods.

Treatments	Concentrations or Exposure time
Sodium bicarbonate	0.9% (w/v)
Potassium sorbate	0.2% (w/v)
Ultraviolet	10 min at 336 nm
Ascorbic acid	0.2% (w/v)
Citric acid	0.5% (w/v)
Sodium benzoate	0.2% (w/v)
Control	Deionized water

3 Results and discussion

3.1 Control of *Aspergillus niger* growth *in vitro*

As shown in Table 2, at 24 h after inoculation, the most effective method for inhibiting mycelial growth was sodium bicarbonate, since no growth was observed during this time. The highest mycelial growth was observed in control with an average of 11.40 mm, however, it could be observed, on average, mycelial growth of 10.50 mm in citric and ascorbic acids. While the smallest growth occurred with sodium benzoate (9.39 mm), potassium sorbate (9.80 mm), and UV (9.90 mm) at 24 h. At 48 h, sodium bicarbonate showed the lowest mycelial growth, with a mean of 10.40 mm; the highest growth was observed in ascorbic acid, with an average of 15.40 mm and 15.10 mm for potassium sorbate; UV presented a mycelial growth of 13.74 mm, without presenting differences with the control. Finally, at 72 h, more significant mycelial growth was observed in control with an average of 20.50 mm and potassium sorbate with 19.78 mm, sodium benzoate had a growth of 14.10 mm, and UV radiation of 15.70 mm, and sodium bicarbonate of 15.99 mm.

The statistical analysis showed significant statistical differences ($p < 0.001$) between sodium bicarbonate and all the treatments evaluated, including the control at 24 h. At 48 h, there were only differences between sodium bicarbonate and ascorbic acid ($p < 0.05$), this presented the highest mycelial growth at this time. Finally, at 72 h, the control showed the highest mycelial growth with a statistical difference ($p < 0.05$); on the other hand, sodium benzoate showed the lowest growth at that time.

Concerning the inhibition of mycelial growth of *A. niger*, it was found that sodium bicarbonate presented the best results at 24 and 48 h, and sodium benzoate at 72 h. Sodium bicarbonate can raise the pH of the medium where the microorganism develops, inactivating extracellular enzymes and generating a cellular alteration or disruption that affects the sporulation of the fungus; these effects are mainly attributed to the impact of the sodium cation (Alvindia, 2013; Palou et al., 2001). Ratnayake et al. (2009) evaluated the effect of sodium bicarbonate, calcium chloride, sodium benzoate, and citric acid in *A. niger* presented in wood apple (*Feronia limonia* (L.) Swingle); finding that sodium bicarbonate completely inhibited the growth of the fungus and increasing the shelf-life of the fruit in 28 days; whereas sodium benzoate and the other preservatives affected the germination of *A. niger* spores, but without the efficiency of sodium bicarbonate. The effect of sodium benzoate on the inhibition of *A. niger* has been related to physiological, homeostatic, and metabolic distortion; its attempt to overcome these adverse conditions leads to stress, causing metabolic exhaustion and subsequent cell death (Nwafor & Ikenebomeh, 2009). Regarding UV radiation, this radiation was able to control the growth of the fungus, with good results in 48 h and 72 h. Something similar was observed by Jun et al. (2003) who used pulsed UV light to inactivate spores of *A. niger* in corn meal with a 4 $\log_{10}$ reduction in the number of viable spores. The mechanism of inactivation of the microorganism with UV has been attributed to an irreversible change in DNA (Moreau et al., 2013).

Table 2. Inhibition of mycelial growth (mm) of *Aspergillus niger in vitro.*

Treatments	Time		
	24 h	48 h	72 h
Control (mm)	11.40 ± 2.88^{a}	14.80 ± 3.55^{a}	$20.50 \pm 4.40^{a,b}$
Sodium bicarbonate (mm)	0.00 ± 0.00^{b}	$10.40 \pm 2.59^{a,b}$	15.99 ± 2.28^{a}
Citric acid (mm)	10.50 ± 2.51^{a}	15.00 ± 3.77^{a}	19.40 ± 4.99^{a}
Sodium benzoate (mm)	9.30 ± 3.97^{a}	12.90 ± 2.96^{a}	$14.10 \pm 2.08^{a,c}$
Ultraviolet light (mm)	9.90 ± 2.77^{a}	13.74 ± 5.46^{a}	15.70 ± 6.63^{a}
Ascorbic acid (mm)	10.50 ± 3.34^{a}	$15.40 \pm 3.10^{a,c}$	18.80 ± 4.54^{a}
Potassium sorbate (mm)	9.80 ± 3.55^{a}	15.10 ± 5.00^{a}	19.30 ± 6.82^{a}

[a, b, c] Different letter in the same column indicates that the means are statistically different between the treatments ($p \leq 0.05$). The experimental results were expressed as mean ± Standard Deviation.

3.2 Control of the growth of *Aspergillus niger* in corn dough

Considering sodium bicarbonate´s potential to inhibit *A. niger* observed in the *in vitro* study, three concentrations of this salt (1%, 1.8%, and 2.7%) were evaluated in a corn dough inoculated with the fungus.

As evidenced in Table 3, at 24 h, no incidence of *A. niger* was observed in the corn dough, included in the control. At 48 h, an incidence rate of 80% was obtained for the corn dough with 1% of sodium bicarbonate. For 1.8% and 2.7% concentrations of sodium bicarbonate, no incidence of *A. niger* was observed, unlike the control, which at 48 h presented 100% incidence. After 72 h, *A. niger* incidence was 100% for the lowest concentration (1%) and the control, however, for 1.8% and 2.7% concentrations, no incidence was observed (Figure 1). The statistical analysis showed significant statistical differences ($p < 0.001$) between control and sodium bicarbonate concentrations (1.8% and 2.7%), and between lower sodium bicarbonate concentrations (1%) with 1.8% and 2.7% concentrations at 48 h and 72 h (Table 3).

Table 3. Percentage of incidence of *Aspergillus niger* in the corn dough, at different times of growth.

Sodium bicarbonate concentrations	Time		
	24 h	48 h	72 h
1% (w/w)	0.0%[a]	80%[a]	100.0%[a]
1.8% (w/w)	0.0%[a]	0.0%[b]	0.0%[b]
2.7% (w/w)	0.0%[a]	0.0%[b]	0.0%[b]
Control	0.0%[a]	100%[a]	100.0%[a]

[a, b, c] Different letter in the same column indicates that the means are statistically different between the treatments ($p \leq 0.05$).

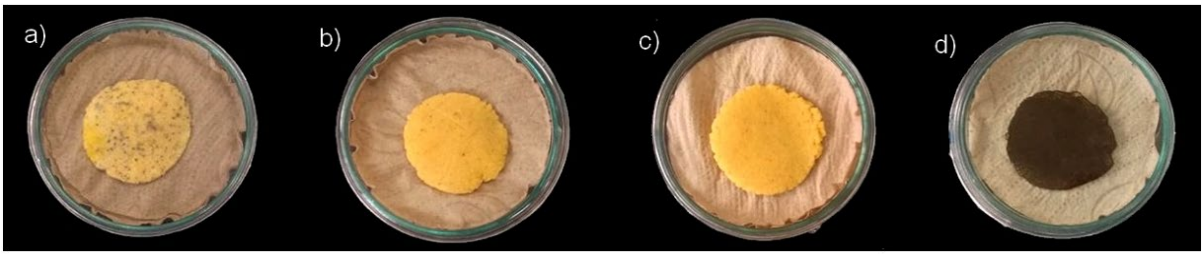

Figure 1. Mycelial growth of *Aspergillus niger* (GIBI_00056) in corn dough at 72 h after inoculation. (a) Sodium bicarbonate at 1%; (b) Sodium Bicarbonate at 1.8%; (c) Sodium Bicarbonate at 2.7%; and (d) control.

Some studies have shown the potential for the control of *Aspergillus* spp., using sodium bicarbonate in fruits and corn. Ratnayake et al. (2009) carried out a study to control *A. niger* in Wood apple (*F. limonia*), using different salts including sodium bicarbonate at concentrations of 1, 2, 3, 4, and 5% (w/v), the results showed that a low concentration of salt promoted the growth of the fungus. However, 2% and 3% of sodium bicarbonate concentrations inhibited the fungus up to 70%, while between 4% and 5% of sodium bicarbonate, the inhibition of *A. niger* was 100%. In corn, sodium bicarbonate has been evaluated at a concentration of 4% for the control of *Aspergillus* spp; however, it was insufficient to inhibit growth, and it was not sensory accepted at the concentration evaluated (Samapundo et al., 2007). Montville & Shih (1991) assessed the inhibition of *A. ochraceus* with 1% and 2% of sodium bicarbonate in re-hydrated corn up to 23% of humidity; the authors found that this salt had an inhibitory effect on the fungus. Castro-Ríos et al. (2021) recently evaluated chemical treatments like sodium bicarbonate, electrolyzed water, and physical treatment ultrasound and UV in the reduction of inhibition in the control of mycelial growth of *A. niger* in corn grains and reduction of Aflatoxins *in vitro*; sodium bicarbonate achieved complete inhibition of the fungus and a reduction of 87.03% of aflatoxins. The inhibition of microorganisms by bicarbonate has been attributed to an impact on germination, mycelial growth, and inactivation of enzymes, due to an increase in pH in the medium (Alvindia, 2013; Smilanick et al., 1999).

4 Conclusions

The results indicated significant effects of sodium bicarbonate and sodium benzoate on *A. niger in vitro*; there was no significant effect on fungal inhibition with UV and the other GRAS preservatives (potassium sorbate, citric acid, and ascorbic acid).

The lowest contamination incidence of *A. niger* in corn dough was with sodium bicarbonate at the medium and highest concentration (1.8 and 2.7%); and the highest incidence for the control without sodium bicarbonate. These results showed that sodium bicarbonate had the potential to retard and inhibit *A. niger* growth in a corn dough.

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