

# QualiNut

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## Analysis of Aflatoxin Producing Moulds by Aspergillus Flavus and Parasiticus Agar (AFPA)



Training course

25-27 October 2006, EMBRAPA Acre, Rio Branco, Brazil

# Food Borne Fungi



Moulds & yeasts



grains, nuts, beans, fruits, vegetables, meat and processed food such as cheese, bread, jams, cookies...

# Moulds & yeasts



In field, in  
growing plants



During storage  
and processing



# Mould and yeast spoilage of foods

- off-flavours,
- discolouration,
- rotting,
- lowered nutritional value,
- mycotoxin production,
- formation of pathogenic or allergenic propagules

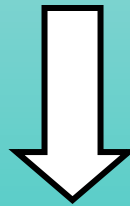


Economic losses and health hazards for  
Producers, consumers and handlers

# Environmental requirements

In general:

- ~ pH 2 - 9
- 10 - 35°C (0 - >50°C)
- water activity ( $a_w$ ) of 0.85 or less. Yeasts generally require a higher water activity.



- obligate aerobes
- Cannot synthesise carbohydrates
- Assimilate organic nitrogen

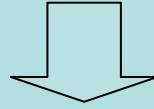
# Food borne fungi

different species have different growth requirements



different compositions and other environmental factors of foods favour different species

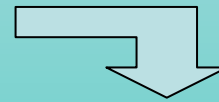
# Kingdom Fungi



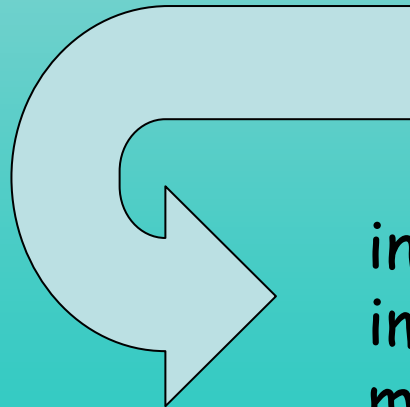
## Food borne fungi

three subkingdoms

- Zygomycotina
- Ascomycotina
- Deuteromycotina .....

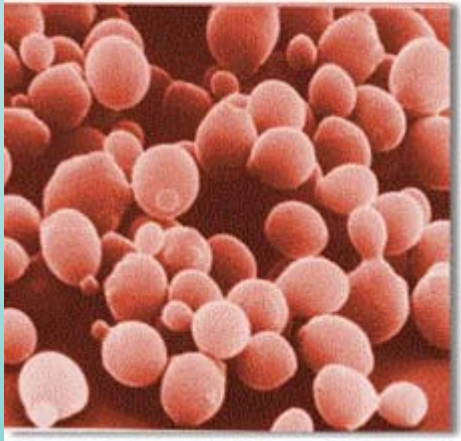


*Aspergillus*  
*Fusarium*  
*Penicillium*

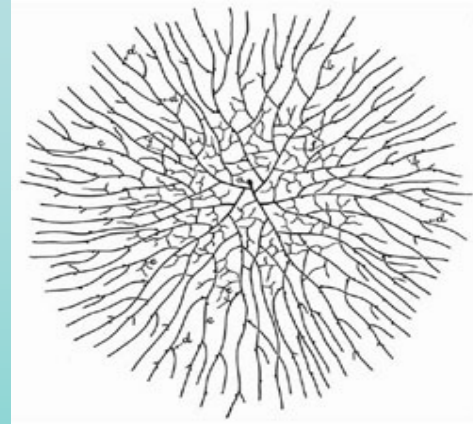


include the most important mycotoxin producing mould species in foods

yeasts



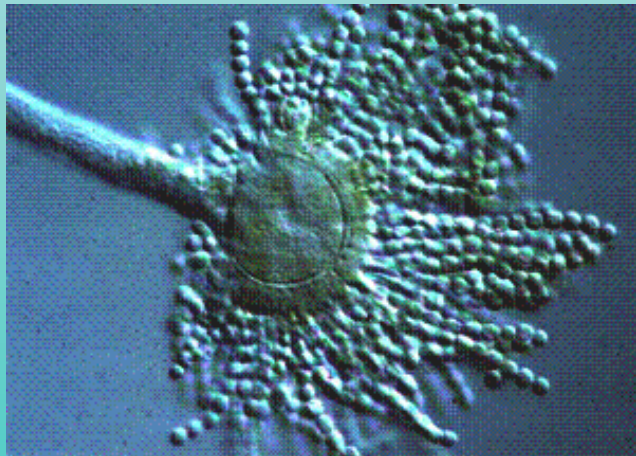
moulds



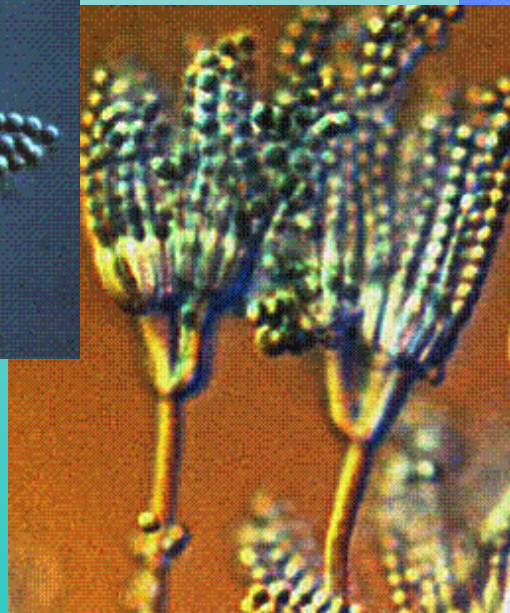
Eukaryotic

Vegetative (asexual) or  
sexual reproduction

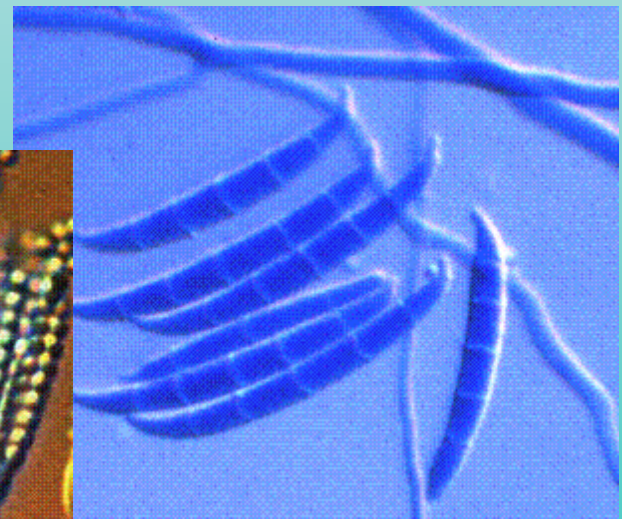
Deuteromycotina - vegetative reproduction through conidia (= asexual spores) or hyphal fragments



*Aspergillus*



*Penicillium*



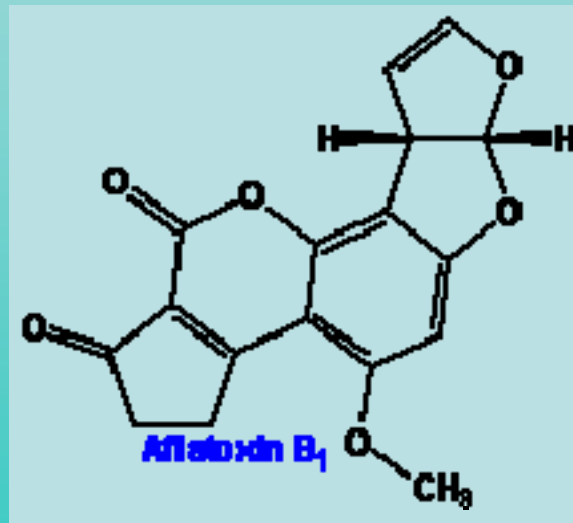
*Fusarium*

1960

"Turkey X disease"



Aflatoxin from *Aspergillus flavus*



aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub> (M<sub>1</sub>) most important

# Mycotoxin production

The presence of potentially toxinogenic moulds is no guarantee for mycotoxin production

- The physiological/nutritional requirements for mycotoxin production are generally more specific compared to the requirements for growth
- For many potentially toxinogenic species not all strains are capable of producing mycotoxins

# Aflatoxin producing moulds in foods

*Aspergillus flavus*

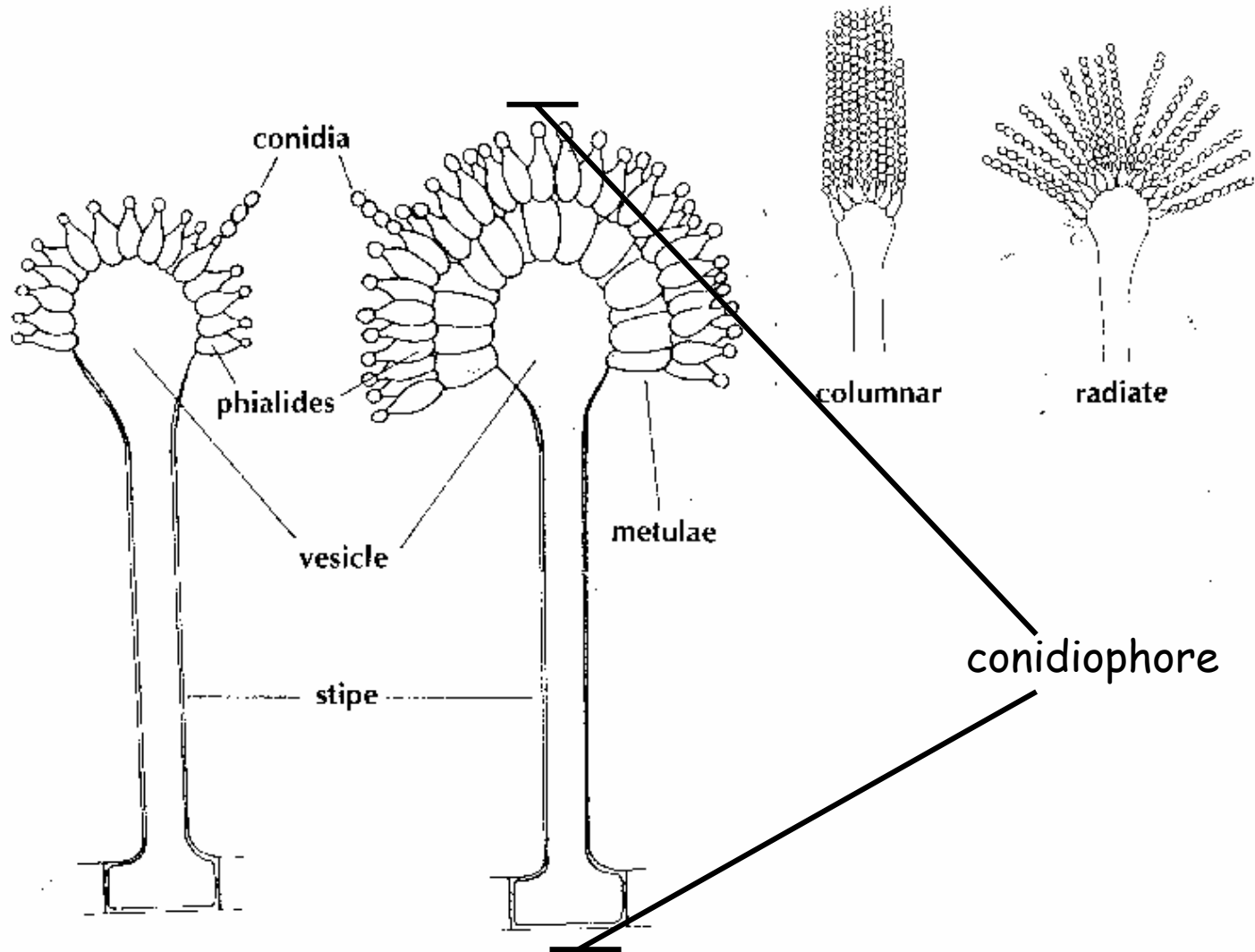
*A. parasiticus*

(*A. nomius*)

Products that are grown in tropical countries



# Aspergillus morphology

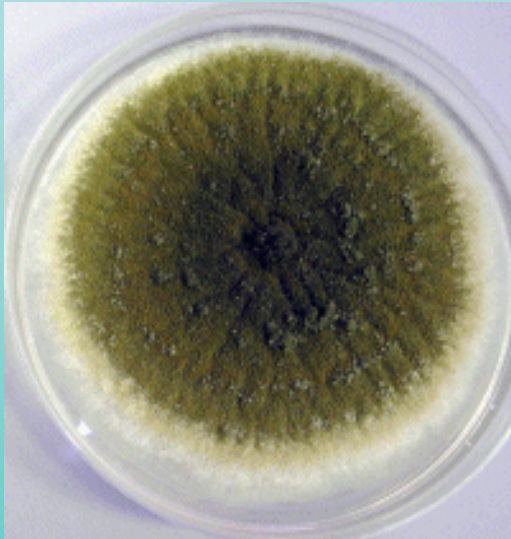


# *Aspergillus flavus*



- The most important aflatoxin producer in foods and feeds
  - Not all strains are capable of producing aflatoxins
    - Only produces the B - aflatoxins

# *Aspergillus parasiticus*



- Less common than *A. flavus*
- Nearly all strains are capable of producing aflatoxins
- Produces aflatoxin B<sub>1</sub>, B<sub>2</sub>, G<sub>1</sub>, G<sub>2</sub>

# Requirements for growth and toxin formation

## *A. flavus*

	Growth	aflatoxin formation
Temp. max (°C)	43 – 48	37
Temp. min (°C)	10 – 12	13
Temp. optimum (°C)	33	16 – 31
a <sub>w</sub> min	0.78 – 0.84	0.82
pH range	2.1 – 11.2	
pH optimum	3.4 – 10 (peak at 7.5)	

## *A. parasiticus*

	Growth	aflatoxin formation
Temp. max (°C)	42	40
Temp. min (°C)	12	12
Temp. optimum (°C)	32	
aw min	0.80 – 0.82	0.86
pH range	2.4 – 10.5	3 – 8
pH optimum	3.8 – 8	

# Macroscopic and microscopic features

Macroscopically very similar  
→ Yellow-green to green

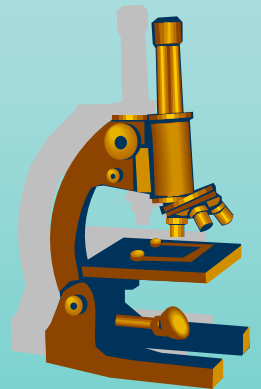
## Microscopic differences

### *A. flavus*

- Large vesicles 30-50  $\mu\text{m}$
- Often with metulae
- Conidia of different size and shape
- Conidia finely rough or smooth with thin walls

### *A. parasiticus*

- vesicles rarely > 30  $\mu\text{m}$
- Rarely bear metulae
- Conidia globose
- Conidia very rough with thick walls



# Aspergillus Flavus and Parasiticus Agar - AFPA

- selective for aflatoxin producing mould species



- Potentially aflatoxin producing moulds are easily differentiated from other species on this medium by their bright orange reverse
- The colour is a result of a chelate bonding between aspergillic acid and ferric salts

- Incubation time 42-48 hours in 30°C

- Sporulation on AFPA is rather poor
  - Aflatoxin is not produced on AFPA
- Dichloran is added to the medium to inhibit fast growing fungi.
  - Antibiotics are added to prevent bacterial growth.
- Confirmations can be made on coconut extract agar (CEA) → fluorescence...
- ...or on yeast extract sucrose agar (YES) -  
→ chemical analysis

## AFPA - Limitations

- AFPA is not suitable for general quantification of moulds. For this, other media like DRBC (Dichloran Rose Bengal Chloramphenicol Agar) or DG18 (Dichloran 18 % Glycerol Agar) should be used.
- AFPA can only be used for detection of live fungi

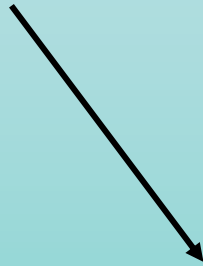
## Note!

- *A. sojae* and some strains of *A. sclerotium*, that form aspergillic acid but not aflatoxins, also form an orange reverse on AFPA. However, these species are very rare in foods.
- *A. niger* grows at the same rate as *A. flavus* and *A. parasiticus* and may form a yellow, but not orange, reverse on AFPA. *A. niger* forms black conidia after 48 hours of incubation.
- After > 48 hours *A. ochraceus* (orange reverse) and *A. tamaritii* (brown reverse) may be a source of confusion.

# Plating techniques - AFPA

Direct plating

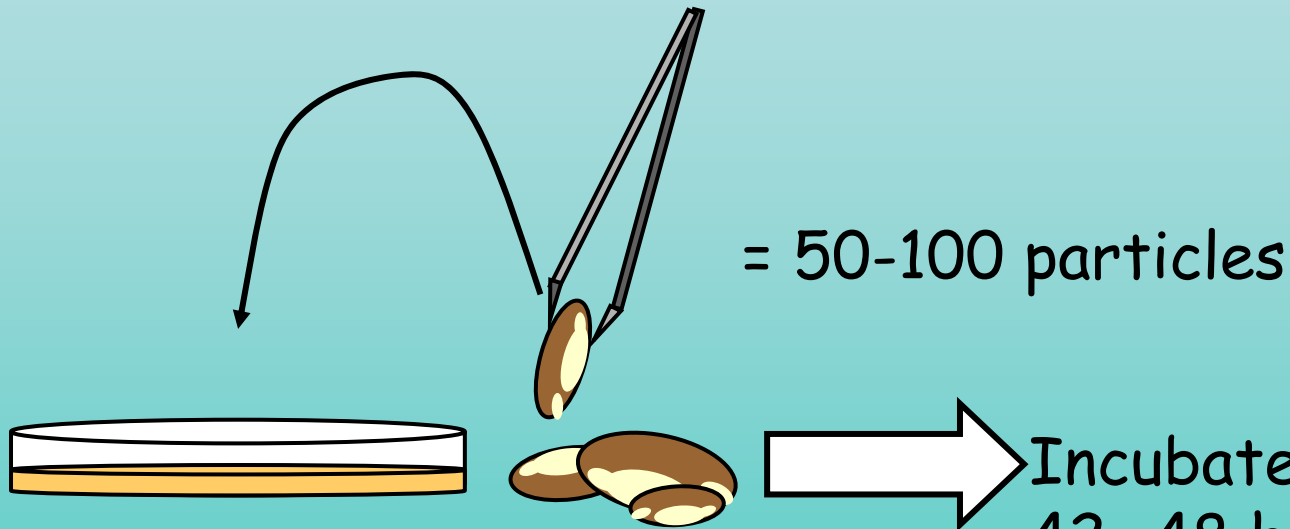
Dilution plating



- For particulate foods such as grains and nuts
- With surface disinfection (hypochlorite, 0.4% active chlorine) to detect fungi that have actually invaded the food
- Or, without surface disinfection e.g. for fungi that are expected to follow through the production chain

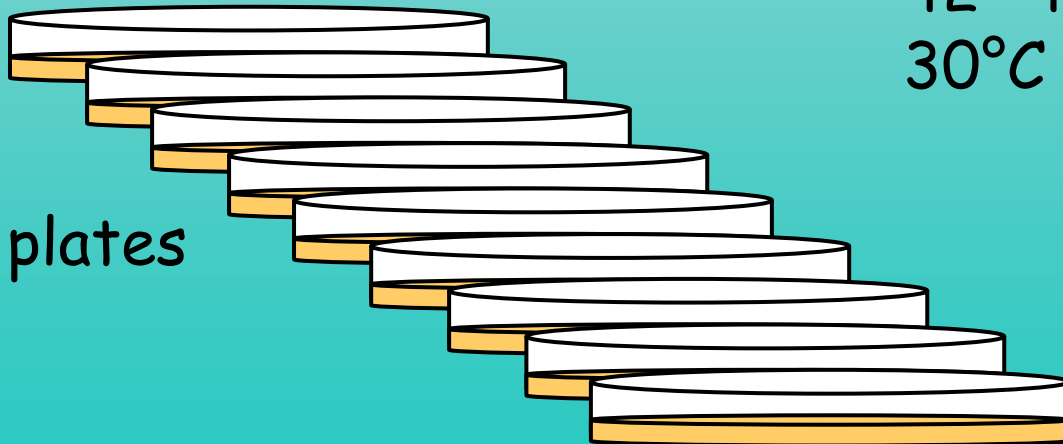
# Direct plating - AFPA

5-10 particles/plate



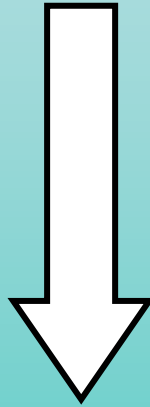
Calculate %  
infection

10 plates



## Direct plating - AFPA

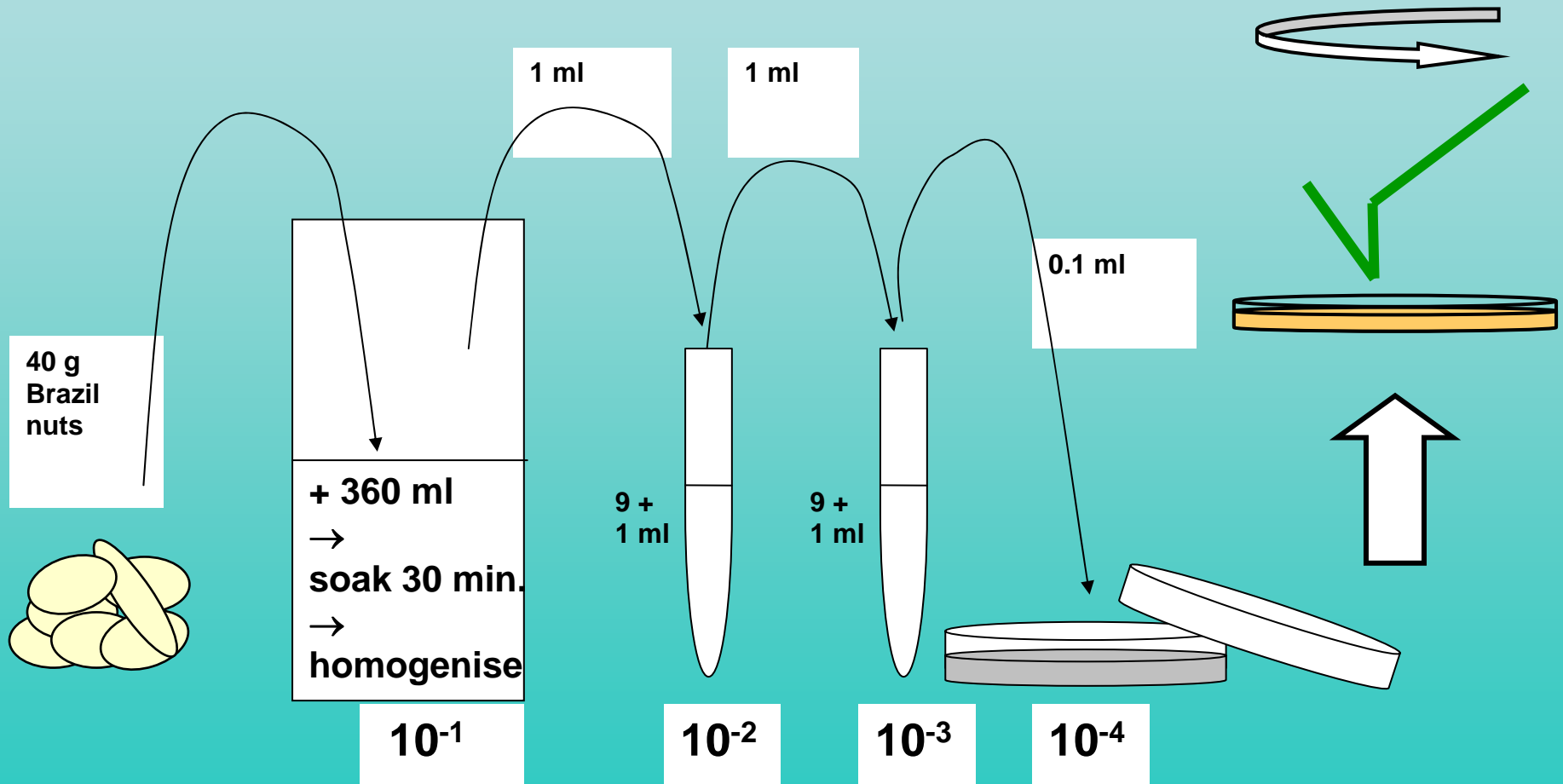
- Not correlated to fungal biomass



% frequency

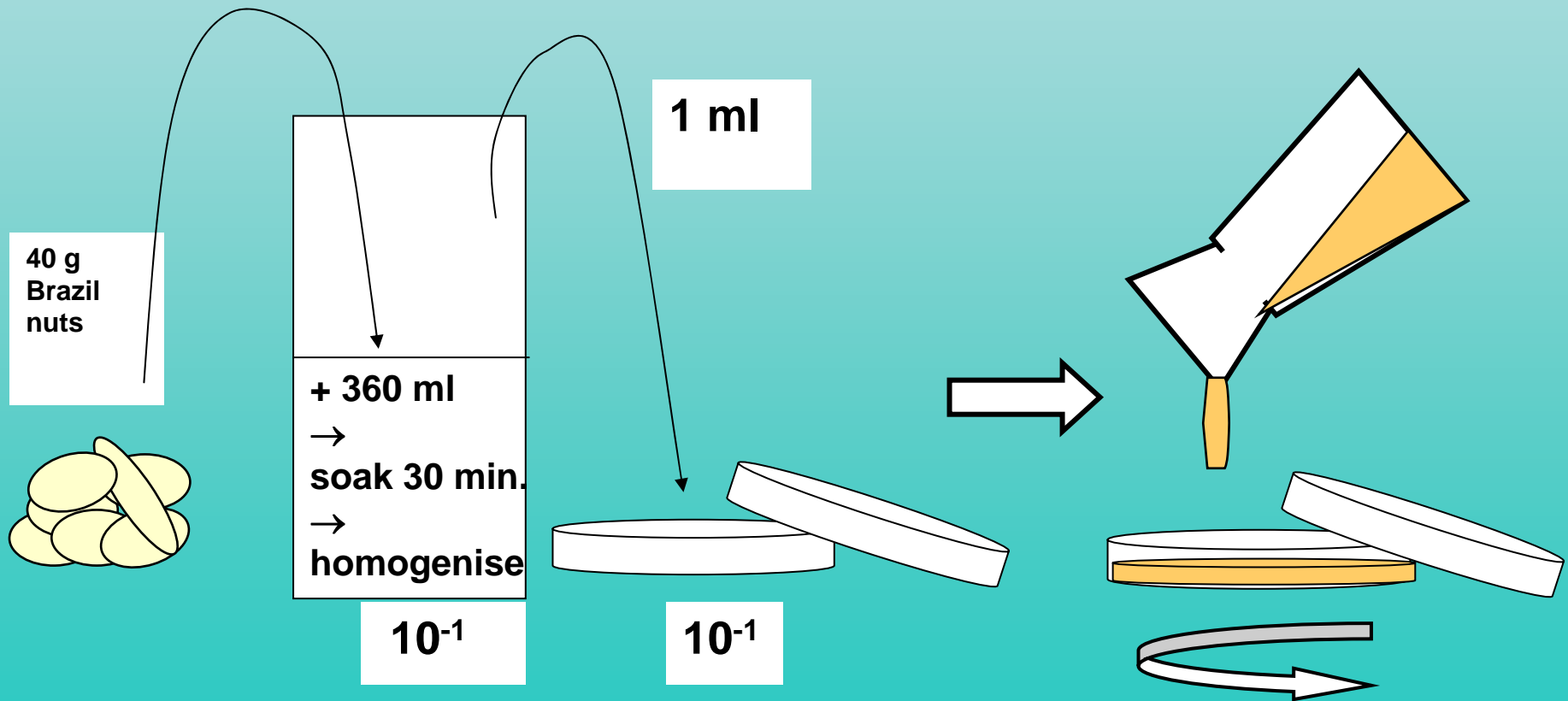
# Dilution plating - AFPA

## 1. Spread plating



# Dilution plating - AFPA

## 2. Pour plating



# Dilution plating - AFPA

- Incubate 42- 48 h 30°C

- Calculate the number of colony forming units per gram (cfu/g)



- Result correlated to fungal biomass

When the concentration is expected to be low

For example:

- Pour plate the first dilution
- Use a lower initial dilution, e.g. 1:5
- ...or, do both





# Isolation



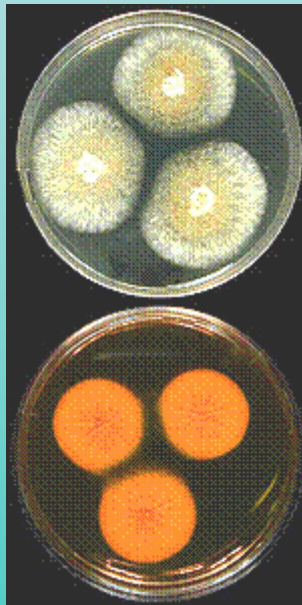
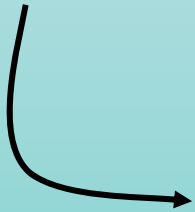
1. Malt extract agar (MEA) - Microscopy and/or short term storage



2. Czapek agar (Cz) - Colony colour



3. Coconut extract agar (CEA) or yeast extract agar (YES) for aflatoxin production



AFPA



# Long term storage

1. A few months → Slant agar  
(e.g. MEA)  
store in 1-4°C



2. Several years → Freeze-drying

# Safety !

- When handling dusty and heavily infected material, wear a facemask and gloves.
- When handling cultures, preferably work in a ventilation hood or similar that will transport dust, spores and volatile compounds away from your face.
- Avoid opening Petri dishes, but if it is necessary, do it carefully and away from your face
- Avoid sniffing cultures
- Use wetted loops when isolating fungi from sporulating cultures
- Wear a laboratory coat when working in the laboratory
- Unwanted cultures and other infected material should be autoclaved before being disposed of
- Keep the laboratory clean - use 70% ethanol for disinfecting
- Discard unwanted cultures regularly