

THE RELATIONSHIP BETWEEN FUNGAL BIOMASS, ERGOSTEROL AND GRAIN SPOILAGE

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ABSTRACT

The ergosterol content and fungal biomass of Alternaria alternata, Eurotium amstelodami and Penicillium aurantioogriseum was determined on both malt and wheat extract broths and wheat extract agars at 0.98 and 0.94 a_w over periods of up to 21 days. The ergosterol content differed between species, nutrient substrate and a_w. Correlation in liquid culture between fungal biomass and ergosterol was poor, while that on the solid substrate was markedly better.

Grain samples from an experimental ambient grain drier were subsequently examined at various heights to determine changes in CFUs and ergosterol. The water content (w.c.) of the grain varied from 15% at the bottom to about 23-24% w.c. at the top. The number of CFU, visible moulding, and ergosterol content increased with height in the drier. The dominant fungal species were Penicillium and Eurotium spp. and there was a good correlation between number of CFU and ergosterol.

INTRODUCTION

Ergosterol (ergosta-5,7,22-trienol) is the predominant sterol of most fungi in the classes Ascomycetes and Deuteromycetes (McCorkingdale et al., 1969; Demel and Kruyff, 1976). Most of the fungi involved in spoilage of agricultural stored products fall within these two classes. This sterol is considered to be a good potential indicator of fungal spoilage as it is only found in the cell walls of fungi and is not present in plant, animal or other microbial material. Other methods of assessing fungi include Adenosine 5' - triphosphate (ATP) and chitin have been used but are not specific to fungi. ATP is present in all living cells, making differentiation between fungal and grain activity difficult while chitin is also present in insects.

Seitz et al., (1979) showed that ergosterol was a more sensitive and more rapid method than the chitin assay for detecting Alternaria and Aspergillus spp. on milled rice. Experiments with wheat and maize also demonstrated that a good correlation could be obtained between ergosterol content and the number of colony forming units (CFU) and dry matter loss (Seitz et al., 1982a; b). The amount of ergosterol does however vary with substrate and with individual fungal species (Nout et al., 1987).

The objectives of this study were to determine the relationship between growth of important grain fungi and ergosterol content in both solid and liquid culture under different water availability conditions. The sensitivity of the technique to assess initiation of moulding was subsequently determined on wheat grain of different water contents obtained from an ambient drier.

MATERIALS AND METHODS

Fungal isolates

The fungi tested in this study were Alternaria alternata, Eurotium amstelodami and Penicillium aurantiogriseum and were isolated from wheat grain.

Media preparation

Liquid culture studies involved using malt extract broth (2%) and wheat extract broth (2%). Wheat extract broth was prepared by homogenising 50g of dry wheat in a household blender and adding 500 ml of distilled water. After steaming the mixture for 1h the resultant wheat extract was strained (twice) through muslin and made up to 1l with distilled water. The solution was diluted to give a 2% concentration of wheat extract.

Wheat agar plates (2%) were made by the addition of homogenised wheat to distilled water and agar. The water activity (aw) of all media was modified to 0.98 and 0.945 by the addition of NaCl (Lang, 1967).

Fungal inoculation and measurement

A.alternata and P.aurantiogriseum were grown on 2% malt extract plates and E.amstelodami on 2% malt 10% salt plates for 7-8 days at 25°C to provide spore inoculum for the experiments. Spore suspensions were prepared in 0.1% (with a wetting agent) water agar and passed through sterile glass wool to remove mycelial fragments. For liquid culture studies 1 ml of a 10⁷ spore concentration was added to 100ml of prepared liquid medium. Liquid cultures were placed on a rotary shaker at 120 rpm and incubated at 25°C for up to 14-21 days. Samples were periodically removed and destructively sampled for fungal biomass and ergosterol concentrations. Three replicates per treatment were used at each sampling time for each treatment.

2% wheat agar plates were inoculated with spore using a fine loop. Plates were incubated at 25°C for up to 16 days. Six replicates were removed on each occasion, three for ergosterol determination and three for biomass determination. The diameter of all colonies were determined in two directions to determine growth rate.

Biomass determination

Liquid cultures were filtered through Whatman 541 filter paper under vacuum. The fungal biomass and filter paper was then dried at 105°C for 24h and weighed. Fungal colonies on solid agar plates were removed by melting the agar in 250ml water in a microwave until the colonies flated freely (Nout et al., 1987). The colonies were carefully placed on weighed cellulose acetate membrane filters and dried at 105°C for 24h.

Ergosterol extraction and analyses

A mycelial mat from liquid culture experiment was placed in a flat-bottomed boiling flask to which 20 mls 80% methanol and 20% hexane (HPLC grade) was added. The flask was shaken for 30 min on a rotary shaker and the extract filtered through Whatman 541 filter paper into a 50 ml measuring cylinder and made up to 30 ml. This solution was then poured into a 100ml screw capped bottle to which 3g KOH was added. The bottle was placed in a water bath for at 70°C for 20 min. The tube was cooled to room temperature and the mixture transferred to a separating funnel where 5 ml

of water was added. After vigorous shaking the layers were allowed to settle and the upper hexane fraction collected. This process was repeated twice with 5ml of hexane. The hexane was evaporated to dryness using a rotary evaporator. The residue was dissolved in 1ml methanol (HPLC grade) and filtered through a 0.2 micron filter immediately prior to HPLC analysis.

The HPLC system included a guard column (50mm x 4.6 Spherisorb C18 ODS2 5micron); main column (250mm x 4.6 Spherisorb C18 ODS2 5micron) and 100% methanol (HPLC grade) as the solvent. The flow rate was 1.5ml min⁻¹ and the ergosterol detected at 280nm under at a column temperature of 30°C. A 100ul of sample, of both pure ergosterol at different concentrations, and experimental samples were injected onto the column. Ergosterol peaks appeared after 10.5 min. For the standard curve, the correlation coefficient between ergosterol concentrations and peaks area units was 0.9711 for direct input of ergosterol and 0.9948 when taken through the extraction procedure.

Fungal assessment of wheat grain

The colonisation of wheat grain of different water contents from an ambient drier was determined by both directly plating grain onto 2% malt and 2% malt 10% salt agars and serial dilution of grain washings onto the same agars. Plates were incubated at 25°C for 8 days before identification and enumeration of fungi.

RESULTS AND DISCUSSION

Table 1 shows the amount of ergosterol produced mg⁻¹ biomass after 7, 14 and 21 days growth in wheat extract broth. With *A.alternata*, a higher amount of ergosterol was produced mg⁻¹ biomass at 0.98 than 0.94 a_w. These was a rapid increase after 14 days but after 21 days the amount of ergosterol mg⁻¹ biomass decreased again. This pattern was also followed by *E.amstelodami* and *P.aurantiogriseum*. Of the three fungi, *P.aurantiogriseum* produced the highest ergosterol mg⁻¹ biomass, particularly after 7 and 14 days incubation. Correlations between biomass and ergosterol content on both malt and wheat extract broth showed that there was a positive correlation for *E.amstelodami* (malt broth, 0.8750) but only at 0.98 a_w while on wheat extract broth there was poor correlation for all three fungi at both a_w levels tested. The decrease in ergosterol content mg⁻¹ biomass after 21 days incubation suggested that autolysis of cultures may have occurred perhaps due to nutrient limitation. These results do however suggest that the age of the fungal culture will influence the ergosterol content.

Previous studies on the ergosterol content of *Rhizopus oligosporus* in liquid culture found a similar range of concentrations (2 - 24 ug mg⁻¹ biomass) depending on culture age (Nout et al., 1987). Ergosterol did not vary with temperature in this study but was found to vary with substrate composition, although water availability was not considered. Seitz et al (1979) found variations of 3.8 - 4.4 ug mg⁻¹ ergosterol in *A.alternata*, 2.3 - 3.3 ug mg⁻¹ biomass in *A.flavus* and 4.9 - 5.9 ug mg⁻¹ in *E.amstelodami* grown on moist milled rice at 25°C.

Table 1. The effect of water activity on the amount of ergosterol present g⁻¹ fungal biomass of three fungi grown in wheat extract broth for up to 21 days. The results are means of three replicates.

Time (days)	Fungus species	Water activity	ug ergosterol mg ⁻¹ biomass		
			7	14	21
	<u>Alt.alternata</u>	0.98	11.5	20.5	12.3
		0.94	9.8	11.1	10.0
	<u>Eur.amstelodami</u>	0.98	16.6	29.1	21.5
		0.94	18.0	15.6	10.5
	<u>Pen.aurantioqriseum</u>	0.98	36.4	63.5	19.0
		0.94	33.6	42.2	23.3

Alt, Alternaria; Eur, Eurotium; Pen, Penicillium

Figure 1 shows the increase in colony radius of A.alternata, E.amstelodami and P.aurantioqriseum at 0.94 a_w over a period of 16 days. The biomass (dry weight) of all three fungi increased in a similar way. There was a good correlation between radial growth and biomass with correlation coefficients of 0.9745 for A.alternata, 0.9573 for E.amstelodami and 0.9715 for P.aurantioqriseum. The changes in the mean ergosterol content of single colonies of each fungus is shown in Figure 2. The increase in ergosterol was greatest for E.amstelodami, followed by A.alternata and P.aurantioqriseum. There was however a decrease in ergosterol content between 12 and 16 days growth. This may have been due to physiological changes, including sporulation processes. The correlation coefficients for radial growth and ergosterol content were 0.8916 for A.alternata, 0.8922 for E.amstelodami and 0.8048 for P.aurantioqriseum. Previously, Matcham et al (1985) working with Agaricus bisporus, the cultivated mushroom, obtained a good correlation between biomass and ergosterol content on moist rye grain.

Wheat grain from an ambient grain drier had different water contents with the driest grain at the bottom and the wettest grain at the top. The water contents varied from about 15% to 23.5%. The dominant fungal groups are shown in Table 2. Both field and storage fungi were isolated from the driest grain while the wetter grain was colonised only by Aspergillus, Eurotium spp. and Penicillium spp. The changes in total numbers of CFU g⁻¹ grain and the changes in ergosterol content are shown in Figure 3. Generally, the total fungal populations and the ergosterol content increased in similar ways. A good correlation coefficient (0.9533) between the total numbers of CFU and the ergosterol content g⁻¹ grain was obtained. This suggests that ergosterol may reflect the level of grain spoilage quite accurately. However, more information is required to determine whether ergosterol could be an effective means of detecting fungal activity in grain prior to the onset of visible growth.

Figure 1. Increase in radial growth (mm) of three fungi grown on 2% wheat agar at 0.94 a_w and 25°C.

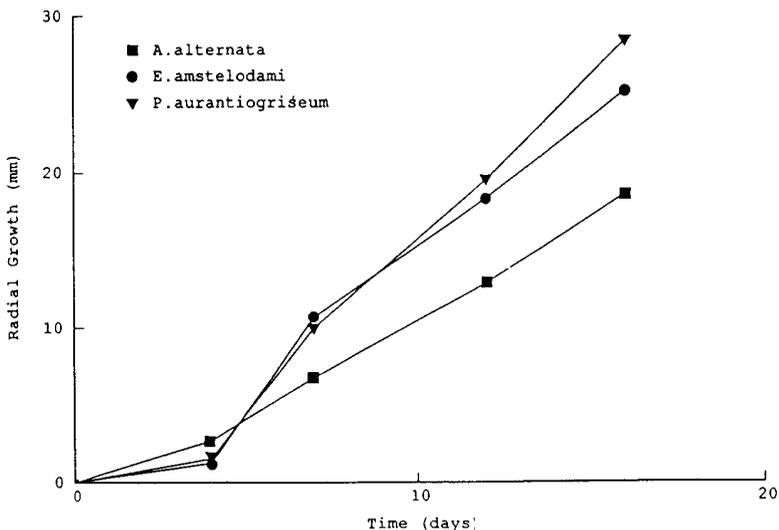


Figure 2. The mean changes in ergosterol content of single growing colonies of three different grain fungi on 2% wheat agar at 0.94 a_w and 25°C.

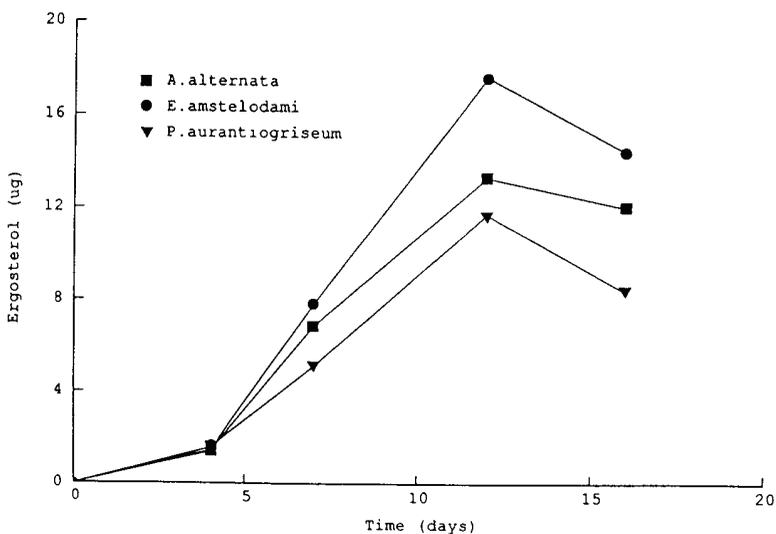
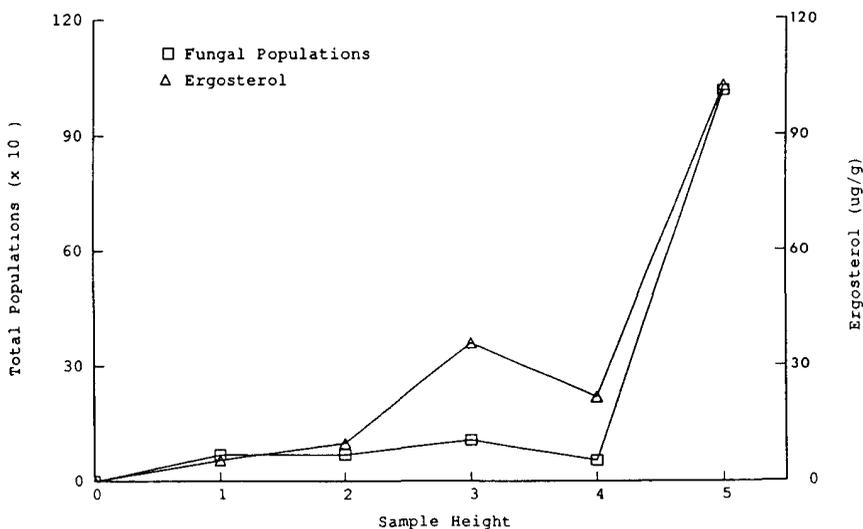


Table 2. The percentage (%) fungal colonisation of wheat grain from different heights in an experimental ambient drier plated on 2% malt and 2% malt 10% salt agars.

Bin position	bottom		middle		top
Sample number	1	2	3	4	5
Water content (% wet weight)	15.3	21.0	21.4	21.7	23.5
Fungi isolated					
<u>Epicoccum nigrum</u>	20	0	0	0	0
<u>Alternaria alternata</u>	10	0	0	0	0
<u>Cladosporium</u> spp.	15	0	0	0	0
<u>Penicillium</u> spp.	80	100	100	100	100
<u>Aspergillus</u> and <u>Eurotium</u> spp.	10	90	100	100	20

Figure 3. Comparison of the total fungal populations (number of colony forming units, CFU) and ergosterol content obtained from wheat grain of different water contents and with different degrees of moulding. Samples which were visibly moulded are indicated (M).



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RELATIONS ENTRE LA BIOMASSE FONGIQUE, L'ERGOSTEROL
ET LE DEVELOPPEMENT DES MOISSISSURES

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RESUME

Après différentes durées d'incubation, on a mesuré la teneur en ergostérol et la biomasse fongique de *Alternaria alternata*, *Eurotium amstelodami* et *Penicillium aurantiogriseum* dans des milieux de culture à 0,98 et 0,94 a_w . La teneur en ergostérol différait entre les espèces, le substrat nutritif et l' a_w , ces facteurs étant peu corrélés sur une période d'incubation allant jusqu'à 21 jours.

La teneur en ergostérol et le nombre de colonies formant des unités (CFU) ont aussi été comparés pour le blé humidifié à 0,80, 0,85 et 0,88 a_w , et stocké jusqu'à 28 jours à 25 C. Le contenu en ergostérol était bien corrélé avec les CFU des champignons de stockage par g de grain, mais pas avec les espèces du champ. On discutera de la valeur du contenu en ergostérol en tant que mesure de la colonisation fongique des grains.