Agriculture and
Agri-Food Canada

An overview of Ochratoxin A, associated fungal species and implications for Canada's grain industry

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Canada

a) Proposed Health Canada maximum limits (MLs):

raw cereal grains*	5 ng OA/g;
directly consumer grains (i.e. rice, oats, pearled barley):	3 ng OA/g;
derived cereal products (flour**):	3 ng OA/g;
derived cereal products (wheat bran) :	7 ng OA/g;
breakfast cereals :	3 ng OA/g;
grape juice (and as ingredients in other beverages) and related products:	2 ng OA/g;
dried vine fruit (currants, raisins, sultanas):	10 ng OA/g;
baby foods and processed cereal-based foods for infants and young children :	0.5 ng OA/g
dietary foods for special medicinal purposes intended for infants:	0.5 ng OA/g

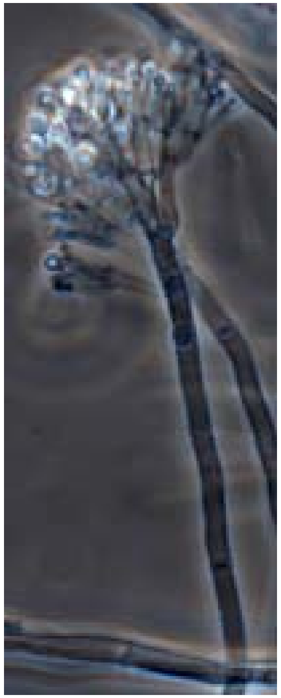
*These levels have been established taking into consideration the reducing effect of processing or redistribution.
**For bread, pastries and other flour-based foods, Health Canada considers these guidelines to pertain to the flour portion. In the future, based on further monitoring data (ongoing HC studies), the Department may consider modifying these MLs, or introduce MLs for products not yet covered, if necessary.

www.hc-sc.gc.ca/fn-an/consult/limits-max-seuils/myco_consult_ochra-eng.php

T. Kuiper-Goodman, C. Hilts, S. M. Billiard, Y. Kiparissis, I. D. K. Richard, S. Hayward. 2010. Health risk assessment of ochratoxin A for all age-sex strata in a market economy. Food Additives and Contaminants 27: 212–240.

Overview

- Historical background
  - The toxin and the moulds
- Biology and taxonomy of *Penicillium* and *Aspergillus*
  - Development of modern species concepts
    - Mycotoxins
  - DNA based identification
- Ochratoxin A in Canada and internationally
  - Storage issues
- Future directions

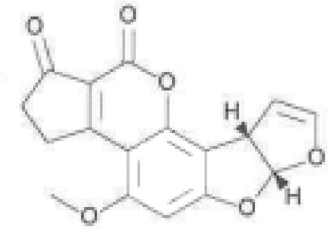


Penicillium

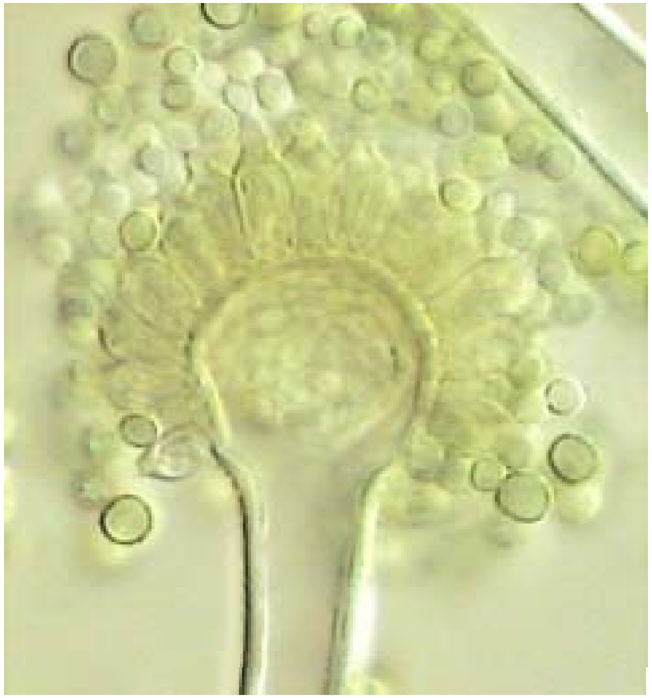
Aspergillus

Aspergillus flavus

the aflatoxin mould

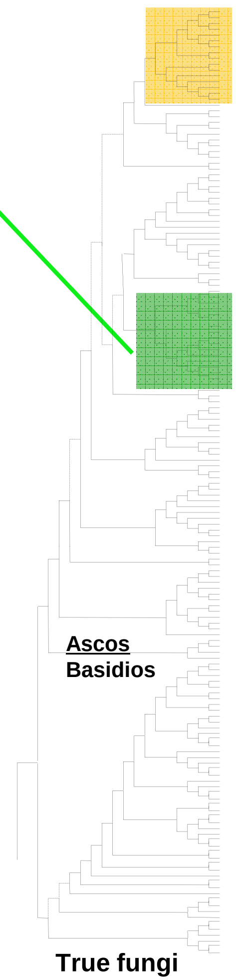


I: Aflatoxin B_1



Penicillium

- ~300 known species
  - > 1000 expected
- **Storage fungi**
- **Dry, electrostatic spores**
- Diverse mycotoxins
  - Ochratoxin A
  - Patulin
  - Griseofulvin



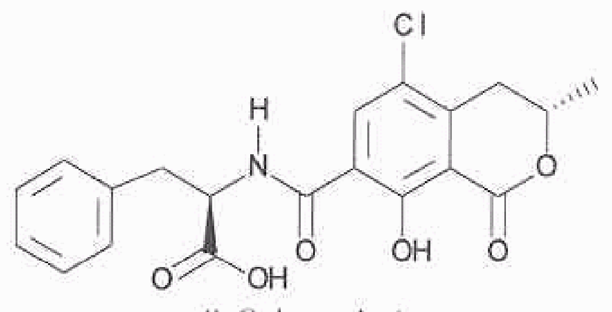
Fusarium

- ~150 known species
  - > 750 expected
- **Field fungi, plant pathogenic**
- **Slimy spores**
- More uniform mycotoxins
  - Trichothecenes
  - Zearalenone
  - Fumonisins

Biology of *Penicillium* and *Aspergillus*

- The quintessential moulds
- Common agents of biodeterioration in soil, wood, dung and man-made commodities
- Talented producers of secondary metabolites
  - High ‘creativity index’
- Plant diseases, especially spoilage during storage
- Common in indoor air, causing allergies
- Ecological significance possibly over-estimated
  - ability to grow on standard microbiological media
- ***P. roqueforti*** **makes 1–10 billion spores per Petri dish**

Ochratoxin A



II: Ochratoxin A

- A fungal toxin
- Abbreviations: OTA or OA
- Regulated in North America and Europe for many years
- Kidney cancer in humans
  - Group 2B *possible* human carcinogen
    - International Agency for Research on Cancer (IARC)
- Found in grains, in particular barley, oats and beer, and other commodities
- Detectable in blood sera of all Canadians
- Terrible confusion about what fungal species produce the toxin
  - Misidentification of moulds
  - Misidentification of toxins

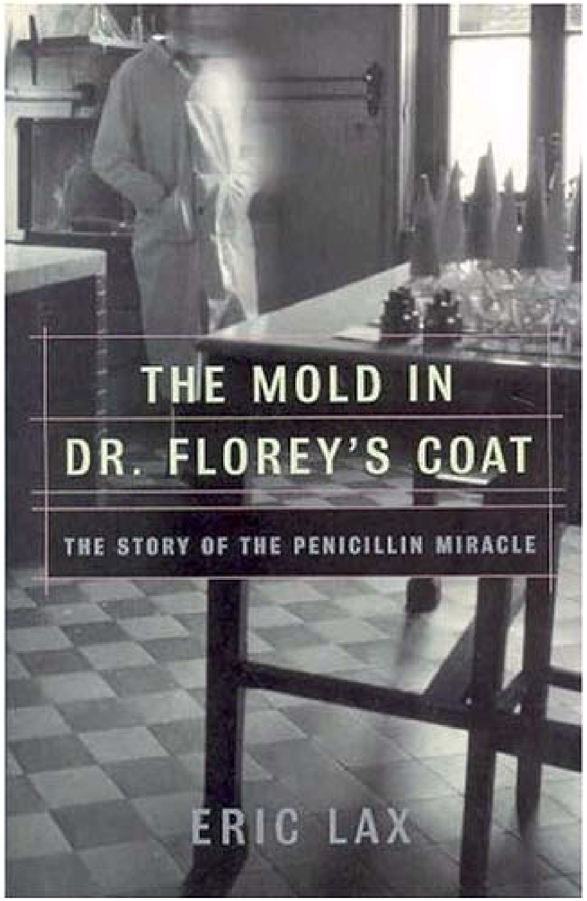
Penicillium as the taxonomist's nightmare

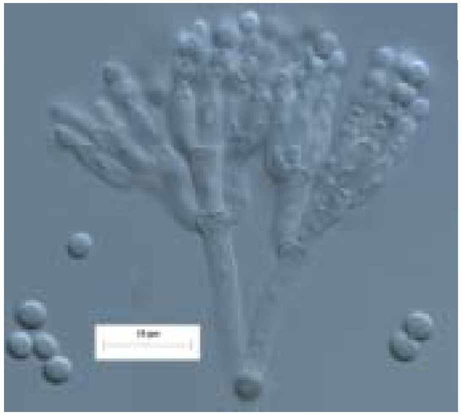
"Alive and actively growing, they have individuality as pronounced as their capacities for evil, but that individuality, color, odor, and habit or growth are evanescent as frost designs on a window pane in winter."

Charles Thom 1930

Penicillium chrysogenum

The penicillin mould





Penicillium expansum

Blue mould of apple

Penicillium camembertii

the Brie mould

Penicillium roqueforti

the blue cheese mould

Penicillium roqueforti

the blue cheese mould

Major Mycotoxins

- Mycophenolic acid
- PR-toxins
- Roquefortine C

Photos Rob Samson

Penicillium history

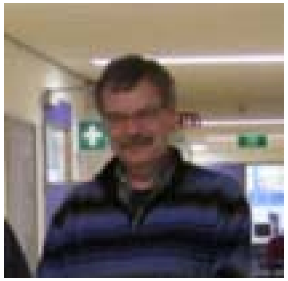
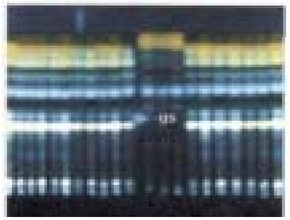
- **1809** Described by Link
- **1930** Monograph by Thom
- **1949** Monograph by Raper & Thom
- **1979** Monograph by Pitt

} **Microscopy and culturing**

- **1980s** **Secondary metabolites & isozymes**
- **1990s** DNA sequencing
- **2000s** Multigene phylogenies, molecular diagnostics
- **2010s** Genomics

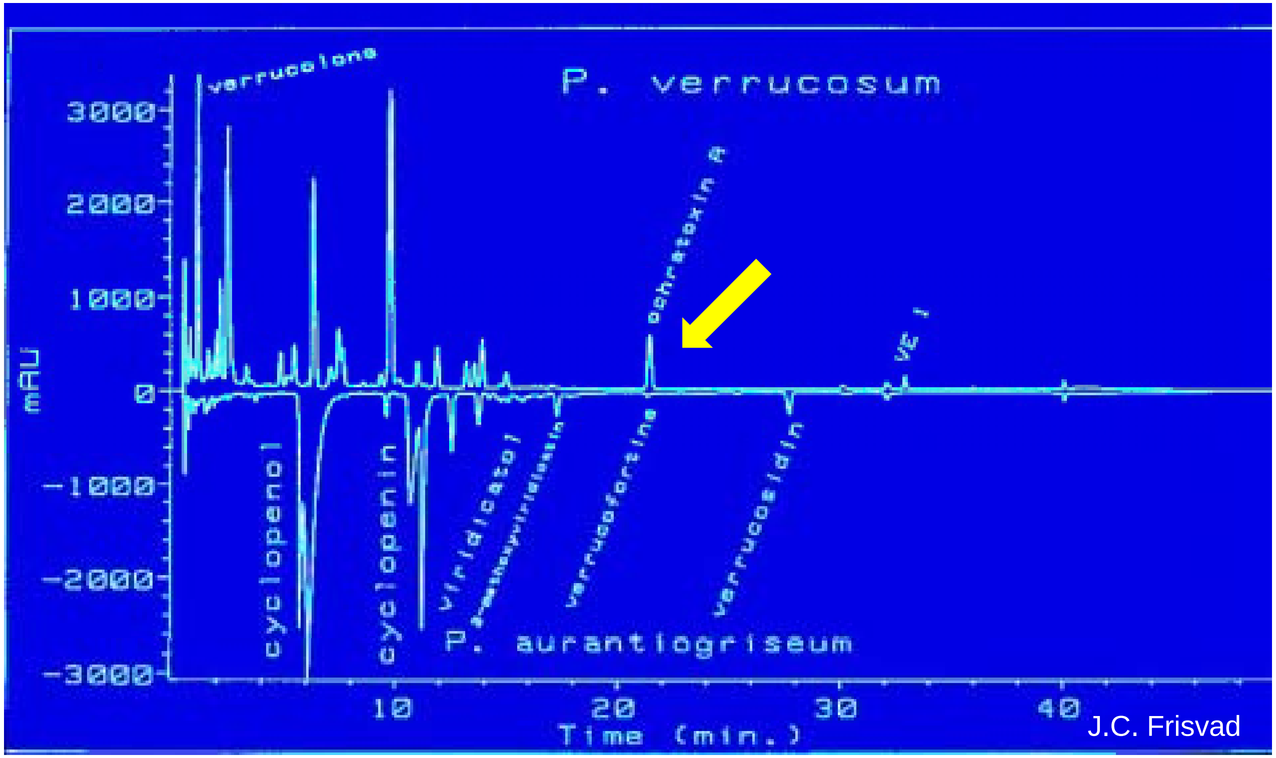
Modern species concepts (1)

- Mid-1980s,
  - Jens Frisvad *et al*., Denmark
- Hypothesis
  - Species have constant, reproducible secondary metabolite profiles
- Secondary metabolite profiling
  - Thin layer chromatography
  - then HPLC
  - then GC-MS
  - then LC-MS
- Result
  - Division of existing morphological species
  - Identification using mycotoxin profiles

Penicillium, Aspergillus & secondary metabolites

- Produce all major groups of secondary metabolites
- ~ 60-100 secondary metabolites per species



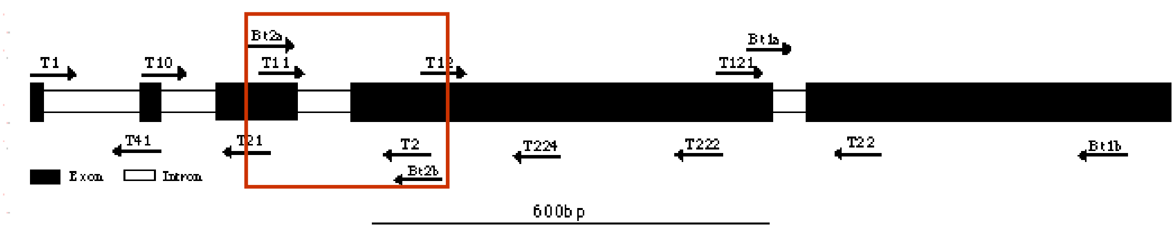
Ochratoxin A production in *Penicillium*

- *Penicillium viridicatum* var. *cyclopium* (PVC)
- *Penicillium viridicatum* chemotype
- ***Penicillium verrucosum***
  - Divided into 2–3 species
    - *P. verrucosum* – grains
    - *P. nordicum* – meat
    - *P. lumpii* – dried fish (not described)

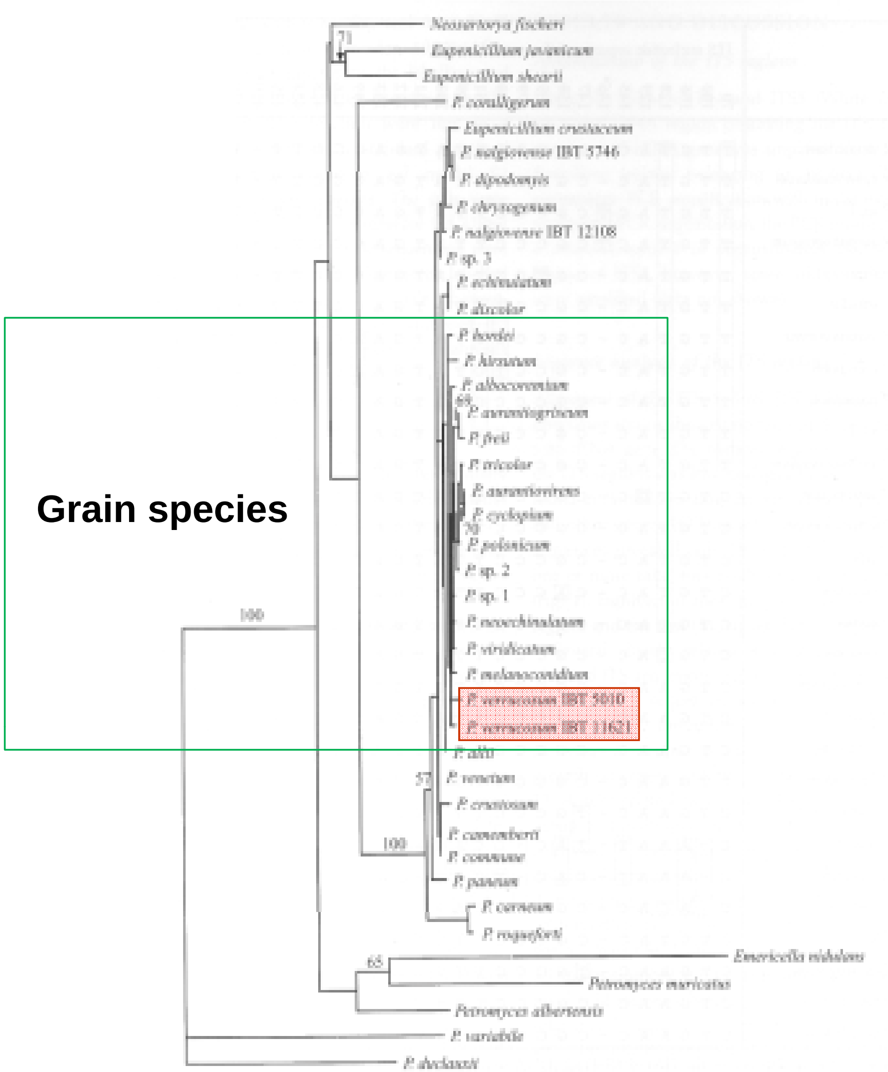
- **All claims of ochratoxin A production in other *Penicillium* species are unproven**

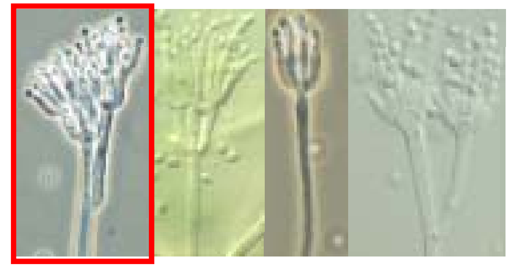
Modern species concepts (2)

- Early 1990s
- DNA-based taxonomy
  - Ribosomal operon sequencing (ITS, LSU)
    - Peterson, USDA, Peoria
  - Protein gene sequencing
    - Seifert lab, Canada
- Good (but not perfect) correlation of phylogenetic species with mycotoxin groups



nuclear beta-tubulin gene





ITS Gene Tree

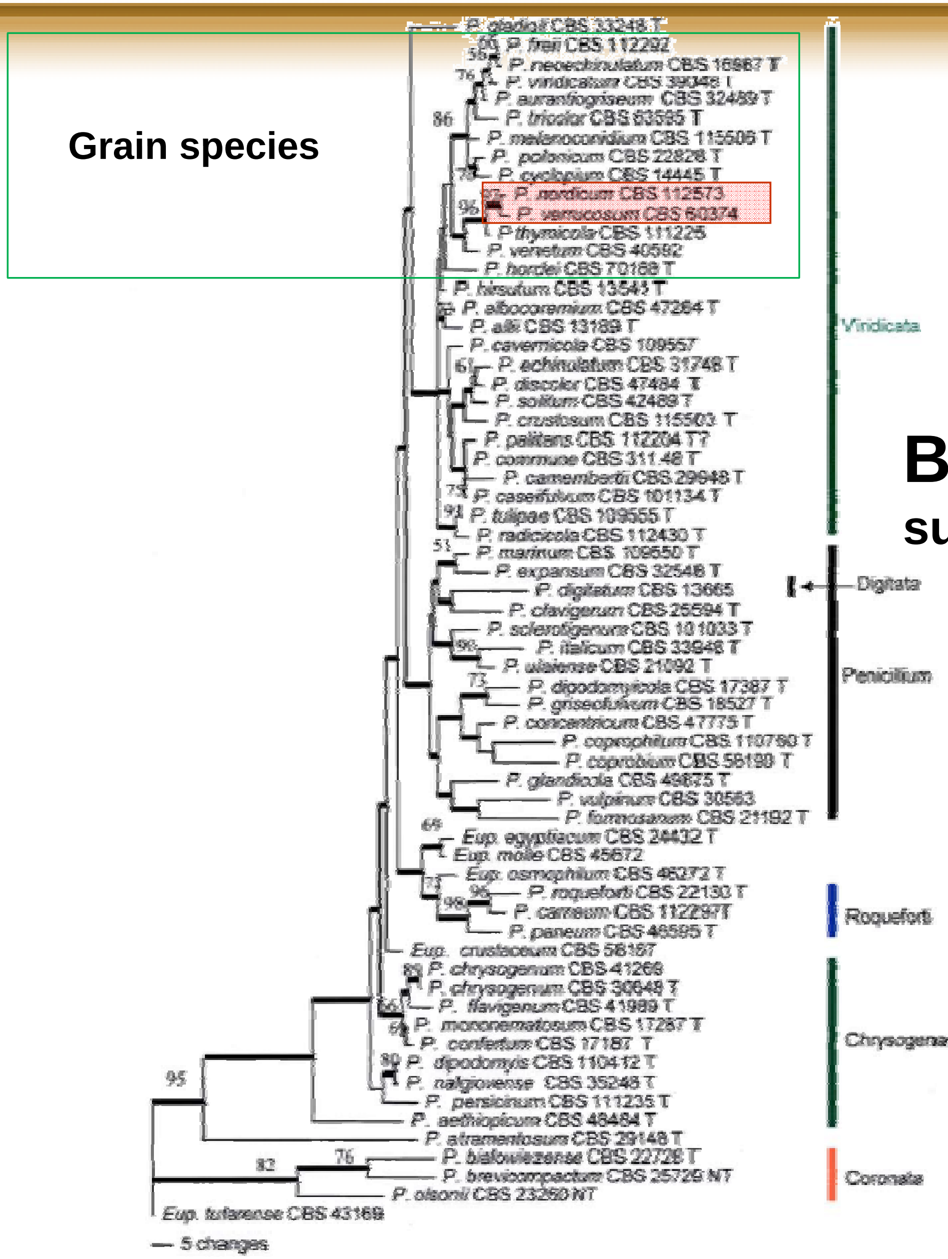
subg. *Penicillium*

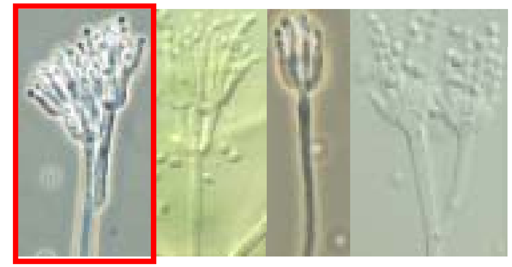
- Variable sites ~ 20%
- DNA barcoding

P Skouboe, JC Frisvad, JW Taylor, D Lauritsen, M Boysen, L Rossen. 1999. Phylogenetic analysis of nucleotide sequences from the ITS region of terverticillate *Penicillium* species. Mycological Research 103: 873–881.

Beta-tubulin Gene Tree

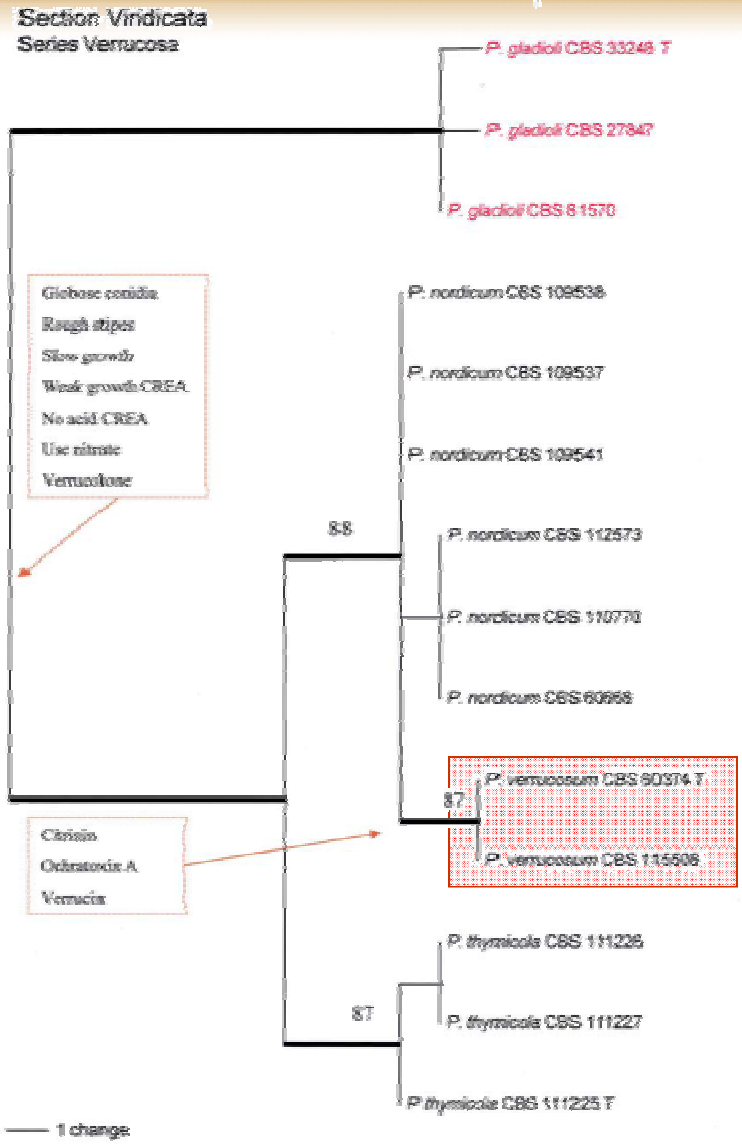
subg. *Penicillium*

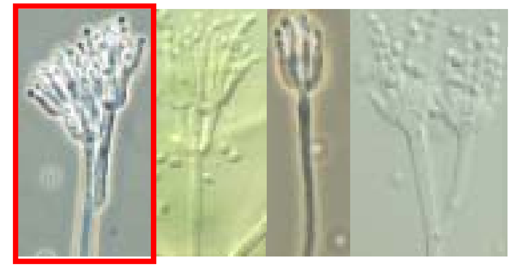




- Variable sites ~ 66%

RA Samson, KA Seifert, AFA Kuijpers, JAMP Houbraken, JC Frisvad JC, 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial beta -tubulin sequences. Studies in Mycology 49: 175–200.



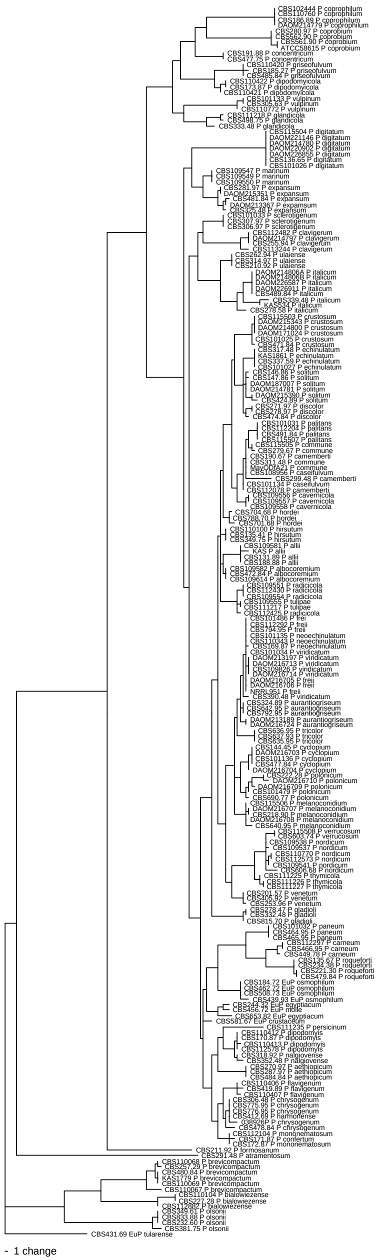


Beta-tubulin Gene Tree

subg. *Penicillium,* Sect. *Viridicata,* Ser. *Verrucosa*

Ochratoxin A group

RA Samson, KA Seifert, AFA Kuijpers, JAMP Houbraken, JC Frisvad JC, 2004. Phylogenetic analysis of *Penicillium* subgenus *Penicillium* using partial beta -tubulin sequences. Studies in Mycology 49: 175–200.



Molecular Assay Development

from beta-tubulin sequences

Successes

P. roqueforti complex (3 spp.)
Citrus penicillia (4 spp.)
P. brevicompactum complex (3 spp.)

Assays design failed

P. aurantiogriseum complex (5 grain spp.)
P. camemberti complex
- *P. commune*
- *P. camemberti*
- *P. caseifulvum*
- *P. palitans*

***P. verrucosum* complex**
- **ochratoxin A**

P. chrysogenum
- penicillin, IAQ

P. expansum
- apples, patulin

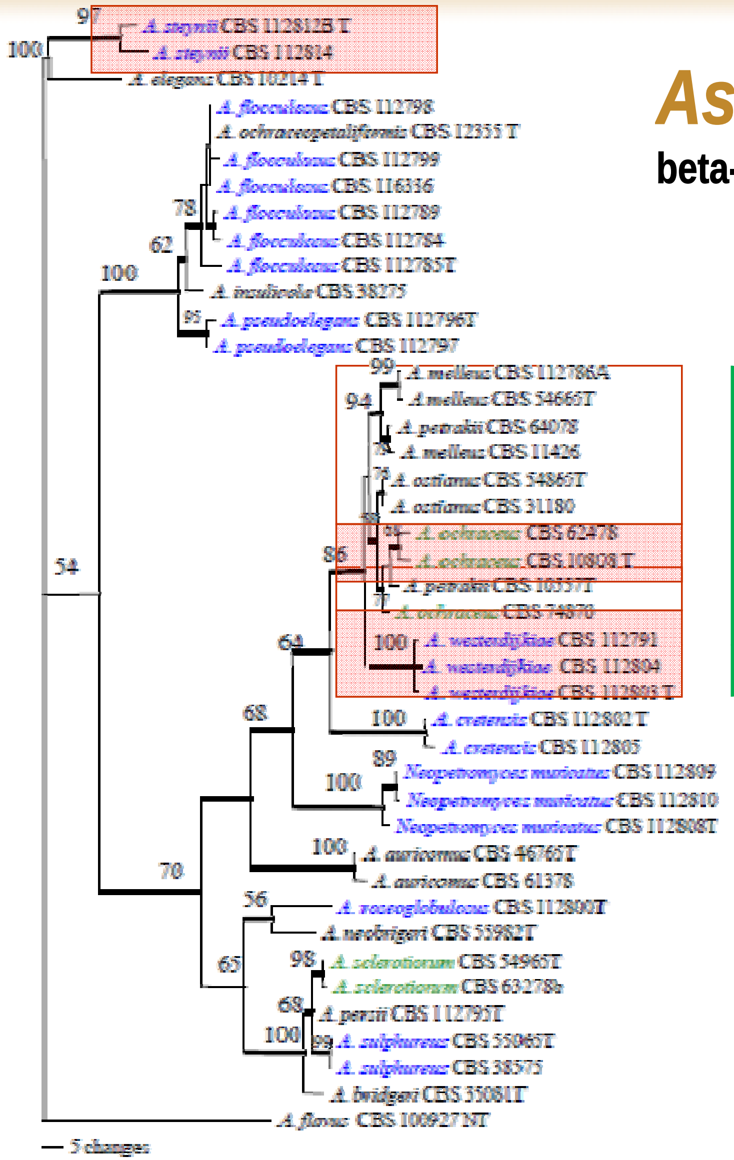
Seifert *et al.* unpublished

The changing ochratoxin A landscape

- Historical concerns in Europe
  - Pork products
  - Beer
- High OTA human blood titers
- High endemic kidney cancers
  - Balkans
- High OTA levels in wine and coffee
  - increased scrutiny of imports

Aspergillus ochraceus complex

beta-tubulin gene tree



OTA group

J.C. Frisvad, J..M. Frank, J.A.M.P. Houbraken, A.F.A. Kuijpers, R.A. Samson. 2004. New ochratoxin A producing species of *Aspergillus section Circumdati.* Studies in Mycology 50: 23-43.

Aspergillus niger complex

beta-tubulin gene tree

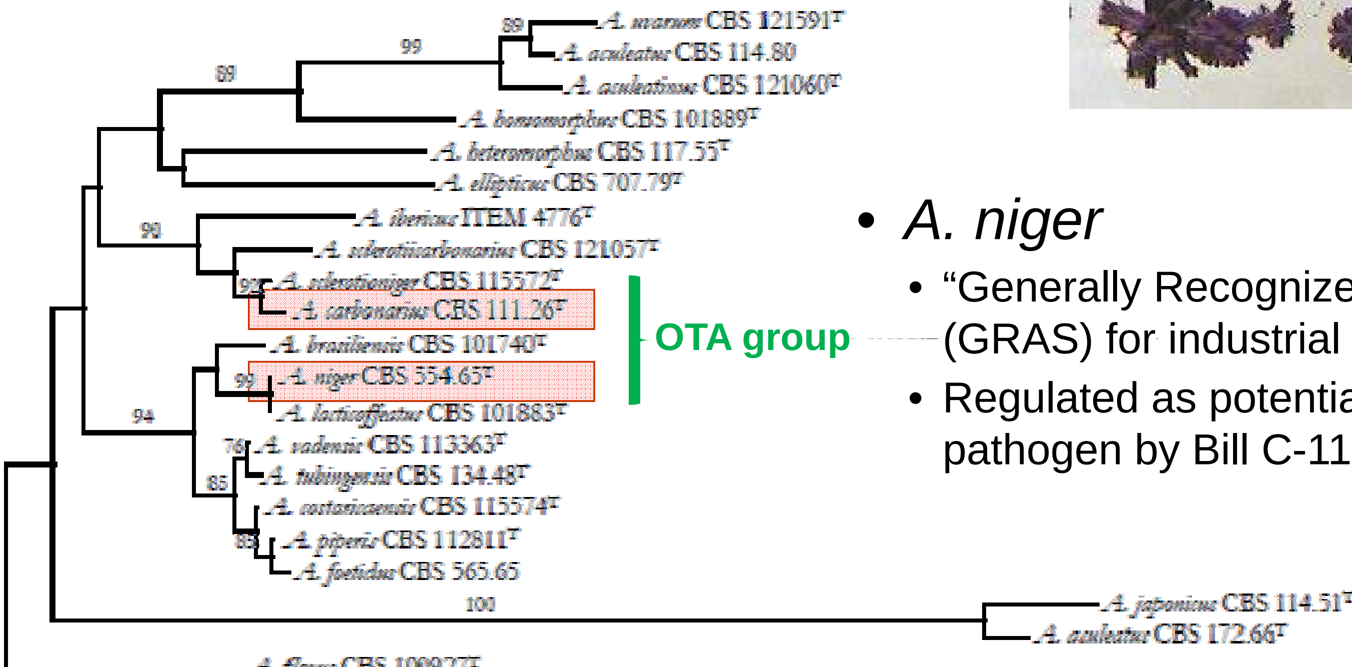


Fig. 9B.

- *A. niger*
  - "Generally Recognized as Safe" (GRAS) for industrial use
  - Regulated as potential human pathogen by Bill C-11

R.A. Samson, P. Noonim, M. Meijer, J. Houbraken, J.C. Frisvad and J. Varga. 2007. Diagnostic tools to identify black aspergilli. Studies in Mycology 59: 129-145.

Determination of ochratoxin A in wine and grape juice

Source and number of samples

Country	Year of analysis	Red wine	White wine	Red grape juice	White grape juice
Algeria	2001–02	1(1)a			
Canada	**1999–2000**	**15(1)**	**16(6)**	**24(3)**	**12(1)**
	2000–01	**6(1)**	**6**	**–**	**–**
	2001–02	**15(1)**	**21(1)**	**13**	**7**
Cyprus	1999–2000	–	1(1)	–	–
England	1999–2000	–	–	1	–
France	1999–2000	3(1)	1	–	–
	2001–02	6	6		
Greece	1999–2000	7(6)	9(6)	–	–
	2000–01	3(2)	4(3)		
Italy	1999–2000	3(3)	3(1)	–	–
	2000–01	6(4)	10(2)		
Spain	1999–2000	1(1)	2	–	–
	2000–01	6(3)	4(1)		
Turkey	2000–01	1(1)	1(1)		
USA	1999–2000	–	–	1	2
	2001–02	11	12	7	4
Total number > LOQ	1999–2002	25	22	3	1
Grand total	1999–2002	84	96	46	25

Table 1 from: W. Ng, M. Mankotia, P. Pantazopoulos, Introduction R. J. Neil and P. M. Scott. 2004. Ochratoxin A in wine and grape juice sold in Canada. Food Additives and Contaminants, 21: 971–981.

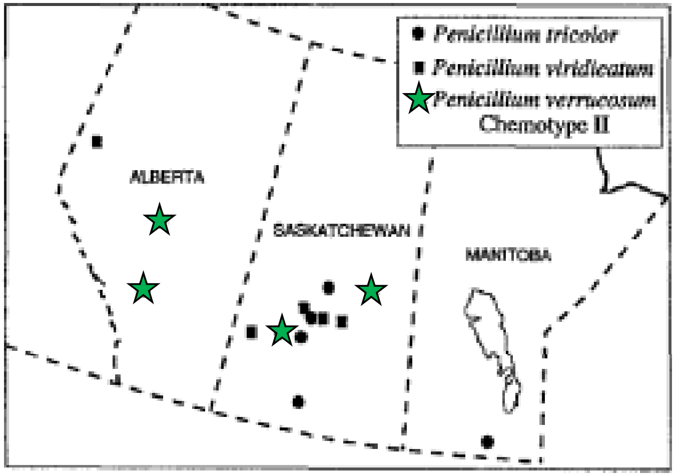
Searching for *Aspergillus carbonarius* in southern Ontario

Penicillium verrucosum in Canada

Table 1. *Penicillium* species isolated from cereal grains delivered to primary elevators from farms in western Canada

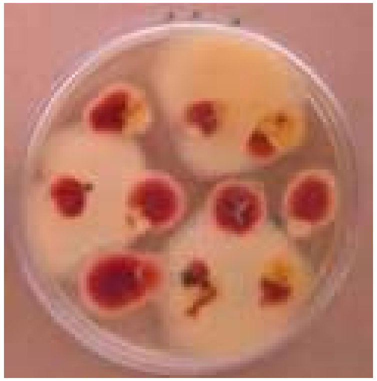
Penicillium species	No. of isolates			
	Manitoba	Saskatchewan	Alberta	Total
Nephrotoxigenic species				
P. aurantiogriseum	14	35	12	61
P. freii	18	4	5	27
P. tricolor	1	5		6
P. verrucosum Chemotype II		2	2	4
P. viridicatum		3	1	4
Non-nephrotoxigenic species				
P. aurantiovirens			3	3
P. chrysogenum	1	2	1	4
P. corylophilum	1			1
P. crateriforme		1	2	3
P. griseofulvum	16	11	2	29
Total	51	63	28	142

- *P. verrucosum* grows slowly
- Overgrown by other species
- Many other *Penicillium* species in grain



J.T. Mills, K.A. Seifert, J.C. Frisvad, D. Abramson. 1995. Nephrotoxigenic *Penicillium* species occurring on farm-stored cereal grains in western Canada Mycopathologia 130: 23-28.

Ecology of *Penicillium verrucosum*



DYSG
Dichloran Yeast Extract
Sucrose 18% Glycerol agar

- Presently no molecular assay
- Monitored with selective medium
  - laborious
  - low sensitivity
- In Denmark
  - Irregular distribution in fields and soils (0-300 CFU/g soil)
  - Sporadic recovery from harvested grain
  - Hot spots in stored grain
    - related to moisture, temperature

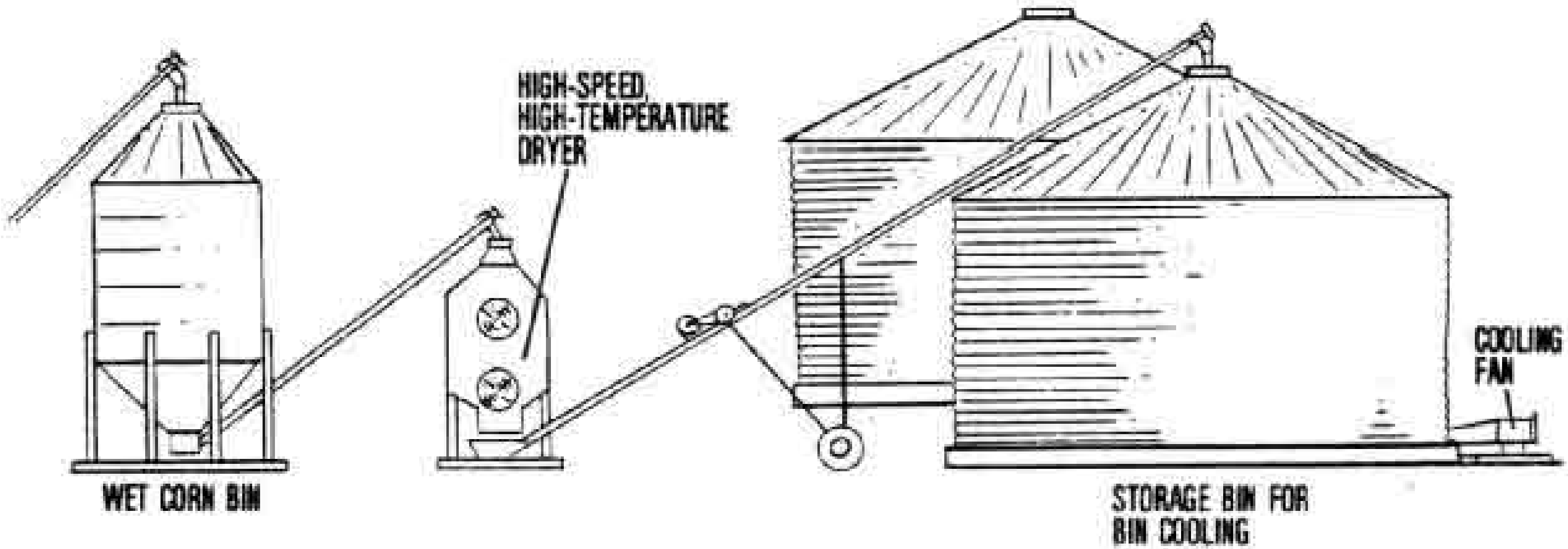
S. Elmholt. 2003. Ecology of the ochratoxin A producing *Penicillium verrucosum*: Occurrence in field soil and grain with special attention to farming system and on-farm drying practices. Biological Agriculture and Horticulture, 20, pp. 311-337.

Factor affecting ochratoxin-A during storage

Good Storage Practice	Impact
Good Store Design and Condition Ensure stores are well designed and maintained	High
Good Harvest and Store Hygiene Clean harvest and store machinery	High
Timely Harvest Service and maintain equipment	High
Adequate Drying Capacity Ensure adequate drying capacity is available	High
Rapidly Dry Grain Rapidly dry grain to below 18% moisture content	High
Rapidly Cool Grain Rapidly cool grain to below 15°C	High
Continued Drying Dry to 15% moisture content for long-term storage	Medium
Continued Cooling Cool grain in winter months to below 5°C	Medium
Monitor Temperature and Moisture Content Continue monitoring temperature and moisture content	Low
Monitor Insect and Mite Activity Use traps and sieving to monitor insects and mites	Low

UK Food Safety Agency. 2007. The UK Code of Good Storage Practice to Reduce Ochratoxin A in cereals.

A. Schaafsma



Good store hygiene	Timely harvest	Ensure adequate drying capacity; rapidly dry and cool grain	Carry out continued drying and cooling	Monitor temperature, moisture content and insect activity

Storage Time →

Mycopathologia (2006) 162: 215–223
DOI 10.1007/s11046-006-0055-4

Ochratoxins: A global perspective

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[2]USDA ARS Western Regional Research Center, 800 Buchanan St., Albany, CA , 94710 , USA

Abstract

Ochratoxins have been overshadowed by better-known mycotoxins, but they are gaining importance. Here we consider ochratoxins in the context of aflatoxins, which are better understood than ochratoxins on many levels. We review recent work on taxonomic distribution, contamination of commodities, biosynthesis, toxicity and regulatory aspects of ochratoxins. We focus on ochratoxins in coff… key commodity in ochratoxin research and regulation.

Key words: *Aspergillus*, coffee, food safety, mycotoxin, ochratoxin, *Penicillium*

What are ochratoxins?

Ochratoxins are fungal secondary metabolites produced by several species of *Aspergillus* and *Penicillium*. They consist of an isocoumarin moiety and a phenylalanine moiety linked by an amide bond [1]. Ochratoxin A (OTA) is chlorinated, which is unusual for natural products (Figure 1). Ochratoxin B, which is not chlorinated, and ochratoxin C, the ethyl ester of OTA, are less toxic and less common [1]. Most studies on ochratoxins have therefore focused on OTA.

Present and past importance of ochratoxins

In January 2006, the owner of a major Italian grain mill was arrested and charged with importing 58,000 tonnes of Canadian wheat contaminated with 15 μg/kg OTA [2]. The wheat was milled and sold to food processors. Consumers and food processors were disturbed by the news, but human exposure to ochratoxins is nothing new. There is circumstantial evidence for involvement of mycotoxins, including ochratoxins, in historical events, though the evidence for ochratoxins is not as conclusive as for ergot alkaloids.

guardian.co.uk

Polluted pasta causes toxin alarm in Italy

John Hooper in Rome
The Guardian, Thursday 12 January 2006

A larger | smaller

Italians tucked into their pasta yesterday with a little less relish than usual after learning that 58,000 tonnes of wheat infected with a powerful natural toxin had been milled into flour and sold on to the market.

Police arrested Francesco Casillo, the director of the Molino Casillo Francesco, yesterday on charges that he imported the wheat from Canada last September which was contaminated with cancer-causing toxins.

Consumer associations and manufacturers of wheat products clamoured to know what … the east coast, where it entered Italy, a spokesman for the local consumers federation … damage the health of unwitting consumers."

A statement from the Italian pasta manufacturers association said Italian pasta was "absolutely safe and innocuous".

"It is unacceptable that a mountain of grain should have been allowed to damage the health of unwitting consumers."

Notice

This archive of previously issued food recalls and allergy alerts is provided for reference and research purposes.

Users should note that the products listed in the archive have been subject to removal from the marketplace or appropriate corrective action. Food recalls or allergy alerts are not an indication of the food safety status of products produced at a later date.

HEALTH HAZARD ALERT

HEINZ MIXED CEREAL FOR BABIES MAY CONTAIN OCHRATOXIN A (OTA)

OTTAWA, December 10, 2009 - The Canadian Food Inspection Agency (CFIA) and Heinz Canada are warning the public not to consume the Heinz Mixed Cereal for babies described below as it may contain elevated levels of Ochratoxin A (OTA).

The following product is affected by this alert:

Heinz Mixed Cereal, a Baby Cereal, Stage 2, From 6 Months
227 g
UPC 0 57000 02516 8
Codes:
BB/MA 10 DE 26 and
BB/MA 10 DE 29

The best before date codes are located on the top left hand corner of the package.

www.inspection.gc.ca/english/corpaffr/recarapp/2009/20091210e.shtml

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2010 Call for Data on Ochratoxin A

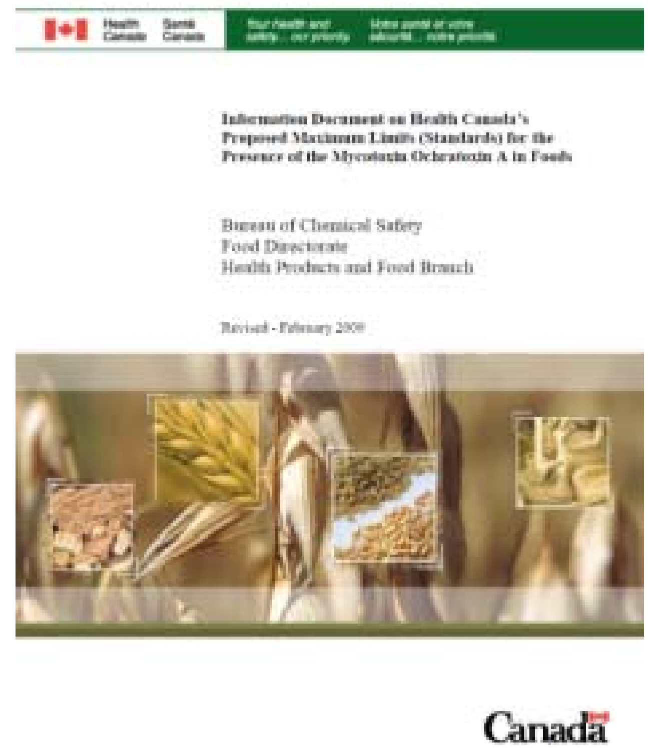
Background

Ochratoxin A (OTA) is a toxic fungal metabolite that causes nephrotoxic, teratogenic, immunosuppressive and carcinogenic effects in a number of animal species. It has also been implicated in the development of a chronic kidney disease in humans known as Balkan Endemic Nephropathy. OTA occurs naturally in low concentrations in many foods, such as cereal-derived staples as well as other food commodities including grapes, raisins, wine, coffee and beer.

On August 29th, 2008 Health Canada's Bureau of Chemical Safety (BCS), Food Directorate, sent pre-consultation letters to targeted stakeholder groups requesting input on Health Canada's proposed maximum limits for ochratoxin A (OTA) in foods. On March 2, 2009, BCS webposted it's Information Document on Health Canada's proposed Maximum Limits (Standards) for the Presence of the Mycotoxin Ochratoxin A in Foods on Health Canada's Food Safety website, requesting further comments on this proposal from the larger stakeholder community. Comments were accepted until 12:00 a.m. EDT on June 1st, 2009.

On December 9, 2009, the Bureau of Chemical Safety's 'Health risk assessment of ochratoxin A for all age-sex strata in a market economy' was published on-line in the journal *Food Additives and Contaminants* (Volume 27, Issue 2, February 2010, pages 212-240). The article was made publicly available by Health Canada through its website in April 2010.

www.hc-sc.gc.ca/fn-an.../securit/chem-chim/toxin-natur/call-demande-eng.php



Health Canada Santé Canada

Information Document on Health Canada's Proposed Maximum Limits (Standards) for the Presence of the Mycotoxin Ochratoxin A in Foods

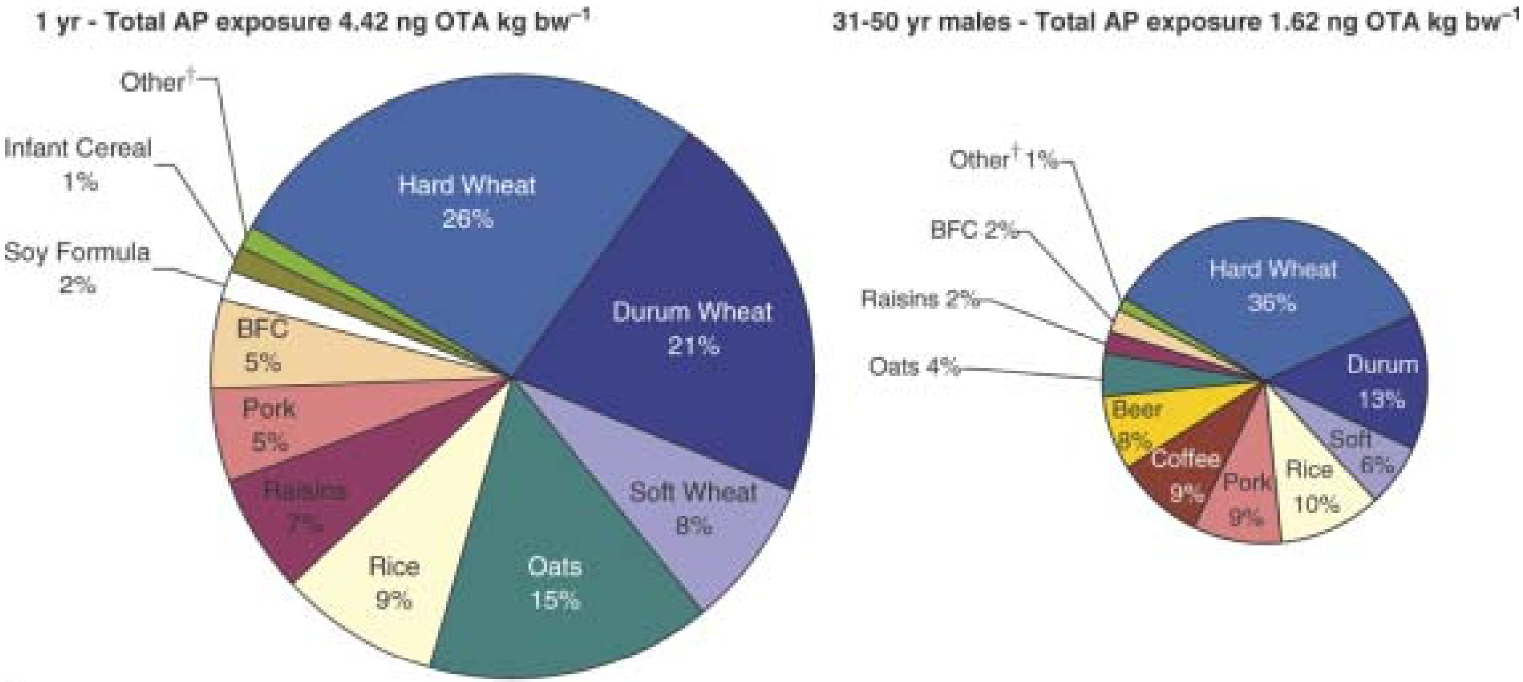
Bureau of Chemical Safety
Food Directorate
Health Products and Food Branch

Revised - February 2009

Canada

… /consult/limits-max-seuils/myco_consult_ochra-eng.php

Ochratoxin A exposure by commodity



† Commodities contributing less than 1% to the total AP exposure to OTA are included in the 'Other' category. For the 1 yr olds, this includes foods such as Barley, Infant Strained Food, Infant Biscuits, etc. For the 31–50 yr old males, this includes commodities such as Wine, Grape Juice, Peas, etc.

T. Kuiper-Goodman, C. Hilts, S. M. Billiard, Y. Kiparissis, I. D. K. Richard, S. Hayward. 2010. Health risk assessment of ochratoxin A for all age-sex strata in a market economy. Food Additives and Contaminants 27: 212–240.

a) Proposed Health Canada maximum limits (MLs):

raw cereal grains*	5 ng OA/g;
directly consumer grains (i.e. rice, oats, pearled barley):	3 ng OA/g;
derived cereal products (flour**):	3 ng OA/g;
derived cereal products (wheat bran) :	7 ng OA/g;
breakfast cereals :	3 ng OA/g;
grape juice (and as ingredients in other beverages) and related products:	2 ng OA/g;
dried vine fruit (currants, raisins, sultanas):	10 ng OA/g;
baby foods and processed cereal-based foods for infants and young children :	0.5 ng OA/g
dietary foods for special medicinal purposes intended for infants:	0.5 ng OA/g

*These levels have been established taking into consideration the reducing effect of processing or redistribution.
**For bread, pastries and other flour-based foods, Health Canada considers these guidelines to pertain to the flour portion. In the future, based on further monitoring data (ongoing HC studies), the Department may consider modifying these MLs, or introduce MLs for products not yet covered, if necessary.

www.hc-sc.gc.ca/fn-an/consult/limits-max-seuils/myco_consult_ochra-eng.php

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The way forward

- Robust identification of mycotoxigenic fungi and mycotoxins
  - Detection and identification of moulds without culturing
    - Real-time PCR assays from genomic studies
  - Cheaper, higher throughput, accurate mycotoxin assays

- Ecological and 'epidemiological' studies
  - Amplification of low incipient contamination from soil or in storage bins is probably a significant issue
- Robust Canadian surveys for the fungus and the toxin
  - Keeping in mind the possibility of a role for *A. ochraceus*
- Continued collaboration among the grain industry, plant breeders, and researchers

Agriculture and Agri-Food Canada

Thanks to:

- **The AAFC team:**

Gerry Louis-Seize, Sarah Hambleton, André Lévesque, Peter Sholberg

- **The CGC team:**

Tom Gräfenhan, Sheryl Tittlemier

- **Ontario colleagues:**

David Miller, Art Schaafsma

- **The CBS team (Netherlands):**

Rob Samson, Ellen Hoekstra, Jos Houbraken

- **The Lyngby team (Denmark):**

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