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Mycology

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Mycology:

Deals with the study of fungi from the lower to the upper ones on the scale of evolution starting from the simple-structure and reproduction fungi to the most complicated ones in terms of their structure or their fruitage bodies. This discipline studies all types of fungi thoroughly including:

1- Studying the external appearance of fungi.

2-Reproduction.

3-living styles.

4-The vegetative structure of their bodies.

5-Propagation structures.

6-Sexual and nonsexual reproduction.

7-Life cycles.

8-Classification.

9-Their harmful and useful effects.

History of Mycology:

Mycology is derived from the Greek term Mycology which consists of two words, Mikes which means Mushroom and Loges which means science. The knowledge of man about fungi is as old as using bread, but this knowledge hasn't found its way into scientific study only after the invention of the microscope by **Livinhook** more than 400 years ago and that the study of fungi had begun to develop by **Antoni Michele** who is considered the founder of fungi science (the father of fungi science) as well as Carolus Linnaeus (1707–1778) (The father of Botany).

Then in 1837 came the scientist Person who was able to improve the microscope and had much more fungi followed by the scientist **Anton de Bary** who achieved many deeds particularly the discovery of many life cycles of fungi among which are **rust fungi**.

Mycology has made use from other sciences such as **Cell scienc**e, **Biochemistry** , **Molecular biology** and other sciences that helped in the development of Mycology through studying their division, their life cycles and the possibility of using them in solving human's problems and his wellbeing. The story of the discovery of Penicillin and other antibiotics are not far from our minds, and time might come when feasts contribute in solving one of the greatest problems of humanity: the lack of nutrition via making use of industrial and agricultural residues in producing **Single Cell Protein (SCP).**

Humanity has witnessed so many disasters caused by fungi. Examples for this is when the Russian army was infected in the 18th century, poisoned with the Ergotism disease because of feeding on flour containing stony bodies of ***Claviceps purpurea***.

Fungi has also caused a disaster in Ireland, t**he Irish Famine 1945-1946** which resulted in the death of more than million people who starved to death because they were infected by potato plants which were died because of blight disease caused by *Phytophythora infestans* and forced more than other million to immigrate to north America.

Fungi also caused another disaster in Bangladesh in 1941 when the rice crop was infected with blight disease caused by *Pyricularia oryzae* resulted in the death of about half million people.

What are fungi ?

It is too hard to give a constant definition of fungi due to the great number of this group, their diversified and overlapped characteristics. Still, the term fungi is referred to the group of the organisms that are characterized by having Eukaryote and Spores-bearing (Spores = germs), non-chlorophyll, sexual and asexual reproduction and their bodies are usually formed of varied linear structures and their cells are surrounded with a cellular wall .These walls contain Chitin . This definition leads to the following:

1. Eukaryotic organisms

2. The fungi's body consists of filaments called hyphae (cells) divided or undivided. Fungi have cells surrounded by a well-formed cell wall.

3. Cell walls contain **Chitin.**

4. Cell membranes contain ergosterol instead of cholesterol

5. Usually unmoving, yet the cells may be moving.

6. Reproduce by germs/spores. A spore is a simple reproductive unit where a new one is yielded through its growth.

7. Fungi do not have stems, roots or leaves and a carrier vessel system.

8. The fungi's body consist of multi-cells filaments and the cells are Eukaryotic.

9. Reproductive structures are different from the vegetative ones have different shapes and they are the base for fungi division except for a small number of vegetative parts(physical) of fungi that look like those of most fungi.

The importance of fungi:

1. Fungi are used in biodeterioration.Therefore, they clean the environment from animal and plant residues which is considered a positive feature. At the same time, fungi destroy skins, clothes and very expensive wood, which are used in the industry of roofs in many countries and cause great material and human losses.

2. The major reason for causing plant and veterinary diseases as well as many diseases for man, Mycoses.

3. Fungi are active in biodegradation due to their out-cellular enzyme potentials that help them in degrade a large number of materials.

4. They are used in producing a large number of materials such as organic acids and citric acid.

5. They are considered a good source of nutrition as a many types of them are planted commercially such as many types of mushrooms, edible fungi such as *Agaricus bisporus*.

6. They have significant benefits in agricultural , forestation and horticulture fields .They are used in biocombating to eliminate fungi, insects or other organisms such as using the fungus *Beauvaria bassiana* in insects combat.

7. Fungi contain a large quantity of proteins, which is a direct nutrition source such as *Fusarium venanatum and Agaricus bisporus*.

8. Many kinds of them produce toxic materials for man and animal, they are called Mycotoxins such as Aflatoxins. The fungus body might contain toxic materials and are called poison fungi (*Claviceps purpurea*)

9. They are considered the base for many industries such as fermentation, an example for that is Saccharomyces (*Saccharomyces: cerevisiae*) .

10. Some kinds of fungi create symbiotic relationships with high class plants and help them to grow better , an example for that is the Mycorrhiza or with Lichens.

11. They are used to produce different types of medicines, vitamins and a number of antibiotics commercially (Penicillin and Cephalosporin) .

At the academic level (the study and the research), fungi are no longer confined to fungi scientists only, rather they have become favored research tools used by scientists of cytology, genetics, biochemistry and molecular biology due to the **following characteristics**:

1. Fast growth rate .

2. Short period of generation for most fungi.

3. Produce (n) Haploid spores, which make their genetic study easy.

4. Grown in small places, may be in a test tube and on simple environments.

5. Give huge number of strains (individuals).

Nutrition and Growth of fungi:

Fungi cannot get food by themselves; rather they depend on the biomaterials they deteriorate or by invading the living cells. They also get their food by degrading materials into simple compounds easy to be absorbed, heterotrophy.

Fungi are divided-according to nutrition- into two main groups:

1. Parasitic Fungi

2. Saprophytic Fungi (Saprotrophic)

The fungi of the first group get their food by parasite on other organisms, while the second group's fungi get their food by degrading organic matter dead (plant and animal residues).

In addition to the previously mentioned groups, there are other ones derived from them as follows:

1. **Obligate parasite fungi**: this type of fungi can't live and grow unless parasite on living cells,

2. **Facultative Parasitic Fungi**: Saprophytic fungi but can parasite on organisms under certain circumstances.

1. **Obligate Saprophytic Fungi**: they are only saprophytic on organic materials and never have the ability to parasite.

2. **Facultative Saprophytic Fungi**: parasite fungi but could be saprophytic under certain circumstances in the absence of the host or the sustainer they parasite on.

Fungi could be symbiotic with other organisms such as plants particularly with the roots of higher-class plants. These structures are called Mycorihiza. The fungus absorbs nutrition materials and deliver them to the plant, and then the plant ensures a suitable environment for the fungus. There are also symbiotic relations between fungi and mosses called mosses.

Fungi differ from most plants for their need to readymade and complicated food materials, (they are heterotrophic organisms, i.e. could not make food by themselves rather they degrade organic materials into simple compounds to get elements and energy out of them). Yet, if they were provided with a carbon source such as (Sugar, Maltose, Glucose), they will be able to use these sugars to make proteins and make use of Nitrogen that could be supplied from a nonorganic source such as Nitrates or from an organic source such as (Amino acids) including the (Arginine) plus making use of mineral salts. The following elements are required by all fungi: (C, O, H, N, P, K, Mg, Mn , S, B, Mo ,Cu, Fe, Zn) and some fungi need Ca but its role has not been identified.

In general, Glucose is considered the best source for Carbon and organic, Nitrogen is the best source for Nitrogen followed by Ammonium compounds and Nitrates.

Most of the fungi are able to make the necessary vitamins required for their growth. Yet, a few number of them need certain vitamins such as Biotin, Thiamine or the origins of these vitamins (Precursor's) which are added to the nutrition environment or medium.

Fungi vary in their nutrition needs as some of them can coexist with an organic medium like the *Penicillium* and *Aspergillus* fungi which have the ability to grow on fruits, skins and seeds provided that an amount of moisture, while other fungi have limited growth such as the fungi that parasite on a certain group or a specified type or a single descent of the sustainer. Generally, the enzymes that the fungi have bound their ability to make use of the nutrition sources upon which most of the fungi exist.

Fungi grow in temperatures range between 0-35°C, but the optimum degree ranges between 20- 30°C. There are fungi that could live under 0°

or above 50°C. Organisms are generally divided-per temperatures suitable for growth- into groups with no boundaries separate between them as follows:

1. **Psychrophilic**: (cold-living fungi): grow between 0° and 10°C and the maximum is 25°C.

2. **Mesophyllic :** optimum temperature for growth is 5°C or more and could reach 70°C or **more and the minimum is 25°C**.

3. **Thermophiles**: (Heat loving): grow in a middle range of temperatures from 25 to 45°C or more .

4. **Hyper thermophiles** : this type prefer growing in 75°C and can grow in higher temperatures as in the *Pyrodictium* species and the optimum temperature for this type is 82°C and could endure up to 110°C.

Fungi prefer to grow in the acidic nutrition (agricultural) medium and pH-6 is considered the best conditions for most fungi.

Though fungi do not need light during growth, however light is important in Sporulation and Spore dispersal. Some of spores-carrying fungi are of positive phototropic where they throw their spores in the direction of the light.

Structure of fungal body

Fungi's bodies greatly vary in size and shape starting from the uni-cell (yeast) which can be seen by microscope only to the big mushrooms' bodies, which represent only the fruit bodies of fungi. The body of fungus may be apparent over the medium it lives in or concealed or sunken into the sustainer's tissues it parasite on.

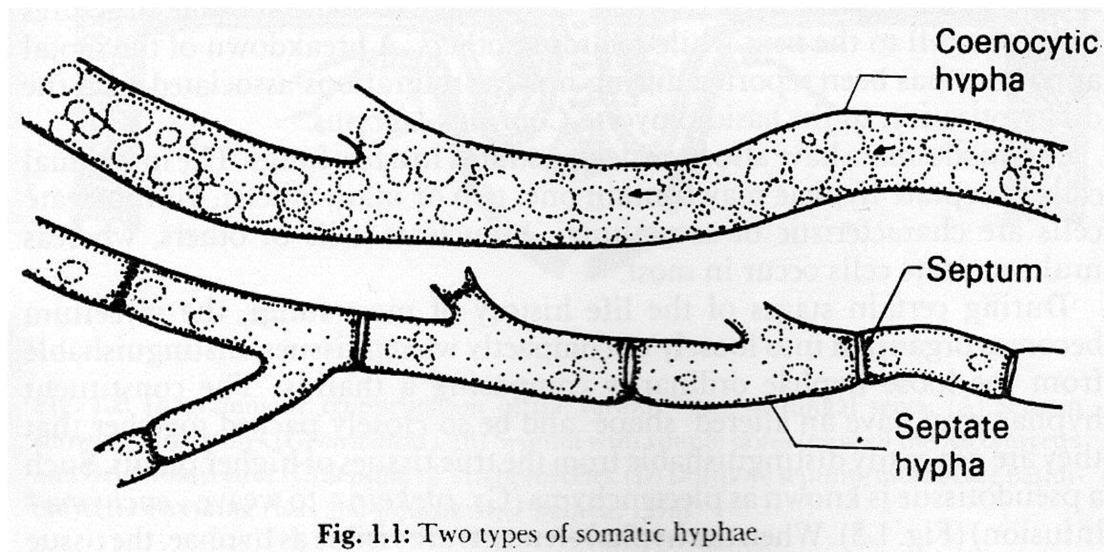


Fig. 1.1: Two types of somatic hyphae.

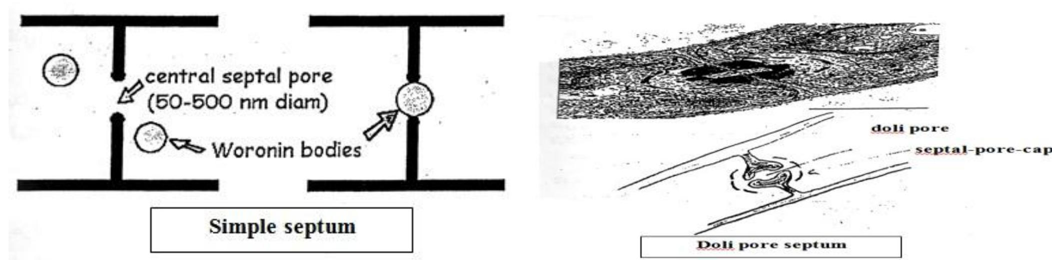
There are many fungi of unicell (Yeast) or might be in the form of (Plasmodium) looks more like animal's body than plant such as in jelly fungi. However, the body in most fungi consists of minute filaments with too many ramifications called the fungal filaments or the Hyphae (the singular form is Hypha). These Hyphae are usually have no color with white cottony appearance or might have different colors (red, orange, black). These filaments form fungal isolation called (Mycelium) and they might be ramified or non-ramified, divided or undivided (Septate or No-Septate) and the latter is called (Coenocytic).

In this case, fungal isolation is tube filaments where Cytoplasm continues to go through without septa and most of them contain nucleus. In Septate fungal cells might be (Uninuclate) or (Binucleate) or

(Multinucleate). Cells are separated from each other by means of Septa (the singular form is septum) and often contain central pores through which the cytoplasmic filaments pass representing the continuation of the living material among fungal cells. Crosswise septa are formed as an internal circle of a wall, then gradually widen towards the center, then the process ends.

The central pore which allows food pass through from one cell to another and sometimes the nucleus and the cavity is formed besides a little formation of septa at the back of the Hyphal tip. The septa is simple in all fungi except in some types of Basidiomycetes and it is a sort of a middle opening in the wall with few simple modifications. Round-shaped bodies might exist on both sides of the pore called **Woronin bodies** play a protection role for the fungus when exposed to inconvenient conditions. These bodies block the pore exposed to bad condition in order to prevent the fungus's content going out and to keep the fungus sound.

In Basidiomycetes, the septum is more complicated as the parts of the transverse wall surrounding the pore swallow up such that take a barrel shape. Moreover, the pore opening is surrounded with fragments of Endoplasmic Reticulum (ER) which take the shape of the arches surrounding the pore called Parenthosome or Septal pore cap , the pore is called Doli pore and the septum is called Dolipore Septum.



Septate Hyphae

Tissue of fungi:

Fungus isolation takes the shape of disassembled filaments unconnected together like many other fungi (*Rhizopus*), but in other types of fungi, the fungal isolation takes the shape of tissue and it might be disassembled and the fungal filament are apparent. This tissue is called, **Prosenchyma Tissue.**

The hyphae cohesive fungal filaments, which is not a characteristic of filaments and appear in a form of cells in the cross section. This tissue is called the **Pseudoparanchyma T .**

The disassembled fungal tissues that resemble the **Plectenchyma** tissues or special structures that might be vegetative or reproductive such as the Strome in ascus fungi or constitute the (Sclerotium) in a great number of fungi including the *Botrytis.*

Fungal Cell:

As in other organisms, the fungal cell consists of the following:

a. Cell wall ,

b. Protoplasm

and consists of:

1. Cell membrane,
2. Cytoplasm,
3. Endoplasm Reticulum,
4. Eukaryotic Nucleolus ,

5. Mitochondria,

6. Golgi apparatus (Dictysomes)

7. stored food stuff (lipids,Glycogen) ,

8. Lysosmetes ,

9. vacuoles .

There are a few major differences between animal, plant, fungal, and protistan cells, and guess what? Here they are:

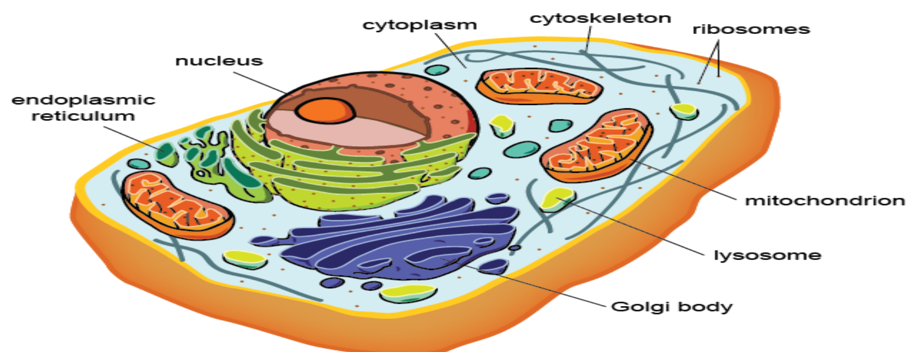
***All* plant cells have**

1. A cell wall made of cellulose ,
2. 2-A large central vacuole ,3-Chloroplasts

Some animal and protistan cells have

1. Flagella ,
2. 2-Cilia

All animal cells have Centrioles Well, that was short.



All fungal cells have

1. A **cell wall** made of chitin. ,
2. 2-Hyphal Apex and hyphal extention.

Hyphal Apex and Hyphal Extension:

Fungal Hyphal have the ability to grow under suitable conditions and naturally the fungal colonies have the ability to grow for many years up to 400 and more (perhaps certain types of Mycelium but not the same cells whose age is more than thousand years).

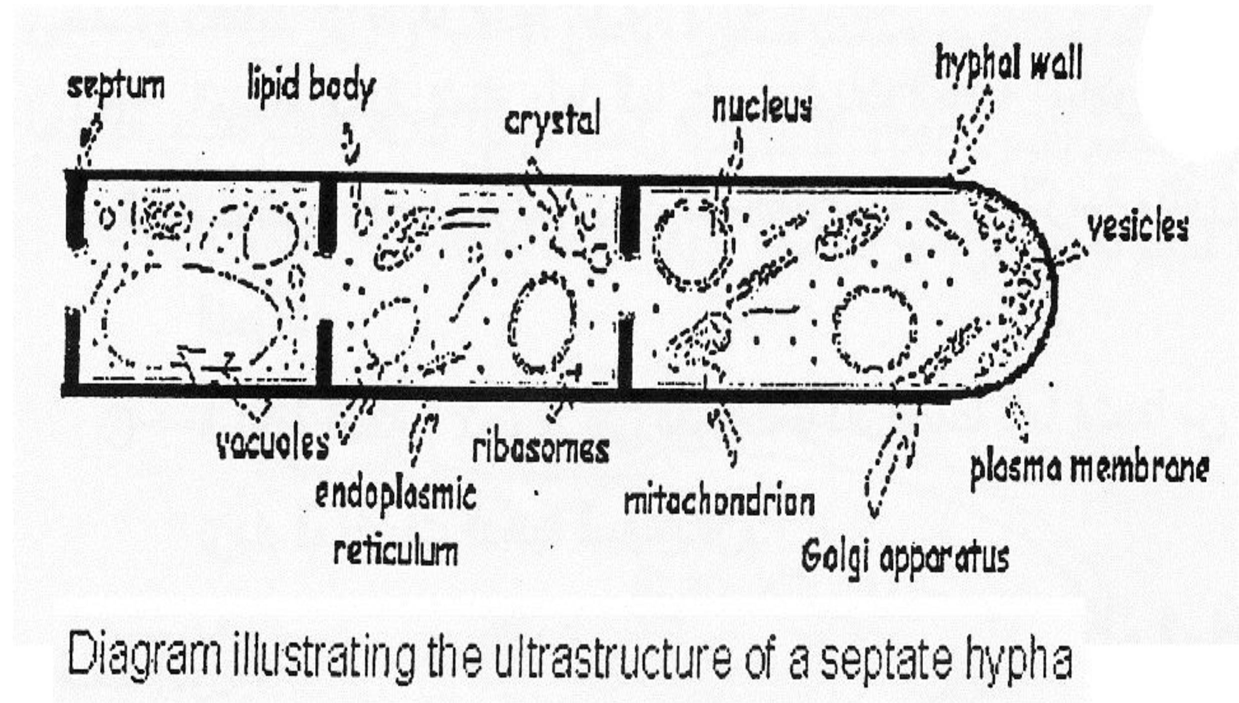


Diagram illustrating the ultrastructure of a septate hypha

Mycelium starts as a short grow germination tube in all directions from the central point and is a round colony. This is applied on industrial solid environments, while in liquid environment the fungi is a ball-shaped colony. But in normal conditions this doesn't happen. Hyphae grow only from the apical zone and there are studies with the assistant of light and electronic microscopes showed that there are three zones for hyphal tip:

1. Apical zone ,

2. Sub-apical zone ,

3. Zone of vacuolation

1. The first zone:

A large number of Cytoplasmic vesicles, which cause the displacement of other contents.

2. The second zone:

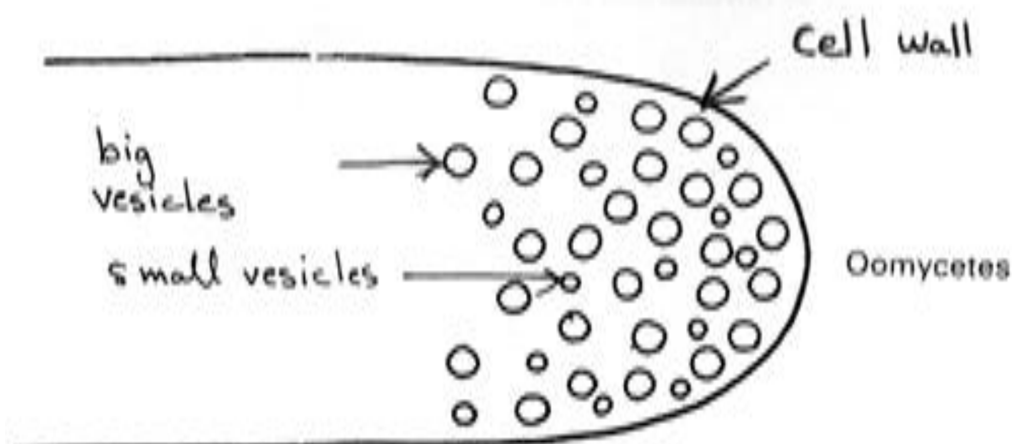
Contains Cytoplasmic cellular compositions such as the nucleus, the Mitochondria, Golgi bodies, Drybosomat and Endoplasm net. The zone is void of vacuoles.

3. The third zone:

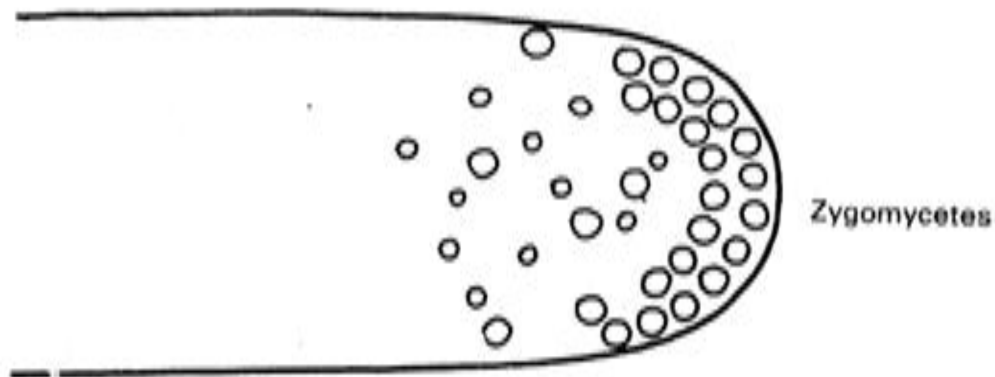
Contains vacuoles and expands whenever we move away from the hyphae end and the lipoid content increases (Cytoplasm).

Vesicles are distributed in the first zone (Apical zone) into **three types as follows:**

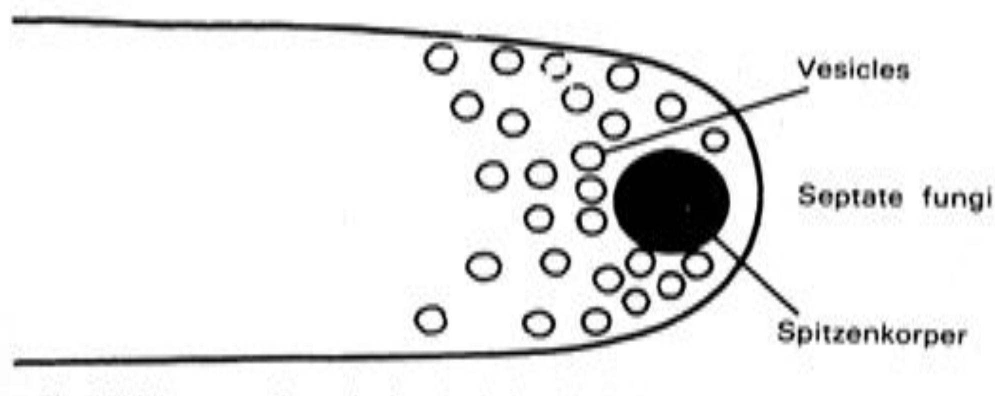
1. The first type (Oomycetes): Vesicles are regularly distributed within each apical zone. They are of two types: small and big.



2. The second type (Zygomycetes): the existence of the small and big Cytoplasmic vesicles at the apex of the hyphae and a part of the vesicles arranges and takes the shape of a crescent lining the apex.



3. The third type: Divided fungi (Ascomycetec, Deutteromycetes, Basidiomycetes), the Cytoplasmic vesicles are roughly of the same size and are regularly distributed in the apex, but the apex of these fungi are characterized by having a circular zone within the vesicles void of vesicles except for small ones, some membrane and Ribosomes. This zone is called by the same name of the black body Spitzenkorper, which is believed to related to the Hyphae growth organization and this body's direction plays a role in the course deviation of the Hyphae during growth.



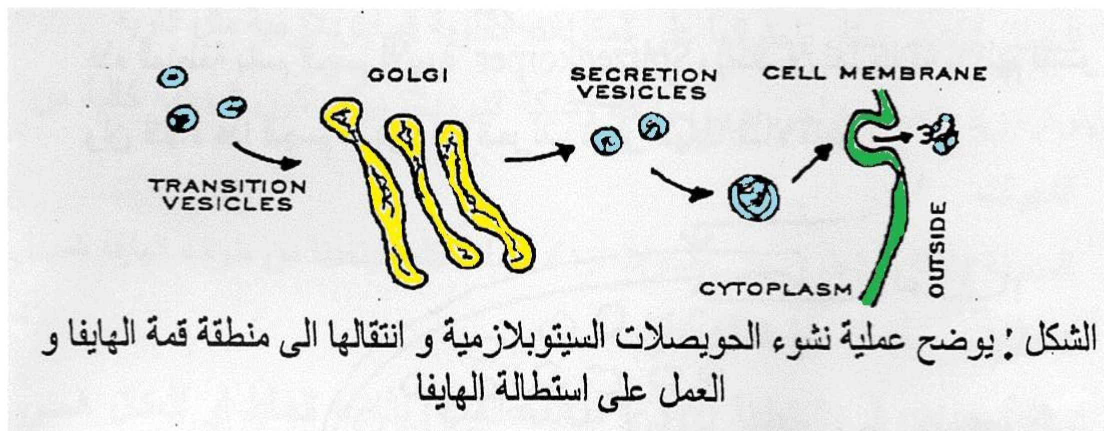
Ascomycetes ,Basidiomycetes

It is believed that the apical vesicles evolve from the Endroplasmic net in the zone under the apical, and then pass through (Golgi body, Dictysome) the stage of maturity. After that, they move up to the apical zone where they either secrete their contents or might integrate with the plasma membrane.

As a preliminary step of the extension of Hyphae, Cytoplasmic vesicles are formed from the Endoplasmic net then Colgi system (Dictosome) in the zone under the apical and the vesicles are loaded with the compositions of the cell wall or its Precursors. Some of them are loaded with enzymes to deteriorate the old wall and others are loaded with enzymes to build the new wall by the compositions loaded with other vesicles, which move and reach:

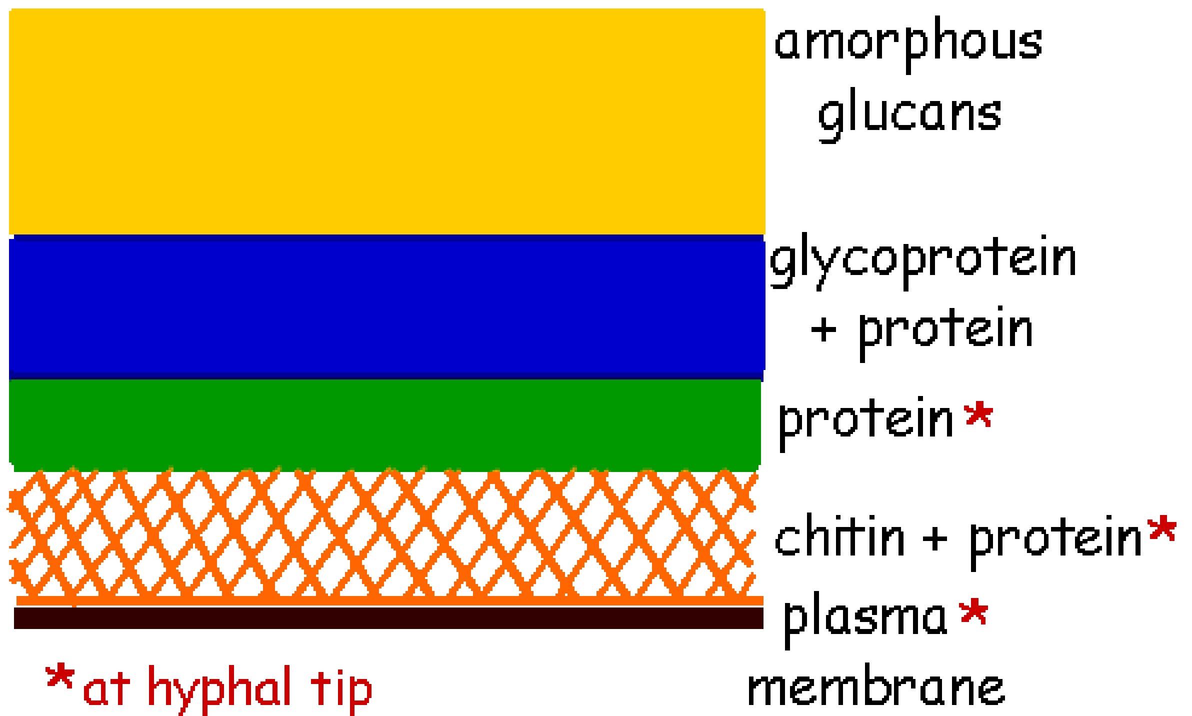
1. The **apical zone** where they unload contents outside the cellular wall zone and the loaded enzymes attack the Microfibrils of the old wall and break them, consequently they lose the ability to resist the osmosis of the Cytoplasm pushing the new membrane of the vesicles towards the new wall. Then the process is repeated whenever new vesicles reach.

2. **Growth zone**: Because of constant deterioration and building process due to addition of new compositions, growth is resulted in Hyphae apical zone.



الشكل : يوضح عملية نشوء الحويصلات السيتوبلازمية و انتقالها الى منطقة قمة الهايفا و العمل على استطالة الهايفا

Cell Wall Composition :



Fungal cell wall is distinguished with its complicated structure and its variant chemical compositions from one fungus to another. Chemical analyses have proved Carbohydrates exist in a shape of multi sugar Polysaccharides plus proteins, fats and other compositions. The most important of the Carbohydrate compositions that are in the form of minute fibers that make the wall solid are the (Microfibiri-CHO, **Chitin**, Chitosan, and Cellulose ??. Carbohydrates might not be in a form of fibers rather in a form of matrix. Moreover, wall compositions are not only different in the engineering of compositions per different fungus, but also per different stages of fungus growth as well as the different environmental factors that surround them. Cellular wall consists of several zones.

Special Issue "Fungal Cell Wall"

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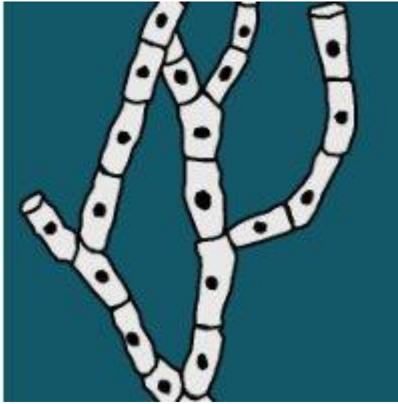

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Guest Editor **Dr. Anne Beauvais** (Institut Pasteur, Paris, France)

Special Issue Website: http://www.mdpi.com/journal/jof/special_issues/cell_wall

Dear Colleagues,

The fungal cell is surrounded by a wall, which acts as a sieve, as well as a reservoir, for effector molecules that play an active role during infection. Considered a living organelle, the cell wall is essential for growth and crucial for resisting host defense mechanisms. Polysaccharides constitute >70% of the cell wall, and their biosynthesis is under the control of three types of enzymes: Transmembrane synthases, cell wall associated transglycosidases, and glycosylhydrolases; these are responsible for the remodeling of the de novo synthesized polysaccharides resulting in the characteristic three-dimensional structure of the cell wall. While certain polysaccharides are species- or genus- specific, some of them, such as β-(1,3)-glucan and chitin, are common to all fungal species, but both display fungus- and morphotype-specific differences in their

concentration and localization. The cell wall is covered by an outer layer or extracellular matrix with hydrophobic, adhesive, and protective properties and plays a major role during infection. Importantly, host recognition of the fungal cell wall is essential for the initiation of the immune response. Moreover, because of their specific composition, the fungal cell wall and its outer layer are unique targets for the development of drugs against pathogenic fungal species.

Dr. Anne Beauvais

Guest Editor

Reproduction of Fungi:

Reproduction is a sort of producing new individuals holding all the properties of fathers. In fungi, there are **two types of reproduction** as follows:

1. Sexual Reproduction

2. Asexual Reproduction

Asexual reproduction is called **Somatic or vegetative** and it doesn't include nucleus combination, reproductive cells or organs.

Sexual reproduction is characterized with the combination of two nucleus and all fungi have the ability reproduce in one way or more ways of asexual reproduction but not all fungi have sexual reproduction ways. At sexual and asexual reproduction, the whole thallus (body of the fungus) might change into a single reproductive organ or more and thus, somatic or reproductive structures don't exist on one individual. These fungi are called the college of fruits (**Holocarpic Fungi**). In the vast majority of fungi, the sexual organ is created from a part of the fungus body and the remaining part are somatic structures. Fungi in this case are called **Eucarpic Fungi** . and are more specialized and high-ranking than the primitive fruits.

Asexual Reproduction :

Means of asexual reproduction are more important for fungi than sexual reproduction regarding propagation for its ability to produce new individuals without opposition that might happen between two individuals and it happens more than once in a single season or in one growth stage whereas sexual reproduction needs the opposition of two pairs of

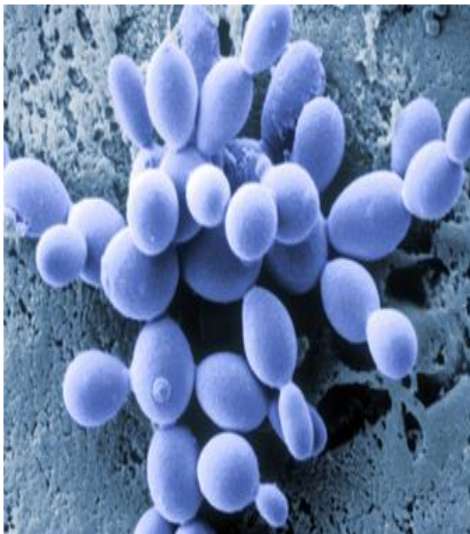
individuals that are sexually compatible. Asexual reproduction gives huge numbers compared to sexual reproduction .

Asexual reproduction occurs in many methods as follows:

1. **Fragmentation:** The fungus body is fragmented into small parts, each one of them can grow to be a new fungus. This method is used in laboratory in most of *Aspergillus* and *Fusarium*.

2. **Fission:** Vegetative cells split into two cells with two nucleuses and each cell gives a new birth as in yeasts.

3. **Budding:** One or more buds grow from the vegetative cell and each bud splits and form a new birth as in yeasts, then another bud might grow on the first one and so forth. Consequently, mycelium is formed called the Pseudomycelium.



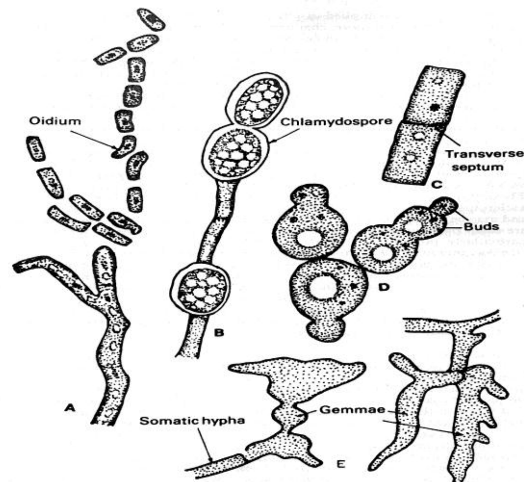


Fig. 1.22: Vegetative reproduction. (A) Fragmentation of hypha to form oidia. (B) Chlamydospores. (C) Fission. (D) Budding. (E) Gemmae formation.

Spores formation:

It could be said that any cell of the fungus can play the role of the spore if it was able to separate and live independently and then it could be considered asexual spores. However, most of the spores bear spores on special bearings varied in shape, size, color and the number of their cells, something made the scientists of fungi classification depend on them in fungi's' diagnosis and division. According to the methods of asexual spores formation, they could be divided into **two basic groups**:

1. Sporangiospores;

2. Conidiospores:

Sporangiospores are borne on bearings called Sporangiophores. These bearings swallow at the end and sometimes interfacial to form a Sporangium surrounded with a thin membrane wall called Peridium within which Protoplasm contains many (n) Haploid uni-nucleus group. The protoplasm splits to multi uni-nucleus parts soon each of them becomes round and together form a large number of asexual spores which could be movable by whips called (Planspores), (Zoospores) and moving spores.

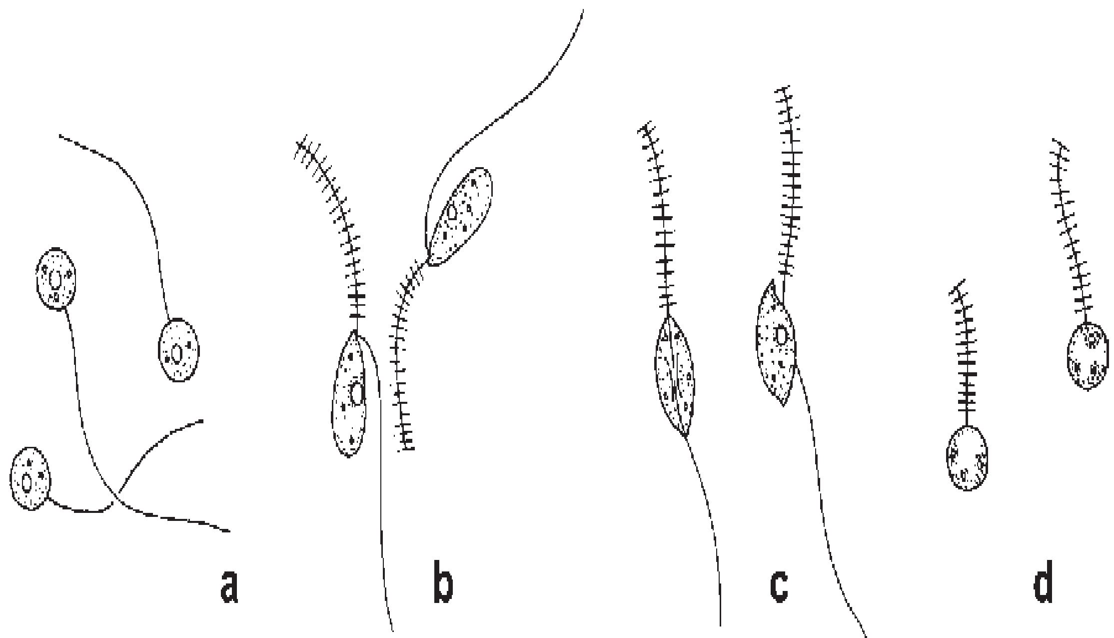
Spores formation:

1-One whip a filament type, back site (Whiplash). E.g. Chytridiomycetes.

2-One whip tensile type front site. E.g. Hypochytridiomycetes.

3-The spore has two whips one is filament whiplash and the other is tensile.
E.g. Oomycetes.

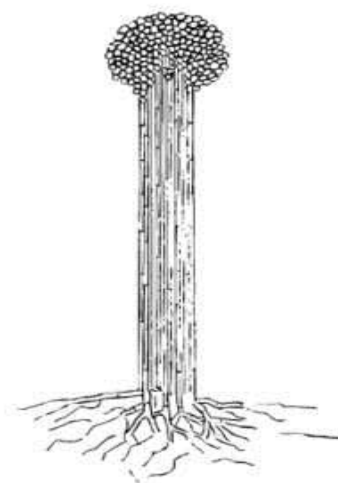
4. The last type of moving spores has two whips of filament type whiplash.
E.g. Plasmodiophoromycetes.



Asexual spores borne with Conidiophores in different ways. They are different spores as there is no membrane or a fragment that surrounds it. Conidiophores could be separate or gathered with each other and are borne either separately or in chains. Conidiophores could gather with each other in different ways and form different asexual fruity bodies as follows:

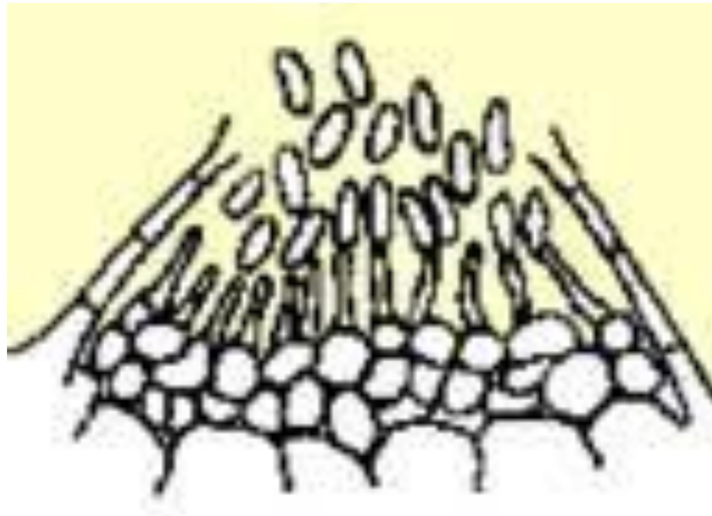
1. Synnemata (synnema) or Coremium:

Conidiophores here take together the shape of a standing unlimited growth column in some cases and Conidia are produced laterally whereas the apex still able to grow such as *Arthrobotryum*.



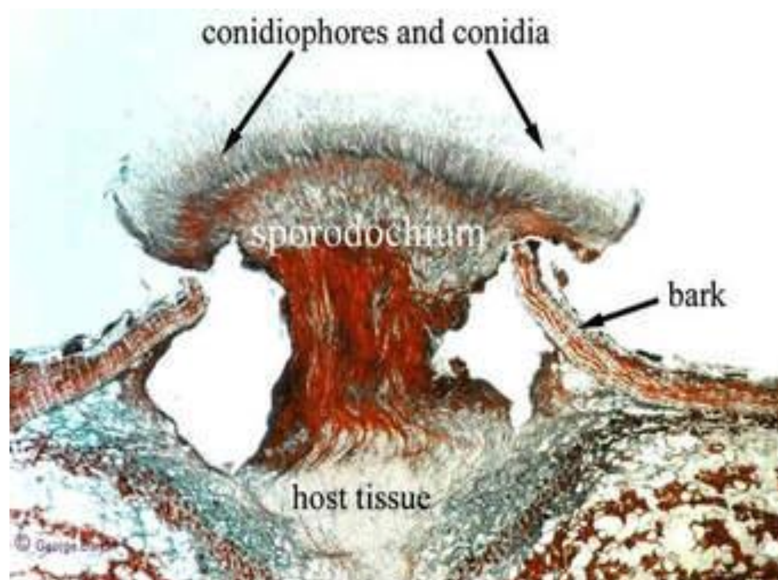
3. Acervulus:

This body is made up of Stroma from which the Conidia is stemmed from vertically. Conidiophores are outside the sustainer's body and the Conidiophores are not arranged. E.g. *Fusarium*.



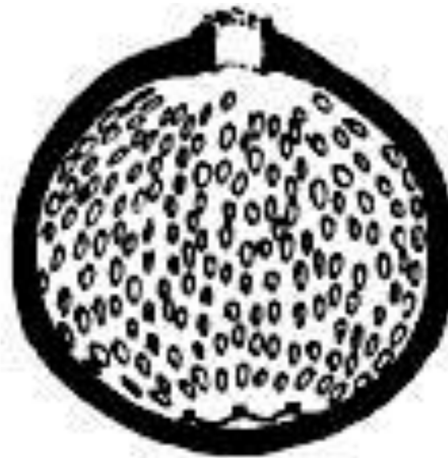
3. Sporodochium:

The Conidiophores are vertically arranged, short and perpendicular on the Synnemata. The sporodochium might contain sterile capillaries called (Setae) or might not. E.g. *Colletotrrichum*.



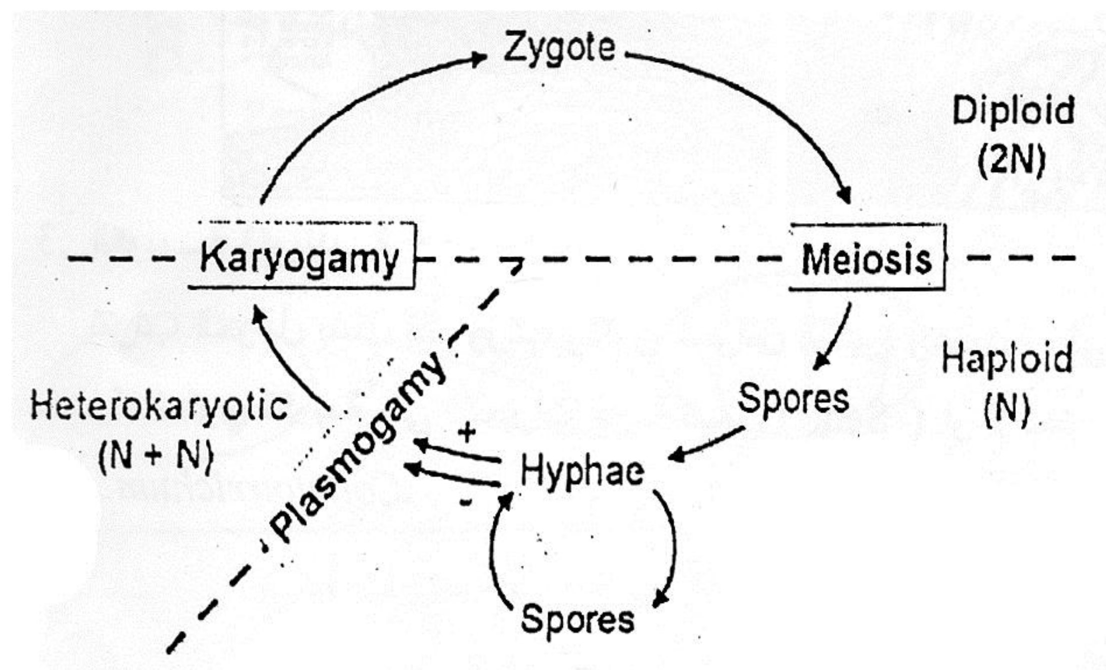
4. Pycnidium:

Short simple Sporespholes surrounded with Pseudoparanchyma wall taking a ball or flask shape. E.g. *Septoria*.



Sexual Reproduction :

This kind of fungi reproduction as in other organisms includes a combination of two nucleus , integrating their genetic contents then redistributing them by Meiosis process. Sexual reproduction process involves **three successive stages as follows**:



Sexual Reproduction in Fungi

1- Plasmogamy

It is the union of protoplasts of reproductive hyphae or cells, one from the male and the other from the female to bring about the nuclei of the two parents close together as a pair. However, the two nuclei do not fuse with each other. Such a cell is called a **dikaryon**. The dikaryotic condition is unique to fungi and may continue for several generations as the two nuclei (dikaryon) divide simultaneously during cell division. These are passed on to the daughter hypha.

2-Karyogamy

The fusion of the two nuclei which takes place in the next phase is called karyogamy. It may immediately follow plasmogamy as in lower fungi, or it may be delayed for a long time as in higher fungi.

3-Meiosis

Karyogamy which eventually occurs in all sexually reproducing fungi is sooner or later followed by meiosis producing four genetically different spores.

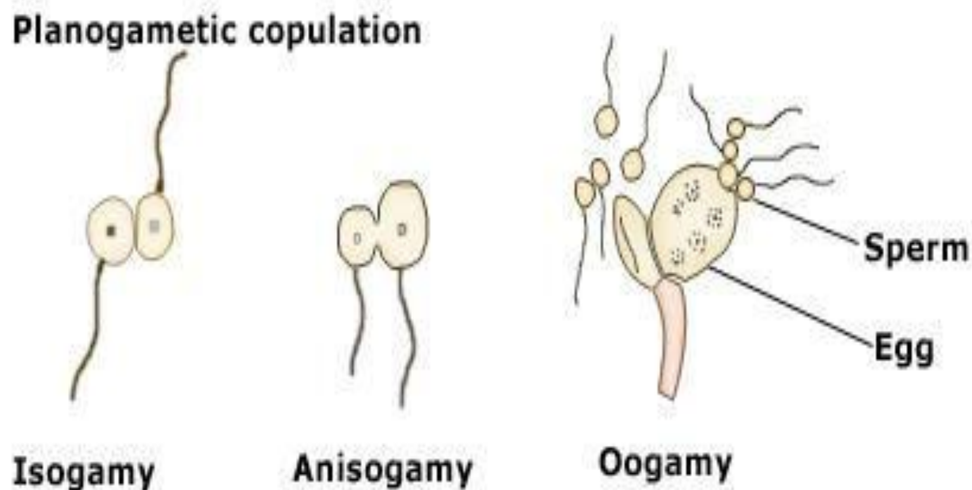
Reproductive organs of fungi are named by the name of the gametes cases (Gametangia, the single form is gametangium). The organs might be specialized sexual cells called Gametes. Gametangia and Gametes might be of the same shape called, Isogametangium or Iso-gametes. But if the male organs are different in shape from the female organs, the female ones are called, Oogonium and the males are called Antheridium. There are many ways or means by which the process of Plasmogamy between two

compatible cells is performed. These are called sexual reproduction ways as the process of sexual reproduction starts with.

Methods of sexual reproduction(Different Methods of Plasmogamy)::

1-Planogametic Copulation

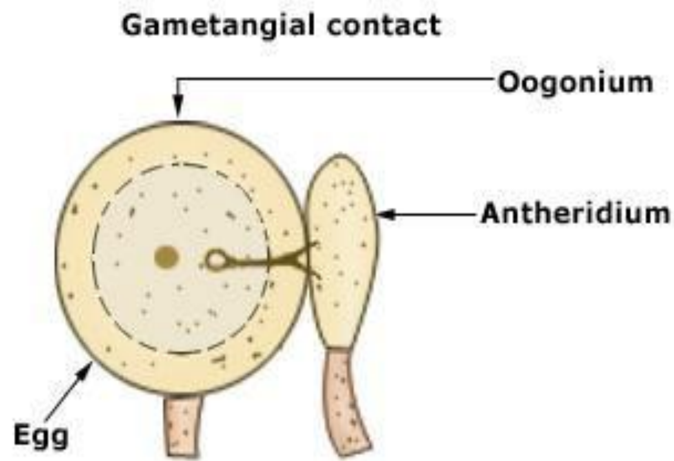
It involves fusion of two gametes. Like in algae sexual union in fungi may be isogamous, anisogamous or oogamous. **Anisogamy** and **oogamy** are together called heterogamous sexual reproduction. Isogamy is the simplest type of sexual reproduction, where the fusing gametes are morphologically similar e.g. *Olpidium* and *Catenaria.* Anisogamy, where the fusing gametes are dissimilar is found in one genus, *Allomyces,* a chitrid. In oogamy as you may recall the motile antherozoid enters oogonium and unites with egg or oosphere forming a zygote. Oogamy is seen in fungi like *Pythium* and *Albugo.*



2-Gametangial Contact

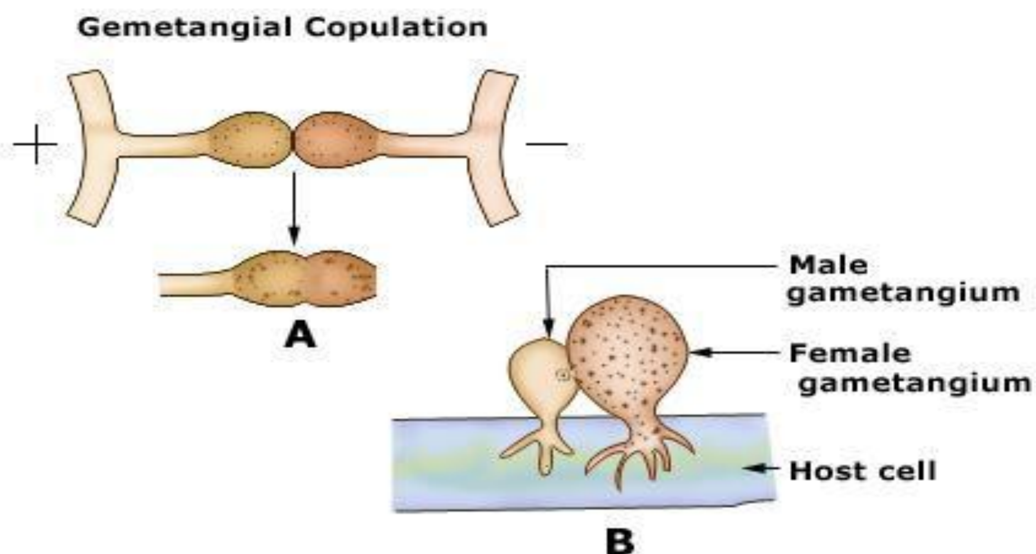
The male gamete is not a separate entity but the nucleus in the antheridium represents the gamete. As you can see in the Fig. 9.6 the oogonium and antheridium form a contact through a tube and one or more

nuclei, inside the antheridium migrate into the oogonium. You may note that in this case the two gametangia do not fuse. It is observed in *Penicllium.*



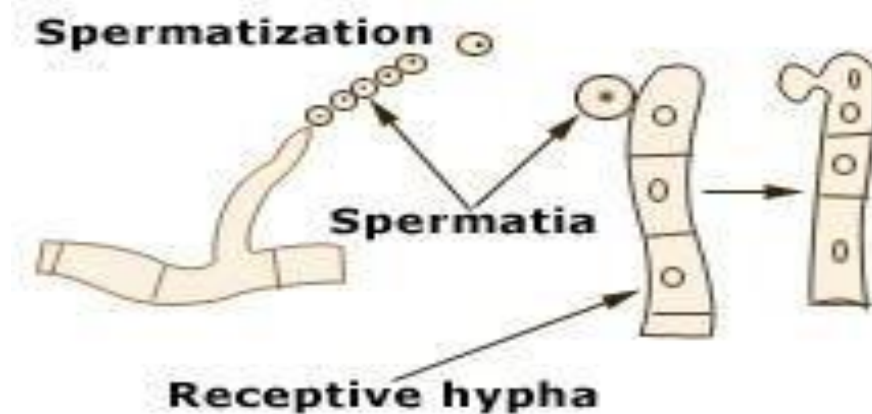
3-Gametangial Copulation

The Go gametangia make contact and the entire contents of the two fuse together and become one e.g., *Mucor* and *Rhizopus.* In some fungi the entire protoplast of one gametangia flows into the other through a pore. Among the two, the recipient is the female and the donor is the male.



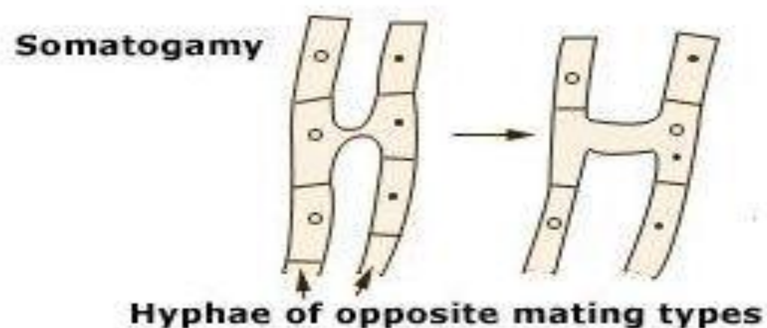
4-Spermatization

This mode is quite remarkable as the minute conidia like gametes called spermatia are produced externally on special hyphae called spermatiophore. Spermatia may develop inside the cavities called spermatogonia. The female cell may be a gametangium, a specialised receptive hypha or even a vegetative hypha.



5-Somatogamy

In higher fungi like Ascomycetes and Basidiomycetes there is a progressive degeneration of sexuality. The entire process is very much simplified by the fusion of two mycelia which belong to opposite strains. The post-fertlization changes result in the production of a fruiting body which is called ascocarp in Ascomycetes and basidiocarp in Basidiomycetes.



Life cycle of fungi

In the life cycle of a sexually reproducing fungus, **a haploid phase** (n) alternates with a **diploid phase**(2n) .The haploid phase ends with nuclear fusion, and the diploid phase begins with the formation of the **zygote** (the diploid cell resulting from fusion of two haploid sex cells). **Meiosis** (**reduction division**) restores the haploid number of chromosomes and initiates the haploid phase, which produces the gametes. In the majority of fungi, all structures are haploid except the zygote. Nuclear fusion takes place at the time of zygote formation, and meiosis follows immediately. Only in *Allomyces* and a few related genera and in some yeasts is alternation of a haploid thallus with a diploid thallus definitely known.

1- **Asexual cycle** (Imperfect fungi – *Penicillium* + *Aspergillus*):
Commonly found in imperfict fungi where the fungi spend the life cycle the (n) phase because these fungi lose sexual reproduction like the *Penicillium* and the (2n) phase doesn't appear in these fungi.

2- **Haploid cycle** (Zycomycetes and Ascomycetes): This cycle is common in Zygomycetes and some Ascomycetes. These fungi are characterized with spending the whole life cycle roughly in the (n) form except for the stage of (2n) zygote formation.

3- **Haploid cycle with restricted dikaryon** (*Neurospora*): This cycle occurs in some types of Ascomycetes as in the life circle of *Neurospora*

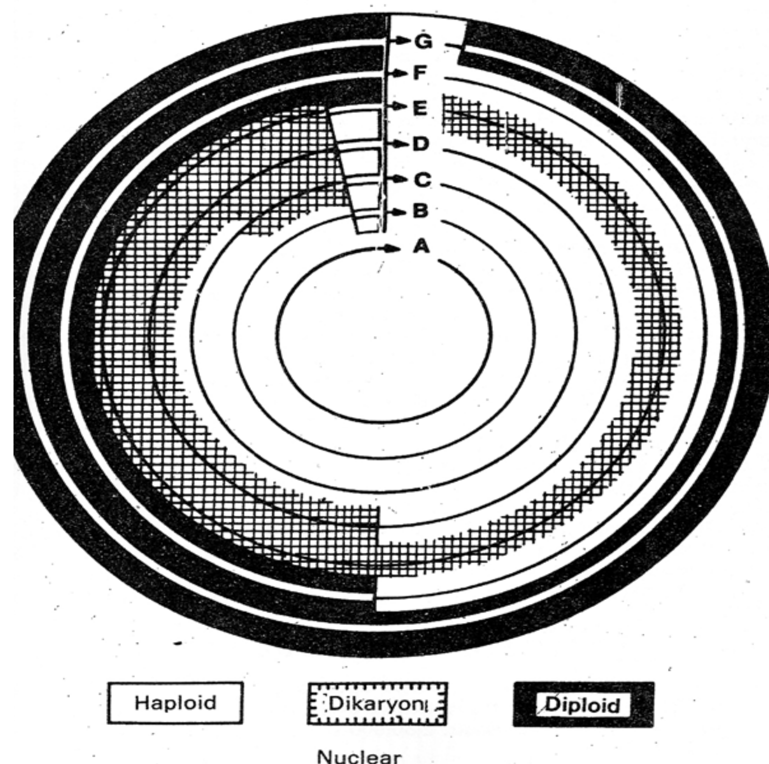
4- **Haploid Dikaryon cycle** (Basidiomycetes): where the two nucleus undiffused directly after the (p) diffusion rather continue for a short time and such the cells be in (n+n) Dikaryon stage and the Hyphae

are dependent on uni-nucleus Hyphae (n) and usually each phase needs different physiological conditions as in *Coprinus.*

5- **Dikaryotic cycle** (Smuts and Yeasts):In this cycle both phases (n) and (2n) meioses each phase to a short period whereas the fungus spends most of its life in a form of (n+n) as in the life cycle of *Ustilago* and *Puccini*

6- **Haploid –Diploid Cycle** (Chytridiomycetes) :In this cycle the two phases succeed clearly as in *Allomyces*.

7- **Diploid cycle (Myxomycetes)** :In this cycle, the dominant phase in the fungus's life is the (2n). This common circle in the vulnerable organisms such as top plants and animals and be only (n) after meiosis directly. Example of fungi of this life cycle is the Myxomycetes.



Life cycles of fungi

Heterokaryosis

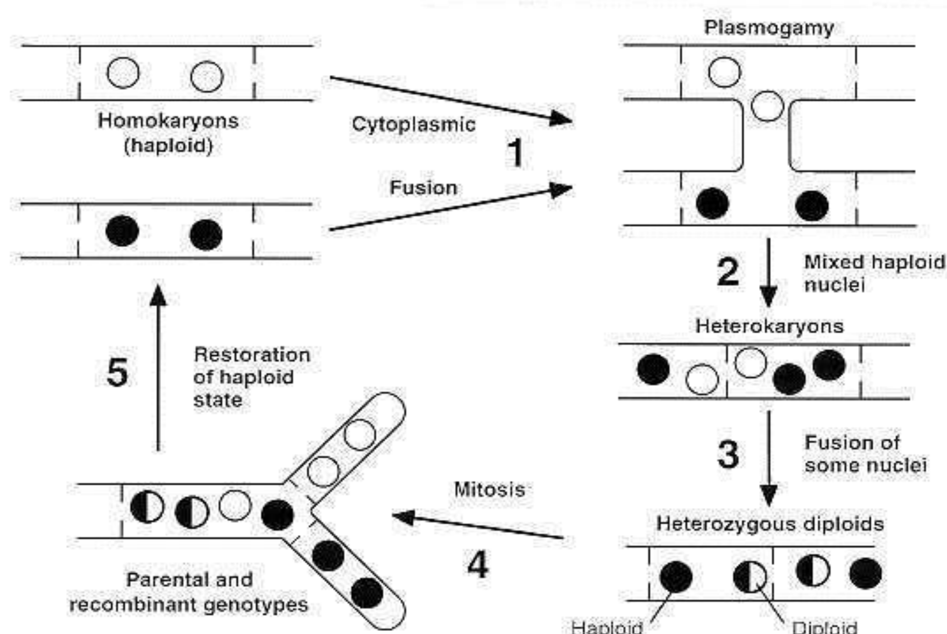
Refers to the presence of **two or more genetically distinct nuclei within the same cell**. While uncommon throughout most of life's kingdoms, heterokaryosis is a hallmark of kingdom Fungi. The fungal subkingdom, Dikarya, which contains two phyla (Ascomycota and Basidiomycota) and 95 % of all known fungal species, is named for its characteristic heterokaryons with exactly two genetically distinct nuclei., the nuclei in these dikaryotic cells fuse and undergo meiosis, resulting in genetically recombined haploid basidiospores or ascospores.

1-Germination of heterokaryotic spores produced heterokaryon hyphae.

2-Introduced different nucleus in monokaryon.

3-Mutation in one nucleus of hypha contain many nucleus.

4- Somatogamy between two hyphae(**genetically distinct nuclei**) or between hypha contain nucleus (haploid = n) and hypha contain (diploid=2n).



Compatibility And Sterility

Introduction

Variation in a population may arise following sexual reproduction. Two compatible isolates of the same fungus may initiate reproduction, and the basic process is remarkably similar in all fungi. The isolates first recognise each other and potentially conjugant regions are induced, probably because of the release and recognition of specific hormones. Growth of one or both towards the other, and contact between the two isolates is followed by fusion of the cells (**plasmogamy**). Nuclei from one pass to the other thallus. Nuclei fuse (**karyogamy**), perhaps multiply and spread, within the second, before the reproductive units are formed. The details of sexual structures, and process and location of **plasmogamy**,

Acording the sexuality fungi divided into 3 types:

1- Monoecious (Hermaphroditic) fungi

2- Dioceous fungi

3- Sexually undifferentiated (Un-differentiated fungi).

Sexual Compatibility:

How do fungi recognise one another, and once aware, get together? Sexual compatibility is determined by mating systems. Various patterns are found. A few fungi are **homothallic**, and are thus capable of forming sexual structures within a single thallus. Most fungi appear to be **heterothallic**, requiring compatible mating types on different thalli for sexual reproduction to be initiated.

Acording the **Compatibility fungi divided into**

A- Homothallic Fungi:

Homothallism enables isolated fungi to go through the sexual process where no compatible mycelia are present. Homothallic isolates are particularly common in isolates of fungi maintained in the laboratory. Particularly successful genotypes may be sustained by homothallism in a sexually reproducing population.

B-Heterothalic fungi

1- Bipolar Mating Types:

The fungi have a variety of compatibility genes involved in outcrossing. The simplest system is where a single locus has two alleles, leading to a sexual interaction where both alleles are involved. Where only **one type of allele** is present, the interaction is incompatible.

Mating types	A	B
A	-	+
B	+	-

(+) **50 % Self-fertile and** (-) **50% Self-sterile**

2-Tetrapolar Mating Types:

In the remaining Basidiomycota, mating is more complex. Compatibility is determined by **two loci or linked regions**, each of which may have many different alleles. The mating system is called tetrapolar if **two loci** determine compatibility. Here, a compatible interaction requires different **alleles** at each of the two loci Tetrapolar interactions are more complicated. This is because the interaction may be staged: one locus determines whether the hyphae combine, the second determines whether the organism produces a sexual structure. For a dikaryon to be fertile, the alleles at each locus must be different.

Mating types	A1B1	A1B2	A2B1	A2B2
A1B1	-	-	-	+
A1B2	-	-	+	-
A2B1	-	+	-	-
A2B2	+	-	-	-
25% Self-fertile and 75% Self-sterile				

3-Secondarily homothallic fungi:

Some of these fungi form secondarily homothallic mycelia in apparently heterothallic mycelia. Each spore may contain a pair of compatible nuclei. Indeed, some mating type alleles are "silent" in the genome of some fungi, resulting in successful mating because the mating type has switched.

Mycotoxin

A **mycotoxin** (from the Greek μύκης *mykes*, "fungus" and τοξίνη *toxini*, "toxin" is a toxic secondary metabolite produced by organisms of the fungus kingdom[3] and is capable of causing disease and death in both humans and other animals.[4] The term 'mycotoxin' is usually reserved for the toxic chemical products produced by fungi that readily colonize crops.

Examples of mycotoxins causing human and animal illness include aflatoxin, citrinin, fumonisins, ochratoxin A, patulin, trichothecenes, zearalenone, and ergot alkaloids such as ergotamine. One mold species may produce many different mycotoxins, and several species may produce the same mycotoxin.

Production

Most fungi are aerobic (use oxygen) and are found almost everywhere in extremely small quantities due to the diminute size of their spores. They consume organic matter wherever humidity and temperature are sufficient. Where conditions are right, fungi proliferate into colonies and mycotoxin levels become high. The reason for the production of mycotoxins is not yet known; they are not necessary for the growth or the development of the fungi. Because mycotoxins weaken the receiving host, they may improve the environment for further fungal proliferation. The production of toxins depends on the surrounding intrinsic and extrinsic environments and these substances vary greatly in their toxicity, depending on the organism infected and its susceptibility, metabolism, and defense mechanisms.

Major groups

Aflatoxins

are a type of mycotoxin produced by *Aspergillus* species of fungi, such as *A. flavus* and *A. parasiticus*.[10] The umbrella term aflatoxin refers to four different types of mycotoxins produced, which are B_1, B_2, G_1, and G_2.[11] Aflatoxin B_1, the most toxic, is a potent carcinogen and has been directly correlated to adverse health effects, such as liver cancer, in many animal species. Aflatoxins are largely associated with commodities produced in the tropics and subtropics, such as cotton, peanuts, spices, pistachios, and maize.

Ochratoxin

is a mycotoxin that comes in three secondary metabolite forms, A, B, and C. All are produced by *Penicillium* and *Aspergillus* species. The three forms differ in that Ochratoxin B (OTB) is a nonchlorinated form of Ochratoxin A (OTA) and that Ochratoxin C (OTC) is an ethyl ester form Ochratoxin A. *Aspergillus ochraceus* is found as a contaminant of a wide range of commodities including beverages such as beer and wine. *Aspergillus carbonarius* is the main species found on vine fruit, which releases its toxin during the juice making process. OTA has been labeled as a carcinogen and a nephrotoxin, and has been linked to tumors in the human urinary tract, although research in humans is limited by confounding factors.

Citrinin

is a toxin that was first isolated from *Penicillium citrinum*, but has been identified in over a dozen species of *Penicillium* and several species of *Aspergillus*. Some of these species are used to produce human foodstuffs such as cheese (*Penicillium camemberti*), sake, miso, and soy sauce (*Aspergillus oryzae*). Citrinin is associated with yellowed rice disease in Japan and acts as a nephrotoxin in all animal species tested. Although it is associated with many human foods (wheat, rice, corn, barley, oats, rye,and food colored with Monascus pigment) its full significance for human health is

unknown. Citrinin can also act synergistically with Ochratoxin A to depress RNA synthesis in murine kidneys.

Ergot Alkaloids

are compounds produced as a toxic mixture of alkaloids in the sclerotia of species of *Claviceps*, which are common pathogens of various grass species. The ingestion of ergot sclerotia from infected cereals, commonly in the form of bread produced from contaminated flour, causes ergotism, the human disease historically known as St. Anthony's Fire. There are two forms of ergotism: gangrenous, affecting blood supply to extremities, and convulsive, affecting the central nervous system. Modern methods of grain cleaning have significantly reduced ergotism as a human disease; however, it is still an important veterinary problem. Ergot alkaloids have been used pharmaceutically.

Patulin

is a toxin produced by the *P. expansum*, *Aspergillus*, *Penicillium*, and *Paecilomyces* fungal species. *P. expansum* is especially associated with a range of moldy fruits and vegetables, in particular rotting apples and figs. It is destroyed by the fermentation process and so is not found in apple beverages, such as cider. Although patulin has not been shown to be carcinogenic, it has been reported to damage the immune system in animals. In 2004, the European Community set limits to the concentrations of patulin in food products. They currently stand at 50 μg / kg in all fruit juice concentrations, at 25 μg / kg in solid apple products used for direct consumption, and at 10 μg / kg for children's apple products, including apple juice.

Fusarium

toxins are produced by over 50 species of *Fusarium* and have a history of infecting the grain of developing cereals such as wheat and maize .They include a range of mycotoxins, such as: the **fumonisins**, which affect the

nervous systems of horses and may cause cancer in rodents; the **trichothecenes**, which are most strongly associated with chronic and fatal toxic effects in animals and humans; and **zearalenone**, which is not correlated to any fatal toxic effects in animals or humans. Some of the other major types of *Fusarium* toxins include:beauvercin and enniatins , butenolide , equisetin, and fusarins.

Occurrence

Although various wild mushrooms contain an assortment of poisons that are definitely fungal metabolites causing noteworthy health problems for humans, they are rather arbitrarily excluded from discussions of mycotoxicology. In such cases the distinction is based on the size of the producing fungus and human intention.[15] Mycotoxin exposure is almost always accidental whereas with mushrooms improper identification and ingestion causing mushroom poisoning is commonly the case. Ingestion of misidentified mushrooms containing mycotoxins may result in hallucinations.The cyclopeptide-producing *Amanita phalloides* is well known for its toxic potential and is responsible for approximately 90% of all mushroom fatalities. The other primary mycotoxin groups found in mushrooms include: orellanine, monomethylhydrazine, disulfiram-like, hallucinogenic indoles, muscarinic, isoxazole, and gastrointestinal (GI)-specific irritants.[22] The bulk of this article is about mycotoxins that are found in microfungi other than poisons from mushrooms or macroscopic fungi.

In indoor environments

Buildings are another source of mycotoxins and people living or working in areas with mold increase their chances of adverse health effects. Molds growing in buildings can be divided into three groups – primary, secondary, and tertiary colonizers. Each group is categorized by the ability to grow at a certain water activity requirement. It has become difficult to identify mycotoxin production by indoor molds for many variables, such as

(i) they may be masked as derivatives, (ii) they are poorly documented, and (iii) the fact that they are likely to produce different metabolites on building materials. Some of the mycotoxins in the indoor environment are produced by *Alternaria*, *Aspergillus* (multiple forms), *Penicillium*, and *Stachybotrys*. *Stachybotrys chartarum* contains a higher number of mycotoxins than other molds grown in the indoor environment and has been associated with allergies and respiratory inflammation.[24] The infestation of *S. chartarum* in buildings containing gypsum board, as well as on ceiling tiles, is very common and has recently become a more recognized problem. When gypsum board has been repeatedly introduced to moisture, *S. chartarum* grows readily on its cellulose face.[25] This stresses the importance of moisture controls and ventilation within residential homes and other buildings. The negative health effects of mycotoxins are a function of the concentration, the duration of exposure, and the subject's sensitivities. The concentrations experienced in a normal home, office, or school are often too low to trigger a health response in occupants.

In the 1990s, public concern over mycotoxins increased following multimillion-dollar toxic mold settlements. The lawsuits took place after a study by the Center for Disease Control (CDC) in Cleveland, Ohio, reported an association between mycotoxins from *Stachybotrys* spores and pulmonary hemorrhage in infants. However, in 2000, based on internal and external reviews of their data, the CDC concluded that because of flaws in their methods, the association was not proven. *Stachybotrys* spores in animal studies have been shown to cause lung hemorrhaging, but only at very high concentrations.

One study by the Center of Integrative Toxicology at Michigan State University investigated the causes of Damp Building Related Illness (DBRI).They found that *Stachybotrys* is possibly an important contributing factor to DBRI. So far animal models indicate that airway exposure to *S. chartarum* can evoke allergic sensitization, inflammation, and cytotoxicity

in the upper and lower respiratory tracts. Trichothecene toxicity appears to be an underlying cause of many of these adverse effects. Recent findings indicate that lower doses (studies usually involve high doses) can cause these symptoms.

Some toxicologists have used the Concentration of No Toxicological Concern (CoNTC) measure to represent the airborne concentration of mycotoxins that are expected to cause no hazard to humans (exposed continuously throughout a 70–yr lifetime). The resulting data of several studies have thus far demonstrated that common exposures to airborne mycotoxins in the built indoor environment are below the CoNTC, however agricultural environments have potential to produce levels greater than the CoNTC.

In food

Mycotoxins can appear in the food chain as a result of fungal infection of crops, either by being eaten directly by humans or by being used as livestock feed.

In 2004 in Kenya, 125 people died and nearly 200 others were treated after eating aflatoxin-contaminated maize. The deaths were mainly associated with homegrown maize that had not been treated with fungicides or properly dried before storage. Due to food shortages at the time, farmers may have been harvesting maize earlier than normal to prevent thefts from their fields, so that the grain had not fully matured and was more susceptible to infection.

Spices are susceptible substrate for growth of mycotoxigenic fungi and mycotoxin production. Red chilli, black pepper, and dry ginger were found to be the most contaminated spices.

Physical methods to prevent growth of mycotoxin-producing fungi or remove toxins from contaminated food include temperature and humidity control, irradiation and photodynamic treatment. Mycotoxins can also be removed chemically and biologically using antifungal/anti-mycotoxins agents and antifungal plant metabolites .

In animal food

Dimorphic fungi, which include Blastomyces dermatitidis and Paracoccidioides brasiliensis, are known causative agents of endemic systemic mycoses.

There were outbreaks of dog food containing aflatoxin in North America in late 2005 and early 2006, and again in late 2011.

Mycotoxins in animal fodder, particularly silage, can decrease the performance of farm animals and potentially kill them. Several mycotoxins reduce milk yield when ingested by dairy cattle.

Mycobacterium is derived from its "fungus-like" nature

In dietary supplements

Contamination of medicinal plants with mycotoxins can contribute to adverse human health problems and therefore represents a special hazard. Numerous natural occurrences of mycotoxins in medicinal plants and herbal medicines have been reported from various countries including Spain, China, Germany, India, Turkey and from the Middle East.[36] In a 2015 analysis of plant-based dietary supplements, the highest mycotoxin concentrations were found in milk thistle-based supplements, at up to 37 mg/kg.

Health effects

Some of the health effects found in animals and humans include death, identifiable diseases or health problems, weakened immune systems

without specificity to a toxin, and as allergens or irritants. Some mycotoxins are harmful to other micro-organisms such as other fungi or even bacteria; penicillin is one example. It has been suggested that mycotoxins in stored animal feed are the cause of rare phenotypical sex changes in hens that causes them to look and act male.

In humans

Mycotoxicosis is the term used for poisoning associated with exposures to mycotoxins. Mycotoxins have the potential for both acute and chronic health effects via ingestion, skin contact, inhalation, and entering the blood stream and lymphatic system. They inhibit protein synthesis, damage macrophage systems, inhibit particle clearance of the lung, and increase sensitivity to bacterial endotoxin.

The symptoms of mycotoxicosis depend on the type of mycotoxin; the concentration and length of exposure; as well as age, health, and sex of the exposed individual. The synergistic effects associated with several other factors such as genetics, diet, and interactions with other toxins have been poorly studied. Therefore, it is possible that vitamin deficiency, caloric deprivation, alcohol abuse, and infectious disease status can all have compounded effects with mycotoxins.

Mitigation

Mycotoxins greatly resist decomposition or being broken down in digestion, so they remain in the food chain in meat and dairy products. Even temperature treatments, such as cooking and freezing, do not destroy some mycotoxins.

Removal

In the feed and food industry it has become common practice to add mycotoxin binding agents such as montmorillonite or bentonite clay in order to effectively adsorb the mycotoxins. To reverse the adverse effects

of mycotoxins, the following criteria are used to evaluate the functionality of any binding additive:

- Efficacy of active component verified by scientific data
- A low effective inclusion rate
- Stability over a wide pH range
- High capacity to absorb high concentrations of mycotoxins
- High affinity to absorb low concentrations of mycotoxins
- Affirmation of chemical interaction between mycotoxin and adsorbent
- Proven *in vivo* data with all major mycotoxins
- Non-toxic, environmentally friendly component

Since not all mycotoxins can be bound to such agents, the latest approach to mycotoxin control is mycotoxin deactivation. By means of enzymes (esterase, de-epoxidase), yeast (*Trichosporon mycotoxinvorans*), or bacterial strains (Eubacterium BBSH 797 developed by Biomin), mycotoxins can be reduced during pre-harvesting contamination. Other removal methods include physical separation, washing, milling, nixtamalization, heat-treatment, radiation, extraction with solvents, and the use of chemical or biological agents. Irradiation methods have proven to be effective treatment against mold growth and toxin production.

Regulations

Many international agencies are trying to achieve universal standardization of regulatory limits for mycotoxins. Currently, over 100 countries have regulations regarding mycotoxins in the feed industry, in which 13 mycotoxins or groups of mycotoxins are of concern. The process of assessing a need for mycotoxin regulation includes a wide array of in-laboratory testing that includes extracting, clean-up and separation

techniques. Most official regulations and control methods are based on high-performance liquid techniques (e.g., HPLC) through international bodies. It is implied that any regulations regarding these toxins will be in co-ordinance with any other countries with which a trade agreement exists. Many of the standards for the method performance analysis for mycotoxins is set by the European Committee for Standardization (CEN). However, one must take note that scientific risk assessment is commonly influenced by culture and politics, which, in turn, will affect trade regulations of mycotoxins.

Food-based mycotoxins were studied extensively worldwide throughout the 20th century. In Europe, statutory levels of a range of mycotoxins permitted in food and animal feed are set by a range of European directives and EC regulations. The U.S. Food and Drug Administration has regulated and enforced limits on concentrations of mycotoxins in foods and feed industries since 1985. It is through various compliance programs that the FDA monitors these industries to guarantee that mycotoxins are kept at a practical level. These compliance programs sample food products including peanuts and peanut products, tree nuts, corn and corn products, cottonseed, and milk. There is still a lack of sufficient surveillance data on some mycotoxins that occur in the U.S.

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The genus *Aspergillus*

is among the most abundant and widely distributed organism on earth, and at the moment comprises 339 known species. It is one of the most important economically fungal genus and the biotechnological use of *Aspergillus* species is related to production of soy sauce, of different hydrolytic enzymes (amylases, lipases) and organic acid (citric acid,

gluconic acid), as well as biologically active metabolites such as lovastatin. Although they are not considered to be major cause of plant diseases, *Aspergillus* species are responsible for several disorders in various plants and plant products, especially as opportunistic storage moulds. The notable consequence of their presence is contamination of foods and feeds by mycotoxins, among which the most important are aflatoxins, ochratoxin A, and, at a less extent, fumonisins. Aflatoxins B_1, B_2, G_1, G_2 are the most toxic and carcinogenic mycotoxins, due to their extreme hepatocarcinogenicity; ochratoxin A is a potent nephrotoxin, it is also carcinogenic, teratogenic, and immunotoxic in rats and possibly in humans; fumonisins are hepatotoxic and nephrotoxic with potential carcinogenic effects on rat and mice. In this chapter we summarize the main aspects of morphology, ecology, epidemiology, and toxigenicity of *Aspergillus* foodborne pathogens which belong to sections *Flavi*, *Circumdati*, and *Nigri*, occurring in several agricultural products and responsible of aflatoxin, ochratoxin A, and fumonisins contamination of food and feed.

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The genus *Fusarium*

includes numerous toxigenic species that are pathogenic to plants or humans, and are able to colonize a wide range of environments on earth. The genus comprises around 70 well-known species, identified by using a polyphasic approach, and as many as 300 putative species, according to phylogenetic species concepts; many putative species do not yet have formal names.

Fusarium is one of the most economically important fungal genera because of yield loss due to plant pathogenic activity; mycotoxin contamination of food and feed products which often render them unaccep for marketing; and health impacts to humans and livestock, due to

consumption of mycotoxins. Among the most important mycotoxins produced by species of *Fusarium* are the trichothecenes and the fumonisins. Fumonisins cause fatal livestock diseases and are considered potentially carcinogenic mycotoxins for humans, while trichothecenes are potent inhibitors of protein synthesis. This chapter summarizes the main aspects of morphology, pathology, and toxigenicity of the main *Fusarium* species that colonize different agricultural crops and environments worldwide, and cause mycotoxin contamination of food and feed.

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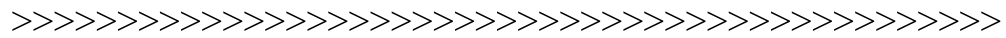
The genus *Penicillium*

are very diverse and cosmopolite fungi, about 350 species are recognized within this genus. It is subdivided in four subgenera *Aspergilloides*, *Penicillium*, *Furcatum*, and *Biverticillium*; recently the first three has been included in *Penicillium* genus, and *Biverticillium* under *Talaromyces*. They occur worldwide and play important roles as decomposers of organic materials, cause destructive rots in the food industry where produces a wide range of mycotoxins; they are considered enzyme factories, and common indoor air irritants. In terms of human health are rarely associated as human pathogen because they hardly growth at 37°, while the main risk is related to ingestion of food contaminated by mycotoxins produced by several species of *Penicillium*. Various mycotoxins can occur in foods and feeds contaminated by *Penicillium* species, the most important are ochratoxin A and patulin; for which regulation are imposed in a number of countries, and at a less extent cyclopiazonic acid. we summarize the main aspect of the morphology, ecology and toxigenicity of *Penicillium* foodborne mycotoxigenic species which belong mainly in

subgenus *Penicillium* sections *Brevicompacta*, *Chrysogena*, *Fasciculata, Penicillium*, and *Roquefortorum*.



The genus *Alternaria*

includes more than 250 species. The traditional methods for identification of *Alternaria* species are based on morphological characteristics of the reproductive structures and sporulation patterns under controlled culture conditions. Cladistics analyses of "housekeeping genes" commonly used for other genera, failed to discriminate among the small-spored *Alternaria* species. The development of molecular methods achieving a better agreement with morphological differences is still needed. The production of secondary metabolites has also been used as a means of classification and identification. *Alternaria* spp. can produce a wide variety of toxic metabolites. These metabolites belong principally to three different structural groups: (1) the dibenzopyrone derivatives, alternariol (AOH), alternariol monomethyl ether (AME), and altenuene (ALT); (2) the perylene derivative altertoxins (ATX-I, ATX-II, and ATX II); and (3) the tetramic acid derivative, tenuazonic acid (TeA). TeA, AOH, AME, ALT, and ATX-I are the main. Certain species in the genus *Alternaria* produce host-specific toxins (HSTs) that contribute to their pathogenicity and virulence. *Alternaria* species are plant pathogens that cause spoilage of agricultural commodities with consequent mycotoxin accumulation and economic losses. Vegetable foods infected by *Alternaria* rot could introduce high amounts of these toxins to the human diet. More investigations on the toxic potential of these toxins and their hazard for human consumption are needed to make a reliable risk assessment of dietary exposure.

Mycotoxin analysis:

- Introduction
- Sampling
- Sample preparation and clean-up
- Analytical techniques
- Multi-mycotoxin methods
- Fast screening methods
- Quality assurance
- Reference materials and intercomparison studies
- Conclusion and outlook
- References

Abstract:

Mycotoxin contamination of cereals and related products used for feed can cause intoxication, especially in farm animals. Therefore, efficient analytical tools for the qualitative and quantitative analysis of toxic fungal metabolites in feed are required. Current methods usually include an extraction step, a clean-up step to reduce or eliminate unwanted co-extracted matrix components and a separation step with suitably specific detection ability. Quantitative methods of analysis for most mycotoxins use immunoaffinity clean-up with high-performance liquid chromatography (HPLC) separation in combination with UV and/or fluorescence detection. Screening of samples contaminated with mycotoxins is frequently

performed by thin layer chromatography (TLC), which yields qualitative or semi-quantitative results. Nowadays, enzyme-linked immunosorbent assays (ELISA) are often used for rapid screening. A number of promising methods, such as fluorescence polarization immunoassays, dipsticks, and even newer methods such as biosensors and non-invasive techniques based on infrared spectroscopy, have shown great potential for mycotoxin analysis. Currently, there is a strong trend towards the use of multi-mycotoxin methods for the simultaneous analysis of several of the important *Fusarium* mycotoxins, which is best achieved by LC–MS/MS (liquid chromatography with tandem mass spectrometry). This review focuses on recent developments in the determination of mycotoxins with a special emphasis on LC–MS/MS and emerging rapid methods.

Keywords: Mycotoxin, analysis, LC–MS/MS, sample preparation, chromatographic methods

Introduction

Mycotoxins are natural, secondary metabolites produced by fungi on agricultural commodities in the field and during storage under a wide range of climatic conditions. About 200 different filamentous fungi species, e.g. *Aspergillus*, *Penicillium* and *Fusarium* species (sp.), have been identified. Several hundred different mycotoxins have been discovered so far, exhibiting great structural diversity, which results in different chemical and physicochemical properties. Aflatoxins and ochratoxins (produced mainly by *Aspergillus* sp.), fumonisins, trichothecenes and zearalenone (produced by *Fusarium* sp.), patulin (produced by *Penicillium* sp.), and ergot alkaloids (produced in the sclerotia of *Claviceps* sp.) receive the most attention due to their frequent occurrence and their severe effects on animal and human health (Bennett and Klich 2003; D’Mello and MacDonald 1997). Mycotoxins are potent toxins and have a wide range of actions on animals and humans, e.g. cyto-, nephro- and neurotoxic, carcinogenic, mutagenic, immunosuppressive and estrogenic effects.

Although mycotoxicoses caused by direct consumption of contaminated food and feedstuffs poses the greatest risk to animals and humans, the entry of mycotoxins or their metabolites into the food chain by "carry over" into milk, animal tissue or eggs, for example, should not be underestimated.

National and international institutions and organisations, such as the European Commission (EC), the US Food and Drug Administration (FDA), the World Health Organisation (WHO) and the Food and Agriculture Organisation (FAO) of the United Nations, have recognized the potential health risks to animals and humans posed by food- and feed-borne mycotoxin intoxication and addressed this problem by adopting regulatory limits for major mycotoxin classes and selected individual mycotoxins. The FAO has compiled comprehensive worldwide regulations and directives regarding mycotoxins in food and feed as of December 2003 (FAO 2004). The Joint Expert Committee on Food Additives (JECFA), a scientific advisory body of FAO and WHO, provides mechanisms for assessing the toxicity of food additives, veterinary drug residues and contaminants, and has recently evaluated the hazards related to several mycotoxins, including fumonisins B_1, B_2 and B_3, ochratoxin A, deoxynivalenol, T-2 toxin, HT-2 toxin, and aflatoxin M_1 (WHO 2002). The report explains the nature of each toxin, including its absorption and excretion, as well as toxicological studies, and it includes general considerations of analytical methods, sampling, associated intake issues and control mechanisms.

The EC has set maximum levels for some mycotoxins, including several aflatoxins, ochratoxin A, patulin, deoxynivalenol and zearalenone, in certain foodstuffs. Maximum levels for fumonisins B_1 and B_2 came into force in October 2007. Consideration of a review of the maximum levels for deoxynivalenol, zearalenone and fumonisins B1 and B2 as well as the appropriateness of setting a maximum level for T-2 and HT-2 toxins in cereals and cereal products should be completed by July 2008 (EC 2006).

The requirement to apply these regulatory limits has prompted the development of a vast number of analytical methods for the identification and quantification of mycotoxins in various samples, such as food, feed, and other biological matrices. The chemical diversity of mycotoxins and their varying concentration ranges in a wide range of agricultural commodities, foods and biological samples poses a great challenge to analytical chemists. The different chemical and physicochemical properties of the mycotoxins require specific extraction, cleanup, separation and detection methods. Therefore, most methods target only individual mycotoxins or at best a group of closely related mycotoxins. These methods are usually based on labour-intensive sample preparation protocols followed by traditional chromatographic separation (mostly liquid chromatography, LC). Gas chromatography (GC) either with electron capture detection (ECD) or mass spectrometric (MS) detection is used in mycotoxin analysis, e.g. for trichothecene or patulin determination, but less frequently than alternative methods. In some cases, fast and accurate screening methods based on enzyme-linked immunosorbent assay (ELISA) are applied instead of the more labour-intensive LC methods. Thin-layer chromatography (TLC) provides a cheaper alternative to LC-based methods and has an important role, especially in developing countries, for surveillance purposes and control of regulatory limits (Gilbert and Anklam 2002). Modern sample clean-up techniques, such as immunoaffinity columns (IAC) or solid-phase extraction (SPE) methods, help to simplify protocols, improve selectivity and, thus, performance characteristics.

To deal with the increasing number of sample matrices and mycotoxins of interest, fast and accurate analytical methods are needed. This demand has led to the development of rapid screening methods for single mycotoxins or whole mycotoxin classes based on immunochemical techniques (e.g. ELISA), biosensors (e.g. protein chips, antibody/protein-coated electrodes)

and non-invasive optical techniques. On the other hand, highly sophisticated multi-mycotoxin methods based on LC coupled to multiple-stage MS are being developed to allow accurate and precise determination and unambiguous identification of mycotoxins without the need for tedious sample preparation and clean-up procedures.

Sample selection and representative sample collection are often underestimated as sources of error. The design of sampling procedures for various mycotoxins and sample materials has been an international concern for several years. (FAO 2004; EC 2006b; FDA 2007). To obtain comparable data, the EC has laid down certain requirements for sampling and performance criteria for analytical methods (EC 2006b). Therefore, the whole analytical method (including sampling, sample preparation, clean-up and final determination) used by enforcement laboratories for the implementation and control of legislation and regulatory limits must be subject to a validation procedure to show that the method produces reliable results and meets the set performance criteria. Several protocols and guidelines for method validation have been published (Thompson 1993; ISO 1994; Eurachem 1998). There are a multitude of analytical methods available that have been validated and accepted by official authorities, such as the European Committee for Standardization (CEN), the Association of Official Analytical Chemists (AOAC International), and the International Organisation for Standardization (ISO) (Gilbert and Anklam 2002, AOAC 2005). Each laboratory should implement quality assurance measures such as frequently checking the accuracy and precision of their methods by analysing (certified) reference materials (CRM) and by regular participation in proficiency testing trials. Although much has been done in the past years regarding the production and certification of reference materials (RM) and calibrants for mycotoxin analysis in various matrices, there is still a need for more RMs appropriate for the different sample matrices and concentration ranges encountered in foods and feeds.

The objective of this review is to summarize recent developments in the determination of mycotoxins with a special emphasis on LC–MS/MS and emerging rapid methods.

Steps of mycotoxins analysis.

The following steps:

1-Sampling,

2-Grinding

3-Extraction,

4-Purification

5-Analysis

Sampling

Sampling plays a crucial part in the precision and determination of mycotoxin levels due to the sometimes very heterogeneous distribution of the toxins in agricultural commodities and products intended for human and animal consumption. The distribution of mycotoxins in the sample material is an important factor to be considered in establishing regulatory sampling criteria. In the past, this has been recognized by many national and international authorities and organisations, such as the EC (2006a), the FDA (2007) and the FAO (2004). In Commission Regulation No. 401/2006 (EC 2006b), the EC has laid down the methods of sampling and analysis for the official control of levels of various mycotoxins in foodstuffs, repealing all former directives and amendments on this subject. It includes methods of sampling and analysis of aflatoxins, ochratoxin A, patulin and *Fusarium* toxins in cereal, dried fruit, fruit juices, must and wine, groundnuts and nuts, spices, milk, coffee, products derived from the above mentioned basic materials as well as baby foods and food for infants and young children. While it can be assumed that mycotoxins in liquid samples are homogeneously distributed, some mycotoxins, especially in fungus-

contaminated grain, may be concentrated in so-called “hot-spots”. Mycotoxins, especially those produced by *Aspergillus* sp., e.g. aflatoxins, can be distributed very heterogeneously in food products with large particle size such as dried figs or groundnuts. The number of contaminated particles may be very low, but the contamination level within a particle can be very high. To obtain the same representativeness for batches of food products with large particle sizes, the weight of the incremental sample taken has to be larger than in cases of batches with smaller particle size. Commission Regulation No. 401/2006 regulates the number of incremental samples to be taken from different places of a lot depending on the weight of the entire lot. This may result in rather large aggregate samples, up to 30 kg in the case of aflatoxin determination in dried figs, groundnuts and nuts. However, it has to be considered that handling and sample preparation of large quantities pose great difficulties in implementing such sampling plans, especially with regard to the validation process of sampling methods. The EC regulation, therefore, stipulates the division of aggregate samples intended for direct human consumption into up to three laboratory samples of ≤10 kg for homogenisation and analysis. This subdivision is not necessary for products intended for further sorting or processing before human consumption or for use as an ingredient (SANCO 2005).

The Grain Inspection, Packers and Stockyards Administration (GIPSA) of the United States Department of Agriculture (USDA) developed general sampling guidelines for grain (GIPSA 1995), rice (GIPSA 1994) and hops (GIPSA 1998). The FDA is constantly updating their Investigative Operation Manual (IOM) that describes general procedures for field investigators and inspectors and includes information on sampling and sampling schedules for various occasions (FDA 2007). It contains a detailed sampling schedule for mycotoxin analysis that lists sample sizes dependent on the type of product and distinguishes between samples taken for surveillance (initial sample) and follow-up samples in case of positive

findings. The minimum total sample size ranges from 4.5 kg up to 34 kg, depending on the heterogeneity expected of the sample type.

Kay (2001) compared a number of approaches for sampling grain, developed by different national and international authorities, and provided an interpretation of the major differences between the methods in terms of lot size, tolerances, sampling techniques, sample size, rates, etc.

Sample preparation and clean-up

Only a few analytical techniques, i.e. optical techniques based on IR spectroscopy (Kos et al. 2003), are capable of detecting mycotoxin contamination directly in ground cereal samples without the necessity of further sample preparation, such as solvent extraction or clean-up. However, the application of such techniques is still limited to screening purposes due to a high matrix dependence and lack of appropriate calibration materials.

Analytical methods based on chromatography or immunoassays usually require solvent extraction to liberate the mycotoxin from the sample matrix, and subsequent clean-up of the extract to reduce matrix effects. Various combinations of solvents, sometimes with the addition of modifiers (e.g. acids, bases, etc.), are used for extraction, depending on the physicochemical properties of the mycotoxins, the sample matrix and the type of clean-up used afterwards (Zöllner and Mayer-Helm 2006). Accelerated solvent extraction (ASE), also known as pressurised liquid extraction (PLE) (Royer et al. 2004; Urraca et al. 2004; Juan et al. 2005; Pallaroni and van Holst 2003, 2004) or microwave-assisted extraction (MAE) (Pallaroni et al. 2002) help to speed-up and automate the extraction process, and offer a robust and time-saving alternative to classical solvent extraction techniques. So far, the high cost of an ASE apparatus has, however, limited the application of this technique in the field of mycotoxin analysis to a few laboratories. Supercritical fluid extraction (SFE), especially with supercritical CO_2 as an environmentally safe extraction

medium, received a lot of attention in the 1990s. The extraction selectivity of the non-polar supercritical CO_2 is influenced by temperature and pressure and can be varied in a wide range by adding modifiers (polar solvents, complexing agents, etc.). Although in the past, SFE has received much attention regarding agricultural applications, only a few papers deal with it as an extraction method in mycotoxins analysis (Huopalahti et al. 1997; Krska 1998; Ambrosino et al. 2004; Liau et al. 2007).

Liquid–liquid partitioning of the mycotoxin containing aqueous acetonitrile/methanol sample extract with hexane is sometimes used for de-fatting or protein precipitation (Sørensen and Elbæk 2005; Kokkonen et al. 2005). For further purification and analyte enrichment, liquid samples and extracts are predominantly submitted to solid-phase extraction (SPE) protocols for which a wide variety of sorbent materials are available. A comprehensive compilation of different clean-up approaches for various mycotoxins has been published by Zöllner and Mayer-Helm (2006). Conventional SPE procedures use reversed-phase (RP) materials (e.g. C_8, C_{18}), strong cation or anion exchangers (SCX, SAX) or polymeric materials with combined properties. Modern clean-up procedures employ multifunctional MycoSep® (Krska 1998; Radová et al. 1998; Biselli and Hummert 2005; Ren et al. 2007) or immunoaffinity columns (IAC) (Krska 1998), although these methods are more expensive than conventional clean-up methodologies. MycoSep® columns contain a mixture of charcoal, ion-exchange resins and other materials and are suitable for aflatoxins, trichothecenes, ochratoxins, zearalenone, moniliformin and patulin (Romerlabs 2007). Mycotoxin specific molecularly imprinted polymers (MIPs) are also considered as a potential and cheaper alternative for clean-up, which, contrary to IACs, do not suffer from storage limitations and stability problems regarding organic solvents. MIPs have been developed with recognition properties towards several mycotoxins including deoxynivalenol (Weiss et al. 2003), zearalenone

(Weiss et al. 2003; Urraca et al. 2006a, b), ochratoxin A (Baggiani et al. 2001; Jodlbauer et al. 2002; Maier et al. 2004; Turner et al. 2004) and moniliformin (Appell et al. 2007).

Currently, there is a strong trend towards the use of IACs in mycotoxins analysis as a clean-up and enrichment technique for sample extracts or liquid samples. IACs contain immobilised antibodies that exclusively retain a certain mycotoxin or mycotoxin class. Due to their high specificity, IACs produce cleaner extracts with a minimum level of interfering matrix components and excellent signal-to-noise ratios compared to less selective SPE sorbent materials. IACs have been developed for most major mycotoxins and mycotoxin classes such as aflatoxins, ochratoxin A, trichothecenes, zearalenone and their metabolites (Zöllner and Mayer-Helm 2006). The AOAC International and the EC have already validated a few IAC methods; however, these address only a limited number of food commodities. For some mycotoxins, such as ochratoxin A, IACs are already used in routine analysis, e.g. coupled with LC with fluorescence detection (FLD). When comparing both conventional clean-up and IAC approaches in the analysis of selected mycotoxins (aflatoxins, B-fumonisins, and ochratoxin A), discrepancies are found for certain food and feed matrices (Castegnaro et al. 2006; Sugita-Konishi et al. 2006). These problems highlight the necessity to validate methods for each complex matrix separately to provide reliable, comparable and traceable analytical data.

Careful selection of the clean-up method is, however, essential for the effectiveness of an analytical method. Immunoaffinity materials are expensive and distinctly less feasible for multitoxin analysis since they are highly specific for only one target mycotoxin (or class). Some scientists even talk about "overkill" when using highly specific clean-up techniques, such as IACs in combination with liquid chromatography with mass spectrometry (LC/MS), since compound-specific detection stands in

contradiction to the multi-analyte detection capabilities of MS (Leitner et al. 2002; Zöllner et al. 1999). However, there are already combined immunoaffinity materials on the market that are specific to a wider range of mycotoxins (MacDonald et al. 2007). It has been shown that, in many cases, the quality of the analytical result does not suffer when conventional SPE approaches are used (Leitner et al. 2002; Reinsch et al. 2005). Of course, this also depends on the selectivity of the MS equipment itself. Single-stage MS in selected ion-monitoring mode might need selective clean-up to remove matrix interferences, while those interferences might not be visible with multi-stage MS in selected reaction-monitoring mode (Zöllner and Mayer-Helm 2006). However, matrix-induced signal suppression or enhancement should always be taken into consideration and can normally be omitted by clean-up of the extract or by using an appropriate calibration method (e.g. matrix-matched calibration standards, standard addition, or the use of adequate internal standards, i.e. isotope-labelled standards, etc.).

Analytical techniques

Conventional analytical techniques

The term ”conventional method” usually refers to a chromatographic separation coupled to a suitable detection system. The currently used quantitative methods for the determination of regulated mycotoxins, such as the fumonisins, zearalenone, type-A (e.g. T2-toxin) and -B trichothecenes (e.g. deoxynivalenol), ochratoxin A and the aflatoxins, in food and feed mainly use immunoaffinity clean-up with high-performance liquid chromatography (HPLC) or gas chromatography (GC) in combination with a variety of detectors, such as fluorescence detection (FLD) with either a pre- or post-column derivatisation step, UV detection, flame ionisation detection (FID), electron capture detection (ECD) or mass spectrometry (MS). Reviews of these methods have been summarized and published elsewhere (Krska et al. 2001, 2005; Krska and Josephs 2001).

From the multitude of available procedures, CEN is trying to standardize methods for mycotoxin analysis. CEN establishes performance criteria for mycotoxin methods usually on the basis of collaborative studies. CEN methods are official reference methods and are used for official control and surveillance and in cases of dispute. CEN–approved methods exist for aflatoxins, ochratoxin A, fumonisins, patulin and deoxynivalenol, for example, in various foods. Further methods for various mycotoxins in feed will be issued in the near future (Gilbert and Anklam, 2002).

Liquid chromatography/mass spectrometry (LC/MS)

Within the last 10 years, liquid chromatography/mass spectrometry has become the universal approach for mycotoxin analysis, as more or less all potential analytes are compatible with the conditions applied during separation and detection. Nevertheless, the breakthrough of this approach did not occur until the mid-1990s, when suitable interfaces, such as atmospheric pressure ionization, became accessible on a routine basis. Compared to conventional detection techniques, such as UV or fluorescence, mass spectrometry offers increased selectivity and sensitivity (although fluorescence detection might be more sensitive for certain mycotoxins, e.g. aflatoxins), unambiguous confirmation of the molecular identity of the analyte and the option to use isotopically labelled substances as internal standards. Furthermore, it is possible to investigate the molecular structure of metabolites and sugar conjugates (such as "masked mycotoxins"; Berthiller et al. 2005b) and to omit time-consuming and error-prone derivatization and clean-up steps. However, it must be kept in mind that a reduction of the sample preparation inevitably emphasizes the Achille's heel of LC/MS, i.e. relatively poor method accuracy and precision due to the irreproducible and unpredictable influence of co-eluting matrix components on the signal intensity of the analytes.

Due to the large number of LC/MS-based methods for the quantitative determination of single mycotoxin classes, their exhaustive examination

goes beyond the scope of this work and, therefore, the interested reader is referred to the reviews of Zöllner and Mayer-Helm (2006) and Sforza et al. (2006).

Multi-mycotoxin methods

In the last few years, increased efforts have been made to develop analytical methods for the simultaneous determination of different classes of mycotoxins using LC–MS/MS. This trend is a result of the discovery of co-occurrence of different toxins and related synergistic toxic effects that raise concerns about the health hazard from contaminated food and feed (Creppy et al. 2004; Speijers and Speijers 2004). In addition, it would be desirable to cover the toxins addressed by Commission Regulation 1881/2006 (aflatoxin B1, B2, G1, G2 and M1, ochratoxin A, patulin, deoxynivalenol, zearalenone, fumonisin B1 and B2, HT-2 and T-2 toxin) with a single method as this increases sample throughput and decreases the costs per analysis. Although mass spectrometry offers sufficient selectivity (especially if tandem-mass spectrometry is applied) and multi-analyte capabilities, its realization in the field of multi-mycotoxin analysis has been hampered mainly by the chemical diversity of the different toxin classes, which include acidic (fumonisins), basic (ergot alkaloids) as well as polar (moniliformin, nivalenol) and apolar (zearalenone, beauvericin) compounds. Therefore, compromises have to be made in the choice of extraction solvent and mobile phase, and the conditions may be far from optimal for certain analytes.

The initial stimulus for LC/MS-based multi-mycotoxin methods came from the field of mycology, where mass spectrometry is used to identify mould species according to their metabolite profile (Smedsgaard and Frisvad 1996). Beside the development of databases dealing with qualitative LC/MS of mycotoxins (Nielsen and Smedsgaard 2003), this has led to early quantitative methods for the simultaneous determination of *Aspergillus* and *Penicillium* mycotoxins in building materials (Tuomi et

al. 2001) and in an artificial food matrix (Rundberget and Wilkins 2002). While the former method suffered from low recoveries of some analytes, excellent accuracy and precision were obtained in the latter case through use of a de-fatting step applied to the raw extract, and use of matrix-matched calibration to compensate for matrix effects. Some years later, this method was applied for the simultaneous determination of aflatoxins, ochratoxin A, mycophenolic acid, penicillic acid and roquefortine C (Kokkonen et al. 2005) after a slight modification of the extraction solvent.

After this initial phase, the focus of multi-mycotoxin analysis shifted to *Fusarium* mycotoxins. Royer et al. (2004) developed a method for the quantitative analysis of deoxynivalenol, fumonisin B_1 and zearalenone in maize, including accelerated solvent extraction, a two-step SPE procedure and internal standards for each analyte. The LODs were below the maximum concentration levels permitted in the EU, but the method suffered from a low recovery for zearalenone. The next generation of methods included several A- and B- trichothecenes as well as zearalenone, and used Mycosep® columns for clean-up of the raw extracts. Zearalanone was used as internal standard for zearalenone in the method of Berthiller et al. (2005a), and Biselli and Hummert (2005) applied matrix matched calibration for this analyte. Cavaliere et al. (2005) added α-zearalenol and three fumonisins to the list of analytes and performed de-fatting and solid-phase extraction of the raw extracts of corn meal. While the efficiency of the extraction step was greater than 84% for all analytes, matrix effects were still present and required matrix-matched calibration.

A method for the simultaneous determination of *Fusarium*, *Aspergillus* and *Penicillium* toxins (ochratoxin A, zearalenone, α- and β-zearalenol, α- and β-zearalanol, fumonisins B1 and B2, T2- and HT2-toxin, T2-triol, mono- and diacetoxyscirpenol, deoxynivalenol, 3- and 15-acetyldeoxynivalenol, deepoxy-deoxynivalenol and aflatoxin M1) was reported by Sorensen and Elbæk (2005). Bovine

milk samples were defatted and after adjustment of the pH, an SPE procedure was applied. Signal suppression/enhancement was minimized and recoveries >76% were obtained. However, the major drawback of this method was the necessity of using two chromatographic runs with different columns and eluents. The two most recent reports, which include a clean-up of the raw extract using MultiSep #226 cartridges, introduced instrumental improvements to multi-mycotoxin analysis in foodstuffs. A time-of-flight mass spectrometer was used by Tanaka et al. (2006), while, in the method of Ren et al. (2007), analysis time was significantly decreased through the application of ultra-performance liquid chromatography. In both methods, recoveries >70% were obtained for all analytes and no significant matrix effects were reported.

As all these methods rely on some sort of clean-up, certain toxin classes are excluded as they are not compatible with the clean-up and/or extraction conditions (for example, the fumonisins are not determined by the methods of Tanaka et al. (2006) and Ren et al. (2007). In particular, neither ergot alkaloids, moniliformin, enniatins nor masked mycotoxins are included in any of these reports. To overcome these problems, some existing methods omit a clean-up of the sample and inject raw extracts into the LC/MS. This clearly increases the demands on the selectivity of the detector as well as on the investigation of matrix effects, especially if complicated food matrices are analysed. Spanjer et al. (2005) determined 22 mycotoxins (including the ergot alkaloid ergotamine) in different food matrices. Samples were extracted with an acetonitrile/water mixture and were diluted with water prior to injection. Matrix effects were investigated for every analyte/matrix combination and validation data obtained that suggested that the analysis of diluted raw extracts is indeed feasible and at the same time sensitive enough for determining most mycotoxin levels set in the legislation. Our own contribution in this field was the quantitative determination of a set of 39 analytes (including moniliformin, beauvericin,

enniatins and masked mycotoxins) in wheat and maize (Sulyok et al. 2006). In both matrices, linear calibration curves were obtained (with the exception of moniliformin) after spiking blank matrices at multiple concentration levels, with coefficients of variance of the overall process of <5.1 and $<3.0\%$, respectively. Significant matrix effects were observed for maize, but these could be overcome by matrix-matched calibration. LODs ranged from 0.03 to 220 $\mu g\ kg^{-1}$ and the trueness of the method was confirmed for deoxynivalenol and zearalenone though the analysis of certified reference materials. Very recently, this method has been extended to the simultaneous determination of 87 mycotoxins and has successfully been applied to mouldy food samples (Sulyok et al. 2007).

In the near future, a strong trend towards multi-mycotoxin methods, which do not involve a clean-up of the sample, can be expected, as these methods can be relatively easily adapted to new analytes and matrices, and the obvious time- and cost-savings compensate for the expense of initial validation. Advances in the technology and in the instrumental design in mass spectrometry will further decrease the influence of matrix effects, which certainly constitute the main drawback of this approach at the moment.

Fast screening methods

Immunochemical techniques

Rapid methods based on immunochemical techniques often have the advantage of not requiring any clean-up or analyte enrichment steps. ELISAs have become routinely used tools for rapid monitoring of most mycotoxins, especially for the screening of raw materials (Gilbert and Anklam 2002; Fremy and Usleber 2003). Although ELISA tests may show a high matrix dependence and possible overestimation of levels, the advantages of the microtitre-plate format are speed, ease of operation, sensitivity and high sample throughput. ELISA test kits are commercially available for most of the major mycotoxins (EMAN 2007).

Alternatives to ELISAs include a number of immunosensors as well as upcoming methods using immunochemical platforms, such as fluorescence polarization immunoassays (FPI) (Ngundi et al. 2005) or surface plasmon resonance (SPR) with mycotoxin–protein conjugates immobilized onto a sensor chip surface (Tüdös et al. 2003). Immunosensors are emerging as a cost-effective alternative for screening and quantitative determination of mycotoxins (Maragos 2004). Array biosensors have been developed using competitive-based immunoassays for the simultaneous detection of multiple mycotoxins, including ochratoxin A, fumonisin B, aflatoxin B1, and deoxynivalenol, on a single waveguide surface by imaging the fluorescent pattern onto a CCD (charge-coupled device) camera (Sapsford et al. 2006). Other formats with fluorescence detection include automated flow-through immunosensors with enzyme-labelled mycotoxin derivatives (Urraca et al. 2005). Electrochemical immunosensors with surface-adsorbed antibodies on screen-printed carbon electrodes have been fabricated for the detection of aflatoxin M1 in milk (Micheli et al. 2005) and, in an array configuration, for the detection of aflatoxin B1 (Pemberton et al. 2006). Affinity-based surface plasmon resonance sensors (SPR) have the advantage of not requiring any labelling of the target mycotoxin (Tüdös et al. 2003) and may become an alternative method for rapid screening, which also enables the simultaneous detection of multiple mycotoxins using serial connected flow cells (van der Gaag et al. 2003). In a further label-free immunochemical approach for the detection of aflatoxin B1 and ochratoxin A, optical waveguide lightmode spectroscopy (OWLS) was used with integrated optical waveguide sensor chips measuring the resonance incoupling angle of polarized light, thus determining the surface coverage (Adányi et al. 2007).

A complementary tool for the screening of cereal samples may be DNA microarray-based chips using PCR followed by microarray colorimetric detection, which has been developed for the fast detection and identification

of 14 trichothecene- and moniliformin-producing *Fusarium* species occurring on cereals (Kristensen et al. 2007).

In recent years, interest in rapid membrane-based immunoassay methods, such as flow-through immunoassays and lateral flow devices (LFDs), has strongly increased due to the need for rapid on-site (pre)-screening. A flow-through enzyme immunoassay was developed for the detection of ochratoxin A in roasted coffee (Sibanda et al. 2002). Requiring no sample preparation other than an extraction step, LFDs allow qualitative or semi-quantitative determination of mycotoxins on one-step strip tests within a few minutes. Such LFDs have been developed for selected mycotoxins, such as aflatoxin B1 (Delmulle et al. 2005) and fumonisin B1 (Wang et al. 2006). The strong interest is furthermore reflected in the increasing number of commercially available test kits for field use, based mostly on direct competitive assays.

Non-invasive techniques

Optical methods, such as Fourier Transform mid-infrared spectroscopy with attenuated total reflection (Kos et al. 2003) or near-infrared transmittance spectroscopy (Pettersson and Aberg 2003), are promising techniques for the fast and non-destructive detection of mycotoxins in grains. The approaches allow sample preparation to be reduced to an absolute minimum and to be integrated into on-line monitoring systems. Nevertheless, since rapid data interpretation is based on the output of chemometric analysis, the high matrix dependence and the lack of appropriate calibration materials are still major restrictions.

Similarly, electronic noses, featuring an array of electronic chemical sensors with pattern recognition systems, have also been developed (Logrieco et al. 2005). In this approach, volatile organic compounds of low molecular weight, which are released by many fungi as products of secondary metabolism, are adsorbed onto the sensor surface and measured with a variety of transduction systems based on electrical-, optical-, or

mass-transduction, such as metal oxide sensors (MOS) and surface acoustic wave sensors (SAW), for example (Olsson et al. 2002).

The high demand for rapid screening methods for mycotoxin analysis reflects the need for fast and cost-effective on-site determination of the level of mycotoxin contamination in food and feed. Immunochemical-based screening methods have shown great potential and are increasingly applied in routine analysis and monitoring of mycotoxins. Nevertheless, although rapid and selective, a loss of sensitivity may have to be taken into account in easy-to-use-assays due to the necessary simplification of the system which usually employ no washing step. Future trends in screening methods include the further development of fast and simple tests requiring no instrumentation and improved detection capability for the simultaneous measurement of multiple mycotoxins.

Quality assurance

Method validation

As mentioned above, a multitude of methods have been published for the determination of mycotoxins in food and feed over the years. However, only a limited number of these publications include performance characteristics data obtained by method validation, which is a prerequisite for the production of reliable results in terms of comparability and traceability. Typical performance characteristics to be evaluated for the validation of a quantitative method are the limit of detection and quantification (LOD/LOQ), linearity, precision (repeatability and reproducibility), selectivity (interference of other compounds and/or matrix components), robustness/ruggedness, working range and trueness/bias (Josephs et al. 2004). Several protocols and guidelines for method validation have been published, e.g. the ISO standard 5725 (ISO 1994) or the guide "The fitness for purpose of analytical methods" (Eurachem 1998).

There are various methods for mycotoxin analysis available that are validated and have been accepted by official authorities such as CEN, the AOAC, and ISO etc. (AOAC 2005; Gilbert and Anklam 2002). CEN usually evaluates the performance criteria of a method on the basis of collaborative studies. Most CEN methods are also AOAC- and ISO-approved. The latest edition of the Official Methods of Analysis (OMA) from the AOAC is available online and contains about 60 validated methods for mycotoxin analysis (AOAC 2005). Gilbert and Anklam (2002) have compiled validated and official analytical methods for the determination of several mycotoxins, including aflatoxins, ochratoxin A, patulin, fumonisins, deoxynivalenol and zearalenone, in various matrices, such as cereals, nuts, milk, fruits and juices, as well as their products, intended for human consumption and animal feed.

Reference materials and intercomparison studies

Reference materials (RM) or certified reference materials (CRM) are materials with a defined sample constitution and known or certified content of analyte(s) along with its uncertainty. (C)RMs play an important role not only during the validation process of a method but also as a measure to assure the quality of analytical data during routine analysis (in terms of trueness, comparability and traceability). (C)RMs can be classified as pure substances (standards), standard solutions (calibrators) or matrix materials (spiked or naturally contaminated). Until recently, the Institute of Reference Materials and Measurements (IRMM) of the EC has been the only provider of mycotoxin CRMs (IRMM 2007). These include RMs for aflatoxins in peanut (BCR 263, BCR 264, BCR 401), compound feed (BCR 375), milk powder (ERM-BC 282, ERM-BC 283, ERM-BC 284), ochratoxin A in wheat (BCR 471), deoxynivalenol in maize (BCR 377, BCR 378) and wheat flour (BCR 396), and zearalenone in maize (ERM-BC 716, ERM-BC 717). The IRMM also provides standard solutions for calibration purposes (calibrators) of deoxynivalenol (IRMM 315) and

nivalenol (IRMM 316) in acetonitrile. The US National Institute of Standards and Technology (NIST) has recently issued a standard RM (SRM 2387) for aflatoxin determination in peanut butter (NIST 2007a). A full compilation of (C)RMs currently available and in production can be found on the homepages of various institutions, e.g. COMAR (2007), CORDIS (2007), IAEA (2007), IRMM (2007) and NIST (2007b). Despite past and current efforts to produce (C)RMs and calibrators for mycotoxin analysis, there is still an eminent need for more RMs that are appropriate for the different sample matrices and concentration ranges, especially with regard to the implementation and monitoring of regulatory limits (maximum levels) of mycotoxins in food and feed set by national and international authorities.

Intercomparison studies (collaborative studies or proficiency testing schemes) play an important role in the validation of analytical methods and the production of RM (Gilbert and Anklam 2002; Josephs et al. 2004) as well as acting as quality assurance tools for laboratories. To improve the comparability and traceability of analytical data in Europe, several intercomparison studies in the field of mycotoxin analysis, especially for *Fusarium* mycotoxins, have been performed in the past 10 years within projects funded by the EC (Josephs et al. 2004; Krska et al. 2005). Additionally, the Food Analysis Performance Assessment Scheme (FAPAS) continually organises proficiency testing schemes in mycotoxin analysis (FAPAS 2005).

Conclusion and outlook

In the laboratory, sample extracts are preferably purified and enriched in a clean-up step procedure using mainly SPE and IAC, with multifunctional IACs currently being put to the test. TLC and LC are still the most frequently employed analytical methods for the (official) determination of mycotoxins; however, LC–MS/MS is increasingly used for the simultaneous determination and identification of large numbers of

mycotoxins, currently up to 87. The use of CRMs and certified calibrants is one of the key issues of quality assurance in the analytical laboratory.

The established state-of-the-art chromatography-based methods for mycotoxin analysis are increasingly being complemented by a number of new screening methods, including LFDs, biosensors and IR-screening techniques, that are fast and cost-effective. Nevertheless, these techniques will have to compete with both classical confirmatory methods and MTP-ELISAs, which are now widely used for mycotoxin screening.