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Research

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Crambe (*Crambe Hispanica* Subsp. *Abyssinica*) Grains Mycobiota and Natural Occurrence of Aflatoxins, Ochratoxin A, Fumonisin B_1 and Zearalenone

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ABSTRACT

Crambe grains are a new feed with high concentrations of proteins and fibers. As there is no control during the pre-harvesting or post-harvesting stages of production other grains, crambe may be contaminated by fungi. Fungal overgrowth may lead to mycotoxins production and nutritional properties decrease of the grains. The aim of this study was to analyze the occurrence of fungi and mycotoxins according to pre-harvesting management. Fungal concentration was higher than that recommended by international regulations (3.4×10^6 to 1.3×10^4 CFU.g^{-1}), suggesting that management in pre-harvesting stages of crambe grains production may expose the animals that will feed on these grains to the risk of contamination by fungal toxins. More studies are required about quality of crambe grains, because may be strongly affected by the exposition to variable environmental conditions. But, considering low mycotoxin incidence and levels founded, the crambe proves to be a safe food to be exploited for animal nutrition.

KEYWORDS: Crambe; Aflatoxins; Ochratoxin A; Fumonisin B_1; Zearalenone; Forage.

ABBREVIATIONS: Afs: Aflatoxins; OTA: Ochratoxin A; ZEA: Zearalenone; UFRRJ: Universidade Federal Rural do Rio de Janeiro; DRBC: Dichloran, Rose-Bengal Chloramphenicol; HPLC: High-Performance Liquid Chromatography; AOAC: Association of Official Analytical Chemists; LSD: Least Significant Difference; ANOVA: Analysis of variance.

INTRODUCTION

Crambe hispanica subsp. *abyssinica* or *Crambe abyssinica* is an oleaginous subspecies belonging to Brassicaceae family plant, original from Mediterranean region. Crambe was introduced in Brazil for the production of biodiesel and to be used as forage. The inclusion of crambe seeds was expected to correct the dry matter content and also increase the crude protein content of the silage (containing 37% of oil, 21% proteins, 13% fibers, magnesium, potassium and

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other minerals),[1] reducing diet costs and potential environmental impacts from the disposal of this material on the environment.[2] But, the presence of glycosylates and the mycotoxins limit the use of crambe in ruminant diets should be limited to 4%.[3] The US guideline was only founded limiting the rational use.[4]

As happens with other cultures, crambe may be contaminated by fungi, which may jeopardize physical and nutritional features of the plant and produce mycotoxins, dangerous for human and animal health. Among mycotoxins, special attention must be paid to aflatoxins (AFs) and ochratoxin A (OTA), produced respectively by fungi of the genus *Aspergillus*, section. Flavi, such as *Aspergillus flavus* and *A. parasiticus* and by fungi of the genus *Penicillum*.[5] According to the Brazilian regulation, maximum concentration of total AFs in grains is 50 ppb.[6]

Monitoring of grains during pre-harvest and post-harvest stages is important to avoid undesirable fungal overgrowth and mycotoxins productions. During post-harvest stage, the inspection of storage conditions is crucial at this stage, as the same important, may be impossible disregards geographic localization of cultures and production systems.[7] Contamination of seeds by mycotoxins producing fungi is mainly associated to the relation between the plant and its endophytic mycobiota and to other biological interactions.[8] The main interaction that may lead to a contamination of crambe can be those with insects, with the soil,[9] farming practices,[10] adverse meteorological conditions during the final stage of grains ripening or grains genotype.[11] Mycotoxins of *Fusarium* species have been found to cause major damage, especially in cereals, and could frequently be associated with pre-harvest cereal contamination. The importance of climatic and meteorological assessment during cultivation to assess contamination risks and create predictive models to control the incidence of toxigenic fungi and mycotoxins[12] and the delay of harvesting of oleaginous species, such as peanut may cause increase of *A. flavus* contamination of pods, production of aflatoxin G_1 (AFG_1), aflatoxins G_2 (AFG_2) and drying environment may cause higher incidence of *A. flavus* in the seeds.[13]

The aim of this research was study the mycobiota of crambe and the natural incidence of aflatoxins (AFs), ochratoxin A (OTA), fumonisin B_1 (FB_1) and zearalenone (ZEA) according to pre-harvest handling of grains.

MATERIALS AND METHODS

Sampling

The experiment was performed at the laboratories of the "Nucleus of mycological and mycotoxicological researches" (*Núcleo de pesquisas micológicas e micotoxicológicas* (NPMM)) of the Universidade Federal Rural do Rio de Janeiro (UFRRJ), Brazil. Crambe grains samples came from the experimental field (2 hectares) of the UFRRJ Department of fitotechnic of Seropédica City, State of Rio de Janeiro, Brazil. The samples used had been collected during harvests performed in 2012 and 2013. The samples from the 2012 harvest had grown in areas either with or without residuals of sunflower plants. The samples from the 2013 harvest had grown in areas with different potassium fertilization (0, 15, 30, 60 or 90 kg K_2O/he) and were harvested with different moistures: 45%, 18% or 9%. During the two harvests drying kept under field conditions, the grains were in uncontrolled environment for drying.

Determination of Water Activity

Grain samples were submitted to water activity (a_w) measurement using Aqualab® cx2 equipment (Decagon Devices Inc., USA).

Mycobiota Count, Isolation and Identification

Mycobiota analysis was performed by decimal dilution in plates, 0.1 ml of each dilution was inoculated in three different culture media: Dichloran Glycerol (DG18) agar for xerophylic fungi, Dichloran, Rose-Bengal Chloramphenicol (DRBC) agar for total fungi estimation[14] and Nash-Snyder Agar (NSA) for isolation of *Fusarium* species.[15] Plates were incubated at 25 ºC for seven days. Quantification was performed as CFUs per gram of sample (10 to 100 CFU/g). Some strains were selected for species identification. Species identification was realized according to macroscopic and microscopic features of samples and according to taxonomic tables.[14-16] Direct plating of grains was also performed. Ten grains of crambe were disinfected and directly distributed on plates containing DRBC, DG18 or NSA agar media. Plates were incubated at 25 ºC for seven days. All plates were assessed and results were expressed as percentage of infected grains. Fungal samples isolated by this technique were classified according to the specific taxonomic keys, described above.

Mycotoxicologic Assessment

Detection and quantification of total AFs, OTA, FB_1 and ZEA was performed with purification in specific VICAM® immunoaffinity columns. Duplicate analysis were performed according to the preconized procedures of any immunoaffinity column (Aflatest, Ocratest, Fumotest e Zeatest Vicam®, Watertown, MA, USA) for HPLC analysis.[17] After samples extraction, screening was performed in a 4ex series VICAM® fluorimeter and successive high-performance liquid chromatography (HPLC) analysis (LC 2000 JASCO® system equipped with fluorescence detector and C18 SUPELCO® silica column). Mobile phase A: water, mobile phase B: acetonitrile/water (95:5, v/v), the flow rate was 50 µL/min. The sample was reconstituted in 400 µL of the starting mobile phase, filtered and 20 µL were injected. External standards for comparison (OTA, FB_1, ZEA and AFs) were calibrated according to Association of Official Analytical Chemists (AOAC), to establishes the detection and quantification limits.

Statistical Analysis

Analysis of variance (ANOVA) was performed to analyze the data. Before performing ANOVA analysis, all data were transformed in Log 10(x+1). Duncan test was performed to compare

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fungal concentration in the different culture media. Least Significant Difference (LSD) Fisher test was chosen to compare mycotoxins quantification data. All statistical analysis were performed using SAS GLM procedure (PROC GLM) (SAS Institute, Cary, NC, USA).

RESULTS

A high variability and heterogeneity of the parameters along the year and moistures percent from studied samples was observed. The a_w, temperatures and precipitation submitted to grains in the different handlings varied, as described: 0.909-0.592, 23.00 °C - 19.00 °C and 25.00-0.20 mm respectively (Table 1).

The mycological research determinate a fungal incidence, with a count in DRBC, DG18 and NSA media varied from 3.4×10^6 to 1.3×10^4 CFU.g^{-1} (Table 2) and the filamentous fungi were predominant over yeasts with an isolated frequency of 65 fungi strains belonging to genera *Aspergillus*, *Fusarium, Penicillium, Cladosporium, Eurotium, Alternaria* and *Curvularia* spp. (Table 3). Couldn´t observes significant difference ($p\geq0.05$) among plate counts of samples for the compared parameters (fertilization and residuals on the ground).

From these samples, were identified the main mycotoxins producing species: genus *Aspergillus*: *A. flavus*, *A. parasiticus*, *A. oryzae*, *A. fumigatus*, *A. niger* aggregated. From genus *Penicillium* were isolated the species: *P. citrinum*, *P. clavatus* and from genus *Fusarium: F. verticillioides e F. chlmydosporum* (Table 3).

Mycotoxins research presents the detected several mycotoxins in studied samples: AFB_1(39.00-4.23 µg.Kg^{-1}), OTA(4.60-4.01 mg.Kg^{-1}) and ZEA(0.76-0.74 mg.Kg^{-1}) (Table 4).

Samples	a_w	MaxT (°C)	MinT (°C)	Rainfall (mm)
C/R; S/R	0.650	20.3	19.4	0.60 mm
C1	0.909	22.5	21.5	0.80 mm
C2	0.592	23.0	21.3	0.20 mm
C3	0.972	20.0	19.0	25.0 mm

Table 1: Mean data of water activity (a_w), maximum temperature (MaxT), minimum temperature (MinT) and rainfall for three days prior to taking the samples from the area with (C/R) and without (S/R) sunflower residue and obtained in three times crops (C1, C2 and C3).

Samples	DG18	DRBC	NSA
C/R	$1.2x10^{6\,a}$	$2.3x10^{6\,b}$	$1.3x10^{4\,c}$
S/R	$2.5x10^{6\,a}$	$3.4x10^{6\,b}$	$1.3x10^{4\,c}$
C1	$7.2x10^{5\,a}$	$4.1x10^{5\,b}$	$\leq1.0x10^{2\,c}$
C2	$1.0x10^{6\,a}$	$4.2x10^{5\,b}$	$\leq1.0x10^{2\,c}$
C3	$2.0x10^{5\,a}$	$7.5x10^{5\,b}$	$\leq1.0x10^{2\,c}$

Limit of Detection (LOD): $\leq10^2$ UFC/g.

Table 2: Fungal counts (CFU.g^{-1}) in three culture mediums (DG18, DRBC, NSA) taking the crambe samples from the area with (C/R) and without (S/R) sunflower residue and obtained in three times crops (C1, C2 and C3).

Areas with and without sunflower residues		
Fungal genus	**Number of samples**	**Frequency (%)**
Aspergillus spp.	13	37.1
Eurotium spp.	3	8.6
Penicillium spp.	3	8.6
Cladosporium spp.	3	7.6
Mucor spp.	2	5.7
Fusarium spp.	6	17.1
Curvularia spp.	2	5.7
Alternaria spp.	3	8.6
Scopulariopsis spp.	1	2.5
Total	36	100.0
Different crops		
Fusarium spp.	15	42.85
Chlmydosporum spp.	10	28.57
Alternaria spp.	10	28.57
Total	35	100.0

Table 3: Frequency (%) of fungal genera in crambe sample from the area with and without sunflower residues and three seasons of crops.

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	Aflatoxins ($\mu g.kg^{-1}$)	Ochratoxin A ($\mu g.g^{-1}$)	Zearalenone ($\mu g.g^{-1}$)	Fumonisin B_1 ($\mu g.g^{-1}$)
S/R	23.00	4.10	0.71	≤0.30
C/R	39.00	4.60	0.76	≤0.30
1° crop				
0	≤0.30	≤0.50	≤0.20	≤0.30
15	≤0.30	≤0.50	≤0.20	≤0.30
30	≤0.30	≤0.50	≤0.20	≤0.30
60	≤0.30	≤0.50	≤0.20	≤0.30
90	≤0.30	≤0.50	≤0.20	≤0.30
2° crop				
0	≤0.30	≤0.50	≤0.20	≤0.30
15	≤0.30	≤0.50	≤0.20	≤0.30
30	≤0.30	≤0.50	≤0.20	≤0.30
60	≤0.30	≤0.50	≤0.20	≤0.30
90	≤0.30	≤0.50	≤0.20	≤0.30
3° crop				
0	6.03	≤0.50	≤0.20	≤0.30
15	4.23	≤0.50	≤0.20	≤0.30
20	4.42	≤0.50	≤0.20	≤0.30
60	6.72	≤0.50	≤0.20	≤0.30
90	7.54	≤0.50	≤0.20	≤0.30

LOD: AFs (0.3 $ng.g^{-1}$), OTA (0.5 $\mu g.g^{-1}$), ZEA (0.2 $\mu g.g^{-1}$), FB_1(0.3 $\mu g.g^{-1}$).
LOQ: AFs (0.6 $\mu g.g^{-1}$), OTA (0.5 $\mu g.g^{-1}$), ZEA (0.6 $\mu g.g^{-1}$), FB_1(1.0 $\mu g.g^{-1}$).

Table 4: Mycotoxin levels in crambe seed samples from plants grown in areas with different levels of potassium fertilization taken from 5 seasons of crops (0, 15, 30, 60 and 90) and area with (C/R) and without (S/R) sunflower residues.

DISCUSSION

The precipitation along the years rising the humidity of the grains and a_w may foster fungal contamination of grains, jeopardizing its use and processing.[19,20] The evaluation of these parameters is a good reference for a predictive condition and modulations.[21]

Crambe grains obtained from plants grown in areas with residuals of sunflowers on the ground showed higher counts on DRBC medium compared to the values practiced by Good Manufacturing Practices (GMP)[22] and Brazil. All samples showed higher concentration of fungi than prescribed by international recommendations and regulations. Grains from the third harvest showed higher fungal contamination, probably because of the heavier rainfall during the three days before the harvest (25 mm) as compared to the others (Table 1). The precipitation and harvest time modulate higher fungal counts.[23]

Observed higher count of fungi in DG18 medium in samples of crambe grains from areas with sun flower residual culture and from samples obtained from the second harvest (C2). The higher contamination with fungi in samples from the second harvest could be associated to the dry weather during the three days before harvesting (0.2 mm of rainfall) as compared to the other samplings.

The high concentration of fungi may affect the quality of feed, causing economic losses and damages to animal health. The importance of meteorological study during harvest to assess the risk of contamination by mycotoxins producing fungal species.[24]

Aflatoxins are produced by fungi of the *Aspergillus flavus* groups, such as *Aspergillus flavus* and *A. parasiticus*, common in grains and in areas with high air humidity.[25] The most important abiotic factors which influence growth and AFs, OTA, FB_1 and ZEA production by such spoilage fungi include water availability, temperature and when grain is moist.[26]

Calcination does not affect the population of *Aspergillus* spp. on the ground, and does not prevent the contamination of hulls and peanuts grains**.** Also describe, periods of lower humidity of the ground are associated to higher frequency of *Aspergillus flavus* isolation.[27]

Researches relative of different partitioning mycotoxins in fractions of cereals, when they are milled and used for different purposes, including human and animal consumption. The relevance of the mycotoxins founded are corresponding with European dates, as show a relevance of incidence of AFs, OTA, ZEA, DON and FB_1 in grains.[28]

The relevance of weather forecast and climate knowledge during harvest and point out the importance of drawing sampling plans and predictive models to assess the risk of contamination by fungi in cultivated areas. The quality of crambe grains may be strongly affected by the exposition to variable environmental conditions. More studies are required on the quality

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of this feed and continuous analysis are necessary to protect the safety of crambe grains and improve its use for Brazilian herd.

CONCLUSION

In relation to the assessed parameters, considering the low toxin levels found and the use of available material for animal feed, the crambe proves to be a safe food to be exploited for animal nutrition. The high counts of fungi and the presence of detectable mycotoxins in the grains suggest critical situations at pre-harvest stage and needs to review and control this practice. The presence of fungi and mycotoxins in crambe grains associated to the exposure to adverse meteorological conditions may cause a risk of contamination to the animals that will feed on these grains.

CONFLICTS OF INTEREST

The authors declare that they have no conflicts of interest.

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