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Interaction between *Tribolium castaneum* (Herbst) and Mycotoxigenic *Aspergillus flavus* Link on Maize Flour †

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Abstract: Cereal grains are part of the most important alimentary sources for humans and must be safely stored, as contamination by living organisms, such as insects and fungi, causes quantitative and qualitative losses. *Tribolium castaneum* is one of the most common insect pests of stored products, and may also produce benzoquinones as a defensive action. This insect presence makes the products more susceptible to the spread of fungi, such as *Aspergillus flavus*, which alters the quality of the grains and may produce mycotoxins, such as aflatoxins. The aim of this work was to evaluate the influence of adults of *T. castaneum* presence on a mycotoxigenic strain of this fungi development and production of mycotoxins, as well as the influence of the fungi on insect adults. Maize flour was exposed to: *T. castaneum* adults; spores of *A. flavus*; both organisms; and only maize as control. In all assays, except control, AFB_1 and total aflatoxins content were above the accepted limit for human food. The ability of these organisms, to thrive under the same conditions and the chemical compounds released by them, makes the interaction between *T. castaneum* and *A. flavus* a subject with great importance to the stored maize safety.

Keywords: Benzoquinones; *Aspergillus flavus*; Mycotoxins; Maize flour; *Tribolium castaneum*

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1. Introduction

Some strains of fungal species can produce mycotoxins, which are secondary metabolites harmful for animals and humans [1]. Besides being a fungus thriving on different commodities [2], *Aspergillus flavus* Link has some mycotoxigenic strains, which may contaminate the commodities with aflatoxins, enabling its consumption by end users, as their effect on animal and human health after consumption are severe [1,3–5].

Tribolium castaneum Herbst is an important pest of stored agricultural products [6], being considered one of the most important key-pest of the stored milled grain, despite being also a model organism, and having its genome completely sequenced [7]. The adults of this insect secrete a mixture of compounds composed of mainly 1,4-benzoquinone: methyl-1,4-benzoquinone, ethyl-1,4-benzoquinone [8–10]. These cuticular secretions may have a defensive role towards predators and microbes, as well as a putative regulatory effect on its own population growth [11–13]. This insect has shown resistance to most classes of insecticides, fact which may be partially attributed to the existence of detoxification enzymes within this insect, that are encoded by insecticide resistance genes, as for example, the cytochrome P450 proteins [9,14–20].

Within this work, the aim was to evaluate the possible influence of the insect presence on the production of aflatoxins by a mycotoxigenic *A. flavus* strain, in maize flour. This is a first step for the understanding this complex interaction.

2. Materials and Methods

2.1. Maize Flour Preparation

For this study, maize collected directly from fields was stored at −4 ℃ and then grinded and sieved to obtain maize flour. Initial moisture content and water activity of the maize flour were determined, using adequate equipment (moisture measurement scale PMB202 ADAM (Adam Equipment, United Kingdom) and Hygrolab C1 (Rotronic, Switzerland)), and an average value is presented, as three replications were used for these determinations.

2.2. Tribolium Castaneum Rearing

Insects were obtained from natural populations, with less than five years of rearing at Entomology Laboratory of the *Departamento de Ciências e Engenharia de Biossistemas* (DCEB) of *Instituto Superior de Agronomia* (ISA), University of Lisbon. The cultures were maintained at 26 ℃ and 65–70 % RH in a mixture of wheat flour and beer yeast (*Saccharomyces cerevisiae* Hansen) in a 95:5 proportion, according to [21]. Mass rearing procedure was done as previously described by the authors [22]. Insects were maintained at a climatic chamber Fitoclima S600, ClimaPlus 400 (ARALAB, Portugal), at 30 ℃ ± 2 ℃ of temperature and 70 % ± 5 % RH. Adults used had eight days old.

2.3. Aspergillus flavus preparation

A mycotoxigenic strain of *A. flavus* from Minho University Mycotheca (MUM-UMinho) was selected and maintained at 4º C in the collections of the Laboratory of Mycology of the *Departamento de Ciências e Engenharia de Biossistemas* (DCEB) of ISA, University of Lisbon.

Suspensions of fungi' conidia were prepared from potato dextrose agar (PDA) plates grown for eight days by rubbing the sporulating surface with a bent needle. After filtering through a 60-μm mesh sieve to remove debris, sterile distilled water was added to the suspension to reach 10^7 conidia/mL concentration [23,24], based upon cell counts using an hemacytometer.

2.4. Assays

For the assays, 40 g of maize flour were placed on glass flasks and autoclavede to eliminate any potential fungi or insect contamination. The assays were divided to have the following: (1) control assays, with only the maize flour; (2) the insect assays, with 80 *T. castaneum* adults; (3) the fungi assays, with the maize flour inoculated with 0.5 mL of the fungal conidia suspensions; and 4) the 'insects + fungi' assays, with both the 80 insect adults and the 0.5 mL of the fungal conidia suspensions. Ten replicates were set for the assay.

The assays were maintained in a climatic chamber at 30 ℃ ± 2 ℃ and 70 % ± 5 % RH for eight weeks. After, fungi development was evaluated by direct observation. Samples of the maize flour were analysed, for the presence of aflatoxins, according to a previously established methodology [25] in the laboratories of the National Institute of Health Dr. Ricardo Jorge (INSA).

3. Results

The assays with insects scored a mortality rate of 50 %, while the assays with insects and fungi score a mortality rate of 98 %.

In terms of fungi development evaluation, the assays with only fungi showed a visible fungi growth. However, there was a higher fungi growth, with the development of caking, in the assays with insect and fungi (Figure 1).

Figure 1. Aspect of final samples of the assays (from left to right): 1 - control (containing only maize flour), 2 - insects (maize flour and *T. castaneum*), 3 - fungi (maize flour and *A. flavus*), and 4 - insects and fungi (maize flour, *T. castaneum* and *A. flavus*).

In all experiments, moisture content as the maize flour was exposed to insects, fungi and 'fungi and insects' was not possible to perform the measurements in safe conditions due to caking and high content of aflatoxins from control.

Regarding mycotoxins analysis the results showed the existence of aflatoxins AFB_1 and AFB_2 above the Limit of Detection (LOD) 0.011 µg/kg and 0.004 µg/kg, respectively and the Limit of Quantification (LOQ) 0.038 µg/kg and 0.013 µg/kg, respectively.

4. Discussion

The insect mortality in the assays 'insects and fungi' denote a possible negative effect of *A. flavus* on insect adults of *T. castaneum*, despite these insects' defensive secretions (benzoquinones). Aflatoxin B_1, the most abundant aflatoxin in those assays, showed to have negative influence on the development and fecundity of *Ahasverus advena* Waltl, a mycetophagous insect which may also attack the food products where fungi are growing, although this insect has more tolerance to aflatoxins [26]. It is important to stress out that *T. castaneum* reaches its maximum benzoquinones excretion about 40 days after adults' emergence [9], and the adults reached the 40 days old during the experiments. Considering that the red flour beetle excretes benzoquinones to compete with other organisms, namely fungi, their presence might have stimulated response by *A. flavus* to produce mycotoxins (or other secondary defensive metabolites). However, this would need further investigation efforts as mycotoxins production is affected by several abiotic and biotic factors [27,28]. The negative effect of fungi on insects corroborates the competitive nature of their relationship [28] within the conditions of the assays of the present study.

The interaction between this insect and mycotoxigenic fungi is still an intriguing field of research, which may have important outcomes regarding innovative control methods of stored products associated insects and fungi. The possible tolerance or resistance mechanism of *T. castaneum* to aflatoxins could be an important contribution to the field of novel mycotoxins control methods in food, which may include the use of enzymes that promote the enzymatic degradation of mycotoxins, and new enzymes are needed [29]. This was the first work evaluating the interaction between *T. castaneum* and *A. flavus* mycotoxin production.

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