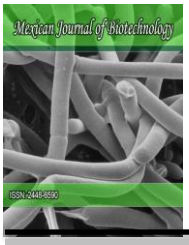

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SHORT COMMUNICATION

Cold plasma: an alternative to reduce the viability of *Aspergillus flavus* conidia in lentil beans

Plasma frio: una alternativa para reducir la viabilidad de los conidios de *Aspergillus flavus* presentes en granos de lenteja

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ABSTRACT

Microbiological food safety is a major issue and the genus *Aspergillus* is of great interest given the frequency of its toxin contamination in grains. This paper describes the use of cold plasma generated with argon and a mixture of argon-nitrogen as a method of sanitizing lentil beans. Lentil beans were sanitized and exposed to *Aspergillus flavus* conidia then four different experimental sets were prepared, using only argon and a mixture of argon-nitrogen to generate plasma at nitrogen flow rates of 1.2, 0.81 and 0.32 L/min. Each lentil bean was exposed for 5, 10 and 15 min to plasma. Assays were performed in triplicate. Beans not exposed to plasma were used as controls. All plasma treatments caused a lethal effect on *A. flavus* conidia within exposure periods of 5 to 15 min. The application of argon plasma showed a $\log_{10}$ reduction of 0.81 (84%) after 15 min. The mixture of argon: nitrogen at 0.81 and 0.32 L/min had a higher lethal effect than argon alone. Although lentil beans sterilization was not completely achieved, an important $\log_{10}$ reduction of 1.43 (96.44 %) and 5.53 (99.99 %) of *A. flavus* conidia was obtained after 15 min of exposure to the plasma generated by argon-nitrogen mixture using

nitrogen at flow rates of 0.81 and 0.32 L/min, respectively. Nitrogen flow rate of 0.32 L/min showed a reduction above 3.0 logarithmic units, so this treatment showed a fungicidal activity. The lowest reduction, 0.3 logarithmic units (50.3 %) was observed at a nitrogen flow rate of 1.2 L/min. Additionally, as a consequence of plasma exposure, conidia of *A. flavus* showed a delay in germination process and also conidia formation was affected. It was concluded that cold plasma could be used as an alternative to sanitize grains and avoid contamination by microorganisms, which cause grain deterioration and affect its nutritional properties.

Keywords: *argon, Aspergillus flavus, cold plasma, contamination, nitrogen,* sanitizing

RESUMEN

La seguridad microbiológica de alimentos es un tema importante, el género *Aspergillus* es de gran interés dada la frecuencia de contaminación de los granos por sus toxinas. El presente estudio describe el uso de un plasma frío generado a partir de argón y de una mezcla de argón nitrógeno como método de sanitización de granos de lenteja. Para el estudio, cada grano fue previamente sanitizado y expuesto a conidios de *Aspergillus flavus*, cuatro diferentes ensayos fueron realizados, usando únicamente argón y una mezcla de los gases argón-nitrógeno para generar el plasma, a una velocidad de flujo de nitrógeno de 1.2, 0.81 y 0.32 L/min. Cada grano fue expuesto durante 5, 10 y 15 min al plasma, cada ensayo fue realizado por triplicado. Como controles, se emplearon granos no expuestos al plasma. Todos los tratamientos con plasma mostraron un efecto letal sobre la viabilidad de los conidios de *A. flavus* en periodos que van de 5 a 15 min. La aplicación del plasma generado por argón mostró una reducción logarítmica de 0.81 (84 %) al final de los 15 min de exposición. El plasma generado con la mezcla de gases argón-nitrógeno a 0.81 y 0.32 L/min presentó un efecto letal mayor en comparación con el plasma generado con argón. Aunque no se logró la esterilización completa de las lentejas, se observó una reducción logarítmica de 1.43 (96.44 %) y 5.53 (99.99 %) de los conidios de *A. flavus* después de 15 min al ser expuestos al plasma generado por la mezcla argón-nitrógeno a velocidades de flujo de nitrógeno de 0.81 y 0.32 L/min respectivamente. La velocidad de flujo de 0.32 L/min mostró una reducción de más de 3 unidades logaritmicas, por lo que este tratamiento tuvo un efecto fungicida. La menor reducción logarítmica de 0.3 (50.3 %) fue observada a una velocidad de flujo de nitrógeno de 1.2 L/min. Adicionalmente, como consecuencia de la exposición al plasma, los conidios de *A. flavus* mostraron un retraso en la germinación y el proceso de conidiación se vio afectado. El uso de plasma frío podría ser usado como alternativa para sanitizar granos y evitar la contaminación por microorganismos que ocasionan el deterioro de los granos y sus propiedades nutricionales.

Palabras clave: *argón, Aspergillus flavus, contaminación, nitrógeno, plasma frío,* sanitización.

1. INTRODUCTION

Safety is a major issue in foods that are stored for long periods, such as grains and cereals which could be contaminated by microorganisms or pests causing deterioration, decreasing their ability to germinate and reducing their nutritional properties (Bucio *et al*., 2001; Ito *et al*., 2001; Hedayati *et al*., 2007). The process of grain contamination can take place throughout different stages sowing, harvesting, storage, transport and final destination (CAC/RCP 51-2003). The most important aspects that affect seed health are the associated fungi that not only decrease seed germination, but also reduce seed vigor resulting in low yield. Seed-borne diseases caused by fungi are relatively difficult to control as the fungal hyphae get established and become dormant (Zafar *et al*., 2014). In Mexico, the NOM-188-SSA1-2002 describes the necessary tests and how they must be applied for detection of aflatoxins in grains, as well as establishing the permissible toxin limits depending the use of the grain. Fungi are of particular interest since, in addition to causing deterioration of food, they can affect human and animal health due to mycotoxin production (Li *et al*., 1998; Razzaghi-Abyaneh *et al*., 2014). Fungal species of *Aspergillus* and *Penicillium* are the most frequently reported in cases of toxin contamination in grains (Ito *et al*., 2001; Selcuk *et al*., 2008). To prevent grain damage by microorganisms, and possible aflatoxin production, different methods of inactivation or elimination of pathogens in grains have been used including hot water, natural compounds, aerated steam, commercial bleach, ethylene oxide and fungicides or sanitizing energy in the form of microwaves, radio frequency, UV radiation, radiation (gamma rays), magnetic energy, electron bombardment, hydrostatic pressure and low energy electrons (Laroussi *et al*., 2000; Gunterus *et al*., 2007; Selcuk *et al*., 2008; Kim *et al*., 2011). However, some methods have considerable disadvantages due to their operating costs, the risk to operators or by altering the food quality or the germination capacity of the grains (Dorner et al., 1992; Maxwell *et al*, 2006). An ideal treatment would be one that with antimicrobial activity over the food surface without leaving any hazardous residue (Selcuk *et al*., 2008). Among the available technologies, the cold plasma has been proposed as a method of sanitizing grains, with advantages such as low temperature, shorter processing times and reduced risk for operators. Plasma is produced by the application of electromagnetic fields to gas and the operating conditions can be set to allow the efficient inactivation of microorganisms while minimizing the damage to the materials subjected to the treatment (Anderson, 1989; Lerouge *et al*., 2000). It is well known that gaseous discharges produce antimicrobial agents like oxygen radicals, free radicals and even UV radiation (Laroussi, 2009; Lee *et al*., 2006; Moisan *et al*., 2002). Additionally, non-thermal argon plasma is under intensive study as an alternative approach to control superficial wound and skin infections within the clinical setting due to the effectiveness of chemical agents is weak for pathogens or biofilm (Hong *et al*., 2009; Bourke *et al*., 2017). Ermolaeva *et al* (2011) reported the considerable

potential for non-thermal argon plasma in eliminating pathogenic bacteria from biofilms and wound surfaces. Thus, the aim of this study was to determine the effectiveness of cold plasma generated from argon and a mixture argon-nitrogen on the viability of *Aspergillus flavus* conidia in lentil beans.

2. MATERIALS AND METHODS

2.1 Microorganism

Aspergillus flavus CDBB-613A was grown on potato dextrose agar (PDA) at 30°C until sporulation. A conidia suspension was prepared adding 20 mL of sterile deionized water to the PDA culture and then harvested by centrifugation at 6000 rpm for 6 min by using a centrifuge 5804 R (Eppendorf) with a F-34-6-38 rotor. The pellet was washed once with sterile deionized water and concentrated by centrifugation as mentioned above. The number of conidia ($1x10^8$ conidia/mL) was counted in a Neubauer chamber using a bright field microscope with a 40X objective.

2.2 Sanitization and lentil beans exposure to conidia

Lentil beans were similar in characteristics such as color, weight, size and surface, and were cleaned from husks and dust. Beans were sanitized with a 10% benzalkonium chloride solution during 10 min, washed with sterile distilled water, dried under a N_2 stream and stored until use. For contamination, 15 µL of *Aspergillus flavus* conidia suspension ($1x10^8$ conidia/mL) were applied on the surface of each lentil bean and them placed in a Petri dish until dryness, later each bean was transferred to the plasma generating apparatus.

2.3 Cold plasma treatment

Four different experimental sets were prepared: a) exposure to argon plasma, b) exposure to a mixture of argon-nitrogen plasma at different nitrogen flow rates (0.32, 0.81 or 1.2 L/min). Each lentil bean was exposed during 5, 10 and 15 min with a distance of 19.72 mm between the plasma-generating pen and the bean surface. Plasma was generated using a voltage of 26.2 V at 20.27 KHz (Nieto-Pérez *et al*., 2011). Assays were performed by triplicate. Beans not exposed to any plasma were used as controls (Leclaire *et al*., 2008). The design of the plasma apparatus allowed only the exposure of one lentil bean per test.

2.4 Assessment of conidia viability

Once lentil beans were treated, microbial density was evaluated by placing treated beans in serial dilution tubes (up 10^{-7}) containing sterile deionized water. Aliquots of 100 µl of serial dilutions were spread on PDA medium - Rose Bengal (35 mg/L) plates and then incubated at 30°C for 72 h or until the appearance of colonies. Colony count was done manually to estimate the number of viable conidia (Selcuk

et al., 2008; Ragni *et al*., 2010; Mitra *et al*., 2014). Fungicidal activity was defined as a reduction of ≥3 $\log_{10}$ (99.9%) of the initial inoculum (Lago *et al.,* 2013; Ballo *et al.,* 2017). The reduction was calculated using the formula.

$$\text{Reduction } \log_{10} = \text{Log } [N_0] - \text{Log } [N]$$

where:
N_0: number of colonies counted in plates without exposure to plasma
N: number of colonies counted in plates after plasma exposure.

Data were statistically analyzed using the Minitab 17 software with Tukey's HSD pairwise comparisons.

3. RESULTS

As shown in Figure 1, the cold plasma generated with argon caused a reduction of *A. flavus* conidia from $8.5x10^7$ to $5.3x10^7$, $3.6 x10^7$ and $1.3x10^7$ conidia/lentil bean after 5, 10 and 15 min respectively, which corresponds to reduction $\log_{10}$ of 0.32 (37%), 0.38 (57.9%) and 0.81 (84.4%). Results do not showed fungicidal activity.

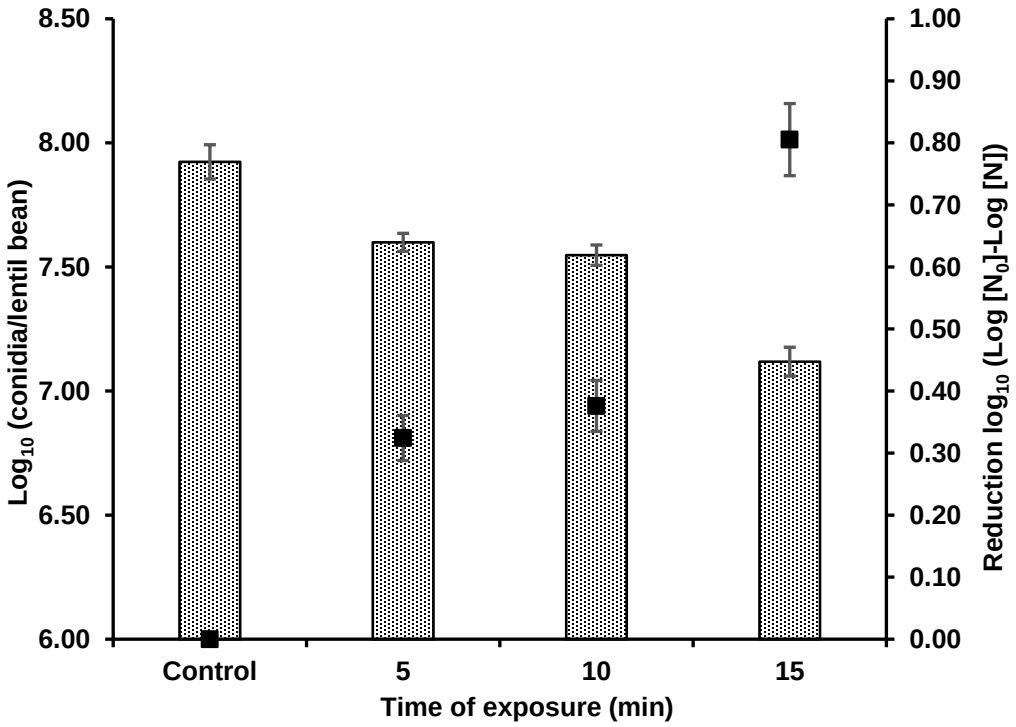

Fig. 1. Conidia viability (Bars) and logarithmic reduction (squares) of *A. flavus* in lentil beans after exposure to argon plasma treatment.

Figure 2 shows that the treatment with the argon-nitrogen plasma also had a lethal effect on the conidia of *A. flavus,* in this case the effect was increased when nitrogen flow was reduced from 1.2 to 0.32 L/min. The use of argon-nitrogen plasma at 1.2 L/min did not have a significant lethal effect on conidial viability after 5, 10 and 15 min of exposure corresponding to a reduction $\log_{10}$ of 0.05 (11.9 %), 0.12 (25.3 %) and 0.3 (50.3 %) (Table 1). Treatment with argon- nitrogen plasma at a flow rate of 0.81 L/min reduced the conidia population from $9.35x10^7$ to $3x10^7$ after 5 min, corresponding a reduction $\log_{10}$ of 0.5 (74%). Treatments for 10 and 15 min of exposure had a logarithmic reduction of 1.13 (89.2%) and 1.43 (96.4%), respectively. The nitrogen flow of 0.32 L/min caused a higher reduction in conidia population with a reduction of $\log_{10}$ of 5.53 corresponding to 99.99% after 15 min of exposure. Viability decrease was also observed for treatment at 5 and 10 min with a reduction $\log_{10}$ of 0.52 (64.3 %) and 1.33 (95.9 %) respectively. Results presented in Table 1 show that the treatment with argon-nitrogen plasma at a flow rate of 0.32 L/min showed a reduction above 3.0 logarithm, this treatment shows fungicidal activity (Lago *et al.,* 2013; Ballo *et al.,* 2017). Based on Mexican Official Law, NMX-BB-040-SCFI-1999, germicidal activity is considered when reduction percentage is 99.999%.

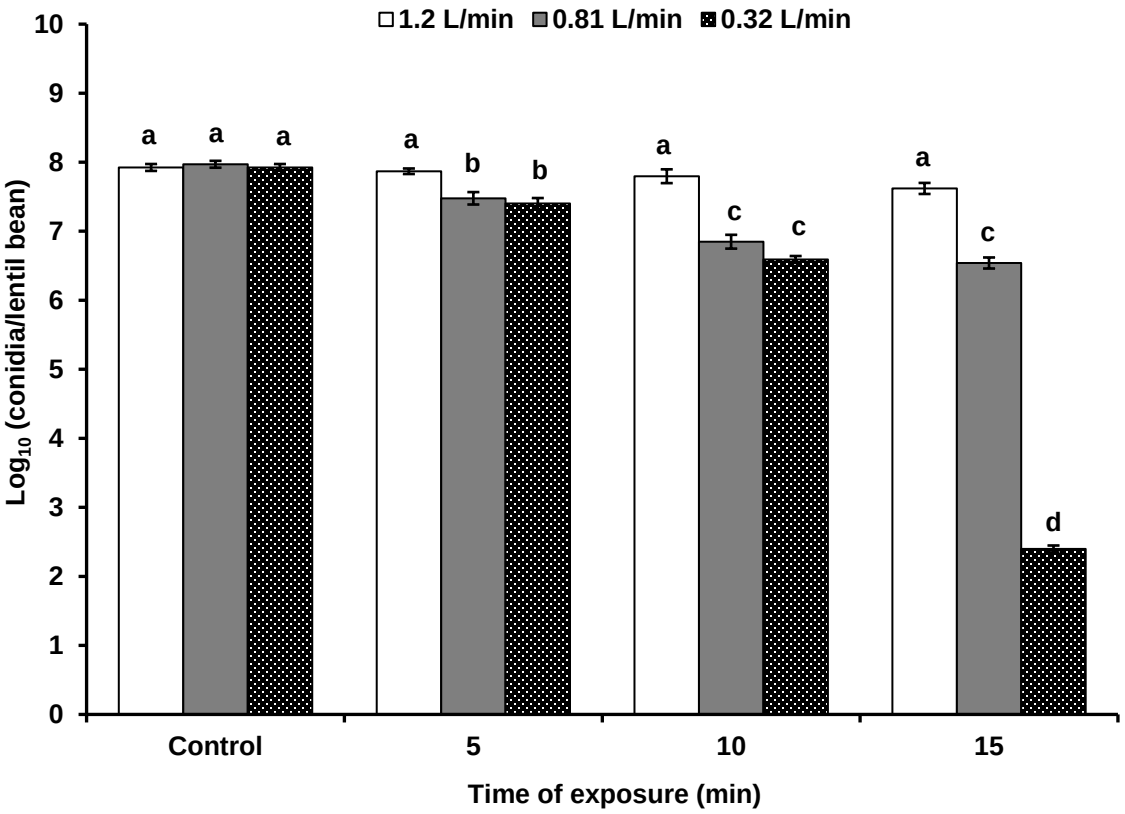

Fig. 2. Viability loss of *A. flavus* conidia in lentil beans using argon-nitrogen plasma at nitrogen flow rates of 1.2, 0.81 and 0.32 L/min. Statistically significant differences (one-way ANOVA with Tukey's HSD ($P < 0.05$)) are indicated by different letters.

Table 1. Reduction $\log_{10}$ (Log [N_0]-Log [N])/Percentage death of *A. flavus* conidia after exposure to argon-nitrogen plasma at nitrogen flow rates of 1.2, 0.81 and 0.32 L/min.

	Nitrogen flow rate (L/min)		
Time (min)	1.2	0.81	0.32
5	0.05/11.905 %	0.50/73.912 %	0.52/64.286 %
10	0.12/25.298 %	1.13/89.226 %	1.33/95.959 %
15	0.30/50.298 %	1.43/96.402 %	5.53/99.999

Additionally, as a consequence of exposure to cold argon-nitrogen plasma at a nitrogen flow rate of 0.32 L/min conidia of *A. flavus* showed a delay in germination and conidia formation was also affected (Fig. 3).

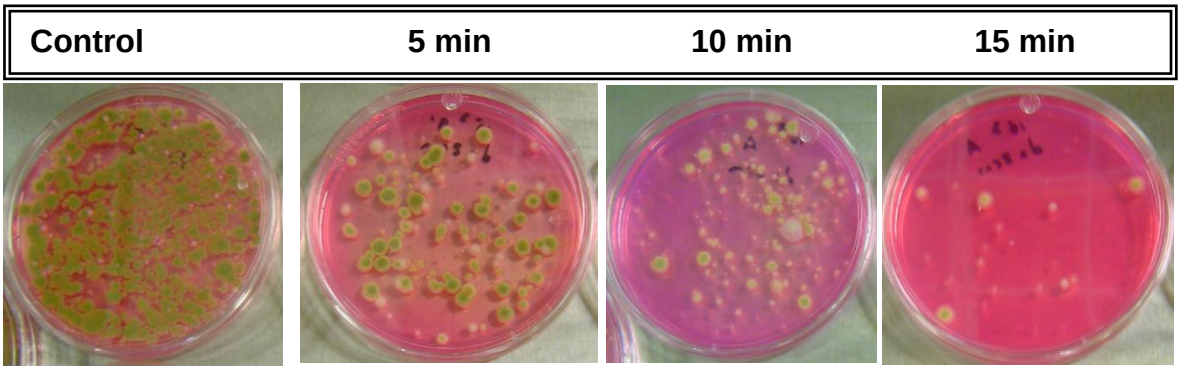

Fig. 3. Growth of *A. flavus* after treatment by argon-nitrogen plasma at a nitrogen flow rate of 0.32 L/min.

4. DISCUSSION

Selcuk *et al.,* (2008) showed that a 15 min exposure to cold plasma generated with air and sulfur hexafluoride decreased the viable population of *Aspergillus spp*. conidia of lentil beans by 85.6%. They also observed that the effectiveness of the plasma treatment varied according to the surface structure of different types of lentil beans, in some lentil beans, a 99% of the fungus elimination was obtained. Some authors (Laroussi, 2009; Lee *et al*., 2006; Moisan *et al*., 2002) have described the antimicrobial mechanism of the plasma due to the synergistic effect of free radicals, ions and UV radiation as a result of the treatment, which cause oxidative stress and DNA damage. Additionally, a process called "Etching" involves the interaction of highly reactive radicals with organic materials generating by-

products that are desorbed from microbial surface and cause drainage of the cell wall. (Lee *et al*., 2006; Hong *et al*., 2009; Moisan *et al*., 2001). It appears that, in general, microorganisms with thin cell walls like Gram-negative bacteria are more sensitive to treatment with plasma (Lee *et al*., 2006; Baier *et al*., 1992). Although the cellular damage to microorganisms caused by cold plasma and free radical treatments is very similar, the former appears to have advantages in terms of security and its lack of inhibition of grain germination (Será *et al*., 2009; Volin *et al*., 2000). The plasma effectiveness as a microbial population control method depends on several factors, including exposure period, gas mixture and gas flow rates. In this study, a lower nitrogen flow rate increased the plasma lethality for *Aspergillus flavus* conidia, and it was more effective than the treatment using argon only plasma. Nitrogen probably permits the formation of excited NO_X molecules that potentiate the UV emissions from the plasma, increasing the cell damage at the level of DNA damage and rupture of macromolecules as it has been reported (Laroussi *et al*., 2000; Maxwell *et al*., 2006; Moisan *et al*., 2002). As a result of plasma exposure, the surviving *Aspergillus flavus* conidia grew more slowly on plates, indicating damage to the fungus wall. The delay in the growth of surviving conidia is an additional benefit of this method for sanitizing lentil beans. Most research on cold plasma has been done in surface sanitization. Future research could be focused on food products that have a complex surface, e.g., skin-on chicken pieces, porous nuts, and stem scar or blossom ends of fruits. The understanding of the action mode of plasma is a key step toward optimization of the technology for specific applications in the food processing industry (Niemira, 2011). Bahrami et al (2016) mentioned that cold plasma has the potential to change the chemical composition of wheat flour, however more investigations are necessary to evaluate the effect of cold plasma on microbial reduction and its effects on chemical composition of grains.

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CONFLICT OF INTEREST

All the researchers listed as authors of the current study declare that there is no conflict of interests regarding the publication of this manuscript.

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