



Original article

Effects of aflatoxin contaminated maize on *Folsomia candida* (Collembola)Borbála Szabó^{a,b,*}, Benjamin Bálint^a, Miklós Mézes^{c,d}^a Szent István University, Department of Zoology and Animal Ecology, H-2100, Gödöllő, Péter K. Street 1., Hungary^b CER Danube Research Institute, H-1113, Budapest, Karolina Street 29, Hungary^c Szent István University, Department of Nutrition, H-2100, Gödöllő, Péter Károly Street 1, Hungary^d MTA-KE-SZIE Mycotoxins in the Food Chain Research Group, H-6400, Kaposvár, Guba Sándor Street 40, Hungary

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ABSTRACT

The aflatoxin B1 (AFB1) producing mould, *Aspergillus flavus*, is spread to more northern regions with global warming. AFB1 can cause genotoxicity, oxidative stress, immunosuppression, and necrosis in the liver, kidney, and muscles in vertebrates, but the effect on invertebrates is not well known. *Folsomia candida* (Collembola) is a cosmopolitan species and could be easily bred in the laboratory because of its parthenogenetic reproduction. The aim of the present study was to determine whether AFB1 has a toxic effect on survival and reproduction. Also, we wanted to answer the question of whether the animals prefer to feed on *A. flavus* or yeast. Furthermore, we wanted to determine whether the animals avoid the aflatoxin-contaminated soil or not.

The mortality did not increase because of AFB1, but the reproduction was impaired. In addition, the animals fed on AFB1 contaminated maize but did not prefer it compared to yeast. Moreover, the animals did not avoid the contaminated AFB1 soil. They even preferred it most probably, because *A. flavus* could be high-quality food if the animals consume the not toxin producer hyphae; because of that, the animals could have tried to consume the contaminated hyphae too.

In conclusion, AFB1 contaminated maize is not recommended to be used as green manure in the soil in a native state. Alternative solutions could be the use of mycotoxin contaminated maize as biogas.

1. Introduction

One of the most important safety risks of feed and food supply are mycotoxins, while significant mycotoxin contamination is reported in feed and food despite efforts to control fungal contamination [1]. Aflatoxin B1 (AFB1) is one of the most toxic mycotoxins produced by fungi of the *Aspergillus* genus (*A. parasiticus*, *A. flavus*) [2,3]. Aflatoxins are known for their high toxicity, carcinogenicity, and mutagenicity [2]. AFB1 can cause genotoxicity, oxidative stress, immunosuppression, and necrosis in the liver, kidney, and muscles [3–5]. The distribution of AFB1 producing fungi is well known in tropical and subtropical regions, but spreading to northern regions also found due to global warming [4]. Unfortunately, the AFB1 production could go up until 1000–5000 µg kg⁻¹ in some isolates, which is the thousand fold of the level of the European Union directive of feed and foodstuff [4,6].

Secondary metabolites of moulds, such as mycotoxins, could act as protection against fungivores, or as a competitive advantage against other saprophytic organisms [7,8], but coevolution and some tolerance could have evolved in exposed animals [9–12]. For instance, honeybees

(*Apis mellifera*) are adapted to a low concentration of AFB1, while saprophytic fungi, such as *Aspergillus* sp, often infect bee nests [13]. Also, the fact that when *Folsomia candida* (Collembola) grazing on *A. nidulans*, the secondary metabolite production, sterigmatocystin, a precursor of aflatoxins [14], was increasing seems to prove that mycotoxin acts as an antifungal-feeding metabolite [15].

AFB1 can get into the soil in several ways, such as with infected kernels, plant residues [2], and in dry years, inoculum can disperse into the soil during harvest [16]. Therefore, it is relevant to take the effects of aflatoxin on soil animals into account. *Folsomia candida* Willem, 1902 (Collembola, Isotomidae), is a commonly used model organism in ecotoxicology. It is a cosmopolitan species distributed in organic-matter rich soils and can be easily bred in the laboratory because of its parthenogenetic reproduction [17]. *F. candida* has a role in the humification of organic matter and the regulation of the soil microbial community [18–24].

Most of the researches are done with sterigmatocystin, which is the precursor in the aflatoxin biosynthesis [25]. However, these experiments do not use different concentrations of the toxins, but only toxin

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deficient mutant versus toxin producer wild type mould strain. In an experiment, when *F. candida* was fed with wild type *Aspergillus nidulans* or its Δ *LaeA* gene mutant (which unable to synthesize sterigmatocystin), it was found that those genes which are encoded stress reaction genes (such as isopenicillin-N-synthetase and isopenicillin-N-synthase) and redox reactions (such as chorion peroxidase) are activated when fed with sterigmatocystin contaminated food, but the effects are not as strong as in mammals [26]. However, in other experiments, the growth rate of *F. candida* was positively related to the wild type and negatively to the gene-deficient type, which suggests that not necessarily sterigmatocystin is the most important in the chemical defense [27]. Additionally, sterigmatocystin seems to alter consumer excretion rates, which are necessary for detoxification [28].

The aim of the present study was to determine whether AFB1 has a toxic effect on the survival and reproduction of *F. candida*, or they are adapted to this mycotoxin. Our purpose was also to determine whether the animals prefer to feed on *A. flavus* or yeast and to determine whether the animals avoid the aflatoxin-contaminated soil or not.

2. Material and methods

The model animal, *Folsomia candida* (Collembola, Isotomidae), was obtained from the stock population of the laboratory of the Szent István University, Department of Zoology and Animal Ecology, where the population was kept in the past 35 years. The Collembolans were kept in Petri-dishes (diameter: 9 cm), with a mixture of plaster of Paris and activated charcoal (10:1 vol ratio) [29]. The animals were kept at a temperature of 20 ± 0.2 °C with ~100% humidity in darkness. Petri dishes were watered weekly to maintain the humidity at a relatively constant level. The collembolans were fed ad libitum with dry baker's yeast once per week. During this operation, they were aerated. All phases of the experiment were performed under the above-mentioned environmental conditions.

2.1. OECD reproduction test

The OECD 232 tests were performed on OECD standard soil [30]. The composition of the soil was 74% sand, 20% kaolin clay, 5% sphagnum peat, and 1% calcium carbonate at pH 7.29. Thirty-gram wet soil was used per jar (24.5 g dry soil, 5.5 mL tap water, 40% of Water Holding Capacity). Water holding capacity was measured according to OECD 232 [30]. Survival and reproduction were measured by counting the number of adults and juveniles after 28 days. The mycotoxin contamination was applied by mixing contaminated ground maize into the soil. Aflatoxin B1 was produced by the fermentation of corn grits with a high toxin producer *Aspergillus flavus* Zt41 strain [4]. Aflatoxin content was determined as follows: AFB1 enriched corn grits extracted with 30 mL methanol, allow standing for 24 h in the dark, and then homogenized again for 45 s for thorough extraction. The liquid part was transferred to a 50 mL plastic Falcon tube and centrifuged at 20 °C, 3000 rpm, for 10 min. For the AFB1 measurement, 1 mL of each extract was evaporated and re-suspended in 0.4 mL hexane, which was followed by the addition of 0.1 mL trifluoroacetic acid (TFA) and derivatized at 60 °C for 15 min. Then 0.4 mL of water: acetonitrile (9:1) was added. After mixing, the lower phase was collected, and 3 μ L was applied onto a Prodigy C18 150 X 4.6 mm 5 μ m column (Phenomenex, Torrance, PA, USA) in a modular Shimadzu HPLC system equipped with an RF-20A fluorescence detector (Shimadzu Europe, Duisburg, Germany). The separation was carried out at a flow rate of 1 mL/min using isocratic elution (65:35) of water and a mixture of methanol: acetonitrile (1:1, v/v %). The detector wavelengths were 350 nm and 430 nm for excitation and emission, respectively. The limit of quantification (LOQ) of AFB1 was 0.01 μ g kg⁻¹. The measured AFB1 concentration of maize grits was 4.694 mg kg⁻¹ dry matter.

There is no guidance value for the mycotoxin content for insect feeds or soil contamination; therefore, the lowest concentrations had been

chosen for feeds of food-producing animals as the mean of recommended limits: 14 μ g AFB1 kg⁻¹ maize-based feed material [1]. Therefore, the following mycotoxin concentrations were used: 0, 14, 28, 56, 112, and 224 μ g kg⁻¹ dry soil. There were five replicates for the treated jars and ten for control. The experiments were carried out in disposable plastic jars (100 mL), with ten to twelve days old, synchronised. Ten animals were added to each jar. The amount of maize was added based on the actual mycotoxin content to reach the nominal contamination level in the soil, and the baker's yeast was added ad libitum on the soil surface.

2.2. Food choice test

A food choice test was carried out with the same Collembola population with mycotoxin contaminated ground maize and the standard food, baker's yeast, in Petri dishes (4 cm diameter) on filter paper [31]. The native concentration of the maize was not modified; therefore, it was 4.694 mg AFB1 kg⁻¹ dry matter. There were 20 replicates in each experimental group. The length of the experiment was one week. Twenty to twenty-two days old, synchronised animals were used in the experiment. Each animal was kept individually in the Petri dishes.

2.3. Soil avoidance test

An avoidance test was carried out with the same concentrations as obtainable above in the case of the OECD 232 test (0, 14, 28, 56, 112, 224 μ g kg⁻¹ dry soil) with the same OECD soil in disposable plastic jars (100 mL) [32]. Thirty gram uncontaminated and 30 g contaminated wet soil (24.5 g dry soil, 5.5 mL tap water) separated with a 2 mm gap was placed in a jar in such a way the animals had a choice between uncontaminated versus mycotoxin contaminated soil. Five replicates per treatment and ten per control (uncontaminated soils in both sides) were done. Twenty to twenty-two days old, synchronised animals were used in these experiments from the same population as in the OECD test and the food choice test. Twenty animals were put in each jar into the middle gap, so arriving spot does not bias the outcome. After 48 h, a glass divider was inserted into the dividing gap, and the animals were collected from the soil on both sides with flooding. The test was 48 h long, so feeding was not necessary.

2.4. Statistical analysis

The food choice and the avoidance tests were analyzed statistically with paired *t*-test by using the R Statistical program 3.5.1 [33]. The results of the OECD test were analyzed with the ToxRat® program [34]. The automatic analysis chose Willem's test for the juvenile number and Bonferroni Chi-square test for mortality testing. The first lowest concentration significantly different from control was accepted as the lowest observed effect concentration (LOEC). LC₅₀ (50% lethal concentration) and EC₅₀ (50% effective concentration) values and standard errors were calculated by ToxRat® too.

3. Results

The statistical results of the mortality test are in Table 1. In the OECD test, the mortality did not follow a trend, most probably due to the high variances as visible in Fig. 1.; therefore, neither LOEC nor LC₅₀ value could be calculated. There was no statistical difference in mortality from the control group. The juvenile number was significantly affected by aflatoxin treatment, and as Fig. 2 shows, every concentration decreased the juvenile number significantly. At the highest concentration, the juvenile number was very low, ranged between 8 and 40 juveniles. The lowest concentration, 14 μ g kg⁻¹ was the LOEC, and EC₅₀ was determined as 15.17 μ g kg⁻¹ (confidence limit: 0.001- out of concentration range).

In the food choice test, both food types were consumed, but yeast was

Table 1

The statistical results of the *Folsomia candida* OECD test. Concentration is the nominal soil concentration. The values depict the difference from the control. Significant differences are bolded. If the t-value is positive, the parameter is higher in that concentration. If the t-value is negative, the parameter is lower in that concentration.

Concentration	Mortality		Reproduction	
	t-value	p-value	t-value	p-value
14 $\mu\text{g kg}^{-1}$	2.3	0.990	-4.6	< 0.001
28 $\mu\text{g kg}^{-1}$	1.9	0.969	-4.8	< 0.001
56 $\mu\text{g kg}^{-1}$	0.5	0.684	-2.8	0.009
112 $\mu\text{g kg}^{-1}$	2.6	0.995	-4.7	< 0.001
224 $\mu\text{g kg}^{-1}$	2.3	0.990	-8.0	< 0.001

Aflatoxin Survival

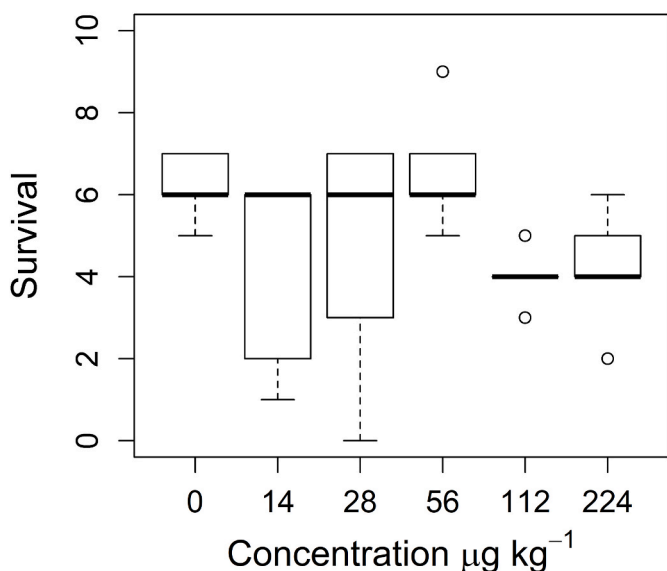


Fig. 1. The number of survived *Folsomia candida* adults of 28 days long OECD tests. The band inside the box is the median. The bottom and the top of the box are the first and third quartiles. The ends of the whiskers are the minimum and maximum excluding outliers. Open circle: outlier (more than 3/2 times of the upper or lower quartile).

preferred over aflatoxin ($t = 3.71$, $p = 0.001$). This is also visible in Fig. 3, while the consumption points (x coordinate fecal pellets around yeast, y coordinate fecal pellets around aflatoxin-contaminated maize) are in the field of baker's yeast and not on the side of aflatoxin-contaminated maize.

The statistical results of the avoidance test can be seen in Table 2 (avoidance data in Table 3.) and showed that the animals did not avoid the contaminated soil. On the contrary, the animals preferred the mycotoxin contaminated soil from 28 $\mu\text{g kg}^{-1}$ aflatoxin equivalent treatment. So the lowest concentration did not show preference or avoidance behavior, while every other concentration has indicated an almost 100% preference for aflatoxin-contaminated soil.

4. Discussion

The mortality was not affected by the AFB1 contamination, but the reproduction decreased significantly. Moreover, the animals are not able to avoid contaminated sites and consume contaminated food too.

AFB1 is one of the most toxic mycotoxins for vertebrates [2]. The mean recommended limit of AFB1 in feed is 14 $\mu\text{g kg}^{-1}$ dry matter [1]. While we did not found different mortality from the control, it would

Aflatoxin reproduction

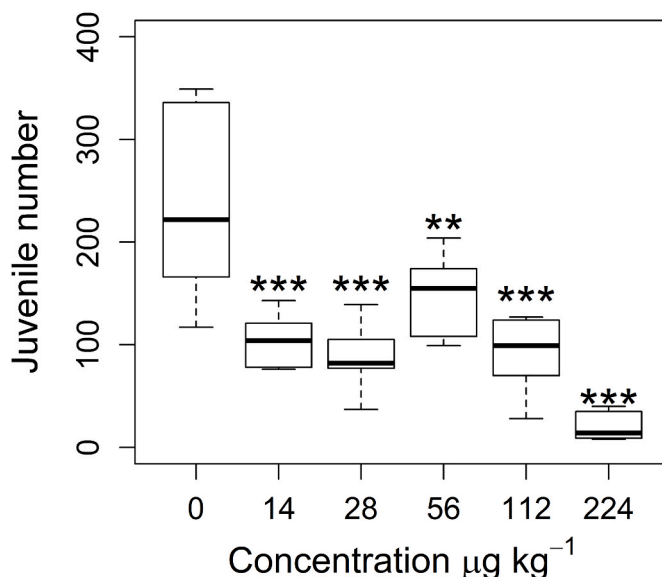


Fig. 2. The number of *Folsomia candida* juveniles of 28 days long OECD tests. The band inside the box is the median. The bottom and the top of the box are the first and third quartiles. The ends of the whiskers are the minimum and maximum excluding outliers. Open circle: outlier (more than 3/2 times of the upper or lower quartile). ** $p = 0.009$; *** $p < 0.001$.

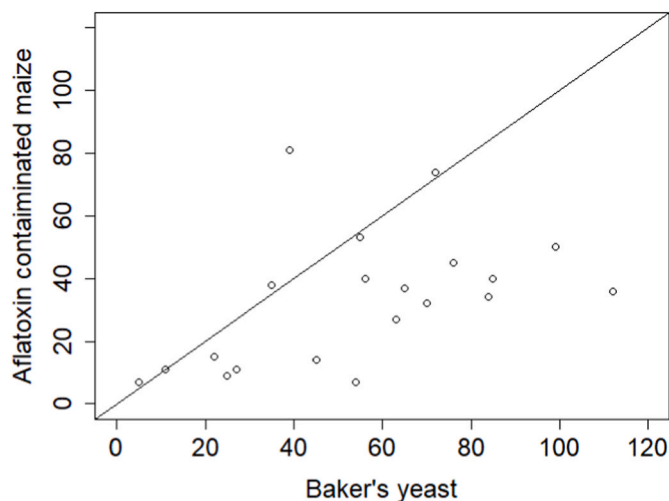


Fig. 3. Results of a week-long food choice test with *Folsomia candida*. The x-axis is the number of faecal pellets on the side of the baker's yeast, and the y-axis is the number of faecal pellets on the side of aflatoxin-contaminated maize with an $x = y$ line.

Table 2

The results of the avoidance test with *Folsomia candida*, where the concentration is the concentration, which was offered versus uncontaminated soil. Significant differences are bolded. The t-value is positive if the control side was preferred and negative if the contaminated side was preferred.

Concentration	t-value	p-value
Control	0.1	0.929
14 $\mu\text{g kg}^{-1}$	-1.8	0.109
28 $\mu\text{g kg}^{-1}$	-9.6	<0.001
56 $\mu\text{g kg}^{-1}$	-27.5	<0.001
112 $\mu\text{g kg}^{-1}$	-22.7	<0.001
224 $\mu\text{g kg}^{-1}$	-18.4	<0.001

Table 3

Data on the avoidance test with *Folsomia candida*, where the concentration is the concentration, which was offered versus uncontaminated soil. The numbers are the living adults found on the side of the uncontaminated soil or the side of contaminated soil.

Concentration	Uncontaminated soil	Contaminated soil
Control	11	9
Control	14	6
Control	7	7
Control	5	13
Control	10	9
Control	8	12
Control	13	8
Control	8	11
Control	10	10
14 µg kg ⁻¹	11	6
14 µg kg ⁻¹	7	12
14 µg kg ⁻¹	7	10
14 µg kg ⁻¹	4	16
14 µg kg ⁻¹	8	11
28 µg kg ⁻¹	0	20
28 µg kg ⁻¹	1	18
28 µg kg ⁻¹	0	16
28 µg kg ⁻¹	0	20
28 µg kg ⁻¹	4	12
56 µg kg ⁻¹	0	17
56 µg kg ⁻¹	0	19
56 µg kg ⁻¹	0	20
56 µg kg ⁻¹	1	18
56 µg kg ⁻¹	2	18
112 µg kg ⁻¹	0	17
112 µg kg ⁻¹	0	15
112 µg kg ⁻¹	0	14
112 µg kg ⁻¹	0	14
112 µg kg ⁻¹	0	17
224 µg kg ⁻¹	0	14
224 µg kg ⁻¹	0	12
224 µg kg ⁻¹	0	13
224 µg kg ⁻¹	0	11
224 µg kg ⁻¹	0	15

seem that the used concentrations are not toxic for *F. candida*. However, the number of juveniles significantly decreased. The EC₅₀ was very close to the lowest concentration: 15.17 µg kg⁻¹, which means that even at the recommended limit, it could decrease the collembolan population to a great extent. It is possible that the hatching rate of the animals decreased, while aflatoxin has genotoxic traits [6]. AFB1 transforms into AFB1 oxide what binds to DNA guanine, causing mutations when encountering a polymerase, also initiating strand brakes [35].

AFB1 did not cause mortality with the used concentration range (14–224 µg kg⁻¹), and the natural AFB1 production could be even 1000–5000 µg kg⁻¹ [4], which would halt the reproduction (there was barely reproduction at 224 µg kg⁻¹). Most probably, it may cause high mortality too. In addition, it is an alarming result that the animals consumed the polluted maize and preferred the contaminated soil. Most probably, *A. flavus* has high nutrient content for collembolan and possibly the fungus does not produce mycotoxin in every hypha, but mostly in the reproduction parts [36], so the animals visit also the toxin contaminated site, which could act as an ecological trap. The driver of the evolution of mycotoxin contaminated fungi could have been fungal grazers, such as collembolan [7,37]. While the Δ *LaeA* gene mutant (lack of sterigmatocystin synthesis) *A. nidulans* biomass decreased, the *F. candida* grazer gained a reproductive advantage [7]. However, in other experiments, the growth rate of *F. candida* was positively related to the wild type and negatively to the gene-deficient type, so it is possible that beside sterigmatocystin, other defensive chemicals could cause a trade-off between growth and reproduction [27].

AFB1 could get into the soil in greater amounts with contaminated plant residues after harvesting [2], but its degradation is possible in the soil with microbial enzymes [38–43]. For instance, the supernatant extract of *Stenotrophomonas maltophilia* is able to degrade 82.5% of AFB1

[39]. Another microorganism, *Streptomyces cacaoi* subsp. *asoensis* (K234) strain could degrade 88% of AFB1 without the formation of harmful side products [44]. In addition, *Rhodococcus rhodochromis* NI2 proved to be the most effective AFB1 degrader (99.98%) with the least negative biological effect from 33 *Rhodococcus* isolates [45]. The extracellular enzyme producer, *Pleurotus ostreatus*, produces ligninolytic enzymes like laccase and manganese peroxidase, which can degrade many hazardous environmental chemicals, such as mycotoxins. Two strains was found to degrade AFB1 very effectively: *P. ostreatus* MTCC 142 (89.14%) and *P. ostreatus* GHBBF10 (91.76%) [46]. However, biodegradation methods would be too expensive for agricultural use.

5. Conclusions

In conclusion, AFB1 contaminated maize residues are not recommended for ploughing, while in the upper layer of the soil, it causes impaired reproduction of *F. candida*, most probably because of the high sensitivity of juveniles. Alternative solutions could be the use of mycotoxin contaminated maize residues for biogas production.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ejsobi.2020.103233>.

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