

THE RELATIONSHIP BETWEEN FUNGI GROWTH AND AFLATOXIN PRODUCTION WITH ERGOSTEROL CONTENT OF CORN GRAINS

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Submitted: December 22, 2000; Returned to authors for corrections: July 11, 2001; Approved: January 17, 2002

ABSTRACT

The relationships between fungal growth and ergosterol content and between aflatoxins B₁ and B₂ production and ergosterol content were verified in corn grains. In the first experiment, fungal growth and ergosterol content were monitored during incubation of corn grains presenting water activities of 0.85_{a_w} and 0.92_{a_w} at 25°C over a period of 18 days. For the Taiúba variety, the fungi growth and ergosterol content increased more rapidly for 0.92_{a_w} than 0.85_{a_w}. Maximum ergosterol levels were 2.8 and 4.6 µg/g, respectively, for 0.85_{a_w} and 0.92_{a_w}. For the Cargill hybrid 606, a more pronounced increase in fungal growth was verified just at the end of the incubation period, mainly for 0.92_{a_w} when an accentuated increase in ergosterol content was also observed. Maximum ergosterol levels detected were 1.6 µg/g and 5.8 µg/g, respectively, for 0.85_{a_w} and 0.92_{a_w}. There was a significant correlation between ergosterol content and log of CFU g⁻¹ for 0.92_{a_w} but not for 0.85_{a_w}. In the second experiment, samples of corn grains of the Taiúba variety at 0.87_{a_w} and 0.95_{a_w} were inoculated with a toxigenic *Aspergillus flavus* strain and incubated at 25°C. Ergosterol levels reached maximum values of 12.1 and 73.4 µg/g, respectively, for 0.87_{a_w} and 0.95_{a_w}. In both water activities, content aflatoxin B₁ followed the same trend as ergosterol. For the aflatoxin B₂ this trend was not observed. Ergosterol assay appears to be a useful test to measure fungal growth and to indicate the possibility of aflatoxin production in corn grains.

Key words: corn grains, ergosterol, fungal growth, aflatoxins

INTRODUCTION

Corn, the main cereal crop in Brazil, is extremely susceptible to fungal deterioration and mycotoxin contamination due to the climatic conditions that prevail during harvesting, drying and storage and also to the intrinsic characteristics of this cereal (4). The level of contamination of grains by fungi is therefore, an important aspect to be considered when evaluating the quality of corn for human or animal consumption. The most common methods for detection and fungi quantification in grains are the serial dilution technique and the direct plating of grains. However, these methods are time consuming and do not detect non-viable mycelia fragments and heat damaged mold structures, which

indicate prior invasion by fungi and possible presence of mycotoxins (2). The use of faster chemical methods is becoming more necessary. The ergosterol method has been proposed as an alternative for estimating fungal growth. It is fast and presents high sensitivity and specificity. However, more information is needed on the relationship between ergosterol content and fungal growth as measured by the serial dilution technique traditionally used to evaluate fungi in foods. Furthermore, the relationship between ergosterol content and mycotoxins production needs to be determined. Tothill *et al.* (13) verified the ergosterol content in naturally moldy wheat grains presenting 0.85 and 0.95 water activities incubated at 25°C up to 10 to 16 days and observed that the ergosterol content increased more

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rapidly in grains at 0.95 a_w than at 0.85 a_w . There was a significant correlation between ergosterol content and total fungal population (CFU.g⁻¹) at 0.95 a_w but not at 0.85 a_w . Schnürer and Jonsson (8) also verified a positive correlation between colony forming units and the ergosterol concentration in wheat. The correlation level using DG18 medium (0.95 a_w) was higher than using MEA (0.99 a_w). The determination of ergosterol is also valuable in correlating fungal activity to synthesis of fungal secondary metabolites such as aflatoxins, alternariol and zearalenone (10,14). Gourama and Bulleman (3) verified a positive correlation between fungal growth and aflatoxin B₁ production in rice stored under controlled conditions. Thus, the objectives of this work were to verify the relationship between mold growth (measured by the serial dilution technique) and the ergosterol content and between the production of aflatoxins and the ergosterol content in corn grains.

MATERIALS AND METHODS

Raw material

The experiments were conducted with corn grains of the Taiúba variety produced at the Experimental Station of Capão Bonito of the Instituto Agronômico de Campinas-S.P.- Brazil (IAC) and the hibrid Cargill 606 produced at the Experimental Station of Campinas of IAC. The two corn samples were freshly-harvested and sun-dried. They were cleaned and homogeneized in the Boerner homogeneizer and kept frozen in plastic bags at -20°C until used. The initial moisture content of the samples were 14.4% (wet basis) and 14.8% (wet basis), respectively, for the Taiúba variety and for the hibrid Cargill 606.

Experimental methodology

The relationships between fungal growth and ergosterol contents were verified for the Taiuba variety and for the Cargil hibrid 606 during incubation at 25°C for 18 days. Enough amounts of sterile distilled water were added to 3kg of grains in order to modify the a_w to 0.85 and to 0.92, equivalent to 17% and 20% water content. The grains were kept at 4°C for five days and shaken regularly to obtain a uniform water content. For each water activity, 300g of grains in glass recipients were placed in desiccators with saturated solution of potassium chlorate (85% relative humidity) or sodium tartrate (92% relative humidity) (15). The desiccators were incubated at 25°C for 18 days. The samples were periodically removed for analyses of ergosterol and total fungal populations. Three repetitions were carried out for each treatment.

The relationship between aflatoxin production and ergosterol content was verified in the Taiúba variety by inoculating the grains with a strain of *Aspergillus flavus* that produced B₁ and B₂ aflatoxins, isolated from a corn sample. Cultures of the organism were grown on CZAPEK agar slants (Oxoid) for a week. Spores were harvested with an inoculating

loop and suspended in a tube containing sterile phosphate buffer solution with 0.05% Tween 80. The spore suspension was adjusted to contain 10⁷ spores/ml. Spore numbers were determined using a Neubauer chamber and plate counts of the suspension were determined on AFPA medium (7).

For each water activity 300g of corn grains were inoculated with 1 ml of the spore suspension containing 10⁷ spores/ml. The glasses with the grains were shaken for 5 minutes and placed into dessicators with saturated solution of potassium sodium tartrate (87% of relative humidity) or lead nitrate (95% relative humidity) (15). The dessicators were incubated at 25°C for 18 days. Samples were periodically removed for analyses of ergosterol, total fungal populations and aflatoxins. Three repetitions were carried out for each treatment analyses.

Fungi

Fungi were enumerated by homogenizing 25g of grains in 225 ml of peptone water, serial diluting 1ml in 9ml of the same diluent and spreading 0.1ml aliquots on DRBC (Difco, Detroit, Mi., U.S.A) and DG18 (3) agar media. In the second experiment, only DG18 was used. Plates were incubated for 5 days when fungi were counted.

Ergosterol determination

Extraction and analyses of ergosterol were done using a method based on Seitz *et al.* (9) with some modifications. One hundred ml of methanol were added, to 50 grams of a ground sample mixed for 2 minutes in a blender and filtered through a filter paper Whatman 41. The residue in the filter paper was transferred to the blender to which 50 mL of methanol was added and agitated for one minute. The mixture was filtered and the procedure repeated again. The mixture was then saponified with 20 grams of potassium hydroxide and 50 mL of ethanol in a water bath at 70°C for 30 min. The bottle was cooled to room temperature and 50 mL of water was added. The mixture was transferred to a 200 mL separating funnel to which 100 mL of petroleum ether was added. After shaking, the mixture was allowed to settle and the upper petroleum ether fraction was collected. This process was repeated with 50 mL of petroleum ether and this solvent was evaporated to dryness using a rotary evaporator. The residue was dissolved in 2 mL of acetonitrile/isopropanol and injected into a HPLC chromatography (Shimadzu LC-10AD). For the chromatography, the conditions were: binary solvent system (LC-10AD), Rheodyne valve with a 20 μ L sampling lift; guard column, Lichrospher 100-RP18 (4 x 4 mm, 5 μ m) (Merck, Germany); monomeric column Lichrospher 100-RP18 125 x 4mm, 5 μ m) (Merck, Germany); isocratic mobile phase of acetonitrile-isopropanol (70:30, v/v), flow of 1.0 mL/min; diode array detector (SPD-M10A), fraction collector (FRC-10A), oven column (CTO-10A) and software (CLASS-LC10) for the peaks integration. Absorbance spectra were taken in the range between 190 and 300 nm and the cromatograms at 282 nm.

The ergosterol peak was eluted at about 15 minutes. All solvents were of P.A. grade for extraction and sample preparation and HPLC grade for chromatography analyses. The solvents were filtered and deaerated in an ultra-sonic apparatus before use. The quantification was made by external standardization (ergosterol from Sigma, United States) and the calibration curve was built from 1.0 to 20.3 µg/ml. The curve was linear and covered the concentration range of the samples. The method recuperation was in average 95%.

Aflatoxins

Aflatoxins analyses were carried out by thin layer chromatography according to the methodology described by Soares and Rodrigues-Amaya (11).

Statistical analyses

Correlation coefficients were calculated using the Statistics software (12).

RESULTS AND DISCUSSION

Figs. 1 to 4 show the changes in ergosterol content and total fungal populations on DRBC and DG18 agar media for the Taiúba Variety and Cargill Hybrid 606 corn grains. The results were not consistent for the two corn samples used in this study. For the Taiúba variety the fungal growth and ergosterol content increased more rapidly at 0.92_{a_w} than at 0.85_{a_w}. Ergosterol reached maximum values of 2.8 and 4.6 µg/g for 0.85_{a_w} and 0.92_{a_w}, respectively.

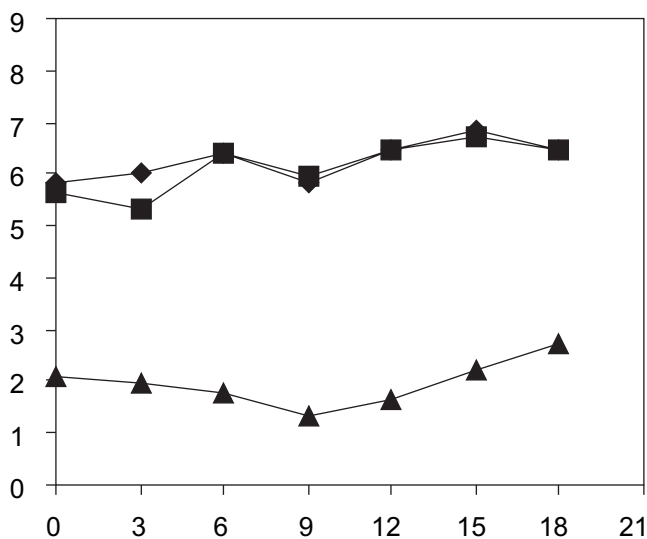


Figure 1. Changes in ergosterol content and total fungal populations in corn of Taiúba variety incubated at 0.85 a_w for 18 days ■ log CFU DG18 ◆ log CFU DRBC ▲ ergosterol (µg/kg).

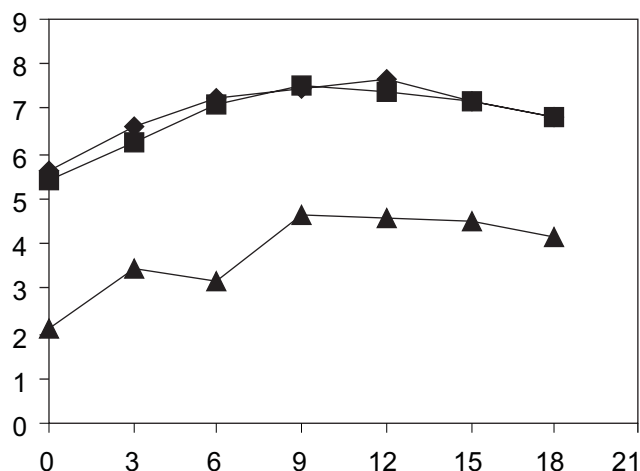


Figure 2. Changes in ergosterol content and total fungal populations in corn of the Taiúba variety incubated at 0.92 a_w for 18 days ■ log CFU DG18 ◆ log CFU DRBC ▲ ergosterol (µg/kg).

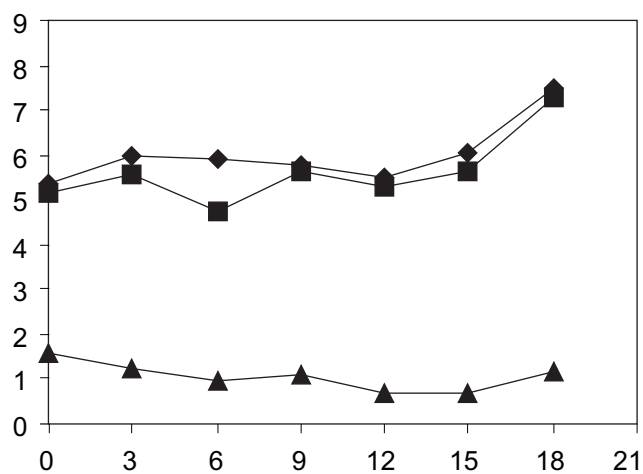


Figure 3. Changes in ergosterol content and total fungal populations in the corn hybrid Carill 606 incubated at 0.85 a_w for 18 days ■ log CFU DG18 ◆ log CFU DRBC ▲ ergosterol (µg/kg).

For the Cargill hybrid 606 a more pronounced increase in fungal growth was verified by the end of the incubation period in both water activities and an acentuated increase in ergosterol content was observed at 0.92 a_w. Maximum ergosterol levels were 1.6 and 5.8µg/g, for 0.85_{a_w} and 0.92_{a_w}, respectively.

In both corn samples a significant positive correlation (probability level of 5%) between ergosterol and fungal growth was observed at 0.92_{a_w} but not at 0.85 a_w in the two media studied (Table 1). A positive correlation between numbers of colony forming units and ergosterol concentration was also found for wheat grain naturally contaminated and stored at 0.95_{a_w} (13),

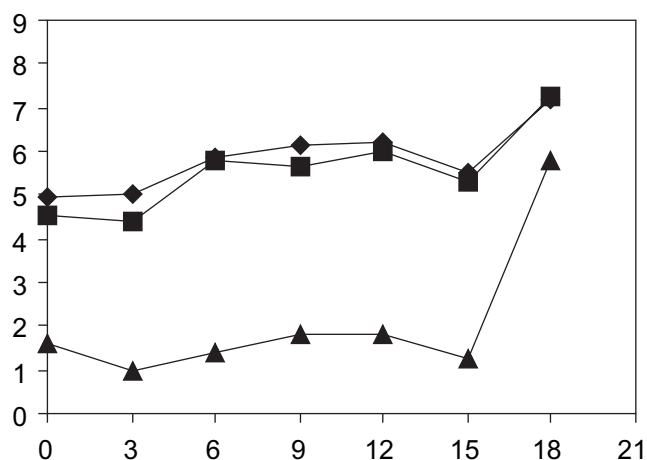


Figure 4. Changes in ergosterol content and total fungal populations in the Cargill hybrid 606 incubated at 0.92 a_w for 18 days ■ log CFU DG18 ◆ log CFU DRBC ▲ ergosterol ($\mu\text{g}/\text{kg}$).

Table 1. Correlation coefficients of the relationship between ergosterol concentration and total colony forming units (CFU) for naturally contaminated corn grains.

Type of grain	Water activity	Correlation coefficients	
		DRBC	DG18
Taiúba variety	0.92	0.73 ($p < 0.05$)	0.72 ($p < 0.05$)
	0.85	0.38 n.s.	0.22 n.s.
Cargill 606	0.92	0.88 ($p < 0.05$)	0.78 ($p < 0.05$)
	0.85	0.52 n.s.	0.24 n.s.

n.s. = not significant ($p > 0.05$).

partially moulded wheat grain (6) and in swedish grains (8). Schnürer and Jonsson (8) also found a higher correlation when numbers of CFUs were determined on DG18 than on MEA. In this present study, no differences in correlation were found between the two media studied. Lesage *et al.* (5) found that in drier grains, ergosterol only increased markedly after 15 days at 0.84 a_w and after 60 days at 0.74 a_w . In our experiment, no pronounced increase in ergosterol content was verified over the 18 days incubation period at 0.85 a_w in both corn samples. Cahagnier *et al.* (1) showed that ergosterol levels in good quality wheat and barley were about 4 to 5 $\mu\text{g}/\text{g}$, while for maize the content was much lower, at approx. 0.5 $\mu\text{g}/\text{g}$. They suggested ergosterol thresholds for initiation of spoilage of about 10 to 12 $\mu\text{g}/\text{g}$ for wheat and barley and 5 to 8 $\mu\text{g}/\text{g}$ for maize.

The relationship between ergosterol and aflatoxins production was verified by monitoring corn samples inoculated with a toxigenic strain of *Aspergillus flavus* and stored for 18 days. The effects of water activity on fungal populations and ergosterol content are summarized in Tables 2 and 3. The log of

Table 2. Total fungal populations, ergosterol content and aflatoxins production in corn grains (Taiúba variety) with 0.87 a_w stored at 25°C up to 18 days.

Days	0.87 a_w			
	log cfu/g (DG18)	Erg ($\mu\text{g}/\text{g}$)	AFB ₁ ($\mu\text{g}/\text{kg}$)	AFB ₂ ($\mu\text{g}/\text{kg}$)
0	5.4	2.1	traces	traces
3	7.3	2.5	traces	traces
9	7.5	3.6	13.1	traces
12	8.0	8.6	427.6	20.5
15	7.5	12.1	701.0	nd
18	5.5	4.9	59.4	nd

n.d.= not detected; traces= < 2 $\mu\text{g}/\text{kg}$.

Table 3. Total fungal populations, ergosterol content and aflatoxins production in corn grains (Taiúba variety) with 0.95 a_w stored, at 25°C up to 18 days.

Days	0.95 a_w			
	log cfu/g (DG18)	Erg ($\mu\text{g}/\text{g}$)	AFB ₁ ($\mu\text{g}/\text{kg}$)	AFB ₂ ($\mu\text{g}/\text{kg}$)
0	5.4	2.1	traces	traces
3	8.2	13.0	traces	traces
9	8.3	24.9	62.0	traces
12	8.3	73.4	640.7	22.4
15	7.5	12.1	nd	nd
18	8.1	4.9	nd	nd

n.d.= not detected; traces = <2 $\mu\text{g}/\text{kg}$.

counts of colony formig units per gram increased, in general, in the first 12 days, reaching a maximum of 8.0 and 8.3, for 0.87 a_w and 0.95 a_w , respectively. The fungi growth was higher at 0.95 a_w , as expected. After reaching the maximum value, the counts, in general, began to decrease. The same pattern was observed for the ergosterol levels, that reached maximum of 12.1 $\mu\text{g}/\text{g}$ and 73.4 $\mu\text{g}/\text{g}$ after 15 and 12 days of storage, respectively, for 0.87 a_w and 0.95 a_w . Aflatoxin B₁ was detected at the ninth day of storage for both water activities while aflatoxin B₂ was detected only in the twelfth day. After reaching the, maximum values there was an accentuated degradation of aflatoxin B₁. The highest aflatoxin B₁ concentration was reached more rapidly at 0.95 a_w than at 0.87 a_w . After incubation for 18 days we observed a partial degradation of aflatoxin B₁ at the 0.87 a_w and after 15 days a total degradation was observed. The assays that showed the highest ergosterol levels were the same where the highest aflatoxin B₁ contamination was detected. In general, we observed a good correlation between ergosterol content and aflatoxin B₁ production, that is, increases in ergosterol contents were followed by increases in aflatoxin B₁ production. The highest ergosterol level corresponded to the treatment where aflatoxin B₂ was detected (12 days of incubation for both water

activities). Gourama and Bullerman (2) also verified a positive correlation between fungi growth and aflatoxin B₁ production in rice under controlled conditions.

Ergosterol assay appears to be a useful test to measure fungal growth and to indicate the possibility of aflatoxin production in corn grains. In our study we verified that substrate condition can also affect this relationship as the ergosterol increase was much faster in the Taiúba variety sample which was of poorer quality than the hibrid Cargill 606.

ACKNOWLEDGMENTS

This work was supported by grants from Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP). The authors thank Dr. Katumi Yotsuyanagi for the statistical analyses.

RESUMO

Relação entre o desenvolvimento fúngico e a produção de aflatoxinas com o teor de ergosterol em milho em grãos

A relação entre o crescimento fúngico e o teor de ergosterol e entre a produção de aflatoxinas B₁ e B₂ e o teor de ergosterol foi verificada em milho em grãos. No primeiro experimento, o crescimento fúngico e o conteúdo de ergosterol foram monitorados durante incubação das amostras de milho com 0,85 e 0,92 de atividade de água (Aa) a 25°C por um período de 18 dias. Para a variedade Taiúba o crescimento fúngico e o conteúdo de ergosterol aumentaram mais rapidamente a 0,92 de Aa do que a 0,85 de Aa. Os níveis máximos de ergosterol atingidos foram 2,8 e 4,6 µg/g, respectivamente, para 0,85 e 0,92 de Aa. Para o híbrido Cargill 606, um crescimento fúngico mais pronunciado foi verificado somente ao final do período de incubação principalmente na Aa de 0,92, quando também verificou-se um acentuado aumento do teor de ergosterol. Os níveis máximos de ergosterol detectados foram 1,6 µg/g e 5,8 µg/g, respectivamente, para as Aa de 0,85 e 0,92. Para as duas amostras verificou-se uma correlação positiva nos dois meios de cultura utilizados entre o conteúdo de ergosterol e a contagem total de unidades formadoras de colônias (UFC) g⁻¹ de grãos na Aa de 0,92 mas não na de 0,85. No 2º experimento, amostras de milho da variedade Taiúba com 0,87 e 0,95 de Aa foram inoculadas com uma cepa toxigênica de *Aspergillus flavus* e incubadas a 25°C. Os níveis de ergosterol alcançaram valores máximos de 12,1 e 73,4 µg/g, respectivamente, para as

Aa de 0,87 e 0,95. Em ambas Aa testadas, a produção de aflatoxinas e o teor de ergosterol apresentaram as mesmas tendências, ou seja, o aumento nos níveis de ergosterol foi acompanhado pelo aumento na produção da aflatoxina B₁. Para a aflatoxina B₂ essa tendência não foi observada.

Palavras-chave: milho em grãos, ergosterol, crescimento fúngico, aflatoxinas

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