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## 2 Fungal Spoilage of Crops and Food

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### I. Introduction: Food Is an Ecological Resource

Food products are a rich nutrient source that will attract both bacterial and fungal colonizers. As such, the food product can be regarded as an **ecological resource**. After successful colonization of the product, its nutritional properties are altered. When the nutritional value, structure, and taste of the product are negatively influenced, this colonization is called food spoilage. It can be accompanied by the production of toxic secondary metabolites which may result in grave medical problems, and is an issue that needs our continual awareness with

respect to food safety. This will be the topic of another chapter in this book.

In other cases, colonization with a number of food-borne microorganisms is beneficial with respect to nutritional value and prolonged storage of the food product, which is dubbed as food fermentation. These two aspects of food colonization are two sides of the same coin.

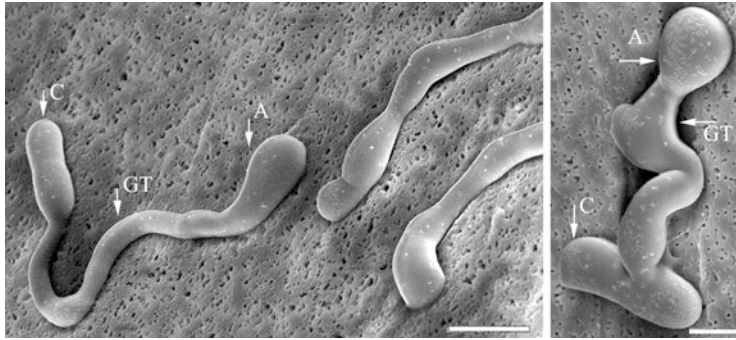
Food spoilage is a major threat for our food stock and is responsible for **enormous losses worldwide**, which makes it a research area that is very relevant with respect to the **increasing demand on food** during the next decennia. Knowledge concerning the specific mechanisms that occur during food spoilage might generate novel insights that result in increased net amounts of food without an increase of land use.

This chapter highlights fungal spoilage, including the fact that it deals mostly with **plant-based food products**. Fungi are the main degraders of the sturdy plant cell wall components that otherwise would accumulate within the ecosystems of the world. Prior to spoilage, the fungi can be present on or inside the crop in low numbers, or as survival structures. Spoilage fungi can also be introduced to an empty habitat if the food is previously treated by pasteurization treatments.

Food products include two main groups, namely **living crops and processed food**. Colonization of food products is hence very diverse. This chapter evaluates different fungal-food relationships. At first, the relationship between the living crop and fungi is illustrated. Then the association of fungi with different types of processed food is described. Different preservation techniques make the food product a difficult environment to colonize, although it is also a rich medium. Only fungi

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**Fig. 2.1.** Conidia (C) of a *Colletotrichum* species germinate on an avocado surface. The germ tube (GT) differentiates into an appressorium (A) that is firmly attached

to the plant cell wall (Micrograph made by Jan Dijksterhuis, CBS-KNAW Fungal Biodiversity Centre, The Netherlands). Bars are 10 and 5  $\mu\text{m}$  respectively

that can survive certain adverse conditions including high osmolarity and heat can successfully spoil processed food. Different aspects of stress resistance are addressed in this chapter, including osmotolerance, protective compounds inside cells, and heat-resistant structures.

Several books on food spoilage fungi summarize many different aspects of fungi and food. Pitt and Hocking (2009) and Samson et al. (2004) provide overviews on the taxonomic description and specificity of food spoilage fungi, and Dijksterhuis and Samson (2007) highlight numerous aspects of the relation between food and fungi including spoilage and fermentation.

## II. Infection of Living Crops: Post-Harvest Diseases

The relation between fungi and living agricultural crops can be regarded as **plant-pathogenic** in nature, which includes a **complex communication** between parasite and host. Some of these fungi enter intact crop cells without direct killing the host. They initially **establish a fungus-host interface** as a biotrophic fungus that can exhibit prolonged survival in a quiescent state, which can be **followed by a necrotrophic** infection stage in which plant tissue is killed and lesions develop. The **true necrotrophic fungi** start to kill plant tissue directly upon entering the host. The so-called

**opportunistic fungi** cause infections of fruit, vegetables, or flower bulbs by entering cracks, wounds or natural orifices on the surface of crops. The total number of fungal species involved in post-harvest diseases is much larger than ever can be covered in this short overview; we would like to illustrate some principles of infection and the fungi involved.

### A. Anthracnose as an Example of Complex Crop-Fungus Interaction

Members of the fungal genus *Colletotrichum* (teleomorph; *Glomerella*) cause **anthracnose** disease in a wide range of fruits and vegetables. For instance, the species *C. gloeosporioides* (*Glomerella cingulata*) infects **over 100 different host species**. These fungi produce highly specialized structures called **appressoria** that provide fungal entry into healthy plant tissue (Fig. 2.1). Appressoria generate enormous turgor pressure, which enables the penetration peg formed on it to breach the sturdy plant cell walls with pure mechanical force (Bechinger et al. 1999). In the case of infection of the climacteric fruit of the tomato with *C. gloeosporioides*, the fungus actually waits for the right moment to infect. The **plant hormone ethylene** that is produced by the tomato during senescence is an important trigger for proper infection (Flaishman and Kolattukudy 1994).

On avocado, appressoria are formed on unripe fruit, and penetrate the plant cell wall. The **subcuticular hyphae** then become

**quiescent until ripening of the fruit has occurred** (Prusky and Lichter 2007). Quiescence is correlated with the presence of an **antifungal compound, AFD**, which is degraded by the enzyme lipoxygenase. Indirectly, AFD levels are controlled by the antioxidant epicatechin which is present in the avocado peel and acts as a lipoxygenase inhibitor (Ardi et al. 1998).

Synthesis of the antifungal compound is correlated with the expression of a  $\Delta 12$ -fatty acid desaturase (Wang et al. 2004). Interestingly, a cold treatment that resulted in increase of unsaturated fatty acid also gave rise to increased antifungal diene (AFD). *C. gloeosporioides* also reacts to **other chemical triggers**, namely the host surface wax in the case of anthracnose of avocado fruit (Podila et al. 1993). Analysis of wax fractions showed that differentiation of appressoria was maximal in the presence of certain long-chain fatty acid alcohols. That this aspect of communication is also very specific is illustrated by a strongly lowered appressorium formation by wax from other fruit (jade wax). Another basic requirement for appressorium formation is **the presence of a surface** (Kim et al. 1998). Thus, different thigmotropic (sense-reactive) and chemical signaling pathways cooperate during differentiation and infection. Wax- and ethylene-dependent signaling pathways are not identical, but share two proteins that must be phosphorylated (Kolattukudy et al. 1995). Unripe avocado tissue is also able to react on the presence of fungal elicitor with **the formation of ROS**; during ripening this ability is almost absent (Beno-Moualem and Prusky 2000).

Following the initial stage of infection, the fungus resumes growth and develops from a biotrophic parasite characterized by fungal cells that are compatible with living plant cells towards a necrotrophic parasite that actively kills the host cells. This is characteristic for a **hemibiotrophic lifestyle** (as reviewed in Prusky and Lichter 2007; Prusky and Kolattukudy 2007; Münch et al. 2008). Necrotrophic hyphae are thinner than biotrophic hyphae, and produce a variety of plant-cell-wall-degrading enzymes, and also produce other factors that lead to cell death such as reactive oxygen species or secondary metabolites.

A typical phenomenon which is correlated with the onset of the necrotrophic stage is the accumulation of ammonium at the leading edge of the developing lesion (Alkan et al. 2008, 2009), as is observed with *C. coccodes* (on

tomato), *C. gloeosporioides* (on avocado), and *Alternaria alternata* (on persimmon fruit). This **alkalinization** is induced by the usually low pH of the host fruit, and is a prerequisite for the activation of a host NADPH oxidase, a ROS-producing enzyme. The presence of ROS increases local cell death, a hallmark of necrotrophic growth.

Tissue alkalization in *C. gloeosporioides* also results in the secretion of a pectate lyase (encoded by PELB) via increased expression of the transcription factor PacC. Loss of function mutants of the latter factor have shown strongly reduced pathogenity and pectate lyase secretion (Miyara et al. 2008). In addition, *C. gloeosporioides* forms a laccase in the avocado peel that is able to degrade epicatechin and thus shortens the period of quiescence in case of some active isolates of the fungus (Guetsky et al. 2005). Thus, hemibiotrophic pathogens are able to establish complex interactions with the host.

## B. True Necrotrophs

**Necrotrophs directly start to kill plant tissue upon entering** the host and some of them develop into **broad spectrum pathogens** that destroy many different and large amounts of vegetables and fruits upon harvesting. *Botrytis cinerea* is fungus that causes widespread infection of grapes, strawberries, and other fruits, as well as vegetables. The fungus enters the host by means of appressorium-like structures or via wounds.

The appressoria can breach intact plant tissues, and require the presence of tetraspanins (the gene *BcPls1*) for successful penetration (Gourgues et al. 2004). Homologues of these specialized membrane proteins are also found in the plant parasite *Magnaporthe grisea* that forms similar appressoria as *Colletotrichum*.

After entrance into the plant tissue, the fungus starts to kill host cells with the help of toxic secondary metabolites such as botrydial. There is evidence that the fungus uses the host hypersensitivity response for further infection (as reviewed by Choquer et al. 2007). The fungal genome of *B. cinerea* contains families of plant-cell-wall-degrading enzymes, and up to 12 different lipases have been identified (van Kan 2006). In particular, the enzymes involved in

pectin degradation, including endopolygalacturonases, are important for *B. cinerea*, and hosts with high pectin contents are an excellent target for the fungus. In tomato fruit, the activity of expansins and polygalacturonases produced by the host loosens the plant cell wall during ripening. Transgenic tomato strains that did not have these activities showed clearly reduced and delayed infection development after inoculation with *B. cinerea* (Cantu et al. 2008). These findings show that the **interaction between necrotroph and the host is considerably more complex** than thought before (Amselem et al. 2011), and that there exists a balance between host and pathogen. *B. cinerea* is an avid producer of **oxalic acid** inside the lesion. This organic acid stimulates cell-wall-degrading enzymes, and also has a strong calcium-chelating activity that helps to **destabilize the pectin network** in which calcium ions are embedded (van Kan 2006; Prusky and Lichter 2007). Oxalic acid production is even more a hallmark of infection by another widespread necrotrophic pathogen *Sclerotinia sclerotiorum* (Kim et al. 2007; Hegedus and Rimmer 2005) that is related to *B. cinerea* (Amselem et al. 2011). This fungus is notorious as a post-harvest pathogen of carrot, sunflower seeds, and bean pods among 400 plant species, most of them dicots.

Here, oxalic acid also modulates the hypersensitivity response, including programmed cell death around the pathogen, in delaying the oxidative burst and prevention of callose deposition at the leading edges of the lesions. (Williams et al. 2011)

### C. Opportunistic Fungi

**Opportunistic fungi can grow well without plant hosts as saprotrophs** on decaying plant material or in soil. They also infect crops mostly without the help of specialized infection structures, and need a natural opening or a wound in the outer layer of the crop. Despite their dual growth mode, they can develop into **true pests of harvested crops**. Opportunists can also enter via the **dying leaves of the flower** before the fruit is fully grown (Snowdon

1990). For tomatoes, fungi often develop first on the remnants of the leaves present on the fruit (the so-called calix), and then colonize the tissues of the fruit (Smid et al. 1996). **Careful handling of crops** directly after harvesting is vital for the quality of the product. The more small wounds that are introduced by, for instance, rough treatment of the crop, the more damage occurs as a result of post-harvest diseases.

For example, the fungus *Alternaria alternata*, a post-harvest pathogen of many vegetables and fruits (Thomma 2003), can attack apples via the calyx tube and causes core rot in susceptible cultivars (Niem et al. 2007). *A. alternata* also causes rot of persimmon fruit, melon, and tomato, and is a fungus that alkalinizes the host tissue.

An important factor in the disease is an endo-1,4- $\beta$ -glucanase that is more highly expressed in the presence of cell-wall polymers and a higher pH (above 6, Eshel et al. 2002). The fungus can survive in a quiescent state in plant material, and enters plant tissues that are weakened as a result of senescence or wounding, but the formation of small appressoria is not ruled out.

Like all other fungi involved in post-harvest rot, growing hyphae of the opportunistic fungi release enzymes that degrade the plant cell wall, which results in **dry or wet rot** of the food crop. This depends on the selection of enzymes formed by the pathogen. **Cellulolytic enzymes** do not disrupt the pectin middle lamella, and therefore do not dissociate plant cells, which results in a more preserved structure of the tissue after infection known as dry rot (as reviewed in Prusky and Kolattukudy 2007). **Pectin-degrading enzymes** destroy the connection between the cells, resulting in maceration and wet rot. The variety of the secreted polysaccharide-degrading enzymes is large. The variability of these enzymes have been reviewed (De Vries 2000; De Vries and Visser 2001; Pel et al. 2007) in the case of the fungus *Aspergillus niger*. The genome of this fungus contains ORFs of 131 secreted carbohydrate active enzymes, which illustrates the versatility of the tool box to degrade plant cell walls.

*A. niger* is a cosmopolitan fungus, and causes serious opportunistic infections in onions and hyacinth bulbs.

In citrus fruit, the fungi *Penicillium italicum* and *P. digitatum* (blue and green rot of citrus respectively) cause the most serious and widespread rots of these crops. Other opportunistic fungi on these fruits are *Alternaria alternata*, *A. niger*, *Fusarium spp.*, *Geotrichum candidum*, and *Trichoderma viride* (Snowdon 1990). In apple, *P. expansum* is a post-harvest problem of similar magnitude. *P. italicum*, *P. digitatum*, and *P. expansum* are all able to acidify the host tissue and form citric acid in liquid culture (Prusky et al. 2004). In citrus and apple fruit, citric acid and gluconic acid accumulate, and expression of an endopolygalacturonase (*pepg1*) was highest at pH 4.0.

The fungi have a preference for ammonium as the nitrogen source. Ammonium levels had dropped sharply in decaying tissue because of uptake by the fungal cells, which excrete  $H^+$  and lower the pH inside the lesion. The production of gluconic acid was accompanied with the expression of a glucose oxidase gene (*gox2*), and virulent isolates showed more of both (Hadas et al. 2007). Interestingly, GOX activity, gluconic acid accumulation, and decay dropped significantly when oxygen levels dropped to 10 % or lower. This indicates that gluconic acid and not citric acid is an important factor for disease.

That opportunistic fungi are markedly adapted to infection of harvested crops is illustrated by conidia of *P. digitatum* that germinate quicker and in higher numbers in the presence of volatiles that surround wounded oranges (Eckert and Ratnayake 1994). Interestingly, the strongest stimulation was observed when the “authentic” volatile mixture was applied, and was invariably lower in preparations of single compounds or mixtures with concentrations above and below that of the wounded oranges.

Microconidia of *Fusarium oxysporum* f. sp. *tulipae* cause a devastating dry rot in tulip bulbs, and only germinate in wounds on the surface of the bulbs and are not able to grow on undamaged epidermis, even when it is very close to a wound (Fig. 2.2).

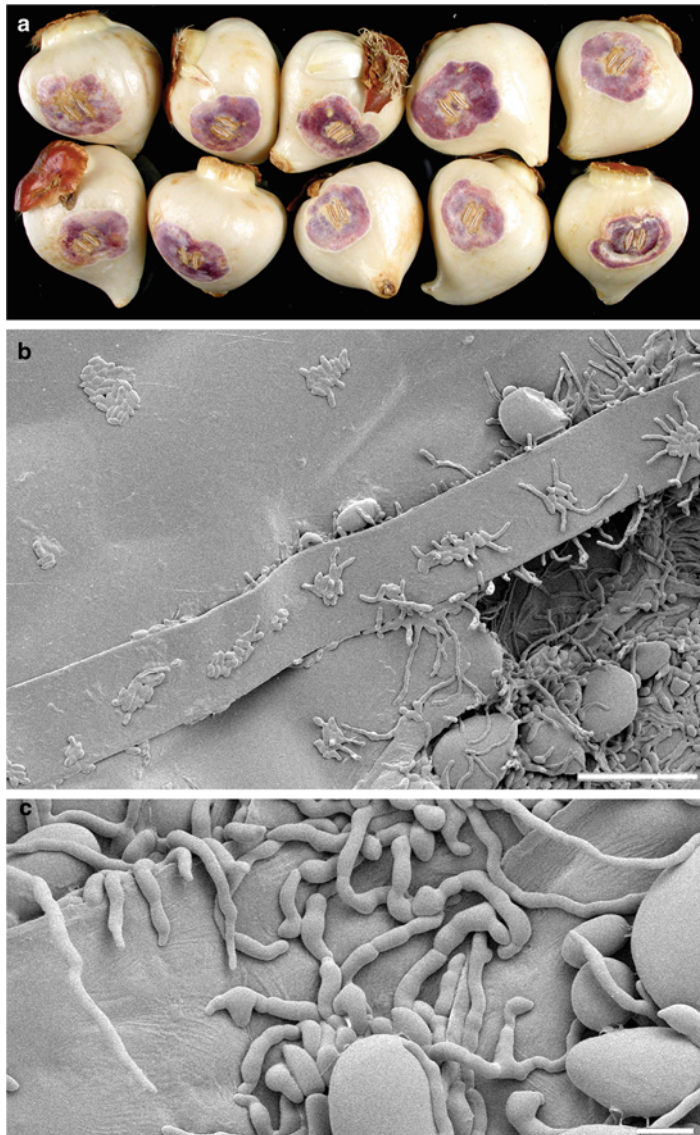
Hyphae then grow out underneath the epidermis and a lesion is formed. At later stages, numerous microconidia are formed on the surface of the bulbs. Dependent on environmental factors such as humidity and temperature, fungal cells stop developing but remain alive and can enter a quiescent stage. When tulip bulbs are planted out upon storage, fungal development resumes after many months, and during outgrowth of the bulbs leads to infection of plant tissues and subsequent death (Dijksterhuis, van der Lee and de Boer, unpublished results). The quiescent stage is called latency, and is an important aspect of post-harvest diseases; and the mechanisms of prolonged survival of fungal cells during this stage are a potential important research topic in order to develop novel strategies to prevent damage to food products.

### III. Processed Foods: Spoilage as Colonization of a Medium

Many processed foods contain vegetables, fruits, and other plant material that are treated to make the nutrients more available for the human digestive system. In a way, these foods are comparable to plant-based media that are used in microbiological laboratories, and as such can be colonized and spoiled by fungi. Spoilage fungi can enter the food via the basic components of the product. For example, some spoilage fungi enter the product via added spices (small pieces of plant material). In other cases, they are introduced during the food production chain or subsequent storage. In particular, airborne spores can enter food products that are not effectively shielded. Airborne contamination is characterized by the simultaneous outgrowth of more species of fungi in a product. The density of fungal spores in the (indoor) air varies greatly, and is correlated with the ability of certain fungal species to form large numbers of them. The propagules then enter food and crop, and can cause damage.

In the case of *Penicillium expansum* infecting apple, high spore densities in the air are probably caused by growth of the mould in high concentration in rotten organic material in orchards (Borner 1963). Fungi also develop inside buildings (where storage occurs), and their proliferation is then often related to leakage,





**Fig. 2.2.** Post-harvest infection of tulip bulbs. (a) Tulip bulbs are infested with conidia of *Fusarium oxysporum* f. sp. *tulipae*, and develop purple stained lesions after 120 h following inoculation. (b) Microconidia do not germinate outside a

wound in the epidermis of the bulb. (c) Germination of conidia on plant cell wall and starch granules (Photographs made by Jan Dijksterhuis, CBS-KNAW Biodiversity Centre, The Netherlands). Bars are 20 and 5  $\mu\text{m}$  respectively

flooding, condensation, and humidity. Occupants inside homes also contribute to mould growth as a result of activities generating humidity (cooking, breathing) in combination with the obstruction of venting of the building caused by, for instance, the insulation of buildings. Therefore, the composition of the

**fungal indoor mycobiota is very dynamic and correlated with and depending on human activity** (Flannigan et al. 2011; Adan and Samson 2011). Previous indoor food spoilage may grossly enhance the inoculum pressure on newly introduced food products. For example, in Dutch cheese warehouses, *Penicillium*

*discolor* commonly occurs, and can cause serious spoilage when poor hygienic conditions increase the sporulation of this fungus.

**Massive production of conidia can be regarded as a vital strategy** for dispersion of a number of important food-borne fungi. The order *Eurotiales* includes many relevant food-spoilage fungi (Samson et al. 2004; Pitt and Hocking 2009), with an emphasis on the genera *Paecilomyces*, *Penicillium* and *Aspergillus*. With respect to food spoilage, *Aspergillus* seems to be more suitable for tropical areas than *Penicillium*, which is observed more in temperate areas.

Fungal spoilage organisms can **build up considerable biomass** in certain areas of the food production chain, and when not sufficiently cleaned act as a **recurrent source** of contamination. In this way a “house flora” can develop inside certain factories; e.g., *Penicillium roqueforti* which causes spoilage in rye bread factories, and *Fusarium oxysporum* in dairy products. *Geotrichum candidum* is known as the “machinery mould” or “dairy mould”, and is responsible for slime building in processing equipment and off-smells in finished products (Wildman and Clark 1947).

In time, **different preservation techniques** are developed with the aim of discouraging fungal development in the food product. These include fermentation, addition of salts or high concentrations of sugars, pickling, drying, cooling, the addition of preservatives or a heating treatment before packaging. More recent techniques include modified atmosphere packaging and the application of high-pressure treatment (Barbosa-Cànovas 1998; Smelt 1998) of the food product, but heat-resistant ascospores clearly show survival of treatment (Butz et al. 1996; Palou et al. 1998). In addition, high-pulse fields are applied to food products in order to evaluate if these are able to kill spoiling organisms.

Novel food-preserving techniques include the application of the preservatives sorbate and benzoate on the surface of fruits and vegetables, and maybe also to processed food by the use of edible coatings (Valencia-Chamorro et al. 2010; Mehvar et al. 2011).

In addition, biocontrol agents such as the yeast *Pichia anomala* are an interesting option that counteracts spoilage fungi in case of high-moisture feed grain under airtight conditions (Petersson et al. 1999). The latter is in fact similar to the use of fermentation as stated at the beginning of this chapter.

In certain aspects, the ecological niche of processed food products therefore can be regarded as an **extreme environment with rich nutrients**. This is of evolutionary interest; the fungi that are able to overcome these stresses are heavily rewarded.

### A. Association of Fungal Species with Food Products

It was already recognized by Johanna Westerdijk in 1949 that there might be an **association between specific fungal species with certain food products or crops**. For instance, *P. expansum* is specific for pomaceous and stone fruits, while the species *P. italicum* and *digitatum* cause damage to citrus fruit. The adaptability of the fungal species to overcome the restrictions of the crop or the limitations introduced by preservation techniques determines the dominance of the species in relation to the relevant food product.

Food parameters are **surprisingly restrictive to the spectrum of species which are able to grow and thus spoil the individual food types**. Normally, less than ten and often one to three species are responsible for spoilage (Frisvad and Filtenborg 1988, 1993; Frisvad et al. 2007b). Table 2.1 shows a survey of different classes of food products and associated fungi (see also Frisvad et al. 2007b). Frisvad et al. (2007a) have described the **importance of accurate identification of spoilage fungi**. The wrong identification will blur the development of a conclusive scheme of food spoilage and disturb research on the (cellular) mechanisms responsible for this specificity as well as the specificity of mycotoxin production. Knowledge of these parameters will lead to novel tailor-made preservation strategies.

**Fungal culture collections** may play an important role in this development. As an

**Table 2.1.** Most common associated fungal species (From Frisvad et al. 2007, with courtesy of Taylor and Francis, CRC Press)

Crop	Product	Fungal species
Beans & peas	Black beans, cowpeas	<i>Alternaria alternata</i> , <i>Aspergillus flavus</i> , <i>Asp. ochraceus</i> , <i>Asp. parasiticus</i> , <i>Fusarium proliferatum</i> , <i>Penicillium citrinum</i>
Cereal	Maize	<i>Asp. flavus</i> , <i>Asp. niger</i> , <i>Asp. ochraceus</i> , <i>F. graminearum</i> , <i>F. proliferatum</i> , <i>F. verticillioide</i> , <i>P. citrinum</i>
	Rice	<i>Asp. flavus</i> , <i>Asp. niger</i> , <i>P. citrinum</i>
	Rye bread	<i>Eurotium repens</i> , <i>Eur. rubrum</i> , <i>P. carneum</i> , <i>P. paneum</i> , <i>P. roqueforti</i>
	Sorghum	<i>Alt. alternata</i> , <i>Asp. flavus</i> , <i>F. verticillioide</i> , <i>F. semitectum</i> , <i>P. citrinum</i>
	Wheat bread	<i>Asp. flavus</i> , <i>Eur. repens</i> , <i>Eur. rubrum</i>
	Wheat, rye, barley, oat	<i>Alt. tenuissima</i> and <i>infectoria</i> sp.-grps., <i>Asp. flavus</i> , <i>Asp. parasiticus</i> , <i>F. avenaceum</i> , <i>F. culmorum</i> , <i>F. graminearum</i> , <i>P. aurantiogriseum</i> , <i>P. cyclopium</i> , <i>P. freii</i> , <i>P. melanoconidium</i> , <i>P. polonicum</i> , <i>P. verrucosum</i>
Cheeses	Hard cheese	<i>Asp. versicolor</i> , <i>P. commune</i> , <i>P. discolor</i> , <i>P. nalgiovense</i> , <i>P. solitum</i>
Coffee	Coffee—monsoon	<i>Asp. candidus</i> , <i>Asp. niger</i> , <i>Asp. tamarii</i>
	Coffee—traditional	<i>Asp. carbonarius</i> , <i>Asp. steinii</i> , <i>Asp. westerdijkiae</i> , <i>P. citrinum</i>
Fruit	Citrus	<i>Alt. tangelonis</i> , <i>Alt. tenuissima</i> sp.-grp., <i>Alt. turkisafrica</i> , <i>P. digitatum</i> , <i>P. italicum</i>
	Dried fruits	<i>Asp. carbonarius</i> , <i>Asp. flavus</i> , <i>Asp. niger</i> , <i>Asp. ochraceus</i> , <i>Xeromyces bisporus</i> , <i>Wallemia sebi</i>
	Fruit juice	<i>Byssoschlamys nivea</i> , <i>B. spectabilis</i> (= <i>Paecilomyces variotii</i> ), <i>Eupenicillium</i> spp., <i>Neosartorya</i> spp., <i>Talaromyces</i> spp.
	Grapes	<i>Asp. carbonarius</i> , <i>Asp. niger</i> , <i>Asp. tubingensis</i> , <i>P. expansum</i>
	Pomaceous & stone	<i>Alt. arborescens</i> sp.-grp., <i>Alt. tenuissima</i> sp.-grp., <i>F. lateritium</i> , <i>P. crustosum</i> , <i>P. expansum</i> , <i>P. solitum</i>
		<i>P. nalgiovense</i> , <i>P. nordicum</i> , <i>P. olsonii</i> , <i>P. chrysogenum</i> , <i>Eurotium</i> spp.
Meat	Sausages	<i>Alt. arborescens</i> sp.-grp., <i>Asp. flavus</i> , <i>Asp. niger</i> , <i>Asp. tamarii</i> , <i>F. acuminatum</i> , <i>F. avenaceum</i> , <i>F. semitectum</i> , <i>P. crustosum</i> , <i>P. discolor</i>
Nuts	Almonds, hazelnuts, pistachio, walnuts	<i>Alt. alternata</i> , <i>Asp. versicolor</i> , <i>P. citrinum</i> , <i>P. expansum</i>
		<i>Asp. flavus</i> , <i>Asp. niger</i>
Oil crop	Olives	<i>Alt. alternata</i> , <i>Asp. flavus</i> , <i>Asp. niger</i> , <i>Asp. parasiticus</i> , <i>F. verticillioide</i> , <i>F. semitectum</i>
	Peanuts	<i>P. brevicompactum</i>
	Sunflower	<i>P. allii</i> , <i>P. glabrum</i> , <i>Petromyces alliaceus</i>
Vegetables	Ginger	<i>Alt. alternata</i>
	Onion & garlic	<i>Asp. flavus</i> , <i>Asp. parasiticus</i> , <i>Asp. tamarii</i>
	Pepper—bell	<i>Alt. alternata</i> , <i>Alt. solani</i> , <i>F. coeruleum</i> , <i>F. sambucinum</i>
	Pepper—black	<i>Alt. alternata</i> , <i>Alt. subtropica</i> , <i>Alt. tenuissima</i> sp.-grp., <i>P. expansum</i> , <i>P. olsonii</i> , <i>P. tularense</i> , <i>Stemphylium eturmiunum</i> , <i>Stemphylium solani</i>
	Potatoes	<i>Botryosphaeria rhodina</i> , <i>F. verticillioide</i> , <i>P. sclerotigenum</i>
	Tomatoes	<i>Asp. flavus</i> , <i>Asp. niger</i>
	Yams	
	Yam chips	

example, Samson and Frisvad (2004) developed a taxonomy of *Penicillium* subgenus *Penicillium* based on a polyphasic approach that includes macro- and microscopic morphology, growth characteristics, DNA sequences, and the excretion of secondary metabolites. More recently, similar approaches have been applied to the genera *Aspergillus* (Samson and Varga 2007)

and other genera within *Penicillium* (Samson and Houbraken 2011). *Penicillium* and *Aspergillus* belong to the most abundant fungi in air, and are dominant fungi with respect to food spoilage. The correct classification and deposition of the identified strains in a culture collection may become an important element in our struggle to prevent food spoilage.



## B. Food-Spoiling Fungi

One of the main preservation strategies is the **increase of the osmolarity** caused by the addition of sugar or salt. Several yeasts (*Debaryomyces hansenii*, *Zygosaccharomyces rouxii* and *Z. bailii*) are very osmotolerant, and can grow in environments with extremely high concentrations of sugar and salt.

Ecologically, *D. hansenii* is a marine fungus (Clipson and Jennings 1992), but it is also isolated from penguin droppings, anthills, and soils. It is observed in hypersaline habitats and subglacial ice (Gunde-Cimerman 2009). It spoils **brine foods and contaminates fruit powders**, but is also the dominant yeast species isolated from dry cured meat products, and plays a role in taste development by the production of specific volatile compounds (Andrade et al. 2010).

The *Zygosaccharomyces* species, including the extremely osmotolerant species *Z. rouxii* and *Z. bailii*, cause **major food spoilage** in many different products including **fruit juices, sauces, carbonated soft drinks, and ketchup** (Pitt and Hocking 2009). These yeasts have a reputation of being able to grow on a very high concentration of sugars (e.g., 5 M glucose for *Z. rouxii*, Martorell et al. 2007) and form high amounts of carbon dioxide, but also degrade food preservatives as sorbic acid. They are halotolerant, but to a lesser extent than *D. hansenii* (Lages and Silva-Graça 1999).

Other filamentous fungi can also grow at **very low water activities** (*Eurotium amstelodami*, *Wallemia sebi*, *Aspergillus penicilloides* and *Xeromyces bisporus*). *E. amstelodami* is known for spoilage in **corn silos**, where it can develop at 15–16 % moisture levels, and *A. penicilloides* is probably the pioneer species for fungal spoilage of **stored grains** (Pitt and Hocking 2009). *W. sebi* grows on **dried figs, dates, chocolate, and fruit bars**; *X. bisporus* is the most xerophilic fungus known to date, and develops on pure marzipan (Williams and Hallsworth 2009; Vinnere-Pettersson et al. 2011; Leong et al. 2011).

Fungi that are present on food products or are introduced via the food production chain are inactivated by heat treatments. Spoilage can

occur **after pasteurisation treatments** when heat-resistant fungi survive high temperatures. Fungi that cause damage worth millions of dollars in the fruit-juice industry are, among others, *Byssoschlamys nivea (fulva)*, *Talaromyces flavus (macrosporus)*, and *Neosartorya fischeri* (as reviewed by Tournas 1994). These are soil-borne fungi, and fruits that develop in contact with soil (such as strawberries) are more prone to contamination. These fungi can survive temperatures of 85 °C for time intervals that are markedly longer than those used for pasteurization treatments. The heat resistance is conveyed by **sexual ascospores** that are candidates to be the most stress-resistant eukaryotic described to date (Dijksterhuis 2007). **The dormant state of these spores is broken** by temperatures used for pasteurization.

Storage of food products in refrigerators is often accompanied by the presence and growth of the so-called **psychrotolerant fungi** (cold-tolerant fungi). These fungi often belong to the genera *Alternaria*, *Fusarium*, *Penicillium*, and *Cladosporium*. In addition, *Botrytis cinerea* is also a fungus that develops well at a surprisingly low temperature (Hoogerwerf et al. 2002).

Several fungi (including filamentous species and yeasts) are able to **degrade the important food preservative sorbate** by the action of enzymic activity. In particular, the species *Penicillium roqueforti* and *Paecilomyces variotii* are notorious for the spoilage of rye bread, drinks, and margarine that contain sorbic acid, benzoic acid, and propionic acid.

*Aspergillus niger* is also capable of degrading sorbic acid, but is not a major spoiler of these products. This illustrates the subtle interplay of different parameters during food spoilage.

Low-oxygen packaging is a relatively novel method of keeping products free of spoilers. However, some fungi have traits that make them suitable for **development under very low oxygen and/or high carbon dioxide**. These include *Saccharomycopsis fibuliger* and *Hyphopichia burtonii*, the “chalk molds”, well-known from products such as pre-baked bread.

In addition, the fungi *P. roqueforti* and *Fusarium oxysporum* are able to grow at very low levels of oxygen. The main question is: are these fungi able to grow anaerobically, or are they microaerophilic?

The following paragraph elaborates on some cellular traits which these organisms have developed as an answer to adverse conditions that are similar to the typical difficulties of growing in food.

## IV. Coping with Adverse Conditions

### A. Osmotolerance

The number of yeast species that are involved in food spoilage is small compared to their total number, and include **extreme osmotolerant organisms**. Lages and Silva-Graça (1999) summarize 33 yeast species that have a maximum tolerance above 2 M NaCl. The yeast *D. hansenii* grows at sodium chloride concentrations up to 2.5 M, and growth is stimulated in 0.5 M (as reviewed in Prista et al. 2005). During mid-exponential growth, *D. hansenii* **accumulates high concentrations of sodium** (approx. 750 mM) and potassium (300 mM, Prista et al. 1997). For this reason, the yeast species can be regarded as a “**sodium includer**”, in contrast to “sodium excluders” that have a strategy of keeping the internal concentration of the sodium ion low (Prista et al. 2005). While higher internal concentrations of sodium are beneficial for the biological performance of the organism, one can state that *D. hansenii*, is not only halotolerant, but is also a halophilic organism.

During salt stress, different **transporter proteins located in the plasma and vacuolar membrane** (Prista et al. 2005) are active in both efflux and influx of protons, sodium, and potassium ions.

*D. hansenii* expresses two P-type ATPases, DhENA1 and DhENA2, one specific for sodium efflux at higher pH. When expressed in *S. cerevisiae* without any Na<sup>+</sup> efflux activity, these proteins were able to recover growth of the yeast in NaCl containing media (Almagro et al. 2001). Alternatively, the DhHAK1 and DhTRK1

transporters enable the cell to take up monovalent cations, especially potassium (Martínez et al. 2011).

These observations indicate that *D. hansenii* realizes halotolerance as a result of the **interplay of different transport processes**.

In addition, another major process is important for the salt-tolerance of these cells, namely the **accumulation of compatible solutes** (Jennings and Burke 1990). The term compatible means that high intracellular concentrations of certain solutes are compatible with enzyme functioning. For fungi, the most common solutes are the polyols **glycerol**, erithreitol, arabinitol, and **mannitol**, as well as the disaccharide **trehalose**.

Remarkably, the **type of solute may change with the growth phase**; arabinitol (arabitol) is the major solute present in the cells of *D. hansenii* in the stationary growth phase (Adler and Gustafsson 1980). During mid-exponential growth, this yeast accumulates glycerol up to approx. 35 % dry weight of the cells in 16 % (w/v) NaCl (Adler et al. 1985). At the beginning of the stationary phase, a major portion of the glycerol leaks out of the cells. Simultaneously, glycerol is actively taken up by the cell and metabolized. This could be the result of the activity of a **proton/glycerol symporter** or even a still-putative sodium/glycerol symporter (Lucas et al. 1990; Prista et al. 2005). The precise functioning of the compatible solutes in *D. hansenii* clearly has its enigmas, as the yeast preferentially accumulates trehalose at low salt levels and glycerol at high salt (2.0 M or higher, Gonzalez-Hernandez et al. 2005).

*Z. rouxii* also **accumulates high levels of glycerol, but is better able to retain it inside the cell** (Hosono 2000). *Z. rouxii* can still grow in 875 g sugar/l and at pH 2.5 (Membre et al. 1999) or in 3.1 M NaCl (Hosono 2000). In addition, *Z. bailii* is also **extremely tolerant to organic acids** such as sorbic and benzoic acid (Steels et al. 2000; Martorell et al. 2007).

*Z. rouxii* expresses a proton-ATPase in combination with a Na<sup>+</sup>/H<sup>+</sup> antiporter to remove sodium ions from the cell (Watanabe et al. 1991, 1995). A *S. cerevisiae* strain that was very sensitive to salt stress was made more osmotolerant with the antiporter genes (ZrSod2 and ZrSod22) from *Z. rouxii* (Iwaki et al. 1998). When the antiporter was deleted from *Z. rouxii*, the organism could not grow on medium with high salt concentration, but was still able to develop on very high concentrations of sugar (Watanabe et al. 1995). Recently,

ZrNha1 was identified, an antiporter that is thought to be indispensable for maintaining potassium homeostasis (Pribylova et al. 2008).

However, how do these yeasts realize growth even at sugar concentrations **above 5 M (90 % w/v)**? Is it their ability to ferment sugars at **high rates**, even in the presence of oxygen, a factor in their survival (Leyva et al. 1999)? Under anaerobic conditions, the yeast can also grow exponentially, with vigorous fermentation given that the medium is complex (as food and beverages are; Rodriguez et al. 2001). *Z. rouxii* employs two unique uptake systems for fructose molecules (Leandro et al. 2011), and is called **fructophilic** as it can transport these molecules with a higher capacity. Alternatively, the structure of the cell wall is very responsive to growth conditions, and is found to be variable with salt tolerance (Pribylova et al. 2007). For example, it might be that a more elastic cell enables the cell to deal with these straining conditions.

The moderate osmotolerant fungus *Geotrichum candidum* is an important spoilage organism (**dairy, vegetables and fruit**) and can heavily contaminate food production chains. It forms numerous one-celled arthrospores, and cultures show some resemblance with yeasts in development and morphology. Arabitol, a sugar alcohol, accumulates in *Geotrichum* species in 1 M NaCl. It reaches amounts above 30–40 % dry weight, which decreases slightly at the stationary phase of culturing. One species of *Geotrichum* (out of five), however, accumulated mannitol as a compatible solute in similar amounts. Mannitol is also formed during the stationary growth phase without salt (Luxo et al. 1993).

## B. Xerophilic Fungi

We discussed osmotolerance in relation to high salt and sugar concentrations in growing media. Strictly speaking, all halophiles and osmophiles are **xerophiles**, fungi that develop at low water activity. For living organisms, the availability of water molecules is a prerequisite for development. Water availability is restricted

as a result of **high concentrations of solutes** in the growth medium, and also when the **relative humidity in air** is low, when fungi grow on inert surfaces, a situation common in indoor conditions. The latter are conditions that may also prevail during large-scale storage of cereals. A number of filamentous fungi are able to grow in the presence of low amounts of water, including *Wallemia sebi*, *Eurotium amstelodami*, and related species, *Aspergillus penicilloides* and the most xerophilic organism known to date, *Xeromyces bisporus*. The genome of *W. sebi* has recently been sequenced (Padamsee et al. 2012), and revealed adaptations to osmotic stress.

Recently, a number of studies have appeared (Williams and Hallsworth 2009; Chin et al. 2010) that reflect on the role of intra- and extracellular solutes on survival and growth at low water activities. Williams and Hallsworth (2009) studied growth of xerophilic fungi on different media containing solutes with varying degrees of **chaotropic activity**. These solutes weaken macromolecular interactions and disorder cellular structures, while **kosmotropic solutes** stabilize these interactions. Chaotropic solutes include **glycerol**, magnesium chloride and **fructose**, and kosmotropic solutes are ammonium sulphate and **sucrose**.

The authors observed that growth media with very low water activity and relatively low chaotropic activity showed relatively better growth of xerophilic fungi. The authors asked the question whether the chaotropicity of glycerol-supplemented media at very low water activity limited hyphal growth, and whether a more kosmotropic environment might result in growth at even lower water activities. Indeed, the authors realized growth of *A. penicilloides* at a water activity of 0.647 on a neutral medium, compared to 0.653 for *X. bisporus* in 7.6 M glycerol. Chin et al. (2010) evaluate the influence of chaotropy on growth at low temperatures, and observe that *Eurotium herbariorum* grows much better in fructose (chaotropic) at 1.7 °C, and best on sucrose at 30 °C. The extreme halotolerant yeast *Mrakia frigida* even can grow slowly at −5 °C in 1.1 M glycerol, which is not possible in 0.73 M sucrose (kosmotropic). Conidia (spores) of *X. bisporus* from glycerol containing cultures survive freezing treatments better than conidia from sucrose-containing medium, which are better survivors after heat and high-pressure treatments.

The rationale behind this is that **chaotropic solutes counteract the rigidity of macromolecular interactions at low temperatures** (and drying?), and kosmotropic solutes stabilize these interactions at high temperatures. This **link** between xerophilic and psychrophilic growth habit is nicely illustrated in the isolation of fungi from high-altitude Nepalese soil (Petrović et al. 2000). Biophysical relationships between water, macromolecules, and solutes reveal novel aspects of cellular survival under adverse conditions.

### C. Fungal Survival Structures

This brings us to fungal structures that are used **for dispersion in time and space**; fungal spores. Fungal spores are extremely variable; the way they are formed and their shape are still very important for the determination of fungal genera and species. They bear different names such as sporangiospores in the case of *Zygomycetes* (reviewed by Dijksterhuis and Samson 2006), ascospores when they are sexual and conidia when they are asexual (see for reviews Chitarra and Dijksterhuis 2007; Magan et al. 2012). Some fungal ascospores belong to the strongest eukaryotic cells described to date (Dijksterhuis 2007). **Fungal genera that are important for food spoilage such as *Aspergillus*, *Paecilomyces* and *Penicillium* are also avid sporeformers** (Berbee et al. 1995).

Some conidia are **hyaline** and are dispersed by **water splashes**, others are **airborne** and have to survive conditions of **drought** during transport through the air and are moderately stress-resistant. Interestingly, the method of dispersion of the conidia is correlated to **membrane composition** (ergosterol level) or **cytoplasmic parameters** (viscosity of the cytoplasm). Van Leeuwen et al. (2010) showed that hyaline, water dispersed conidia of *F. oxysporum*, and *Verticillium fungicola* showed less resistance to the antifungal natamycin, lower cytoplasmic viscosity and higher staining of ergosterol (with filipin, van Leeuwen et al. 2008) compared to airborne conidia of *A. niger* and *P. discolor*. Extremely heat-resistant ascospores of *Talaromyces macrosporus* and *Neosartorya fischeri* exhibit the **highest cytoplasmic viscosity** (Dijksterhuis et al. 2007, Wyatt et al., unpublished results).

In fungal spores, often a combination of mannitol, a polyol, and trehalose, a disaccharide is observed, e.g., in the species *Aspergillus oryzae*, *A. nidulans*, *A. niger*, and *P. rubens* (Horikoshi and Ikeda 1966; D' Enfert and Fontaine 1997; van Leeuwen et al. 2013b; Bekker et al. 2012). Trehalose accumulation is an important factor in yeast **heat tolerance** and protection of cell components (Wiemken 1990). Both **membranes** (Crowe et al. 1984) and **proteins** (Hottiger et al. 1994) are **stabilised** by trehalose. Hallsworth and Magan (1994, 1996) showed that different **environmental conditions** during cultivation **influence the solute composition** inside conidia in the case of insect pathogenic fungi.

Optimal conditions might result in stronger spores, which have higher **shelf lives** if they are used for biological control of insect pests. The principles found in the entomopathogenic species may also apply for fungal species occurring on food, with the difference that now conidia maybe have to be eradicated as effectively as possible. Primary models for the inactivation of fungal spores are reviewed (Dijksterhuis et al. 2012), and can be used for different sporocidal conditions including heat, drying, or vapour treatments (Dao and Dantigny 2009; Dao et al. 2010).

**Fungal colonization of food is often initiated by the deposition of conidia** on the product, with the prerequisite that the **dormant state is effectively broken** and germination can occur. The **transition** from a dormant conidium towards a vegetative growing fungal hyphae includes changes of the cell wall (Tiedt 1993; Fontaine et al. 2010), breakdown of compatible sugars (including trehalose and mannitol) (D' Enfert et al. 1999; Fillinger et al. 2001; van Leeuwen et al. 2013b), reorganization of the transcriptome including major mRNA breakdown, and strong upregulation of specific gene categories (van Leeuwen et al. 2013a). Water is a basic compound needed for cellular functioning, and when added to spores directly influences the formation of polyribosomes (Bonnen and Brambl 1983). **Isotropic growth** of conidia of *A. niger* occurs after incubation in distilled water (Morozova et al. 2002), and conidia of *A. oryzae* germinate on water agar (Sakamoto et al. 2009). Nutrients such as phosphate,



amino acids, glucose, and their combinations reactivated dried sporangiospores of the temperate fungus *Rhizopus oligosporus* to a different extent, but rich media were far more effective in this (Thanh and Nout 2004; Thanh et al. 2005). Small amounts of glucose or ammonium sulphate in water resulted in **germ tubes** on conidia of *A. niger* and in branched mycelium when combined. Other conidia (*A. nidulans*) specifically need carbon sources such as glucose for germination (d'Enfert 1997; Osheroov and May 2000).

A micro-array analysis of *A. niger* conidia clearly indicates that the expression of genes involved in protein synthesis changes most during the first 2 h of germination, which can be seen as the major strategy for early germination (van Leeuwen et al. 2013a). Osheroov and May (2000) already treated conidia of *A. nidulans* with various inhibitors, and only the protein synthesis inhibitor cycloheximide prevented isotropic growth, in contrast to inhibitors of other cell processes.

**Germination of conidia is also affected by volatile compounds** such as, for instance, the volatile 1-octen-3-ol. This compound is produced by fungi during crowding of conidia of *Penicillium paneum* and *A. nidulans* (Chitarra et al. 2004; Herrero-Garcia et al. 2011), and in the absence of germination when spores are present in high densities. 1-Octen-3-ol is also produced by *A. niger* (Karlshøy et al. 2007). It is hypothesized that 1-octen-3-ol acts as a **fungal self-inhibitor** that prevents premature germination of conidia on conidiophores.

The compound had a profound influence on protein expression patterns (Chitarra et al. 2005), blocked isotropic growth, but had only mild physiological effects on germinating conidia in solution. **Volatiles also activate germination of conidia** in the case of *Penicillium digitatum*, which causes post-harvest citrus rot. Conidia, when provoked with volatiles from damaged oranges, showed enhanced germination (Eckert and Ratnayake 1994).

## D. Heat Resistance

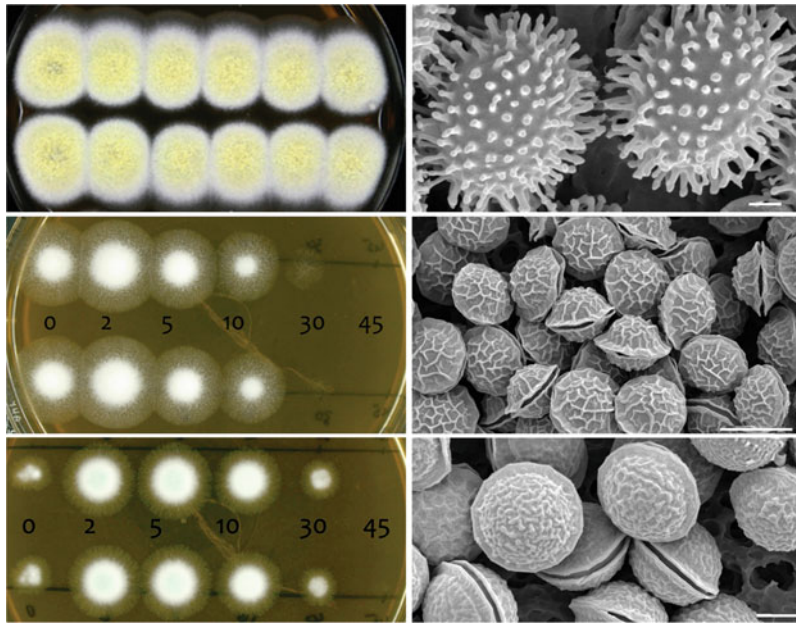
Fungal survival structures can be regarded as more or less heat-resistant compared to vegetative cells, and conidia, sclerotia, chlamydospores, and ascospores survive heat treat-

ments between 55 °C and 95 °C. Yeast ascospores isolated from soft drinks and fruit products (mainly *S. cerevisiae*, *Z. bailii* and *chevalieri* strains) had D<sub>60</sub> values that were 25–350 times higher than those of the corresponding vegetative cells (Put and De Jong 1980). Recent measurements in our laboratory show that **heat resistance of ascospores above 70 °C** (see Fig. 2.3) is a common trait that occurs in all fungal genera of the *Eurotiales*: *Eupenicillium*, *Neosartorya*, *Eurotium*, *Hami-gera*, *Xeromyces*, *Byssoschlamys*, *Thermoascus*, and *Talaromyces*, and most probably occurs in more fungal clades (e.g., *Neurospora* and *Daldinia* species). Ascospores of the fungus *Talaromyces macrosporus* survive at 85 °C for 100 min (Dijksterhuis and Teunissen 2004), which is similar to some bacterial spores (e.g., *Bacillus subtilis*). Ascospores of *Neosartorya spinosa* and *Byssoschlamys spectabilis* shows similar heat resistance (Houbraken et al. 2008; Wyatt and van Leeuwen, unpublished results).

The **heat resistance** of ascospores in food products generally **increases with the sugar concentration** of the surrounding medium (Splittstoesser and Splittstoesser 1977; Beuchat 1988a; King and Whitehand 1990). Additional factors are **pH** and the presence of **organic acids** such as those present in fruits or used for preservation, which counteract heat resistance of ascospores, but only at low values of pH (lower than 4).

Benzoic and sorbic acid had effects on *T. flavus* and *N. fischeri* ascospore heat resistance (Beuchat 1988b; Rajashekhara et al. 1998). Combination of different factors may lead to some unpredictable variations in heat resistance. For instance, *N. fischeri* exhibited a far higher heat resistance in 0.1 M phosphate buffer (pH 7.0) than in grape jellies with large amounts of cane sugar (pH 3.1–3.3, Beuchat and Kuhn 1997), and *B. nivea*, *B. fulva*, and *N. fischeri* were approximately twice as heat resistant in tomato juice (pH of 4.2) as in phosphate buffer (pH 7.0, Kotzekidou 1997).

Factors inside the ascospore are important for heat resistance, for instance as a result of the **age of the culture** in case of *N. fischeri*, *T. flavus*, and *B. nivea* (Conner and Beuchat 1987; Beuchat 1988a; Casella et al. 1990) or the **growth temperature** of the



**Fig. 2.3.** Heat resistant fungi survive 75–85 °C for periods that are longer than conventional pasteurization times. *Left panel*; ca. 5,000 ascospores were inoculated in a small droplet on agar after a heat treatment for 0, 2, 5, 10, 30, and 45 min (from left to right). Top (*Talar-*

*omyces macrosporus* at 75 °C, middle *Neosartorya fischeri* and bottom *Neosartorya hiratsukae*, the latter at 85 °C). The *right panel* shows images of the ascospores as taken with low temperature scanning electron microscopy. Bars are 1, 5 and, 2 μm from top to bottom

spore-generating colony (Conner and Beuchat 1987; King and Whitehand 1990).

Harvested and washed ascospores also showed maturation in case of *T. macrosporus* (i.e., increase of heat resistance in time, Dijksterhuis and Teunissen 2004) when stored at 30 °C. This phenomenon did not occur at 10 °C, suggesting a temperature-dependent acquisition of resistance. Furthermore, King and Whitehand (1990) report higher heat resistance of *T. macrosporus* on solid medium, and also the type of medium used is important (Beuchat 1988a). Finally, heat resistance varies with the fungal isolate used (Bayne and Michener 1979; Beuchat 1986; King and Whitehand 1990).

Conner et al. (1987) investigated the nature of heat resistance. They studied younger and older ascospores of *N. fischeri* that had increasing heat resistance. Ascospores showed **changes in the inner cell wall** region during aging. Older spores contained more mannitol and trehalose. Polyols and disaccharides may play an important role in heat protection as compatible solutes. Recent work indicates that additional compatible solutes may also exist in heat-

resistant **ascospores** made by different fungal species (Wyatt et al., unpublished results). HPLC studies showed that ascospores of *T. macrosporus* contain **very high concentrations of trehalose**, up to 15–20 % of the wet cell weight (that is, 24–32 % of the dry weight, Dijksterhuis et al. 2002). The **low water content** of the spores (38 %) introduces a **very high viscosity** inside the spores as measured by means of EPR (electron paramagnetic resonance) studies (Dijksterhuis et al. 2007). When these spores are in solution at room temperature no **glassy state** occurs, but under dry conditions this may occur. A glassy state is an amorphous phase characterized by very low movement of the molecules inside the cytoplasm. A sudden lowering of the temperature or a reduction of the water content might introduce a glass transition situation inside the cell, which virtually brings all processes in the cell to a stand still.

**Constitutive dormancy** of ascospores includes a **metabolic block**, a **barrier to the penetration of nutrients**, or the production of

a **self-inhibitor** (as defined by Sussman and Halvorson 1966), or as a result of a **specific physical state** of the cytoplasm such as a glassy state. Ascospores often need a robust physical signal such as heat for breaking of dormancy, where the number of viable counts after treatment is increased by several log cycles (e.g., *Eurotium herbariorum* at 60 °C, Splittstoesser et al. 1989). For ascospores of *Talaromyces flavus*, activation is observed at 80 °C and, at 85 °C, activation is followed by killing (Beuchat 1986).

These extreme characteristics may also favour a very long shelf life of the ascospores; they can be still viable for up to 17 years in the case of *T. flavus* (Nagtzaam and Bollen 1994). At lower temperatures, activation fails, and only low numbers of germinated spores are observed. Remarkably, the speed of activation increases with higher temperatures with *T. macrosporus* (Kikoku 2003). Apart from heat, also a **drying treatment** can result in activation. For *N. fischeri*, the dormant state can be broken by a drying treatment of 18 h at 40 °C (Beuchat 1992), but *T. flavus* ascospores did not show a release of dormancy. Heating at 50 % r.h. (dry heat treatment) at 95 °C (for 30 or 60 min) activated *N. fischeri* ascospores, but the temperature of the wetting or recovery buffer was crucial for the viable count obtained (Gomez et al. 1989, 1993).

Further, **high pressure treatments** (6,000 Bar) that are used for non-thermal “pasteurization” of a number of food products can activate ascospores of *T. macrosporus* to germinate (Reyns et al. 2003; Dijksterhuis and Teunissen 2004), in which physical disruption of the thick outer cell wall may play a role.

Recent work at our laboratory indicated that a cell-wall protein is related to the permeability of the cell wall of ascospores and dormancy of these spores (Wyatt et al., unpublished results).

Could activated ascospores resume dormancy again when cytoplasm is forced into a glassy state by a sudden lowering of the temperature or drying to very low water levels? Heat-activated spores of *T. macrosporus* after cooling in liquid nitrogen or kept at −20 °C directly germinated upon introduction into conducive conditions (Dijksterhuis and Samson 2006). Further, ascospores remained dormant after drying, and could be effectively activated by a

heat treatment after resuspension in buffer. These findings indicate that irreversible changes occur during breaking of dormancy.

## E. Food Preservatives

**Food preservatives** are added to food products in order to prevent outgrowth of fungi. The food additive **sorbic acid** is widely used in the food industry as a preservative of low pH sugar-containing products (Stratford et al. 2012). The minimally inhibitory concentration of sorbic acid is dependent on the density of the inoculum of conidia in the case of *A. niger* (Plumridge et al. 2004). Sorbic acid delays conidial germination and lowers the cytoplasmic pH, but at 3 h of germination, conidia start to **degrade the preservative** (Plumridge et al. 2010) and resume development. This is the result of the activity of a **phenylacrylic acid decarboxylase** encoded by the *padA1* gene and a putative 2-hydroxybenzoic acid decarboxylase encoded by *ohbA1* (Plumridge et al. 2008). One of the characteristic degradation products of sorbic acid is **1,3-pentadiene**, which has a kerosene-like smell. Several **osmophilic yeasts** including *Z. rouxii* and *D. hansenii* are able to perform degradation of sorbate, as well as several *Penicillium* species and *Trichoderma* (Cheng et al. 1999; Casa et al. 2004; Pinches and Apps 2007). Yeast cells also strongly upregulate the expression of transporters in answer to weak acid stress, which is imposed on the cell by sorbic acid, including Pdr12 that specifically removes the anions from the cells (Piper et al. 2001).

Another preservative, **natamycin**, is used for the protection of cheese and sausages against fungal development (Stark 2007). This polyene antibiotic binds to ergosterol, which is most available at growing tips of germinating spores and vegetative hyphae (van Leeuwen et al. 2008, 2010) and blocks active fungal growth. Natamycin is active at very **low concentration** (micromolars), and affects many species of fungi. Other polyenes such as amphotericin B and nystatin form complexes that lead to leakage at the plasma membrane, but natamycin does not permeabilize cells

(Te Welscher et al. 2008), but acts directly on different aspects of cellular physiology and membrane trafficking and fusion. It inhibits endocytosis in germinating conidia of *P. discolor* (Van Leeuwen et al. 2009) and fusion of prevacuolar compartments in *S. cerevisiae* (Te Welscher et al. 2012). Very recent work shows that natamycin inhibits transport of amino acids and sugar into cells in a reversible manner, and via a hitherto unknown mechanism (Te Welscher et al. 2012). In natamycin-containing solutions, development of the spores was halted at or before isotropic growth (Van Leeuwen et al. 2013b).

A micro-array study on treated conidia of *A. niger* showed that 8-h-treated cells showed certain similarities with dormant conidia compared to the controls that had formed germ tubes. These included the elevated presence of transcripts of protective proteins (heat shock proteins, dehydrins, LEA-like proteins), genes involved in glyoxylate cycle, fermentation, glycerol, trehalose, and mannitol synthesis. Conidia of *A. niger* and *P. discolor* are able to survive 20 h in a concentration of natamycin of ten times the minimal inhibitory concentration, and germinate at high percentages after removal of the antifungal compound (Van Leeuwen et al. 2010). Mann and Beuchat (2008) suggest a combinatory use of reduced concentrations of organic acids and natamycin to control those fungi that degrade sorbic acid.

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