Short Communication

Effect of CO_2 modified atmosphere packaging on aflatoxin production in maize infested with *Sitophilus zeamais*

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Abstract

The weevil *Sitophilus zeamais* (Motschulsky), the maize weevil, is a pest of stored maize that can cause feeding damage and lead to the proliferation of toxigenic fungi. The application of modified atmospheres with a high concentration of CO_2 is an alternative method for the control of *S. zeamais* and the inhibition of fungal growth. The objectives of the study were to determine the effect of *S. zeamais* infestation, grain damage and grain moisture content on aflatoxin production by *Aspergillus flavus* on maize, and the impact of high CO_2 modified atmosphere packaging on pest infestation and aflatoxin production. Mycotoxin production was only recorded when maize was infested with *S. zeamais* and had *A. flavus* inoculum. However, production of mycotoxins was not recorded when the maize was mechanically damaged and stored at 18% moisture content, indicating that the biological activity of the insect was determinant in the production of mycotoxins. The high CO_2 modified atmosphere packaging tested (90% CO_2, 5% O_2 and 5% N_2) prevented mycotoxin production.

Keywords: *Aspergillus flavus*, Mycotoxins, Maize weevil, Stored product pests, Control

1. Introduction

One of the main pests of stored maize is *Sitophilus zeamais* Motschulsky, the maize weevil (Lamboni and Hell, 2009). This insect pest causes rupture in the grain which can cause proliferation and dispersal of toxigenic fungi. Fungi belonging to the genus *Aspergillus*, *Fusarium* and *Penicillium* can be associated with stored maize (Fleurat-Lessard, 2017). According to Mc Millian et al. (1980) maize infested with weevils exposed to spores of *Aspergillus flavus* Link (Eurotiales: Trichocomaceae) can double the overall grain infection by the fungi. This fungus can produce mycotoxins (aflatoxin B1) and cause serious foodborne infections.

An alternative method to the application of chemicals for the control of *S. zeamais* in stored foods is the use of modified atmospheres (MAs) with a high content of carbon dioxide (CO_2) (Navarro, 2006). The main effect of CO_2 on insects is the permanent opening of spiracles, which regulate their respiration. Consequently, there is a significant loss of water and the insect desiccates. In addition, CO_2 also produces acidification of insect internal fluids, modifications of different metabolic pathways and anaesthetic effects due to the alteration of the permeability of the cell membranes of neurons. The application of modified atmospheres packaging (MAP) with a high concentration of CO_2 (more than 50%) and a low residual concentration of O_2 (less than 3%) has been shown to be effective for the control of *S. zeamais* (Riudavets et al., 2009).

According to Paster (1990), mycotoxin formation by fungi is an aerobic process, which can be blocked when CO_2 levels increase, and may become inhibited at low O_2 concentrations, even when fungal growth is not greatly affected. Giorni et al. (2008) observed that an atmosphere with 50% CO_2 applied to *A. flavus* in artificial media and in maize totally inhibits the production of aflatoxins. Taniwaki et al. (2010) observed a decrease in the growth rate of *A. flavus*, when it was subjected to MAs with 80% CO_2 and

20% O_2. They also detected that, under these conditions, aflatoxin production was totally inhibited.

The objectives of the present study were to determine: a) the effect of *S. zeamais* infestation, grain damage and grain moisture content on aflatoxin production on maize, and b) the impact of integrating conditions of increasing CO_2 atmosphere (up to 90%) with changes in grain moisture content and pest infestation on aflatoxin production.

2. Material and methods

Insects were obtained from stock cultures maintained in brown rice at 25±2ºC and 75±10% r.h.. For the present trials, we reared a specific colony on maize kernels of variety DKC 6666 (Monsanto Company) (16% moisture content, MC) for 3 generations. The Bacteriology and Mycology Service of the Department of Animal Health and Anatomy of the Universitat Autònoma de Barcelona provided the *A. flavus* strain that was used in the trials.

2.1. Effect of *S. zeamais* infestation, grain damage and grain moisture content on *A. flavus* mycotoxin production in maize

This experiment was performed under stabilized environmental conditions of 25±1ºC temperature and 80% relative humidity. Four treatments were conducted; maize kernels at 16% MC without insects and fungi; maize kernels at 16% MC with *A. flavus*; maize kernels at 16% MC with *A. flavus* and *S. zeamais*; and coarsely ground maize grains at 18% MC with *A. flavus*. Five replicates per treatment with 150 g of maize were prepared in ventilated glass cages. Each treatment was isolated in closed plastic boxes (35 x 40 x 55 cm). For the inoculation of *A. flavus*, 0.75 mL of fungal suspension at a concentration of 10,000 spores / mL was introduced into each replicate and mixed with

the maize to obtain a concentration of 50 *A. flavus* spores / g of maize. For the treatments with insects, 225 *S. zeamais* adults were added to each replicate. To simulate damage produced by insects, maize kernels were manually ground with a mortar until the portions were of an approximate measure of a quarter of a grain. Then, ground maize was moistened with tap water until an 18% MC was achieved and spores of *A. flavus* were added. After 17 weeks, the number of *S. zeamais* adults, the number of fungi and yeast colony forming units (cfu) per gram of maize (Silliker Ibérica, SA, Barcelona, Spain) and aflatoxin content (ppb) (AgraQuant® kit, Romer Labs, Getzersdorf, Austria) were assessed in each replicate.

2.2. Effect of MAP on *A. flavus* mycotoxin production in maize

For this experiment, we proceeded in the same way as in the previous experiment, with the difference that an additional treatment was included: maize kernels at 16% MC with *A. flavus* and *S. zeamais* and treated with a MAP of 90% CO_2, 5% O_2 and 5% N_2. The ventilated cages were placed inside 300 x 210 mm and 59 mm-thick plastic bags (Cryovac BB4L). The plastic bags had respective permeabilities to O_2 and CO_2 of 30 and 150 $cm^3/ m^2 day^1 bar^1$ measured under conditions standardized at 23ºC and 0% r.h. The plastic bags were filled with the modified atmosphere, which was previously prepared with a gas mixer (Witt KM 100-3M/MEM, Witten, Germany), using a vacuum packaging machine (Multivac A 300/16, Wolfertschwenden, Germany). It was humidified in a water bubbling container in order to reach approximately 80% relative humidity. A gas analyzer (Abiss model TOM 12, Varennes Jarcy, France) was used to verify the CO_2 and O_2 contents inside the plastic bags. Gas levels were determined at the start and at the end of the exposure with a gas syringe containing samples of 25 ml volume. The modified atmosphere treatment was conducted after 6 weeks of the start of the experiment and for

111 an exposure of three weeks. After the treatment, the ventilated glass cages were again
112 stored in its corresponding plastic box at 25±1 °C and 80% r.h..
113 After 17 weeks, the number of *S. zeamais* adults, the number of fungi and yeast
114 colony forming units (cfu) per gram of maize (Silliker Ibérica, SA, Barcelona, Spain) and
115 aflatoxin content (ppb) (AgraQuant® kit, Romer Labs) were assessed in each replicate.
116 A one-way analysis of variance (ANOVA) was used to compare the number of
117 insects and the aflatoxin content among treatments. The Tukey Multiple Range test was
118 used to compare mean values. Data were transformed before analysis using the log (x+1)
119 function to ensure homoscedasticity and normality of the residuals.
120
121 **3. Results**
122 *3.1. Effect of S. zeamais infestation, grain damage and grain moisture content on A. flavus*
123 *mycotoxin production in maize*
124 The number of *S. zeamais* adults recorded at the end of the experiment in the
125 treatment infested with insects and fungi was 963±180.5. Regarding the growth of fungi,
126 the higher number of colonies was recorded in the treatment infested with insects ($1.6x10^7$
127 of fungi colonies, cfu/g), followed by the treatment with ground maize ($3.4\ x10^6$ cfu/g).
128 In comparison, the other two treatments that had no insects and grain kernels where not
129 damaged the number of fungal colonies was very low, 4100 and 320 cfu / g, respectively.
130 In all treatments, the number of yeast colonies was very low, below 10 cfu / g.
131 Regarding mycotoxin content, in the samples inoculated with insects the amount of
132 mycotoxin were significantly higher than in the other three treatments without insects
133 (F=4.01; d.f. = 3, 37; P<0.05) (Table 1). Mycotoxin production was not significantly
134 different between the treatment with maize kernels manually damaged at 18% MC and
135 infested with *A. flavus* and the other treatments without insects. This result could be

attributed to the different type of damage caused by the insect (during its feeding) and due to manual grinding. In addition, it should be noted that the presence of only *A. flavus* did not favour the production of mycotoxins in the maize samples without insects.

3.2. Effect of MAP on A. flavus mycotoxin production in maize

The CO_2 and O_2 concentrations inside the bags treated with MAP varied slightly throughout the three weeks treatment. The CO_2 concentration at the end of the storage period decreased by 23% compared to its initial content, from 90±0.1% to 67±13.4%. In comparison, the percentage of O_2 remained practically constant throughout the entire test, from 4.9±0.1% to 4.4±2.54%.At the end of the experiment, the samples treated with MAP showed a statistically significant (87%) decrease in the number of *S. zeamais* adults (129±20.3) compared to the samples without MAP treatment (961±5.4) (F=1559.46; d.f.= 1, 9; P<0.001).

The amount of mycotoxin was significantly reduced in the samples treated with MAP compared to the samples without treatment. Mycotoxin production was significantly higher in the samples with the higher number of insects (F=9.52; d.f.= 4; 48; P<0.001) (Table 2). In spite of the presence of a certain number of insects, the concentration of aflatoxins in samples treated with MAP was similar to that of treatments without *S. zeamais*. Neither the presence of the fungi alone without insects nor the increased moisture content in artificially damaged maize favoured significantly the production of mycotoxins.

4. Discussion

The highest concentrations of mycotoxins were found when maize was infested with the weevil *S. zeamais* and had *A. flavus* inoculum. In fact, the concentration of

mycotoxins in these treatments was approximately four times the maximum permitted levels in Europe (5 ppb aflatoxin B1 content in raw cereal grain for human food) (Fleurat Lesssart, 2017). Thus, the results confirm that the presence of a high population of *S. zeamais* can favour the environmental conditions for the development of the fungi (Beti et al., 1995). Sinha and Sinha (1991) observed a significant increase in the *A. flavus* infection and mycotoxin production in wheat samples infested with *Sitophilus oryzae* L. for a period of 3 months. In their experiment, the final concentration of mycotoxins detected was 850 ppb, a very high value compared to the present study (20 ppb, Tables 1 and 2). This could be because in the study of Sinha and Sinha (1991), the initial suspension of *A. flavus* used was 3% while in the present study it was 0.6%. These authors showed that *S. oryzae* adults in contact with samples of infested wheat transported spores of *A. flavus*, and they isolated the fungus from different areas of the insect such as legs, antennae and the surface of the body. Aside from transporting the fungus on the outside, the insect can also carry it inside and contaminate the maize by defecating the spores of the fungus in the grain (Beti et al., 1995).

In the two experiments, the concentration of mycotoxins detected after 17 weeks remained below the maximum permitted level (Fleurat Lesssart, 2017) in the samples where the *A. flavus* fungus was inoculated but in which there was no insect presence, even though r.h. was favourable for the development of the fungus. When comparing the treatments with ground maize kernels at 18% MC in the two experiments with the samples infested with *A. flavus* and *S. zeamais*, the concentration of mycotoxins was much lower. This is probably due to the feeding of insect larvae inside the grains that distributes the fungus spores and increases the surface area in which the fungus can develop and produce more mycotoxins. In addition, insect metabolism causes an increase in temperature and

185 substrate moisture content (Sinha and Sinha, 1991), favouring the development of the
186 fungus and the production of mycotoxins.

187 When a MAP treatment with high concentration of CO_2 and low concentration of
188 O_2 was applied, a 96% decrease of the mycotoxins production was obtained (<1 ppb).
189 This could have been due to the significant decrease in the number of insects observed in
190 these samples. Then, with fewer insects, there was less metabolic activity and
191 consequently lower moisture in the grain to reach the optimal production of mycotoxins
192 by *A. flavus*. According to Riudavets et al. (2009) it is necessary an exposure time of 12
193 days with a MAP with 90% CO_2 for the control of all developmental stages of the rice
194 beetle *S. oryzae*. In the present experiment, after three weeks of treatment with
195 approximately 90% CO_2, some survival of *S. zeamais* was observed. This difference
196 could be explained because of the different relative humidity of the MAs, 80% in the
197 present study and 70% in the study of Riudavets et al. (2009). On the other hand, a low
198 concentration of O_2 and a high concentration of CO_2 could also influence directly the
199 production of mycotoxins by the fungus as demonstrated by Giorni et al. (2008) and
200 Taniwaki et al. (2010).

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234 production by fungi in atmospheres containing 80% carbon dioxide and 20%
235 oxygen. Int. J. Food Microbiol. 143, 218-25.
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238 **Table 1** Mean (±SE) mycotoxin content (aflatoxin, ppb) on maize at different moisture
239 contents (MC) and inoculated with the fungus *A. flavus* and *S. zeamais* adults.

Treatment	Mycotoxin[a] (ppb)
Maize (16%)	1.1±0.37 b
Maize (16%) + *A. flavus*	1.3±0.23 b
Maize (16%) + *A. flavus* + *S. zeamais*	19.6±13.03 a
Maize ground (18%) + *A. flavus*	2.3±0.31 b

240 [a]Values represented with the same letter are not significantly different between them
241 (P<0.05, test Tukey, n=5).

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243

244 **Table 2**. Mean (±SE) mycotoxin content (aflatoxin, ppb) on maize inoculated with the
245 fungus *A. flavus* and *S. zeamais* adults in relation to different moisture contents (MC) and
246 MAP treatment

Treatment	Mycotoxin[a] (ppb)
Maize (16%)	0.6±0.12 b
Maize (16%) + *A. flavus*	0.8±0.04 b
Maize (16%) + *A. flavus* + *S. zeamais*	21.2±6.49 a
Maize (16%) + *A. flavus* + *S. zeamais* + MAP	0.9±0.14 b
Maize ground (18%) + *A. flavus*	1.1±0.09 b

247 [a]Values represented with the same letter are not significantly different between them
248 (P<0.05, test Tukey, n=5).

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