OCCURRENCE AND TOXICOLOGICAL ACTIVITY OF MOLDS ON CEREALS STORED IN AIRTIGHT CONTAINERS

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Abstract

Airtight storage of cereals is not a common practice in West Germany. In 1985, about 500 farms used this form of feed storage, 110 of them being located in Schleswig-Holstein, the northernmost state of the country, with very intensive farming systems.

Mycological investigations were carried out with barley and wheat during the last t wo storage seasons. Despite comparable storage conditions the occurrence, development and species composition of the mycoflora of the kernels varied considerably. Altogether 16 fungal and three yeast species were isolated from cereals stored in airtight structures. Differences in amount, form and distribution of the molds could not be explained as due to abiotic influences during storage. It must be assumed that the primary inoculum at the time of harvest is the key factor for fungal development. This is true particularly for the toxin-producing species Aspergillus versicoler, A. terreus and Penicillium roqueforti and could be confirmed in a model experiment. Of 24 isolates of P. roqueforti, nine produced both mycophenolic acid and PR-toxin, seven formed only PR-toxin, and six did not produce any of the toxins. No patulin could be detected.

Introduction

Airtight storage of cereals has been practiced since ancient times when grain was stored in underground pits (Hill et al., 1983). The scientific elaboration of this form of grain preservation originated in the middle of the last century and modern applications based on this technique were initiated 20-30 years ago in the US and Britain. In Germany the procedure has been introduced for wider application within the last ten years. However, with about 500 airtight silos at present in existence the method is not yet in common practice. In Schleswig-Holstein, the most northern state of Germany with very intensive agromanagement, more than half of the existing airtight silos have been built since 1980.

Though there is a vast literature on the necessary technical equipment and practical applications of airtight storage (Hyde and

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Burrell, 1982), only a limited amount of knowledge is available on the mycological situation in general and with respect to modern agro-production systems in particular (Clarke and Hill, 1981; Hill and Lacey, 1983; Ekstrom et al., 1984). It generally can be observed that intensive mold growth occurs in late spring when as a result of increasing day-night-temperature fluctuations oxygen diffuses into the silo system.

The present two-year investigation was undertaken to obtain an initial evaluation of the fungal population dynamics on grain during airtight storage in an area with a cool maritime climate (Ceynowa, 1986).

Materials and Methods

Standard microbiological methods were used for the quantitative and qualitative investigation of yeasts and molds from barley and wheat grain stored under sealed conditions at the farm level. Media used for microbial determinations were malt-peptone-agar, malt-salt-agar, nutrient-agar, JOFFE-agar. For the identification of the yeast Hansenula anomala a selective medium was developed containing KNO_3 1.0 g/l; KH_2PO_4 0.8 g/l; $MgSO_4 \cdot 7\ H_2O$ 0.5 g/l; K_2HPO_4 0.15 g/l; NaCl 0.1 g/l; $CaCl_2 \cdot 2\ H_2O$ 0.06 g/l; 2 ml 80 % aq. lactic acid; 10 ml micronutrient solution; 10 ml Fe_2SO_4 solution; chloramphenicol 50 ml/l; chlorotetracycline 50 mg/l; agar 20 g/l; pH 5.5.

Grain samples were taken for up to 12 months after harvest. Microorganisms were determined by dilution plate count and direct plating techniques. Fungal enumeration was made from the third day on following incubation at 25° and 37°C respectively. The internal fungal flora is given as the percentage of infested grains (infection rate IR) following surface sterilization (NaOCl; 10-20 min). Counts of dilution plates are given per gram dry matter of grain according to the following formula:

$$\lg \bar{x}_g = \frac{1}{n} \sum_{i=1}^{n} \lg \left(col_i \cdot \frac{90}{FW \cdot pDW} \cdot 10^{DF} \right)$$

where
- $\lg \bar{x}_g$ = logarith of the geometric average of colony forming particles per gram dry matter
- n = number of Petri dishes per dilution (at least 3)
- col_i = number of colonies grown at i dilution
- FW = fresh weight of grain used (always 10 g)
- pDW = percentage dry weight of grain used
- DF = dilution factor (standard solution = 1, next decimal dilution = 2 etc.).

The species identification of the most important storage fungi was made according to their growth characteristics (Aspergillus: Raper and Fennel, 1965; Blaser, 1975; Samson et al., 1984; Penicillium: Raper and Thom, 1949; Pitt, 1979; Ramirez, 1982; Frisvad, 1981). The identifica-

tion of the occurring yeasts was based on their morphological and physiological characteristics (Barnett et al., 1983; Kreger-van Rij, 1984) by using a computer supported programme (FORTRAN).

The determination of the mycotoxin producing capacity was restricted to Penicillium roqueforti and its metabolites PR-toxin, mycophenolic acid and patulin. A number of fungal isolates originating from different sources was grown on YES-medium. The culture filtrates were chloroform extracted, dried by evaporation and redissoluted by methanol. The toxin detection was made after 1-3 weeks incubation by two-dimensional TLC (1st direction: toluene/ethyl acetate/formic acid, 6:3:1; 2nd direction: chloroform/methanol, 9:1) followed by UV observation.

The statistical analysis of the mycological data was made according to the SPSS 9 procedures applying the subprogrammes 'Condescriptive' and 'Nonpar Corr' (Hull and Nie, 1981).

Results

Yeasts

The data given here are part of a comprehensive research programme and deal with the changes of the external and internal mycoflora of cereals during airtight storage. Besides the known decrease in the number of field fungi, the most remarkable change during the early storage phase was the existence of fast growing, white and occasionally hyphal forming yeasts. The most frequent species showing these characteristics was the heterothallic Hyphopichia burtonii (Table 1). This yeast can be obtained from the grain surface where it primarily occurs in the hyphal form. It was the only yeast species which could also be isolated from surface sterilized seeds demonstrating a higher infestation rate with increasing storage time. In wheat H. burtonii preferentially occurs in the embryonic area. With respect to population development there is a correlation between grain moisture content and population density. Following the dry season of 1983/84 H. burtonii could not be detected except in cases of prolonged storage and wet grains harvested in 1982.

The second yeast species occurring in all grain samples is Ha. anomala. The detection of this yeast was made using a minimal medium which allows the growth of beige colonies with 1-2 mm diameter within one week. The development of Ha. anomala on the grain is also influenced primarily by the grain moisture content. The population density, however, remains much lower than for H. burtonii. As another difference between these two yeast species it can be seen that Ha. anomala only grows as an epiphyte on the grain surface and never infects the kernel. In 14-18 % of the grain samples investigated the ascosporogenous Pichia farinosa could be isolated as the third yeast species in both years. Furthermore the population density was rather low with 10^4 particles/g of grain.

Molds

The species spectrum of molds found on airtight stored cereals from Schleswig-Holstein was much greater than that of yeasts. Table 2 gives a summary of the 16 fungal genera or species isolated. Four of these species occur very often. The genus occurring most often was

Table 1 Occurrence of Hyphopichia burtonii and Hansenula anomala on and in barley grains during airtight storage (1984/85)

			Weeks after harvest[1]			
			8	25	43	47
Silo No.1						
	Grain moisture content (%)		19.0	19.4	19.6	22.4
	H. burtonii	lg $\bar{x}_g$	5.1	6.2	7.5	7.7
		IR (%)[2]	8	34	31	46
	Ha. anomala	lg $\bar{x}_g$	3.3	4.9	6.1	7.1
		IR (%)	0	0	0	0
Silo No.5						
	Grain moisture content (%)		17.1	17.1	17.5	18.o
	H. burtonii	lg $\bar{x}_g$	3.5	5.8	7.o	7.1
		IR (%)	n.d.[3]	n.d.	10	98
	Ha. anomala	lg $\bar{x}_g$	n.d.	2.6	3.9	4.6
		IR (%)	0	0	0	0

1 Harvest time: 10 August 1984

2 IR: Infection Rate

3 n.d.: not determined

Table 2 Occurrence of storage fungi on cereal grains following airtight storage

Often occurring storage fungi

	1983/84		1984/85	
	C/J[1]	J	C/J[1]	J
Aspergillus candidus	57[2]	43	5o	37
A. terreus	43	14	5o	16
Eurotium spp. (*A. glaucus* group)	79	68	72	55
Penicillium roqueforti	18	0	37	24

Less often occurring storage fungi

Thermotolerant Mucoraceae (like *Absidia corymbifera*)	11	11
A. flavus	14	3
A. fumigatus	25	8
A. versicolor	7	13
Paecilomyces variotii	25	16
Penicillium spp. (like *P. verrucosum* var. *cyclopium*)	29	21

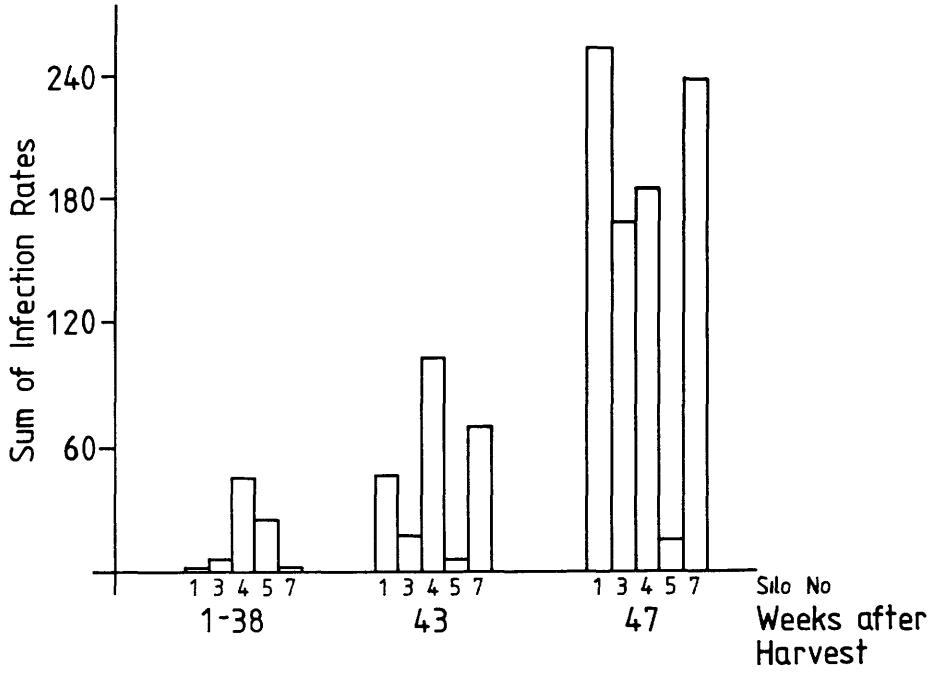
Occasionally occurring fungi

Botrytis cinerea	*Mucor* spp.
Byssochlamys nivea	*Rhizopus stolonifer*
Monascus ruber	*Trichothecium roseum*

[1] C/J: Detection of surface contamination or grain infestation;
J: Detection of grain infestation

[2] Percentage of grain samples drwan during storage showing the specific genus or species

Figure 1 Occurrence of storage fungi following airtight storage in five different silos



Eurotium (anamorph Aspergillus glaucus group) showing an infection rate of 55-68 % and a total surface contamination of the grain samples studied of 71-79 %. The species identification of the complex resulted in E. herbariorum, E. amstelodami and E. chevalieri. P. roqueforti could be isolated from the grain surface in both years while grain infestation was only existent in 1984/85. P. roqueforti could not be obtained earlier than 40 weeks after harvest. At the end of the storage period the infection rate was up to 90 %. Because of the heterogeneity of mold occurrence, Figure 1 gives the sum of the infection rates of grains for 5 silos (nos. 1, 3, 4, 5, 7) for the storage period 1984/85. It can clearly be seen that the distribution of molds is uneven between the silos though they were located closely together. In this respect it is worthwhile to note that silos No. 1 and 5 had been in use for several years while silos no. 3 and 7 were brand new at the beginning of the experiments. And finally it is obvious from this figure that intensive fungal development on airtight stored wheat and barley does not start earlier than 40 weeks after harvest.

Mycotoxins

The problem of fungal contamination of grains used as cattle or pig feed has often been described. Of the fungi occurring on and in cereal grains during these investigations *P. roqueforti* was of particular interest. A total of 27 isolates originating from 19 grain samples from 16 silos collected between 1982 and 1985 were systematically studied for their toxin producing capacity. Because of the simultaneous production of a brown color by 3 isolates these had to be omitted from mycotoxin identification. The remaining 24 isolates were analyzed after 14 days incubation on YES-medium. While 9 isolates were able to produce and secret PR-toxin and mycophenolic acid on the artificial medium, 7 isolates only formed PR-toxin and 2 only mycophenolic acid. Six isolates or 25 % of the strains tested did not produce any toxin. The amount of these two toxins produced was rather small. There was no significant correlation between formatio- of PR-toxin and mycophenolic acid. None of the isolates under investigation were able to produce patulin. According to the toxigenic activity and different physiological tests the isolates of *P. roqueforti* occurring in Schleswig-Holstein can all be grouped to subsection 1 sensu Frisvad and Filtenborg (1983).

Discussion

The present investigations give some information about the mycological potential of cereals stored under airtight conditions in the most northern part of Germany which is characterized by a cool maritime climate and by very intensive agromanagement systems.

The formation of anoxic conditions within the silo largely depends on the occurrence of the field fungi existing on the grain surface or as internal flora. After about 8 weeks of storage these organisms had almost completely disappeared. On the other hand, providing grain moisture content was higher than 16 %, a specific yeast flora could develop simultaneously in all silos studied within this early time. The spectrum consisted of only 3 species showing *H. burtonii* and *Ha. anomala* to be the most important yeasts. Comparable results have been described by Clarke and Hill (1981) for barley in Britain, while Ekstrom *et al.* (1984) found a recognizable yeast population under Swedish conditions, but not earlier than the spring of the year following harvest. The intensive yeast growth prior to mold development gives rise to speculation about the antagonistic activity between these two microbial forms. The present investigation does not support such a hypothesis. More detailed information will be given elsewhere.

Because of the occasionally extremely high population densities of yeasts, toxicological problems could arise after feeding these cereals to pigs. Only limited information is available on this topic. It has been demonstrated that feeding with either conventionally stored or airtight stored cereals did not result in any differences

(Schmidt and Melosch, 1983). But it has also been reported that feeding cereals heavily contaminated by yeasts led to diarrhoea in pigs (hemorrhagic intestinal syndrom). Problems must be particularly expected if secondary fermentation processes start in liquid feeding systems (Bruggemeier, 1985). What kind of metabolites are responsible for these distrubancies is still unknown.

In contrast to the yeast flora mentioned previously the molds growing on airtight stored cereals are not specific to this method of storage. All species isolated are known from literature as grain contaminating fungi and independent of the storage type (Hill and Lacey, 1983). Only P. roqueforti is well adapted to oxygen deficiency. Furthermore there are obviously no geographic differences, at least not within one climatic zone. However, the initial inoculum seems to be of fundamental importance. This can originate from the field or from transport systems as can be seen from the fungal population development in silos never used previously. Of greater importance however, is grain contamination from product residues in uncleaned silos. As a consequence all measures must be adopted to obtain cereals for airtight storage which are free from problems of this nature.

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