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1 Short Communication

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4 Effect of CO₂ modified atmosphere packaging on aflatoxin production in maize infested
5 with *Sitophilus zeamais*

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16

17 **Abstract**

18 The weevil *Sitophilus zeamais* (Motschulsky), the maize weevil, is a pest of stored
19 maize that can cause feeding damage and lead to the proliferation of toxigenic fungi. The
20 application of modified atmospheres with a high concentration of CO₂ is an alternative
21 method for the control of *S. zeamais* and the inhibition of fungal growth. The objectives
22 of the study were to determine the effect of *S. zeamais* infestation, grain damage and grain
23 moisture content on aflatoxin production by *Aspergillus flavus* on maize, and the impact
24 of high CO₂ modified atmosphere packaging on pest infestation and aflatoxin production.
25 Mycotoxin production was only recorded when maize was infested with *S. zeamais* and
26 had *A. flavus* inoculum. However, production of mycotoxins was not recorded when the
27 maize was mechanically damaged and stored at 18% moisture content, indicating that the
28 biological activity of the insect was determinant in the production of mycotoxins. The
29 high CO₂ modified atmosphere packaging tested (90% CO₂, 5% O₂ and 5% N₂) prevented
30 mycotoxin production.

31

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

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36 1. Introduction

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

45 An alternative method to the application of chemicals for the control of *S. zeamais*
46 in stored foods is the use of modified atmospheres (MAs) with a high content of carbon
47 dioxide (CO₂) (Navarro, 2006). The main effect of CO₂ on insects is the permanent
48 opening of spiracles, which regulate their respiration. Consequently, there is a significant
49 loss of water and the insect desiccates. In addition, CO₂ also produces acidification of
50 insect internal fluids, modifications of different metabolic pathways and anaesthetic
51 effects due to the alteration of the permeability of the cell membranes of neurons. The
52 application of modified atmospheres packaging (MAP) with a high concentration of CO₂
53 (more than 50%) and a low residual concentration of O₂ (less than 3%) has been shown
54 to be effective for the control of *S. zeamais* (Riudavets et al., 2009).

55 According to Paster (1990), mycotoxin formation by fungi is an aerobic process,
56 which can be blocked when CO₂ levels increase, and may become inhibited at low O₂
57 concentrations, even when fungal growth is not greatly affected. Giorni et al. (2008)
58 observed that an atmosphere with 50% CO₂ applied to *A. flavus* in artificial media and in
59 maize totally inhibits the production of aflatoxins. Taniwaki et al. (2010) observed a
60 decrease in the growth rate of *A. flavus*, when it was subjected to MAs with 80% CO₂ and

61 20% O₂. They also detected that, under these conditions, aflatoxin production was totally
62 inhibited.

63 The objectives of the present study were to determine: a) the effect of *S. zeamais*
64 infestation, grain damage and grain moisture content on aflatoxin production on maize,
65 and b) the impact of integrating conditions of increasing CO₂ atmosphere (up to 90%)
66 with changes in grain moisture content and pest infestation on aflatoxin production.

67

68 **2. Material and methods**

69 Insects were obtained from stock cultures maintained in brown rice at 25±2°C and
70 75±10% r.h.. For the present trials, we reared a specific colony on maize kernels of variety
71 DKC 6666 (Monsanto Company) (16% moisture content, MC) for 3 generations. The
72 Bacteriology and Mycology Service of the Department of Animal Health and Anatomy
73 of the Universitat Autònoma de Barcelona provided the *A. flavus* strain that was used in
74 the trials.

75

76 **2.1. Effect of *S. zeamais* infestation, grain damage and grain moisture content on *A.*** 77 ***flavus* mycotoxin production in maize**

78 This experiment was performed under stabilized environmental conditions of
79 25±1°C temperature and 80% relative humidity. Four treatments were conducted; maize
80 kernels at 16% MC without insects and fungi; maize kernels at 16% MC with *A. flavus*;
81 maize kernels at 16% MC with *A. flavus* and *S. zeamais*; and coarsely ground maize grains
82 at 18% MC with *A. flavus*. Five replicates per treatment with 150 g of maize were
83 prepared in ventilated glass cages. Each treatment was isolated in closed plastic boxes (35
84 x 40 x 55 cm). For the inoculation of *A. flavus*, 0.75 mL of fungal suspension at a
85 concentration of 10,000 spores / mL was introduced into each replicate and mixed with

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

95

96 **2.2. Effect of MAP on *A. flavus* mycotoxin production in maize**

97 For this experiment, we proceeded in the same way as in the previous experiment,
98 with the difference that an additional treatment was included: maize kernels at 16% MC
99 with *A. flavus* and *S. zeamais* and treated with a MAP of 90% CO₂, 5% O₂ and 5% N₂.
100 The ventilated cages were placed inside 300 x 210 mm and 59 mm-thick plastic bags
101 (Cryovac BB4L). The plastic bags had respective permeabilities to O₂ and CO₂ of 30 and
102 150 cm³/ m² day¹ bar¹ measured under conditions standardized at 23°C and 0% r.h. The
103 plastic bags were filled with the modified atmosphere, which was previously prepared
104 with a gas mixer (Witt KM 100-3M/MEM, Witten, Germany), using a vacuum packaging
105 machine (Multivac A 300/16, Wolfertschwenden, Germany). It was humidified in a water
106 bubbling container in order to reach approximately 80% relative humidity. A gas analyzer
107 (Abiss model TOM 12, Varennes Jarcey, France) was used to verify the CO₂ and O₂
108 contents inside the plastic bags. Gas levels were determined at the start and at the end of
109 the exposure with a gas syringe containing samples of 25 ml volume. The modified
110 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 (1.6×10^7
127 of fungi colonies, cfu/g), followed by the treatment with ground maize (3.4×10^6 cfu/g).
128 In comparison, the other two treatments that had no insects and grain kernels were 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

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

139

140 3.2. Effect of MAP on *A. flavus* mycotoxin production in maize

141 The CO₂ and O₂ concentrations inside the bags treated with MAP varied slightly
142 throughout the three weeks treatment. The CO₂ concentration at the end of the storage
143 period decreased by 23% compared to its initial content, from 90±0.1% to 67±13.4%. In
144 comparison, the percentage of O₂ remained practically constant throughout the entire test,
145 from 4.9±0.1% to 4.4±2.54%. At the end of the experiment, the samples treated with MAP
146 showed a statistically significant (87%) decrease in the number of *S. zeamais* adults
147 (129±20.3) compared to the samples without MAP treatment (961±5.4) ($F=1559.46$; d.f.=
148 1, 9; $P<0.001$).

149 The amount of mycotoxin was significantly reduced in the samples treated with
150 MAP compared to the samples without treatment. Mycotoxin production was
151 significantly higher in the samples with the higher number of insects ($F=9.52$; d.f.= 4; 48;
152 $P<0.001$) (Table 2). In spite of the presence of a certain number of insects, the
153 concentration of aflatoxins in samples treated with MAP was similar to that of treatments
154 without *S. zeamais*. Neither the presence of the fungi alone without insects nor the
155 increased moisture content in artificially damaged maize favoured significantly the
156 production of mycotoxins.

157

158 4. Discussion

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

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

176 In the two experiments, the concentration of mycotoxins detected after 17 weeks
177 remained below the maximum permitted level (Fleurat Lessart, 2017) in the samples
178 where the *A. flavus* fungus was inoculated but in which there was no insect presence, even
179 though r.h. was favourable for the development of the fungus. When comparing the
180 treatments with ground maize kernels at 18% MC in the two experiments with the samples
181 infested with *A. flavus* and *S. zeamais*, the concentration of mycotoxins was much lower.
182 This is probably due to the feeding of insect larvae inside the grains that distributes the
183 fungus spores and increases the surface area in which the fungus can develop and produce
184 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 CO₂ and low concentration of
188 O₂ 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% CO₂ 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% CO₂, 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 O₂ and a high concentration of CO₂ 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).

201

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206 **References**

207 Beti, J. A., Phillips, T.W., Smalley. E.B., 1995. Effects of Maize Weevils (Coleoptera:
208 Curculionidae) on Production of Aflatoxin BI by *Aspergillus flavus* in Stored Corn.
209 J. Econ. Entomol. 88, 1776-1782.

210 Fleurat-Lessard, F., 2017. Integrated management of the risks of stored grain spoilage by
211 seedborne fungi and contamination by storage mould mycotoxins - An update. J.
212 Stored Prod. Res. 71, 22-40.

213 Giorni, P., Battilani, P., Pietri, A., Magan, N., 2008. Effect of aw and CO₂ level on
214 *Aspergillus flavus* growth and aflatoxin production in high moisture maize post-
215 harvest. Int. J. Food Microbiol. 122, 109-13.

216 Lamboni, Y., Hell, K., 2009. Propagation of mycotoxigenic fungi in maize stores by post-
217 harvest insects. Int. J. Trop. Insect Sci. 29, 31-39.

218 McMillian, W.W., Widstrom, N.W., Wilson, D.M., Hill, R.A., 1980. Transmission by
219 maize weevils (Coleoptera, Curculionidae) of *Aspergillus flavus* and its survival on
220 selected corn hybrids. J. Econ. Entomol. 73, 793-794.

221 Navarro, S., 2006. Modified atmospheres for the control of stored product insects and
222 mites. In: Heaps J.W. (ed.), Insect management for food storage and processing,
223 AACC International, St. Paul, Minnesota, USA, pp. 105-145.

224 Paster, N., 1990. Modified atmospheres for preventing molds and mycotoxin production
225 in stored grain. In: Calderon, M., Barkai-Golan, R. (eds.), Food Preservation by
226 Modified Atmospheres. CRC Inc., Boca Raton. Florida, USA. pp: 39-55.

227 Riudavets, J., Castañé, C., Alomar, O., Pons, M.J., Gabarra, R., 2009. Modified
228 atmosphere packaging (MAP) as an alternative measure for controlling ten pests
229 that attack processed food products. J. Stored Prod. Res. 45, 91–96.

230 Sinha, K.K., Sinha, A.K., 1991. Effect of *Sitophilus oryzae* infestation on *Aspergillus*
231 *flavus* infection and aflatoxin contamination in stored wheat. J. Stored Prod. Res.
232 27, 65-68.

233 Taniwaki, M.H., Hocking, A.D., Pitt, J.I., Fleet, G.H., 2010. Growth and mycotoxin
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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237

238 **Table 1** Mean (\pm 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 \pm 0.37 b
Maize (16%) + <i>A. flavus</i>	1.3 \pm 0.23 b
Maize (16%) + <i>A. flavus</i> + <i>S. zeamais</i>	19.6 \pm 13.03 a
Maize ground (18%) + <i>A. flavus</i>	2.3 \pm 0.31 b

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

242

243

244 **Table 2.** Mean (\pm 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 \pm 0.12 b
Maize (16%) + <i>A. flavus</i>	0.8 \pm 0.04 b
Maize (16%) + <i>A. flavus</i> + <i>S. zeamais</i>	21.2 \pm 6.49 a
Maize (16%) + <i>A. flavus</i> + <i>S. zeamais</i> + MAP	0.9 \pm 0.14 b
Maize ground (18%) + <i>A. flavus</i>	1.1 \pm 0.09 b

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

249