State Research Organization for Plant Culture **Report No 1127**

The State Plant Pathology Institute (H. Ingv. Petersen)
The Botanical Department (Arne Jensen)
DK 2800 Lyngby

Aspergillus- and Penicillium species on stored Danish Barley grain
Identification of species of fungi isolated from 40 barley-samples in the harvest year 1970

Aspergillus- og Penicilliumarter på dansk byg.
Artsbestemmelse af svampe isoleret fra 40 bygprøver i høståret 1970

Boldt Welling

Summary.
40 barley samples from the harvest year 1970 were collected during May-July 1971 from different parts of Denmark. After arrival the grains were surface disinfected and incubated on malt agar, salt-malt agar and filterpaper for 8-10 days at a temperature of 24°C.

Identifications of species of the genus *Aspergillus* and *Penicillium* were carried out in relation to this experiment. The dominant species were: *Penicillium cyclopium, P. funiculosum, Aspergillus amstelodami, A. repens, A. flavus* and *A. candidus.*

The results gave a distribution of species which not only depended on the origin of the inoculum and the period of infection but also to a high degree on the substrates on which the fungi were isolated.

SAMMENDRAG

I maj-juli 1971 blev der af høsten 1970 indsamlet 40 bygprøver fra forskellige egne af landet med det formål at undersøge kernerne for svampeflora, spireevne og vandindhold.

I tilknytning til denne undersøgelse blev der foretaget artsbestemmelser af lagersvampene Aspergillus og Penicillium. De hyppigst forekommende arter var: Penicillium cyclopium, P. funiculosum, Aspergillus amstelodami, A. repens, A. flavus og A. candidus.

Artsfordelingen afhænger af inokolummets oprindelse, og under hvilke forhold infektionen er sket; det anvendte substrat, hvorpå svampene er isoleret, har spillet en væsentlig rolle for klarlægningen af mikrofloraen på korn.

Introduction

This paper deals with identifications of species of the genus *Aspergillus* and *Penicillium* to determine which species occurred in the harvest year 1970. The experiment was carried out in relation to a paper published earlier (*Welling* 1972).

In Danish grain quality experiments, very few identifications of species of the storage fungi *Aspergillus* and *Penicillium* have been carried out. This must be considered as a fault. The recent concern over changes in the ecological system (residues of pesticides, alteration in chemical composition) and the growing de-

mand for quality have shown the necessity to know in detail the naturally occurring flora of storage fungi on Danish grain, which is the most important raw material of Danish agriculture. Further the knowledge of a number of effects of the storage fungi (especially production of mycotoxins) should make it desirable, through identifications of species of storage fungi, to prevent growth of such injurious fungi.

In the following paragraphs the different effects of some species found in this experiment will be discussed.

Reduction of the germination power and discolouration of the germs of stored wheat are among effects caused by *Aspergillus amstelodami, A. ruber, A. candidus* and *A. repens* (*Papavizas* and *Christensen* 1960, *Tuite* and *Christensen* 1957). The author's results show the same very high reduction in germination power of barley stored at about 18 per cent watercontent, where species belonging to the *Aspergillus glaucus* group (perf. stage *Eurotium*) were frequently occurring (*Welling* 1969). Culture-filtrate of *Penicillium cyclopium* has the same effect on wheat (*Mirchink* 1957, *Mirchink* and *Aseeva* 1959). The production of fatty acids may be caused by *A. candidus, A. amstelodami* and *A. flavus,* even with great variations between the strains (*Nagel* and *Semeniuk* 1947, *Goodmann* and *Christensen* 1952).

Several of the species are able to produce mycotoxins; for example *P. cyclopium* can produce ochratoxin which is the main cause of the kidney disease »mould nephrosis« in pigs (*Krogh* personal information 1973). *P. viridicatum* can produce citrinin and give rise to »mould nephrosis« (*Krogh* and *Hasselager* 1968). *A. flavus* produces aflatoxin expecially on tropically grown food. Aflatoxin is associated with liverspoilage in animals and may be associated with human livercancer (*Gilman* 1970). However, *Krogh et al* (1966), found that even if *Aspergillus strains,* isolated in Denmark, could produce aflatoxin, the ability to cause mycotoxicose diseases was uncertain. *A. fumigatus* may be injurious to man. It can cause allergic diseases, give rise to infections and grow in the human lung (»Farmer's lung«) (*Lacey* 1969, *Austwick* 1965).

The effects mentioned are specific but, generally storage fungi increases the respiration in the grain which leads to dry loose matter (*Christensen* 1970) and thus may in extreme cases cause fire. Furthermore the odour is pronounced and therefore the estetic value of the grain is reduced.

Methods

Surfacedisinfected grains (1 minute in a 1 per cent sodiumhypochloritsolution) were placed on the surface of malt agar and saltmalt agar (10 per cent sodiumchloride). The pH value was 6,4 and 6,1 respectively. 50 surfacedisinfected grains were placed in a moistchamber (filterpaper diameter 16 cm with 20 ml deionized water pH 7,1 added). The grains on salt-malt agar were incubated in darkness and the others under »black light«. After about one week the grains were observed under the stereomicroscope (6-50x). The incubation-temperature was about 22°C.

The strains used for identification were selected in the following way: From the 3 different substrates (malt agar, salt-malt agar and filterpaper) typical strains were isolated. The number of similar colonies of every type observed was noted. The identification was on the basis of colour and texture.

The identifications of Aspergillus and Penicillium were made according to the manual »*The genus Aspergillus*« by Raper and Fennel (1965) and »*Manual of the Penicillia*« by Raper and Thom (1949) and is mainly based on growth characteristics on Czapek agar after about 2 week's growth at 24°C.

Results

In table 1 the occurrence of the different species in relation to number of samples, frequency and index is listed. The most common *Penicillium* species are:

Tabel 1. Occurrence of Penicillium - and Aspergillus species in stored barley samples in relation to number of grain samples, frequency (per cent) and index[1]

Species of storage fungi	Malt agar samples	frequency	index	Salt-malt agar samples	frequency	index	Filterpaper samples	frequency	index
Penicillium									
P. cyclopium Westling....	14	13	46	5	2	3	2	few[2]	—
P. cyclopium Westling (floccose type).....	15	17	64	7	8	14	0	0	0
P. cyclopium Westling (colour var.).....	2	7	4	6	27	41	2	few[2]	—
P. funiculosum Thom.....	10	14	35	0	0	0	27	23	311
P. hordei Stolk.....	13	5	16	0	0	0	2	2	2
P. urticae Bainier.....	2	few[2]	—	0	0	0	2	20	20
P. palitans Westling.....	1	1	<1	0	0	0	0	0	0
P. viridicatum Westling...	1	1	<1	2	7	4	0	0	0
Aspergillus									
A. amstelodami (M) Thom and Church.....	0	0	0	22	27	149	0	0	0
A. repens (C Da) De Bary.	0	0	0	12	45	135	0	0	0
A. ruber (K.S. and B.) Thom and Church.....	0	0	0	7	12	21	0	0	0
A. flavus Link.....	18	7	31	19	11	52	11	11	61
A. tamarii Kita.....	0	0	0	2	2	1	0	0	0
A. candidus Link.....	3	7	5	20	8	40	3	2	3
A. versicolor Vuillemin....	2	3	2	6	6	14	0	0	0
A. tubingensis (Sc) Mosserray.....	4	5	5	2	1	<1	2	2	2
A. fumigatus Fresenius....	2	3	2	0	0	0	5	1	3

[1]) product of frequency and number of samples per 1000 grains.
[2]) determination very uncertain.

P. cyclopium and *P. funiculosum;* the most common *Aspergillus* species are: *A. amstelodami, A. repens, A. flavus* and *A. candidus.* A further description is given in the following.

DESCRIPTION

The genus Penicillium

Species belonging to Penicillium Biverticillata Asymetrica-Fasciculata group

Most of the strains isolated from barley grains in this investigation belong to the group *Asymetrica-Fasciculata* where an exact identification of species may be somewhat difficult because of the small differences in the characteristics between the species.

This group is characterized by having asymetric penicilli consisting of phialides, metulae and branches, very often one or two branches in addition to the conidiophore; the latter is often coarse and rough. The phialide is a cylindrical or flask-shaped cell, that is narrowed at its apex to a small conidium bearing tube with a diameter about half of that of the basal part. The conidia are mostly globose-subglobose to slightly elliptical. The isolated strains belong to the series where aggregated conidiophores (coremia) occur but where simple conidiophores are dominant. Separation into series is mainly based on growth and colour characteristics.

Penicillium cyclopium Westling

20 strains are identified as *P. cyclopium* Westling. 10 of them agree with the description of this species, given by Raper and Thom (1940), 10 strains differ from the description by having

a much more floccose appearance. 9 strains showed the same morphology, but differ in producing pinkish buff shades in fresh isolates, reminiscent of *Penicillium carneo-lutescens* mentioned as *P. cyclopium colour var.* in table 1. However, after a few transfers they produce the characteristic blue green colony colour of *P. cyclopium.*

The difference between *P. cyclopium* and *P. expansum* is very small and separation is often very difficult. Referring to Raper and Thom (1949) *P. cyclopium* does not rot pomaceous fruits while *P. expansum* does. According to Domsch and Gams (1970) *P. cyclopium* has less complicated penicilli and a thickening at the apex of the phialide.

P. viridicatum Westling

3 strains are identified of the species. Colonies on Czapek agar reach a diameter of 2-2,5 cm after about 2 weeks. The surface appears fasciculate to floccose with the central areas raised about 2-3 mm over the surface. The margin is white and somewhat wrinkled. The colour is bright yellow-green with no blue element and differ at this point from the cyclopium series. Exudate drops appear, small and nearly colourless. Reverse is yellowish-brownish. In one strain a wheellike structure appears.

The conidiophore is rough with a diameter about 3 μ, carrying a Penicilli up to 40 μ in length belonging to the *Biverticillata-Asymmetrica* group. The conidia are subgloose – slightly elliptical with a diameter about 3-4 μ, smooth – slightly roughened.

P. urticae Bainier. (*Syn. P. patulum* Bainier)

3 strains are identified in this species. Colonies attain a diameter of 3-4 cm after 2 weeks, appearing velvety – granular, sporulating all over with conidiophores arising from the substrate in marginal areas and from basal mycelium in central areas. There is some zonation and the margin is not distinct. The odour is a little sourish, not distinct. The colour is in light sandgrey – light green shades, in reverse dull yellowish to brownish.

The conidiophore is smooth, the Penicilli belongs to *Biverticillata-Asymmetrica* and is rather divergent with phialides less than 6 μ in length, characterizing this species. Conidia are subglobose – elliptical with a diameter of about 3 μ.

P. palitans Westling

One strain is identified in this species. The colonies on Czapek attain a diameter of 3-4 cm after 2 weeks, appearing velvet – granulate and in the central areas somewhat floccose. The conidial areas quickly develop in dark-yellow shades.

Reverse is dark vinaceous.

The conidiophore is smooth – slight roughened. Conidia are globose, smooth with a diameter of about 4 μ.

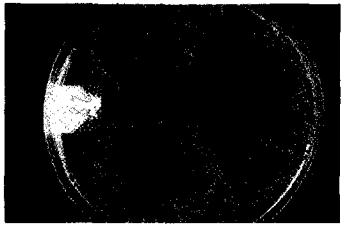
P. hordei Stolk

One strain is identified in this species described by A. C. Stolk 1969. The description by Stolk is mainly based on strains isolated from grains of barley in 1966. Referring to Stolk: »it should be placed in the *P. granulatum* series and is closest to *P. corymbiferum* Westling. The two species can easily be separated from one another by their conidia. Those of *P. corymbiferum* are smooth-walled measuring 2,5-4 μ in diameter whereas those of *P. hordei* are rough-walled and 2-3 μ in diameter.

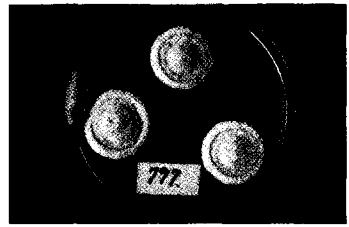
In routine examinations under low magnification (6-50x) this species is easily identified directly on the grains because of its huge, very prominent yellow coremia.

Species belonging to P. Biverticillata – Symmetrica

Species belonging to this group are characterized by having Penicilli consisting of phialides, metulae and branches placed symmetrically around the conidiophore. The general impression of the penicilli is a completely symmetrical

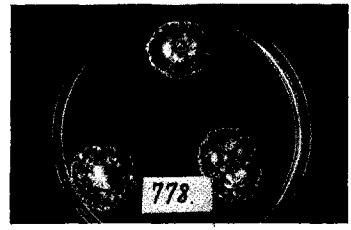


A. tubigensis (Sc) Mosserray



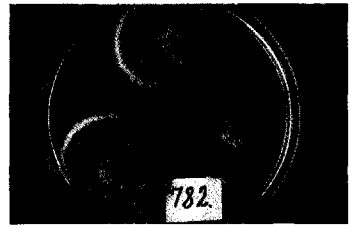
P. cyclopium Westling colour var.

A. tamarii Kita

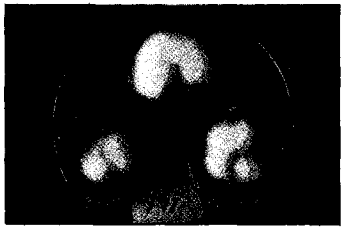


P. cyclopium Westling floccose type

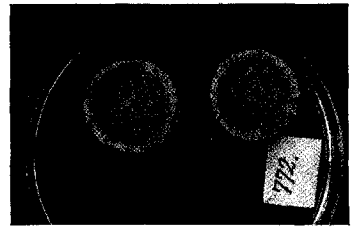
A. flavus Link



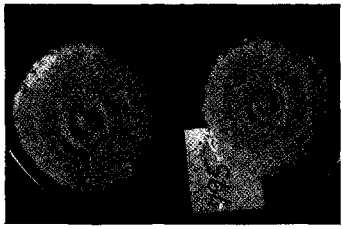
P. cyclopium Westling



P. palitans Westling



P. funiculosum Thom



P. urticae Bainier

P. hordei Stolk

fot. B. Welling

brush, with lanceolate phialides which in a typical way differ from those of asymmetrica. The conidia are very often elliptical.

P. funiculosum Thom
4 strains are identified in this species. Colonies on Czapek agar reach a diameter of 3-4 cm after 2 weeks, appearing velvety – granular, heavily sporulating near the central areas. The margin is raised up to 4 mm with some funiculose mycelium. The central areas are in green shades and the marginal areas in white – yellowish shades. Reverse is in purple-red shades. The odour is fragrant, but not pronounced.

Conidiophores arise from aerial hyphae in central areas and in the marginal areas from trailing hyphae. The surface is smooth – slightly rough. Conidia are mostly elliptical, smooth, measuring 2,5-3 μ.

The genus Aspergillus
Species belonging to *A. glaucus* group. (Perf. stage *Eurotium).*
These species are characterized by numerous cleistothecia giving the colony a yellow appearance when grown on Czapek agar with 20 per cent sucrose.

The identification of the strains are based only on the size and form of the ascospores and could therefore be identified directly by looking at the ascospores taken from the tubes.

15 strains were identified as *A. amstelodami* (*Mangin*) Thom and Church, *A. repens* De Bary, and *A. ruber* (*Konig, Spieckermann* and *Bremer*) Thom and Church.

Aspergillus versicolor group
2 strains are identified as *A. versicolor* (Vuill) Tiraboschi. The species are characterized by having radiate to loosely columnar biseriate heads in shades of light yellow-green, and the vesicle globose – elliptical to spathulate and fertile over the upper half to three-fourth surface.

Aspergillus fumigatus group
2 strains are identified as *A. fumigatus* Fresenius. This group is characterized by having typical columnar parallel arranged heads in shades of blue to dark green, phialides in single series, flask-shaped vesicle, often green pigmented, fertile over the apical part of the vesicle.

Aspergillus flavus group
The group is characterized by globose or columnar heads in shades of yellow-green and brown-green to brown. Uniseriate/biseriate conidial heads. The vesicle is globose and fertile over most of the surface.

3 strains are identified as *A. flavus* Link and one strain as *A. tamarii* Kita. *A. tamarii* differs from *A. flavus* having conidia which are thick-walled and conspicuously roughened by prominent tubercles, whereas those of *A. flavus* are thin-walled and echinulate.

A. niger group
One strain is identified as *A. tubingensis* (*Schöler*) Moss characterized by having radiate heads in greyish-black shades, biseriate conidial heads, vesicles globose and fertile over the whole surface, conidia globose, thin walled, slightly roughened with a diameter of 3,5-4 μ.

A. candidus group
One strain is identified to *A. candidus* Link, which is very easily identified directly under the stereomicroscope because of its persistently white heads. It is characterized by having globose – loosely columnar white heads, conidial heads biseriate, vesicle globose and fertile over the entire surface.

Discussion
As shown in table 1 there is a very great difference in the occurrence of the different species. According to *Gilman* (1957) most of the species found in this experiment occur in soil all over the world and the inoculum in the grain probably comes from the soil, where the distribution of the fungi depends on factors such as food, pH, temperature and competition. The later storage conditions of the grain (watercontent, temperature) decide the manifesta-

tion in the grains. Even after drying of the grain the storage fungi occur nearly with the same frequency (*Welling* 1971).

The registrations in the laboratory depend on several factors. The inoculum may occur as spores sitting on the surface or mycelium in the deeper layers of the grains. When surface disinfection is used the surface contamination is removed and the inoculum will be present as mycelium manifested in the grains. Also the substrate decides which species are registered. For instance *Penicillium cyclopium* is very often isolated on malt agar, seldom on salt-malt agar and does not occur at all on grains in moist-chamber. This must be due to the fact that the 3 substrates used for isolation act selectively on the occurring fungiflora. One should consider grains in moist-chamber as the most natural substrate, but some species do not occur on this substrate. A species like *Penicillium funiculosum* grows abundant in the moist-chamber suppressing the development of others.

The incubation temperature is also a factor of importance. The species *Aspergillus fumigatus* is thermo-tolerant and is able to grow at 50°C but may even grow well at 20°C (*Cooney* and *Emerson* 1964); at a high temperature it is favoured in competition with other fungi. The incubation temperature in this experiment was 22°C, which may explain the small occurrence of this fungus.

The diffenrence in the pH of the substrate (6,1 - 7,1) may not be such that it can explain the difference in occurrence between the species. Difference in accessible food between malt agar and the grains itself may give some explanation.

Furthermore there is a great variation in physiological demands inside the same species. This might explain why one species found in a number of samples is not always found in the same samples on another medium.

A survey like this is not able to give a 100 per cent picture of the occurrence of the different species, because there is always the possibility of errors when registering how many colonies represent the same species based on colour and texture under the stereomicroscope.

It should be noticed that in the paper (*Welling* 1972), the dominant *Penicillium* series on filterpaper has wrongly been identified as *P. purpurogenum* and should be corrected to *P. funiculosum*.

This experiment should be a contribution to a better understanding of the occurrence of storage fungi and provide assistance in future food-quality experiments where the identification of species is necessary to determine the effect of storage fungi on quality. An identification stated os *Penicillium spp.* is insufficient. Furthermore one should take more consideration to the laboratory conditions under which the fungi have been isolated and incubated.

Acknowledgment

The author wishes to thank Centraalbureau voor Schimmelcultures, Baarn, Holland for the possibility to work and receive assistance with the identifications; especially thanks to Dr. A. C. Stolk and Dr. R. A. Samson for valuable help. Laboratory assistant Anitta Idoff carried out the isolations. Economic assistance has been received from the Danish State Board of Plant Culture.

Literature

Austwick, P. K. 1965, Pathogenicity – In the Genus Aspergillus 82-126. Williams and Wilkens, Baltimore.

Christensen, B. H. 1970. Undersøgelser over temperatur og luftfugtighedsforhold i kornlagre i efterårs- og vintermånederne. Kornkvalitetsudvalget under Akademiet for de tekniske videnskaber. Rapport om udvalgets virksomhed. 407-426.

Cooney, D. G. and *R. Emerson.* 1964. Thermophilic Fungi. An account of their biology, activities and classification. p. 161.

Gilman, J. C. 1956. A Manual of Soil Fungi. London 418 pp.

Gilman, G. A. 1970. Fungal spoilage of stored food in the tropics and its possible effect on man. Commonwealth Phytopath. News. part 1. 1-4.

Goodman, J. J. and *C. M. Christensen.* 1952. Lipolytic activity of fungi isolated from stored corn. Cer. Chemistry 29 : 299 - 308.

Krogh, P. and *E. Hasselager.* 1968. Studies on fungal nephrotoxity. Royal Vet. and Agr. College, Copenhagen, Denmark. Yearbook 198-214.

Krogh, P., E. Hasselager, B. Hald and *A. Kjøller.* 1966. Studies on the toxicity of Danish Aspergillus flavus strains. Royal Vet. and Agr. College, Copenhagen, Denmark. Yearbook 129-147.

Lacey, J. 1969. Health hazards from mouldy fodder. World Crops. London. July/August. 1-5.

Mirchink, T. G. 1957. On fungi causing toxicity of turf-podzol soil in various stages of cultivation. Mikroniologiya 26: 78-86.

Mirchink, T. G. and *I. V. Aseeva.* 1959. Fungi as a toxicity factor of turf-podzol soil at different degrees of cultivation. Nauch. Dokl. vyssh. Shk., Ser. biol. Nauk. 1959/2, 206-211.

Nagel, C. M. and *G. Semeniuk,* 1947. Some mould-induced changes in shelled corn. Plant Physiology 22 : 20-23.

Papavizas, G. C. and *C. M. Christensen.* 1960. Effect of invasion by individual species and mixtures of species of Aspergillus upon germination and development of discoloured germs in wheat. Cereal. Chemistry 37 : 197-203.

Raper, K. H. and *D. I. Fennell.* 1965. The genus Aspergillus. Williams and Wilkins, Baltimore. 875 pp.

Stolk, A. C. 1969. Four new species of Penicillium. Antonie van Leeuwenhoek. Journal of Microbiology and Serology 35 : 270-274.

Tuite, J. F. and *C. M. Christensen.* 1957. Moisture content of seed in relation to invasion of the seed by species of the Aspergillus glaucus group and effect of invasion upon germination of the seed. Phytopathology. 47: 323-327.

Welling, B. 1969. Fungusflora and germination at barley. Tidsskr. f. Planteavl 73: 291-308.

Welling, B. 1971. Fungusflora and germination power in mouldy grain – before and after drying. Tidsskr. f. Planteavl 75: 581-585.

Welling, B. 1972. Fungusflora, germination power and watercontent in 40 barleysamples from the harvest 1970. Tidsskr. f. Planteavl 76: 503-510.

Manuskript modtaget den 12. september 1973.