Chapter 5

The germinating spore as a contaminating vehicle

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INTRODUCTION

Fungi can be found in a wide variety of environments, such as in seeds, plants, soil, water, insects, food and food products, and animal products. Phytopathogenic fungi cause damage to the living crops upon storage as *Colletotrichum* that causes anthracnose disease in several fruits and vegetables such as banana, avocado, papaya, and tomato, decreasing their commercial values. Fungal infection of grain, nuts and fruits is often preceded by physical damage caused by insect invasion or mechanical injury during harvest. Fungal growth reduces the nutritional value of storage grains and animal feed and can result in the production of mycotoxins (D'Mello and MacDonald, 1997). Mycotoxins are poisonous, often carcinogenic secondary metabolites of fungi, which are associated with certain disorders in animals and humans (for *Fusarium* on grain see for instance, D'Mello *et al*., 1998; Reid *et al*., 1999).

Food products also become contaminated during processing and handling operations. Processed food can be considered as a complex often plant-based medium that fungi colonise and spoil. Fungal species associated with particular foods correlate with the characteristics and properties of the product (Dijksterhuis and Samson, 2002; Filtenborg *et al*., 2004). The primary cause for the deterioration of rye bread for example are the fungi *Penicillium roqueforti, P. paneum, P. carneum* and *Paecilomyces variotii.* Contaminated commodities, such as cereals, can deteriorate during storage, resulting in enhanced contamination levels of whole wheat flour (Weidenborner *et al*., 2000). In food products, the issue of mycotoxins requires continuous attention, but more recently fungal spores are also increasingly recognized as aeroallergen sources (Green *et al*., 2005). Fungal contamination and the toxic metabolites it forms cause massive economic losses of food. There is a great interest among agricultural, food industrial and medical disciplines to prevent or control fungal contamination. These include different techniques that manipulate the physical environment of the fungus including acidification, increase of the osmotic potential, drying, cooled storage, pasteurisation and the use of modified atmospheres. Some fungal species are able to grow at such adverse conditions and are able to thrive at situations that are meant to be free of spoilage.

Contamination and colonisation of the food products is often by means of survival vehicles including airborne spores. Fungi are known for their capability to produce sexual and/or asexual spores as agents of reproduction, dispersal and survival. Some fungal species predominantly form sexual spores as *Talaromyces* species even without the need of different mating types (homothallic) and ascospores are produced in high numbers, while there is only restricted production of asexual spores. Alternatively, many fungal species do not have a well recognised sexual stage and designated as the Deuteromycetes (mitosporic fungi). This group includes many members of genera as

Aspergillus, Penicillium and *Fusarium,* which are very relevant fungi for food situations (Dijksterhuis and Samson, 2002). Spores play an important role in the life cycle of fungi acting as dispersal or survival spores. Dispersal spores are separated completely from the parent mycelium by different factors to facilitate migration to a new site. They have a moderate capacity for survival in a resting state (dormancy). They are also capable to germinate readily in the presence of nutrients or favourable environmental conditions (Griffin, 1994). In case of *Aspergillus* and *Penicillium*, conidia are formed in chains on specialised spore-forming cells (phialides). Mature conidia have to survive in dry conditions during dispersion through the air current (Dijksterhuis and Samson, 2002). In contrast, survival spores are often produced in lower numbers and may not be separated from the parent mycelium (Carlisle *et al*., 1994). As an example, thick-walled chlamydospores are produced by e.g. *Mucor racemosus*, *F. culmorum* and *Paecilomyces variotii* and typically produced between hyphal cells. Besides, many ascospores are formed inside closed or open fruit bodies (ascomata) that reside within the mycelium and not on specialised structures (conidiophores) that enable the spores to be distributed by air- or water currents. Many fungal species are able to produce different types of spores within one colony as is the case with for example *Fusarium* species (microconidia, macroconidia and chlamydospores) and *Eurotium* species (conidia and ascospores) (Samson *et al*., 2004).

As is stated above, fungal contamination of foods and food products and colonisation and infection of plants and animals is usually initiated by contact of the host with spores (conidia). Contamination by the external environment, e.g. air, water, walls and floors for instance is considered to be the main source of contamination of beef carcasses with *Penicillium*, *Aspergillus*, *Mucor* and *Cladosporium* species (Ismail *et al*., 1995). Additionally spores can be brought on the crop or food product via an encounter with organisms (insects, mites). The germination process is the beginning of fungal colonisation into food and on plants or animals. It involves the initiation of biochemical activities, with an increase of the metabolic rates and induction of morphological changes (Griffin, 1994; d'Énfert, 1997). A better understanding of spore survival and the different processes of spore germination could lead to novel techniques to prevent food spoilage. This chapter describes the germination process of fungal spores and the relation between germination and fungal contamination, mycotoxin production, control methods and the mode of action of antifungal agents. The problem of fungal contamination can be partially confronted with the use of fungal inhibitors of germination and hyphal growth, but spores are less sensitive to different compounds. It is here, that the terms fungistatic and fungicidal have a different meaning. Germination of a spore includes a continuous change from a "stasis"-like situation towards a vegetatively growing hyphal cell expressing processes as active metabolism, expanding cell mass and nuclear division.

LANDING, ADHESION AND WETTING OF THE CONIDIA

The first events of fungal colonisation are the landing of the spores on the substratum and subsequent hydration. Airborne spores are cells that have to deal with drying and rewetting and certainly will possess mechanisms that address the redistribution of cell components that accompany these changes. In *Magnaporthe grisea,* the conidia that are transported through the air have a collapsed appearance as a result of dehydration and this stage is regarded as a normal part of the life cycle of the cell and not as an artefact due to preparation of the cells.. After rewetting these conidia retained their turgid shape (Howard, 1993). A similar feature is visible with dry rust spores immediately after contact with the leaf surface (Deising *et al*., 1992). Upon landing attachment of the spore is important especially in case of the colonisation of plant surfaces, which often have a hydrophobic nature.

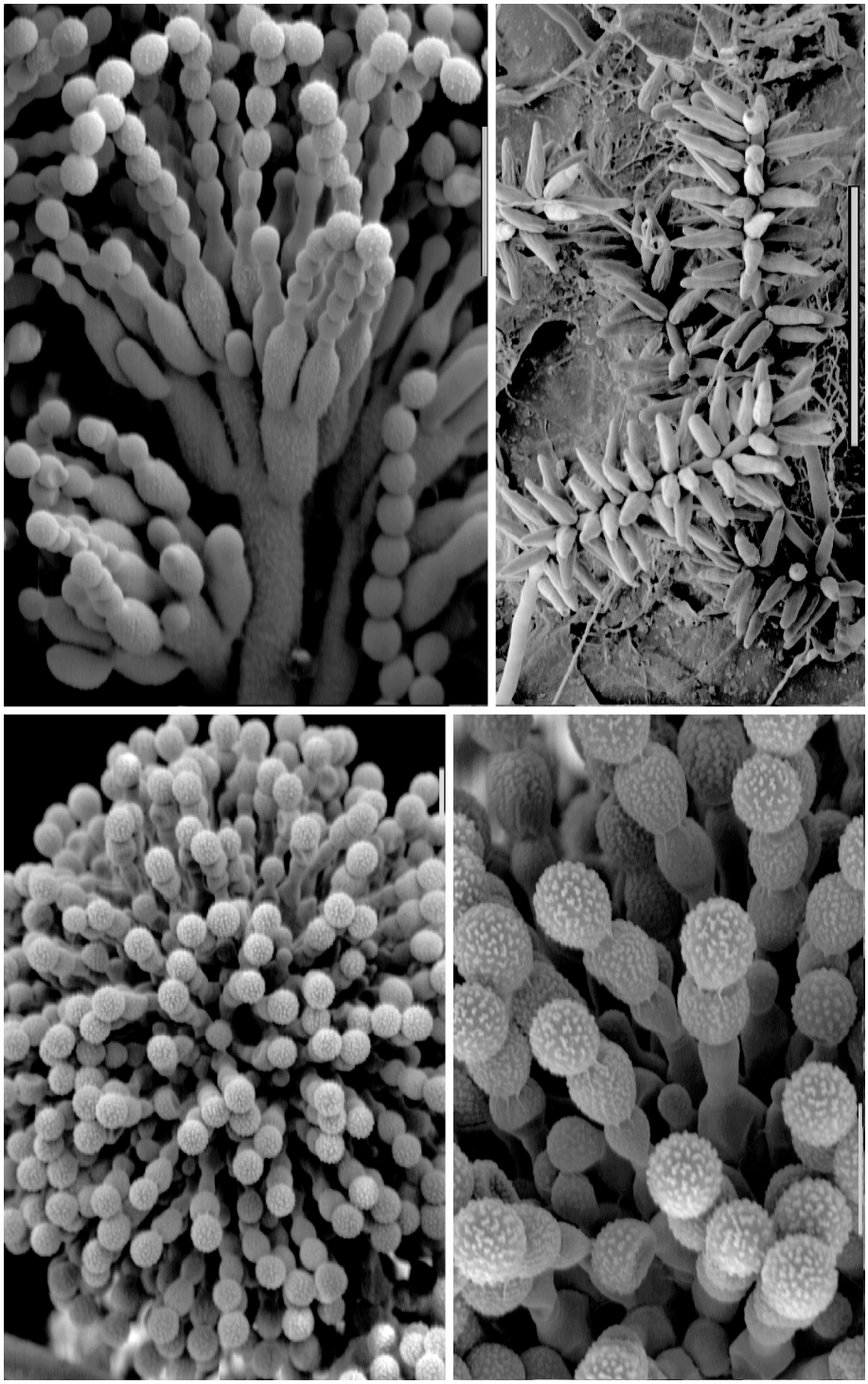

Figure 1. Formation of spores on specialized structures. Top left, a conidiophore of *Penicillium bialovaiense* were spore forming cells (phialides) are clearly visible. Top right, multicelled spores of the fungus *Helminthosporium solani* formed on a spore-bearing structure that is located on potato skin. Bottom left, *Aspergilllus oryzae* forms numerous conidia on a conidiophore were many strings of conidia form a spherical structure. Bottom right, detail of the previous micrograph with visible ornamentation on the conidia.

Some spores possess a droplet of adhesive material as conidia of *M. grisea* that bear a spherical droplet on the tip that literally glues the spore to the leaf surface when it is transported to it (Hamer *et al*., 1988).

Other spores do not have these appendages and attachment must been reached by other means. In case of urediniospores of *Uromyces viciae-fabae,* 10-25% of the spores were able to adhere immediately to different surfaces and were not removed by sterile destilled water (Clement *et al*., 1993). Attachment was higher on hydrophobic surfaces and increased after more than 20 min, which indicates that during development of the spore the attachment to different surfaces increased indicating a "vulnerable" stage where spores can be removed easier. Immediate adhesion of *Botrytis cinerea* conidia was studied by Doss *et al*. (1993) and it was found that dry conidia adhered for 15% to tomato cuticle, but after vapour hydration for 2 min, 93% of the conidia were adhered (were resistant to a jet of nitrogen gas). Washing with water resulted in 37% adhered spores on the cuticle (and 9% on glass). The process was not influenced by lectins or proteases, but detergents had a strong inhibiting effect on adhesion to both hydrophobic substrata polystyrene and tomato. The authors conclude that adhesion is a two-step process with " passive" adhesion mediated by hydrophobic interactions (and also occurring with killed conidia) and subsequent stronger delayed adhesion during swelling and germ tube formation. Upon contact and wetting of the urediniospores *Uromyces viciae-fabae* material accumulation between the spore-cuticle (in this case bean leaves) interface became apparent (Deising *et al*., 1992). This material dubbed " the adhesion pad" plays a role in firmer attachment to and degradation of the (wax) cuticle of the host. The tenacity of adhesion to artificial surfaces of uredospores of *U. appendiculatus* was correlated with the extent of hydrophobicity (Terhune & Hoch, 1993), which was measured after more than 30 min. The black rot fungus of grape, *Phyllosticta ampelicida,* exhibits a complex adhesion pattern towards different substrates including electrochemical attraction (spores have a polyanionic surface i.e. it is negatively charged) and hydrophobic interactions. Remarkably, attachment was a prerequisite in this fungus for subsequent germination, indicating that these phenomena were linked (Kuo & Hoch, 1996). In a number of cases, immediate attachment to the substrate was seen (within seconds) and factors of the imbibing solution (as acidity) where vital here.

Filonov (2001) confirmed that *B. cinerea* conidia become firmer attached to apple skin during swelling and germination. A short ultrasonication treatment removed > 95% of the conidia immediately after addition. During the first 4 h 80% of the spores were recovered, but during the formation of germ tubes attachment to the substrate had increased strongly with 70% attachment after the treatment after 24 h. Filonov (2003) further studied the adhesion and germination of conidia of different fungal species on polycarbonate membranes. Adhesion was assayed after 24 h which means that spores could have germinated very well and that adhesion of germ lings is assayed in such a case. From these studies it became clear that adhesion/germination of *Penicillium expansum*, *claviforme* and *roqueforti* as well as *Botrytis cinerea* was markedly higher and influenced through by the presence of acetate esters. It is tempting to assume that the fruit rotters, *P. expansum* and *B. cinerea* react on volatile constituents of fruits, while adhesion of only these fungi had increased on apple skin with additional esters present in the air. It was also clear that fresh wounds on apples captured 80-100% of the spores compared to approx. 20% on the skin and that the age of the wound was correlated with the firmness of attachment after 4h. Wounds of 24 h old age exhibited 40% recovery of *B. cinerea* conidia after sonication treatment at 150 W for 10s while fresh wounds did not show recovery. In case of *P. expansum* this was 60% vs 20% of the conidia. This observation is very relevant for post-harvest problems while disinfection of wounds directly after formation is in fact very important (see also Filonov, 2004).

The act of wetting alone leads to changes in the conidium as is observed with *Neurospora crassa* (Bonnen and Brambl, 1983). They observed an increase in the fraction of polyri-

bosomes correlated with water harvested conidia while cells obtained in an isoparaffinic hydrocarbon fluid had the same levels of these structures as dry-harvested conidia. This shows that cellular constituents change immediately after contact of the cytoplasm with water. In this case protein synthesis might occur quickly after wetting of the cells. Incubation of sporangiospores of *R. oligosporus* after a 2 hour storage period in buffer (pH 4) showed metabolisation of cFDA (carboxyfluorescein) and germ tube formation in a subpopulation of the cells after 4 hours (Thanh *et al*., 2004), which indicate that spores develop under very poor nutrient conditions upon wetting.

FURTHER STAGES OF GERMINATION

In general water and nutrients are important requirements for proper germination. Many fungal species need external addition of these nutrients for optimal germination, other species often related to plant (leaf) surfaces do germinate in distilled water and have internal deposits of nutrients (as rust-fungi do, e.g. *Uromyces vignae,* see Dijksterhuis, 2003). Leaf pathogenic fungi as *Colletotrichum* species and *Magnaporthe grisea* need a hard surface as one of the requirements for germination and appressorium formation. Similarly, *Botrytis cinerea* germinates on glass surfaces that are hydrophic , but also on rich media. In the latter case conidia rapidly germinate with long germ tubes that soon branch (Doehlemann *et al*., 2006).

Addition of phosphate, amino acids, glucose and combinations of the compounds resulted in increased germination in case of sporangiospores of *Rhizopus oligosporus* (Thanh and Nout, 2004; Thanh *et al*., 2005). Besides, also physical factors can invoke germination; sporangiospores of *Phycomyces blakesleeanus* are activated to germinate by a heat treatment at 50 º C(van Assche *et al*., 1972). When proper nutrients are available the spores continue to develop, which results in isotropic growth also designated as swelling, which is observed in numerous fungal species. *Fusarium culmorum* and *Rhizopus* spores require a carbon and nitrogen source for development. *Penicillium griseofulvum* and *Aspergillus nidulans* conidia need glucose for germination (d'Enfert, 1997; Osherov and May, 2001). In addition, other low molecular weight nutrients as example inorganic salts can activate germination (Griffin, 1994). Uptake and metabolisation of the probe carboxyfluorescein diacetate (cFDA) was strongly increased after the introduction of dried sporangiospores of *R. oligosporus* in malt extract at 37 ºC (Thanh *et al*., 2004, 2005). This was interpreted as a monitor of the beginning of the germination process. After long drying periods (11 months) the spores did show no colony formation on 2% glucose alone (< 1%). More complex media as malt extract, peptone, yeast extract and glucose/peptone medium resulted in much higher numbers of germinated spores (33-36% of the spores) and colonies. The use of the fluorescent probes propidium iodide (PI) indicated that a large subpopulation of the dried spores show PI-related membrane permeabilisation and DNA staining (Thanh *et al*., 2006). However, the dye TOTO-1 was not observed inside the cells. This is remarkable while both PI and TOTO- staining inside the cell was regarded as an indicator of cell death. When dried spores were pre-inoculated in malt extract broth, the majority of the spores stained with cFDA and therefore were metabolically active. This is evidence for a regeneration of a damaged cell population. The fraction of PI positive-TOTO negative (thus damaged) spores increased with storage time. The requirements of germinating spores may clearly differ from other stages of the fungal lifecycle. With *R. oryzae*, sporangiospores germinate readily in malt pepton medium, but germination decreases below a pH of 4,8 (J. Dijksterhuis, unpublished results). Optimal germination was observed at 30º C, while the highest radius of colonies is observed in case at 35º C.

The first obvious change in spore morphology in many fungal species is isotropic growth, also designated as swelling which is observed in case of *Penicillium* and *Aspergillus* species (M.R. van Leeuwen, CBS, unpublished results) and *Fusarium culmorum* macroconidia (Chitarra *et al*., 2005) where the spore starts to swell and

consequently increases its volume. Swelling is not merely water uptake, it is also characterised by changes in the composition of the cell, cell wall growth, and increase in dry weight (Bartnicki-García and Lippman, 1977). Isotropic growth is accompanied by numerous metabolic activities including respiration, RNA and protein synthesis (van Etten *et al*., 1983; Ohja and Barja, 2003), and degradation of trehalose into glucose (Osherov and May, 2001).

Following swelling, cell wall deposition becomes polarized, and the extension occurs at a restricted area at the tip of the developing germ tube (Parton *et al*., 1997). Momany (2002) distinguished different stages of spore germination. First initiation, which inclused breaking of dormancy and the start of isotropic growth. A phase of isotropic growth, which is roughly between 3 and 7 hours is followed by the establishment of an area of polarised growth, which includes the proper positioning of cell wall deposition and directioning of the vesicle transport machinery in which the cytoskeleton, different proteins and the plasma membrane show a precise interplay (Cheng *et al*. 2001). This cooperation results in the outgrowth of a germ tube and later, the formation of a branching mycelium. Extensive studies have been carried out on the germination of unicellular spores, e.g. *Colletotrichum, Aspergillus, Penicillium* and *Rhizopus*, but hardly anything is known about germination of multicellular conidia. (Bourret, 1986; Breeuwer *et al*., 1997; Marin *et al*., 1998; Chaky *et al*., 2001; Leandro *et al*., 2001).

The different stages of conidial development seem to be linked to the different stages of the cell cycle (see Harris, 1999). Isotrophic growth takes place in *A. nidulans* until the first mitosis. After mitosis an axis of polarity is established and maintained in the emergence and elongation of the germ tube (Momany, 2002). Mitosis is also associated with septum formation at the base of the emerged germ tube. Between *A. fumigatus* and *A. nidulans* interesting differences were observed in timing of polarity establishment related to the mitotic state. Pear shape (germ tube emergence) was observed in 22% of the conidia before the first mitosis in case of *A. fumigatus* and not with *A. nidulans*. (Momany and Taylor, 2000). Similar differences are observed with septation and the emergence of a second germ tube and these morphogenetic changes are also related to the nutrient status of the medium. The autors mention that a critical size of cell volume could be an important factor in septum formation. Remarkably, Dijksterhuis *et al*. (unpublished results) have found that a gradual decrease occurs in conidia of different *Penicillium* species with respect to fluid phase viscosity of the cytoplasm to a level that is typical for vegetative cells. In such a case a global and physical parameter might induce cell changes.

SIGNALLING DURING EARLY GERMINATION

Different signalling factors are involved with germination of conidia. In sporangiospores of *Pilobolus longipus,* glucose resulted in a rise of cAMP before germination (Bourret, 1986) and the role of this signalling pathway was also recognised in yeast ascospores (Thevelein, 1984). Fillinger *et al*. (2002) studied the role of adenylate cyclase in the cAMP signalling pathway as well as the downstream kinases schA and pkaA during germination of conidia of *A. nidulans*. A double mutant of pkaA and cyaA (the adenylate cyclase) and the single cyaAΔ mutant exhibited delayed conidial germination (30% in 15 h), but certainly not a complete arrest. Trehalose degradation was blocked in the cyaAΔ and the schaAΔpkaAΔ mutants. This indicates that individual signalling elements of the adenylate cyclase sequence play a role in different aspects of germination, but that they have several targets, which results in the operation of a signalling network. Changing the activity of the signal mediator Ras to dominant activity leads to blocked germ tube formation and resulted in prolonged swelling and multiple nuclei. This Ras-pathway operates independent of the adenylate cyclase pathway/network and dominant activity of Ras results in a defect of polarity establishment. Ras is a member of the small GTPase family and plays and important role in the communication inside different signalling

networks in the cell. Two different GTPase types, a ras and a rho type were studied in the dimorphic fungus *Penicillium marneffei* (Boyce *et al*., 2005). For this study dominant negative and dominant positive transformants were used, while a deletion could not be generated (lethal?). The dominant negative *rasA*^{D125A} and the dominant activated *rasA*^{G19V} both showed less germination after 12 h and the authors state that an increase proportion of the cells are misshapen. This indicates that activity of these molecules above and below a certain level has a bearing on germination. Dominant activation of a rho GTPase and CDC42 homologue *cflA*^{G14V} did undo the effect of *rasA*^{D125A} and resulted in high germination again. Another rho GTPase named *cflB* was deleted and showed some disturbance in conidial germination including not complete germination after 12 h, but a somewhat higher incidence of secondary germ tubes at that stage (Boyce *et al*., 2003).

Zuber *et al*. (2003) studied the effect of alterations of the G-protein α-subunit on germination of conidia at 25º C. This is also a molecule that cycles between a GTP-bound active (signalling) state and a not signalling Gα-subunit. Germination rate was lowest (25% at 27 h) in the Δ*gasC* mutant, also delayed in the *gasC*^{G207R}, which is not signalling (25% at 15 h), but was accelerated in the dominant active *gasC*^{G45R} compared to the wildtype (35% vs 25% in 10 h. These features remained similar under carbon poor situations.

Similarly, in *A. nidulans,* conidia show enhanced germination with a constitutively active *ganB*^{Q208L} mutant and was lowest in the ganB^{G207R}, which is kept in the inactive state (Chang *et al*., 2004).

Calcium is a factor that plays an important signalling-role in cells that settle on a surface These can be Oomycetes as *Phytophthora parasitica* (Warburton and Deacon, 1998) or fungal species as *Phyllosticta ampelicida* (Shaw and Hoch, 2000) or *Colletotrichum gloeosporioides* (Kim *et al*., 1999). The latter two species need a hard surface to germinate and also this features has to be communicated into the cell. Doehlemann *et al*. (2006) have studied germination in *Botrytis cinerea* and found that a disrupted Gα3 subunit reduced fructose dependent germination to approximately 20% of the cells over a long period, but this mutasnt germinated like the wildtype on a hydrophobic polypropylene surface. Deletion of the MAP kinase BMP1 resulted in no-germination at all on hydrophobic surfaces. Buhr and Dickman (1997) observe maximum expression of serine-threonine kinase, calmodulin and protein kinase C prior to germ tube morphogenesis of *C. trifolii*, which illustrates that during germination many different factors play a role and there a complex interplay of signalling routes may depict this picture to the cell.

COMPATIBLE SOLUTES IN CONIDIA

Accumulation of compatible solutes inside living cells serves is thought to protect cells against osmotic stresses and it also is observed after an oxidative or heat shock in germinating conidia of *Aspergillus nidulans* (Fillinger *et al*., 2001). These compounds do not disturb the functioning of proteins and other biomolecules and the complexes formed by them when they are present in high amounts inside the cell, henceforth the name *compatible* solutes (see also Dijksterhuis and Samson, 2002). Trehalose, an α-1,4 non-reducing disaccharide including two linked glucose moieties (α-D-glucopyranosyl-α-D-glucopyrano-side) is an important compatible solute and protects both membranes and proteins (Crowe *et al*., 1984; Hottiger *et al*., 1994; Prestrelski *et al*., 1993; Wolkers *et al*., 1998) against drying and heat. Trehalose is synthesised in yeast cells from glucose by the action of trehalose-6-phosphatase (encoded by *TPS1*) which links two phosphorylated glucose molecules to each other via an UTP-bound energizing step. The resulting trehalose-6-P is dephosphorylated by means of a trehalose-6-phosphate phosphatase (*TPS2*).

Germination of conidia is associated with a degradation of the trehalose pool from 1,2 pg per spore to zero within 120 min and this phenomenon is observed with different types of spores (Thevelein *et al*. 1983; d'Enfert *et al*., 1999; Dijksterhuis *et al*., 2002). Assuming a cell diameter of approximately 3,0 μm (based on

conidia of *A. niger* studied by Tiedt (1993) and a density of conidia above 1,0 gr/ml (otherwise the cells could not be centrifuged so quickly) this results in a weight of one spore of minimally 20 pg meaning that trehalose accumulates to maximally 6,7 % of the cell wet weight. In *A. nidulans* trehalose degradation is performed by an neutral cAMP-activated and calcium-dependent neutral trehalase. Germinating conidia of 2 h show 80% decrease in colony forming units after 20 min at 50°C. A mutant defective of neutral trehalase activity retained levels of trehalose at the maximum level and showed nearly unchanged germination and full heat tolerance for at least 40 min at this temperature (D'Enfert *et al*., 1999). Conidia of *A. nidulans* that had germinated for three hours at 30° C and shifted to 50° C showed accumulation of trehalose within 30 min to a level of maximally 0,8 pg/spore (Fillenger *et al*., 2001). Lower accumulation was observed after addition of 100mM H_2O_2. A tpsAΔ strain of *A. nidulans* was, unable to produce trehalose and surprisingly, the wildtype and mutant showed a similar sensitivity for the stressors and the accumulation of trehalose did not increase the survival of the germlings during short-term exposure. However, germinating conidia of the mutant showed very low colony formation when stored for approx. 15 hours at 44° C, while the wildtype showed no significant decrease. Sustained storage of conidia at 20°C showed gradual decrease in viability during a period of 20 days in case of the mutant, but with maximal germination by the wild type after 50 days. Of course, trehalose only can perform its function when it is present in the cell, it can not restore the damage done to the germling after a heat shock. Yeast cells that were treated with a short heat shock showed a increased *acquired* tolerance to subsequent heat treatment that was associated with the presence of trehalose (De Virgilio *et al*., 1994). Combined these data strongly suggest that the presence of trehalose provides protection against different types of stress and also plays an important role in the longevity of the life of the conidium.

Surprisingly, the *A. nidulans* conidia also contain 0,8 pg mannitol per spore (4 % wet weight) which is degraded to zero in 3 hours time during early germination. Also in conidia of the related fungus *A. niger,* mannitol is an important compatible solute (Ruijter *et al*., 2003). Mannitol is produced by the action of two enzymes mediating a reduction and a phosphatase activity from fructose 6-phosphate via mannitol 1- phosphate. Conidia of *A. niger* contain 10,9% dry weight mannitol and assuming a percentage of water inside the spores of 50% or more this would be approx. 5,5% mannitol (wet weight). For a comparison, stress-resistant ascospores of the fungus *Talaromyces macrosporus* contain approximately 38% water and are regarded as very dense (Dijksterhuis *et al*., 2002). The *A. niger* conidia contain a somewhat smaller quantity of trehalose (3,6% dry weight). The ΔmpdA strain of *A. niger* that is deficient for the mannitol 1-phosphate dehydrogenase has more trehalose (11,5 % dry weight) and reduced mannitol levels (4,0% dry weight). Mutant conidia show 90% viability loss after nearly 1 hour of heating at 50°C while the wildtype survives easily 2h at this temperature. Further, conidia are more sensitive for a freeze-thaw step, lyophilization and a hypochlorite treatment. Interestingly, there is no difference in long-term storage between the wildtype and the mutant, which indicated that trehalose and not mannitol plays an important function this respect. Combined these data may suggest:that a combination of trehalose and mannitol, that are both present in conidia of the two species in approximately equal amounts give protection against different stressors.

That another cell mechanism is connected with the processes of compatible solute accumulation, for instance the expression of heat shock related proteins.

That trehalose plays a role on its own in the process of long-term survival of the spores.

Halssworth and Magan (1994, 1996) provided clear evidence that the growth conditions of the spore-delivering culture in the fungal species *Metarrhizium anisopliae*, *Beauveria bassiana* and *Paecilomyces farinosus* strongly influences the accumulation and composition of compatible solutes inside the conidia. These species are insect pathogens and the spores of

these fungi were used for biocontrol of insect pests and proper storage and survival of the conidia was of great importance. In control situations (in this case Saboraud Dextrose Agar) mannitol was the most dominant solute in the three species (Hallsworth & Magan, 2006). The presence of trehalose, glycerol or starch in the growth medium of the three species highly influenced the internal composition of the spores with mannitol, glycerol, erithr(e)itol and trehalose as main players with total solute levels between 10 and 20% dry weight (Hallsworth and Magan, 1994). These authors observed that glycerol and erythritol dominance inside the spores was correlated with (faster) germination in case of lower water activities (Hallsworth and Magan, 1995). *A. nidulans* conidia were also tested (Hallsworth *et al*., 2003) from PDA media with excess glycerol or KCl. All grow conditions resulted in mannitol levels of 4,4-4,6% dry weight, but glycerol containing medium also showed 6,3 and 2,7% glycerol and erythreitol, two other important compatible solutes in fungi. The KCl conidia showed intermediate levels of the latter (0,35 and 0,64%), and 0,084% and 0,21% in PDA grown cells. The authors observed protection of germination of conidia that contained high levels of ethanol and erithreitol in the presence of ethanol and NaCl (up to 7,5 and 16% wt/vol, respectively). *Penicillium chrysogenum* was grown on pearl barley by Ballio *et al*. (1964) and the harvested conidia showed 10% and 8,3% mannitol and trehalose respectively (dry weight) and 3,0 and 2,7 % glycerol and erythritol which confirm the typical levels of these solutes inside this type of spores. Tabel 1 summarizes very shortly the functions correlated with the different compatible solutes discussed till now.

Tabel 1. Compatible solutes and their function in conidia

Compatible solute	Function inside spore
Mannitol/Trehalose	Protection against heat
Trehalose	Longevity
Glycerol/Erithreitol	Protection during germination at low α_w

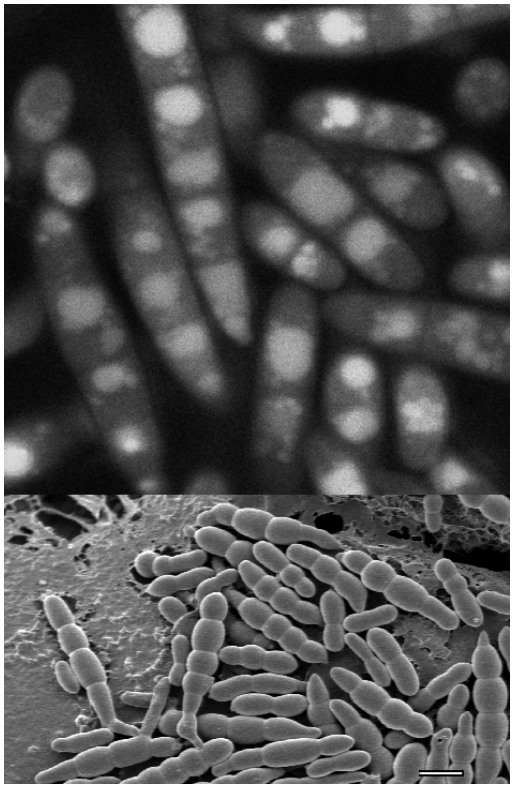

Figure 2. Multi-compartment macroconidia of the fungus *Fusarium culmorum.* Top, cells stained with a fluorescent dye (cFDA-SE), which is used for the measurement of the internal pH of the cells. The cells are studied with the confocal microscope. Bottom, cryo-electron microscopy of germinating macroconidia that clearly show isotropic growth of the individual compartments.

MULTICELLULAR CONIDIA AND INTERNAL pH.

Multicellular conidia are formed by a large number of fungi among them are a number of species very relevant in food situations. *Fusarium* species are important food related (cereals) and plant pathogenic mycotoxin forming fungi. *Alternaria* species are often observed on cereals and form mycotoxins and are related to allergenic reactions. *Helminthosporium solani* causes silver scab on stored potatoes and *Magnaporthe grisea* is the most important pathogen of rice. Germination of these multicellular conidia is only scarcely studied and one could argue if the different cells of such a spore behave as individual conidia "glued" together or

do exhibit a certain differentiation. Is there any ecological reason to produce these structures? It was known that in conidia *M. grisea* and *F. culmorum* germ tubes developed preferably from apical cells and seldom from middle cells (Jelitto, J., 1999; Atkinson *et al*., 2002; Chitarra *et al*. 2005a). The last authors studied *F. culmorum* as a modelsystem of multicellular conidia by means of FRIM (fluorescence ratio imaging) where the internal pH inside spores was followed during germination.

The pH_{in} for *F. culmorum* (pH_{in} 6.4; Chitarra *et al*., 2005a) was higher than *P. paneum* (pH_{in} 5.4; Chitarra *et al*., 2005b), but both within the pH range previously reported for *Rhizopus oligosporus,* which is between pH 5 to 6.5 (Breeuwer *et al*., 1997). The germination of *P. paneum* conidia and *F. culmorum* macroconidia under optimal conditions included both isotropic growth and an increase of intracellular pH of both types of spores and no statistical significant differences among compartments of *F. culmorum* was observed during early stages of swelling. During further stages of swelling and germ tube formation differentiation between the compartments of the macroconidium was statistically sound. After the first stages of swelling the ungerminated middle compartments of *F. culmorum* showed a significant decrease in pH_{in}, which was not associated with an increase in the fraction of vacuoles in the cells. The latter was observed with *M. grisea* conidia (Atkinson *et al*., 2002). The monitoring of the internal pH in multicelled conidia showed higher pH values in apical cells and certainly inside the germ tubes. The pH_{in} in germ tubes of *F. culmorum* (>7.2) was in agreement with the pH_{in} of *M. grisea* germ tubes (7.4) incubated in complete nutrient medium stained with the dye SNARF-1 (Jelitto, T, 1999) or with the cytoplasmic pH found with dextran conjugated dye in hyphae of *Neurospora crassa* (Parton *et al*., 1997). Changes in pH may be associated with differentiation processes as has been reported elsewhere (Inoue, 1985; Stewart *et al*., 1988). These studies do not establish whether an increase in pH_{in} occurs prior to or if it is a product of the metabolic changes occurring inside the cell. Controversial results of internal pH gradient in tip growth hypha have been previously reported (Roncal *et al*., 1993; Jelitto *et al*., 1994; Robson *et al*., 1996; Parton *et al*., 1997).

Taken together these observations suggest that differentiation occurs between the different compartments. The inclination of the apical cells to germinate over middle cells may be started by the difference between the surface-to-volume ratio of apical cells. This may facilitate differences in, for instance, transport processes and henceforth introduce an asymmetry in the development of the conidium. The consistent germination pattern of the macroconidium was changed after treatments of the conidia with sublethal doses of nystatin. Apical cells were preferably targeted by the compound and the distorted germination was counteracted by an increased germination of the middle compartments. This shift indicates an way of communication between the cells and a type of "apical dominance" may be alleviated from the middle cells. The ecological function of such differentiation and communication may be sought in the more versatile response of these cells towards adverse conditions for germination. When the first germination fails another attempt can be done later and if conditions remain unattractive for colonization, the middle cells may differentiate further to long-survival spores as chlamydospores and this was already observed decades ago (French and Nielsen, 1966; Schneider and Seaman, 1974).

FUNGAL GERMINATION AND SELF INHIBITORS

Fungi produce substances during growth that influence their own development. These can be inhibitory substances and then are named self-inhibitors. These compounds inhinit germination of spores or growth of hyphae. For example, germination of spores of *Rhizopus oryzae* shows lower germination when they are present in higher densities. Germination lowers from 67 to 22% after 4 hours of incubation when the density of the spores increases from 10^6 to 7 x 10^7 spores/ml (J. Dijksterhuis, unpublished results). Self-inhibitors have been char-

acterised in many fungal (and non-fungal) genera *Puccinia, Uromyces, Colletotrichum, Dictyostelium, Fusarium* and *Aspergillus* and can be volatile or non-volatile (see for instance, Allen, 1955, Bacon and Sussman, 1973, Barrios-Gonzalos *et al*., 1989). Various self-inhibitors have been isolated and identified after extraction from culture filtrates of fungi (Table 2).

Self-inhibitors also can influence other fungal processes, for example, mycosporine-alanine produced by *C. graminicola* prevents appressorium formation (Leite and Nicholson, 1992, 1993). The self-inhibitors produced by *Glomerella cingulata* and *Dictyostelium discoideum* (not strictly a fungus, but this illustrates that concept of self-inhibition might be widely spread) inhibit protein synthesis (Bacon and Sussman, 1973; Lingappa *et al*., 1973). Self-inhibitors must inhibit spore germination in a reversible manner, after removal of the compound from the spore or its environment, germination is initiated (see also chapter 1 of this book). The major function of self-inhibitors is stated as prevention of premature germination of spores directly after formation when they are located at conidiophores, inside fruiting bodies or on pustules (in case of rust-fungi) and before spore dispersion. This mechanism guarantees that spores only germinate after dispersal into the environment that favour outgrowth to establish a mycelium.

Breeuwer *et al* (1997) studied the mode of action of the self-inhibitory compound nonanoic acid in sporangiospores of *Rhizopus oligosporus*. Nonanoic acid results in both a decrease in internal pH and a lower number of metabolic active cells, but this effect is transient and restoration of the internal pH to normal levels occurs at a concentration of 1 mM. The mode of action of this compound is compared to that of weak organic acids that are used as food preservatives, like sorbate, propionate and acetate. Also spores of other fungal species show similar phenomena in the presence of nonanoic acid.

The intracellular pH of macroconidia of *Fusarium culmorum* fluctuated between 5.4 and 6.5 in the presence of nonanoic acid during a period of 90 minutes at an extracellular pH of 4.0 (Chitarra *et al*., 2005a). The disturbed or fluctuated intracellular pH was recovered

Table 2: Self-inhibitors from fungi.

Fungal species	Chemical compound	References
Aspergillus niger		Krishnan, 1954; Barrios-Gonzales, 1989
Anisogramma anomala		Stone, 1994
Blastocladiella emersonii		Adelman, 1974
Colletotrichum capsici		Louis, 1988
Colletotrichum gloeosporioides	Gloeosporone	Lax, 1985
Colletotrichum graminicola	Microsporine-alanine,	Leite, 1992
Dictyostelium discoideum	N,N-dimethylguanosine	Bacon, 1973
Fusarium oxysporum	Nonanoic acid	Garrett, 1969
Geotrichum candidum		Steele, 1973
Glomerella cingulata		Lingappa, 1973
Hemileia vastatrix	Free organic acid	Musumeci, 1974
Microsporum gypseum		Page and Stock, 1971
Penicillium griseofulvum		Fletcher, 1970
Peronospora tabacina	5-Isobutyroxy-β-ionone	Leppik *et al*., 1972 Page and Stock, 1971
Puccinia graminis var *tritici*	Coumarins and phenolic acids. Methyl-cis-ferulate	Sumere, 1957; Macko, 1971
Puccinia helianthi	Methyl-3,4 dimethoxycinnamate	Macko, 1971
Puccinia antirrhini	Methyl-3,4 dimethoxycinnamate	Macko, 1971
Syncephalastrum racemosum	Nonanoic acid	Hobot, 1980
Tiletia caries	Trimethylalanine	Trione, 1973
Uromyces phaseoli var *typica*	Aspartic and glutamic acid	Wilson, 1958; Stone, 1994; Steele, 1973

twice, indicating that macroconidia had energy enough to pump excess protons out of the cell.

In addition, swelling and germ tube formation of the conidia of *Penicillium paneum* was inhibited and transient collapse of the internal pH of the spores was also observed (Chitarra *et al*., 2005b).

Recently, a volatile self-inhibitor, 1-octen-3-ol, was identified in case of the fungus *P. paneum* (Chitarra *et al*., 2004; Chitarra *et al*., 2005b) that blocked swelling and germination of conidia at a millimolar (4 mM) concentration range and approximately 70% of the conidia had the same size as freshly harvested conidia after 4h, while 80% of the control cells was clearly swollen. 1-Octen-3-ol was initially identified in and above dense suspensions of conidia. Small droplets of very dense (10^9 spores/ml) conidial suspensions placed on thin agar layers showed less than 10% germination after 24 h, which indicate a clear crowding effect. There was some entering of the fluorescent indicators PI and TOTO into the conidia in the presence of 1-octen-3-ol (in case of 20 and 10%) which indicates a mild permeabilization of the plasma membrane. In addition oxygen consumption was slightly lowered and a transient drop in internal pH was observed. Taken together these observations suggest that 1-octen-3-ol has a mild systemic effect on the developing conidial cells. Surprisingly, there were notable differences in the composition of the protein population of treated cells after 5h compared to the controls. So, despite its mild physiological effects a profound influence on protein expression was observed.

1-Octen-3-ol also inhibits other fungal life stages including radial growth of the mycelium of different fungal species. Further, microcycle conidiation was observed in the presence of the compound. One could suggest that 1-octen-3-ol acts as a fungal hormone during development of the fungal thallus. Physiologically, 1-octen-3-ol is a product of the enzymatic breakdown of linoleic acid by the enzyme lipoxygenase and a hydroperoxide. In *Pleurotus pulmonarius,* linoleic acid splits in two compounds, 10-HPOD (10-hydroperoxyocta-decadienoic acid) a precursor of 1-octen-3-ol and 13-HPOD (13-hydroxyperoxy-cis-9,trans-11-octadecadienoic acid)(Assaf *et al*., 1997; Kuribayashi *et al*., 2002, Figure 3.) Together with 1-octen-3-ol, a non-volatile metabolite, 10-oxo-trans-8-decenoic acid (ODA) is formed in this process, which is stated to have an influence on the development of the mushroom. It stimulates growth of the mycelium, stipe elongation, and fruiting initiation during mushroom development and it has been regarded as a growth regulating substance (GRS) produced by gills (Mau *et al*., 1992; Champavier *et al*., 2000). Other linoleic acid derivatives play a role in sporulation phenomena in *Emericella (Aspergillus) nidulans*, which suggest that poly-unsaturated lipid compounds and their degradation products are remarkably important in development of fungi.

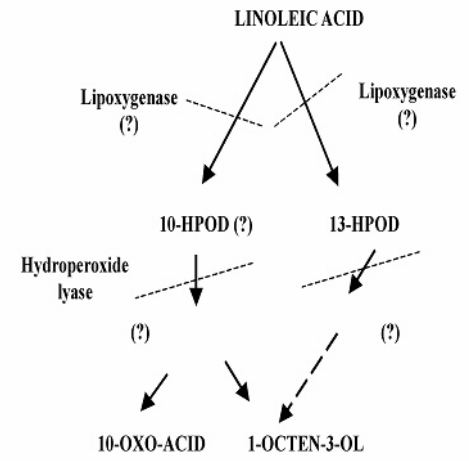

Figure 3. Proposed pathway for the formation of the volatile self-inhibitor 1-octen-3-ol.

Further investigation of the role of self-inhibitors may reveal novel methods for the inhibition of fungal development in food.

ANTIFUNGAL COMPOUNDS

Germination and growth of fungi in food and feed is discouraged by the introduction of different adverse conditions as the use of altered gas composition, low water activities and the presence of organic acids or a combination of these factors. Low oxygen pressure and organic acids for instance are used to preserve grass forage in silos with during ensilage. These conditions lower the metabolism of

fungi and prevent their growth as is also the result of lowering of the water activity of a medium to α = 0.65- 0.86. However, some osmotolerant and xerophilic fungi that are able to grow in the presence of high concentrations of sugar and salt cause spoilage in these conditions (Dijksterhuis and Samson, 2002), but with many commodities including seeds grains, beans and peas prevention of fungal spoilage is successful due to their low water activity if properly dried and well stored.

The main antimicrobial food preservatives are weak organic acids and esters (propionate, sorbate, benzoate and benzoate esters (parabens)), organic acid acidulants (lactic, citric, malic, and acetic acids), inorganic acid preservatives (sulfite), mineral acids (phosphoric and hydrochloric acids) and other compounds as natamycin (Britt *et al*., 1974; Kabara and Eklund, 1991; Gould, 1996, Stark, 2003). Propionate is a highly effective fungal inhibitor used in cheese and bakery products industries. Secbutylamine is commonly used in its free form to preserve fruits against damage by storage fungi such as *Penicillium* and *Aspergillus*. In addition, sorbate prevents fungal growth and decreases mycotoxin biosynthesis by inhibiting the biological pathways responsible for their production. Nowadays, a wide range of antifungal agents is used in combating biodeterioration, preventing or treating fungal disease of plants and or treating diseases in animals and humans (Table 3). The mode of action of these compounds is variable also an important part of the compounds have plasma membrane and cell wall related targets.

As is the case with antibiotics against bacteria novel compounds are actively search at and one potential family of antifungal compounds are the iturins (A-E) that are produced by *Bacillus subtilis*. Iturins are cyclic lipopeptides characterised by the presence of seven α-amino acids (Isogai *et al*., 1982; Latoud *et al*., 1990). Iturins interact with sterols in the cytoplasmic membrane and is similar to that of the antifungal polyene amphotericin B (Maget-Dana *et al*., 1985; Latoud *et al*.; 1996). Other lipopeptides that belong to the iturin group are the bacillomycins D, F, and L, and mycosubtilin (Bland, 1996; Moyne *et al*., 2001). Iturin A reduced the fungal population on seed with variation among the fungal species with respect to their sensitivity, but it is not able to inhibit aflatoxin production of *Aspergillus flavus* (Klich *et al*., 1993, 1994). For instance, *Rhizopus* sp. was known previously not to be sensitive to it (Gould, 1996). This may be explained by the low ergosterol content of the *Rhizopus* sp. membrane (Groll *et al*., 1998). Fungal inhibition was observed in case of post harvest fungal spoilage of peaches and the role of iturins during biological control with *Bacilli* studied (Gueldner *et al*., 1988), An iturin-like compound inhibited the germination of *Penicillium paneum* conidiospores (Chitarra *et al*., 2003). Fluorescence microscopy and FCM revealed that the PI was able to label damaged cells, indicating the permeabilisation of *P. paneum* conidiospores membrane after exposure to the HCl precipitate.

Table 3: Antifungal compounds and their mode of action.

Synthetic antifungals	Mode of action
Benzimidazoles; Griseofulvin	Mitosis
5-Fluorocytosine	Nucleic acid synthesis
Acylalamines	RNA polymerase I
Kasugamycin; Sordarins	Protein synthesis
Carboxamides; Strobilurins	Respiration
-Fosetyl-AL	Phosphate metabolism
Imidazoles; Triazoles; Thiocarbamates	Ergosterol synthesis
Nystatin; Amphotericin B; Natamycin	Plasma membrane
Polyoxin; Nikkomycins; Echinocandins	Cell wall synthesis

EPILOGUE

The fungal spore is a resting phase and as such is not very reactive on antifungal compounds. Killing of spores with other methods than heat is a very difficult task. Germination of spores (conidia) however is a gradual development from resistant and not-responsive cells to germ-tube bearing cells via a number of stages. Knowledge about the sensitivity of these different phases to antifungal compounds is vital to evaluate the potential of fungal spores to form "spoilage time bombs" when the antifungal compound is inactivated due to its stability or diffusion.

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