Fungal heterogeneity; from zones to compartments

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Fungal heterogeneity; from zones to compartments

Heterogeniteit van schimmels: van zones naar compartimenten (met een samenvatting in het Nederlands)

Proefschrift

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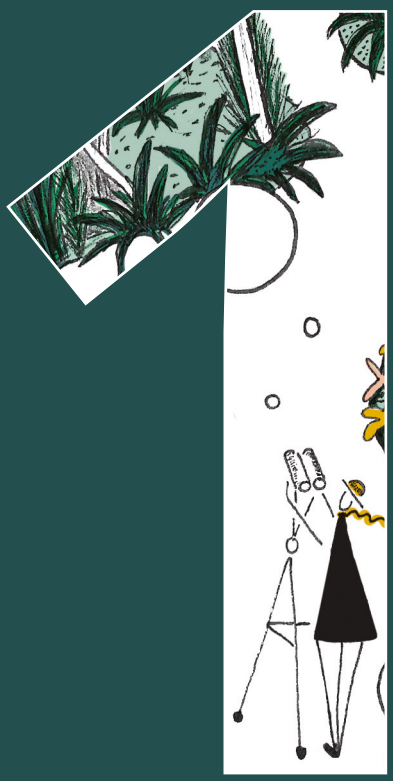
Contents



CHAPTER 1

General Introduction

INTRODUCTION

The fungal kingdom consists of a diverse collection of species that are ubiquitous in nature, where they occupy a wide variety of niches[1,2]. Fungi have a profound impact on life on earth, from a role in nutrient cycling by degrading (in)organic material[3,4,5], to aiding or antagonizing other organisms in their quest for survival[6]. Consequently, fungi have an enormous impact on humans. They create[7,8,9], destroy[10,11], preserve[12-,15], spoil[15-20], protect[21-24], or make ill[25-28] (Table 1). The Janus-faced fungus can be a dedicated partner or an apathetic adversary. Clearly, both faces need to be studied in detail to increase the benefits and to control damage in nature and human society.

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Table 1. Examples of the anthropologic impact of fungi. *Claviceps purpurea* illustrates the duality of the role of fungi in their interaction with humans.

	Fungus	Anthropologic Impact
Create	*Fomes fomentarius*	Use(d) as tinder[7] and to make hats or clothing items[8]
	Pleurotus ostreatus *Trametes multicolor*	Use in composites, as building materials, packaging or acoustic or thermal insulation[9]
Destroy	*Serpula lacrymans*	Causes dry rot in wood structures[10]
	Aspergillus spp. *Stachybotrys chartarum* *Alternaria alternata*	Decreases tensile strength and weight of dry-wall[11]
Preserve	*Rhizopus microsporus var. oligosporus*	Is added to soybeans to produce tempe[12]
	Monilia javanica *Saccharomyces vordermannii* *Mucor indicus* *Rhizopus oryzae*	Used to produce arak, brem and tapai[13]
	Aspergillus oryzae	Production of miso, soy sauce and other Japanese fermented products[14]
	Aspergillus spp. *Mucor spp.* *Rhizopus spp.* *Monascus spp.*	Production of *Huangjiu* and vinegars[15]
Spoil	*Claviceps purpurea*	Infects cereals, hardly impacts yield but produces toxic sclerotia[16,17]
	Zymoseptoria tritici	Leads to up to 53 % of cereal yield losses[18]
	Fusarium spp.	*Fusarium* mycotoxins were detected in up to 89 % of grain samples in the European Union[19]
	All fungi	Annual losses of the five most abundant food crops, due to fungal disease could feed between 596 and 4287 million people per year[20]
Protect	*Claviceps purpurea*	Ergot alkaloids (may) ameliorate post-natal bleeding[21,22]
	Sparassis crispa	Produces the first recognized non-synthetic antifungal [23]
	Penicillium chrysogenum	Produces the first recognized non-synthetic antibiotic[24]
Make Ill	*Claviceps purpurea*	Causes ergotism[17]
	Cryptococcus neoformans	annual mortality rate >0.6 million[25]
	Aspergillus fumigatus	>3 million affected, annual mortality rate >0.6 million[26]
	Candida auris	Multidrug-resistant. Infection leads to 66% mortality rate[27]
	Total all fungi	>1 billion affected annual mortality rate >1.6 million[28]

FUNGAL GROWTH

In nature, fungi display different types of growth. Single-celled yeasts are found in the Dikarya, while zoospores are formed by Aphelidomycota, Rozellomycota, Chytridomyceta, Blastocladiomycota and Olpidiomycota[29] (Figure 1). On the other hand, filamentous fungi are found in all phyla except for the Aphelidomycota and the Rozellomycota[29-31]. Filamentous fungi can form sub-millimeter sized micro-colonies to kilometer-sized subterranean networks of hyphae[32]. Fungi grown under (nutrient) limiting conditions and/or with a high rate of disturbance, generally have sizes in the sub-millimeter scale[33,34]. In contrast, macro-colonies, with sizes larger than a centimeter, can be found in relatively undisturbed, nutrient rich environments such as on fruit or in forest floors[2,35-40]. Mycelia can grow pseudo-two-dimensional when growing on substrates[41-44] or form three-dimensional structures when they grow in substrates like soil or liquid media[33]. The hyphal mode of growth has a higher surface area to volume ratio when compared to yeast cells or zoospores (Text box 1), thus facilitating nutrient uptake. In addition, hyphae provide filamentous fungi with a certain mobility as well as penetrative ability[45]. Hyphae also allow fungi to transport nutrients throughout the mycelium, for instance to metabolically active parts of the mycelium[46].

Prosser[47,48], Trinci[41,49,50], Harris[51] and Rayner[52,53] pioneered the fundamentals of fungal growth, branching, morphology and identity in pseudo-2D colonies. It is generally agreed that fungi have a peripheral growth zone, which is defined by the hyphal length or number of compartments that are needed to maintain the maximum hyphal growth rate. For example, in the case of the ascomycetes *Aspergillus niger* and *Penicillium chrysogenum* this zone was described to consist of 11 or 13 compartments, respectively. In the case of the coenocytic (see compartmentalization of fungal hyphae) mucoromycete *Rhizopus stolonifer* the peripheral growth zone was found to be 8660 μm[41]. Modelling submerged three-dimensional fungal growth in liquid mixed cultures is more complex than that of two-dimensional growth on a solid substrate like an agar medium. Many more parameters affect fungal growth in liquid cultures, like shearing, oxygen availability, level of agitation, number of spores in the inoculum and their coagulation coefficient in the respective medium[54-61]. Depending on these parameters filamentous fungi can grow as dispersed mycelium, as pellets, or in an intermediate state called clumps[62]. Interestingly, the morphology of submerged cultures can influence production of proteins[56] or metabolites by the mycelium[63]. For instance, pellets of *A. niger* produce more citric acid than dispersed mycelium[64,65]. The underlying mechanisms are not yet clear but it may be caused by the effect of the fungal morphology on the viscosity of the medium[66] and/or limited availability of oxygen and nutrients in the center of micro-colonies[67].

TEXT BOX 1.

Surface area/Volume ratio

A high Surface area/Volume ratio means increased exposure to the environment and more opportunities to move waste products or nutrients over a membrane. For a, assumed, spherical yeast with radius r, SV_y^{-1} would be $\frac{3}{r}$. For an apical compartment of a filamentous fungus with total length $h + r$, SV_f^{-1} would be given by $\frac{6(h+r)}{r(2r+3h)}$ This means that:

1. $SV_f^{-1}{}_{h=0}$ equals SV_y^{-1}.
2. When $\lim_{h\to\infty}$, $SV_f^{-1} \to \frac{2}{r}$ meaning hyphae have a SV^{-1} ratio that is maximally $\frac{2}{3}$rds smaller than yeast cells.
3. $\lim_{h\to\infty}$, SV_f^{-1} equals $\frac{2}{3}$.
4. Maximum change per unit of radius has occurred at the point where the vertex is of this function is equal to $\frac{2}{3}$. This is at $h = \frac{2}{9}$. Meaning that the SV^{-1} ratio of hyphae longer than $\frac{2}{9} + r$ µm will decrease slower and slower until $\lim_{h\to\infty}$. 50 % of SV^{-1} ratio change has occurred at $h = \frac{2}{3}r$.
5. This means it is mostly the radius that determines SV^{-1} ratio and h increases only benefit the SV^{-1} ratio.

This makes the cost of long hyphae in terms of SV^{-1} ratio, if any, relatively cheap, given that the major impact of increasing hyphal length occurs relatively soon in hyphal extension. This is also a mathematical explanation of the general lack of yeast-like and filamentous growth intermediates. Since it is either beneficial to remain as a single cell, or if filamentous growth is preferred, limited penalties in terms of SV^{-1} ratio are placed upon the formation of long hyphae.

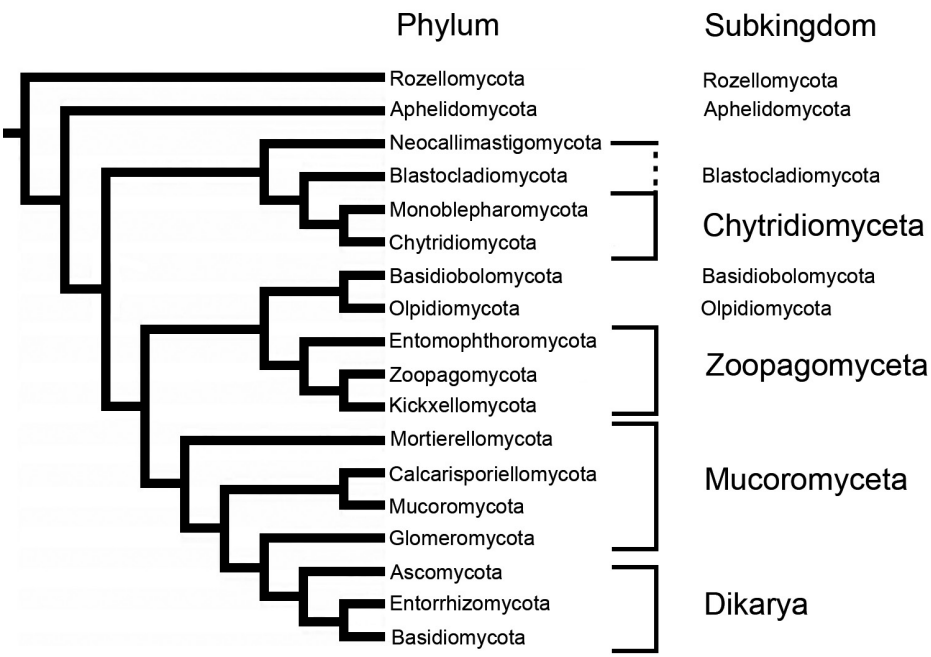

Figure 1. Phylogeny of the fungal kingdom according to Tedersoo et al., 2018.
Basal Clone Group 1, Basal Clone Group 2 and Clade GS01 are omitted. Modified from Lücking, 2019[30]

COMPARTMENTALIZATION OF FUNGAL HYPHAE

Different types of hyphae are found in the fungal kingdom. Hyphae of the Mucoromycota (Figure 1) are generally coenocytic, meaning that hyphae share a cytoplasm. In contrast, hyphae of fungi from the subkingdom Dikarya are divided into compartments by plasma membrane-lined cross-walls called septa. These septa have a central pore with a diameter of 50-500 nm[68,69,70]. In the case of the ascomycetes, these pores enable intercompartmental and interhyphal streaming of cytosol and even organelles. Streaming of cytosol also occurs in basidiomycetes. Yet, inter-compartmental and inter-hyphal translocation of organelles in these fungi is hampered, if occurring at all[71], by elaborate endoplasmic-reticulum derived organelles called septal pore caps that cover the septal pores[72]. Septal pores can be plugged both in the ascomycetes and basidiomycetes thus abolishing inter-compartmental and inter-hyphal cytoplasmic mixing. The septal pore cap is involved in plugging of the septal pores in intact growing hyphae of basidiomycetes[73], while Woronin bodies plug the pores in the ascomycetes[46,74,75]. Environmental conditions impact incidence of septal closure in the basidiomycete *S. commune*[71] and the ascomycetes *A. oryzae* and *A. niger* Chapter 3. For instance, low glucose levels reduce plugging incidence in *S. commune*, while presence of antibiotics, heat shock, and hypertonic shock promote septal closure[71]. Plugging incidence in *Aspergillus* also increases in time. Half of the newly formed apical septa are open, while septa flanking the 10th compartment are always closed[76,77]. It was thus concluded that hyphal compartments transform from a unicellular to a multicellular system[77]. While plugging of septal pores abolishes bulk mixing of cytoplasm, selective transport is still possible via transporters in the septal plasma membrane[46].

Plugging of septal pores in the ascomycetes can largely be abolished by inactivating the *hexA* gene. This gene encodes the HexA protein that forms crystals within the Woronin bodies[78,79]. Growth of the Δ*hexA* strain is reduced in *Neurospora crassa* giving rise to the cytoplasmic bleeding phenotype upon injury of hyphae[78,79]. The Δ*hexA* strain of *Aspergillus flavus* also shows reduced virulence and aflatoxin production[80], while invasive growth within the host is reduced in such a strain of the rice pathogen *Magnaporthe oryzae*. The underlying mechanisms of these phenotypes are not clear yet. Woronin bodies in *M. oryzae* are found near the hyphal tip in infection structures and are not associated with the septum. This suggests an additional role for Woronin bodies in this fungus[81].

FUNGAL HETEROGENEITY

Micro-colonies of *A. niger* within a liquid culture are heterogeneous. This is illustrated by the existence of two sub-populations of micro-colonies that differ in size[82,83] and gene expression[82]. Heterogeneity was also found within these fungal pellets. Total RNA content and expression of 18S rRNA, actin, *glaA*, and *faeA* genes was 45 times higher in the peripheral zone than the central zone[82]. Heterogeneity was also observed between zones of centimeter-wide macro-colonies of *A. niger*[62]. For instance, 40% of all genes are being differentially expressed between five concentric zones of *A. niger* colonies, while 3 % of the genes are only expressed in one of these zones[62,84]. Furthermore, at least three morphologically different zones can be distinguished. Towards the center, mycelial thickness increases and different hyphal layers within the different zones are observed[62]. Heterogeneity can even be observed within a zone of a colony. A sub-population of hyphae at the outer part of *Aspergillus* colonies secrete more proteins[86]. These hyphae also have a higher transcriptional and translational activity[87,88] but have a similar growth rate compared to the sub-population of hyphae that secrete low amounts of protein. From this it was concluded that high such activities are needed for protein secretion, while low such activities suffice to maintain growth rate[87]. Notably, heterogeneity between hyphae could not be observed in *A. oryzae*[76] and *A. niger*[46,89] after inactivation of the *hexA* gene. This was explained by the fact that these strains can no longer block inter-compartmental and inter-hyphal streaming thus preventing mixing of cytosol. Thus, Woronin bodies maintain hyphal heterogeneity by plugging septal pores[45,75,76,89].

OUTLINE OF THIS THESIS

It was the aim of this thesis to study functionality of heterogeneity between micro-colonies, between and within zones of micro- and macro-colonies, and even between compartments of single hyphae.

In **Chapter 2** it is described how pellet diameter of *A. niger* affects protein secretion in liquid shaken cultures. Expression of *gfp* from the promoters of the glucoamylase gene *glaA*, the feruloyl esterase gene *faeA* and the α-glucuronidase gene *aguA* occurred in a peripheral shell and was related to micro-colony size within a liquid shaken culture. Results imply that the highest productive micro-colonies would have a radius ≤ the width of this shell. If pellet sizes were to be constrained to these radii, secretion of glucoamylase, feruloyl esterase and α-glucuronidase would be 54-91 % more efficient under ideal circumstances.

Chapter 3 assessed whether plugging incidence of pores of *A. niger* and *A. oryzae* is affected by environmental conditions and whether hyphal heterogeneity promotes survival of the colony when exposed to stress. Plugging incidence of septa of *A. oryzae* was affected by heat treatment, low pH conditions, and C- and N-starvation, while that of *A. niger* was affected by exposure to heat, high pH, and hypertonic conditions. Growth of the *A. oryzae* and the *A. niger* Δ*hexA* strains was severely reduced after exposure to 45 °C. In addition, growth of the *A. oryzae* Δ*hexA* strain, but not the *A. niger* Δ*hexA* strain, was affected by carbon starvation. Notably, growth of the sub-population of *A. oryzae* wild-type hyphae with low transcriptional and translational activity was less affected by temperature stress than those with high such activities. In contrast, all hyphae of the *A. oryzae* Δ*hexA* strain were sensitive to heat stress. It is therefore posed that hyphal heterogeneity, arising from septal closure, increases adaptability of *Aspergillus* to changing environmental conditions in nature, in particular to heat stress.

Chapter 4 studied heterogeneity between compartments within hyphae of *A. niger.* It was shown that apical compartments of wild-type and Δ*hexA* strains of *A. niger* strain are capable of maintaining maximum growth rate after they are severed from the rest of the mycelium by dissection in the second hyphal compartment. This shows that the peripheral growth zone in *A. niger* can be reduced to the apical compartment. This raised the question what the function is of the sub-apical compartments if they are not needed for optimal apical growth. The first eight subapical compartments of wild-type *A. niger* but not of the *A. niger* Δ*hexA* strain were shown to assume apical growth after their apical located compartment was dissected. These sub-apical compartments thus serve as a backup system to maintain growth at the colony periphery. This back up system is pivotal in nature because of the life style of fungi to continuously explore their surrounding substrate that may prove hostile.

Single apical compartments of *P. chrysogenum* and *S. commune* and the 500-µm-apical zone of *R. stolonifer* coenocytic hyphae were also able to maintain their growth rate after laser dissection (**Chapter 5)**. Single second compartments of *A. niger*, *P. chrysogenum, S. commune* and the 500-1000-µm sub-apical zone of *R. stolonifer* hyphae were also capable of growing but their growth rate was severely impacted. Together, the peripheral growth zone of the fungi studied in this chapter is restricted to a single compartment or to ≤500 µm. In addition, results show that subapical hyphal fragments can function as a back-up in the case of catastrophic damage to the rest of the mycelium.

Results are summarized and discussed in **Chapter 6**.

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CHAPTER 2

Spatial induction of genes encoding secreted proteins in micro-colonies of *Aspergillus niger*

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ABSTRACT

Aspergillus niger is used by the industry to produce enzymes and metabolites such as citric acid. In liquid cultures, it can grow as a dispersed mycelium or as micro-colonies with a width in the micrometer to millimeter range. Here, it was assessed whether expression of genes encoding secreted enzymes depends on mycelium morphology. To this end, expression of *gfp* from the promoters of the glucoamylase gene *glaA*, the feruloyl esterase gene *faeA* and the α-glucuronidase gene *aguA* was related to micro-colony size within a liquid shaken culture. Hyperbolic functions were found to fit the data. This implied that the genes encoding the secreted proteins are expressed in a shell at the periphery of the micro-colony, which was confirmed by confocal microscopy. Modelling predicted that the width of these zones was 13 to 156 µm depending on growth medium and micro-colony diameter. Together, data indicate that the highest productive micro-colonies are those colonies that have a radius ≤ the width of the peripheral expression zone.

INTRODUCTION

Filamentous fungi form mycelia that consist of a network of hyphae that grow at their tips and that branch sub-apically. Mycelia of aspergilli can reach a diameter in the sub-millimeter scale (micro-colonies) to centimeter scale (macro-colonies) depending on the size and the composition of the solid substrate. For instance, *Aspergillus* forms micro-colonies on wheat kernels, whereas macro-colonies can be formed on fruits or bulbs of plants. In liquid shaken cultures, mycelium of *Aspergillus* grows dispersed, as micro-colonies, or in an intermediate state called clumps[1]. Dispersed mycelium consists of small networks of hyphae, while micro-colonies, also known as pellets, consist of a clear central and outer zone[2]. Notably, micro-colonies of *Aspergillus niger* produce more citric acid when compared to dispersed mycelium[3,4]. Why micro-colonies are more productive is not yet clear. It may be caused by the effect of the fungal morphology on the viscosity of the medium being high and low during dispersed growth and growth as micro-colonies, respectively[5]. At the same time, availability of oxygen and nutrients may impact productivity. Diffusion of these compounds would be sufficient in the case of dispersed mycelium while it would be limiting in the center of micro-colonies[6].

So far, it is not known whether morphology of the mycelium affects production of secreted proteins. As a first step, it was assessed whether micro-colony diameter affects expression of genes encoding secreted proteins in *A. niger*. To this end, the highly expressed genes[7] encoding feruloyl esterase FaeA[8], α-glucuronidase AguA[9] and glucoamylase GlaA[10] were used as model genes of xylanolytic and amylolytic activity. Data show that these genes are expressed in the outer part of micro-colonies only. Results imply that small micro-colonies of *A. niger* produce more secreted proteins per biomass than large micro-colonies.

RESULTS

Effect of mycelium transfer on micro-colony size

Wild-type strain N402 was grown in CMX (complete medium with xylose) for 16 h followed by a transfer to either CMX, CMM (complete medium with maltose), MMX (minimal medium with xylose) or MMM (minimal medium with maltose). Biomass of micro-colonies increased during a 4 h growth period from 0.6 g to 0.98, 0.84, 0.90 and 0.76 g, respectively. In the case of CMX, CMM and MMX mean micro-colony diameter increased from 948 µm to 1091, 1065 and 987 µm, respectively (Figure 1). In contrast, mean micro-colony diameter was reduced by 19 µm after transfer to MMM. The size distribution of micro-colonies was also altered after transfer to

the different media. In all cases, the mode was shifted to a higher diameter by ~163 µm (Figure 1). However, a higher number of small micro-colonies were found as well upon transfer to MMX and MMM (Figure 1D, E). This is illustrated by plotting the frequency of micro-colonies with diameters <2.5th, >2.5th <25th, >25th <75th, >75th <97.5th and >97.5th percentile (Figure 2). Transfer from CMX to MMX resulted in an increased proportion of micro-colonies with the smallest 2.5 % as well as largest 25 % diameters. At the same time, the frequency of micro-colonies of median sized micro-colonies was reduced. This shift was exacerbated when CMX grown micro-colonies were transferred to MMM, while transfer of micro-colonies from CMX to CMX only led to an increase in proportion of the largest 25 % of micro-colonies and a decrease in proportion of the median and smaller 25 % of micro-colonies. These findings were confirmed by multinomial logistic regression (data not shown). Together, data show that transfer to fresh medium promotes radial growth of N402 micro-colonies and, depending on the medium, can also result in partial micro-colony fragmentation.

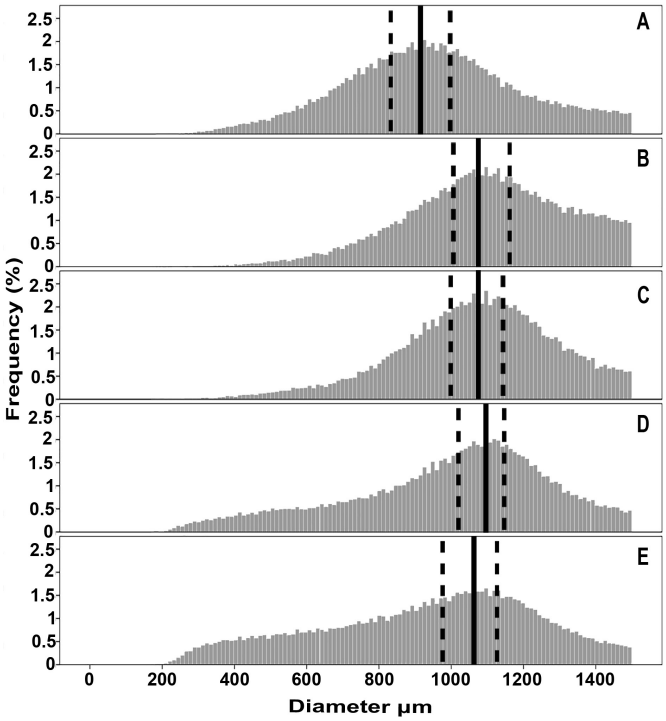

Figure 1. Size distribution of N402 micro-colonies that were not transferred to fresh medium (A) or were transferred from CMX to CMX (B), CMM (C), MMX (D) or MMM (E). Solid black lines flanked by dashed black lines represent the modes of the micro-colony populations and their 95 % confidence intervals, respectively.

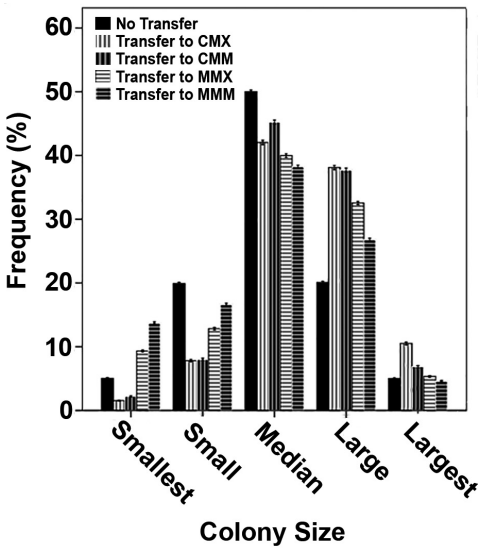

Figure 2. Proportions of size categories of N402 micro-colonies that were not transferred to fresh medium or were transferred from CMX to CMX, CMM, MMX or MMM. Error bars represent 95 % confidence intervals.

Like wild-type N402, biomass of strains expressing *gfp* from the *glaA*, *faeA* or *aguA* promoter increased upon transfer of 16 h old mycelium of non-inducing CM to inducing MM or CM. Biomass of the *glaA::GFP*, *aguA::GFP* and *faeA::GFP* strains had increased in MM from 0.71 to 0.78 g, 0.73 to 0.83 g and 0.73 to 0.93 g during a 4 h period, respectively. When transferred to inducing CM, biomass of the *glaA::GFP*, *aguA::GFP* and *faeA::GFP* strains had increased to 0.85, 1.47 and 1.49 g, respectively. Also in these strains, fragmentation occurred when mycelium was transferred to fresh medium (Figure 3). In fact, mean micro-colony diameter of *glaA::GFP* (1361 µm), *aguA::GFP* (1142 µm) and *faeA::GFP* (1230 µm) was reduced by 140, 145 and 250 µm, respectively, after a 4 h transfer to inducing MM. This reduction was 180, 130 and 79 µm for *glaA::GFP*, *aguA::GFP* and *faeA::GFP*, respectively, after transfer to CM. Transfer of reporter strains to inducing MM or CM coincided with a decrease in proportion of the largest 25 % of micro-colonies and an increase in proportion of the smaller 25 % of micro-colonies (Figure 4). Proportions of median sized micro-colonies also increased for the *aguA::GFP* and *faeA::GFP* strains. In contrast, the *glaA::GFP* strain showed a marked increase in the proportion of median sized micro-colonies. Increase and decrease of proportions of size categories were of lesser magnitude after transfer of fluorescent strains to inducing CM. Diameters of small, median and large micro-colonies of the *faeA::GFP* and *aguA::GFP* strains had reduced after transfer to inducing MM. For *glaA::GFP*, diameter of median and large microcolonies had also reduced

after transfer to inducing MM. For the *faeA::GFP* and *aguA::GFP* strains transferred to inducing CM the smallest 2,5 % of micro-colonies had increased in diameter, while small and median micro-colonies had reduced diameter. Large micro-colonies of *aguA::GFP* were also reduced in diameter, while large micro-colonies of *faeA::GFP* showed a slight increase in diameter. Micro-colony diameter of the *glaA::GFP* strain was reduced for median and large sized colonies but had increased for small micro-colonies. These findings were confirmed by multinomial logistic regression (data not shown). Together, biomass of the reporter strains increased after transfer to inducing medium but size of micro-colonies was reduced by micro-colony fragmentation.

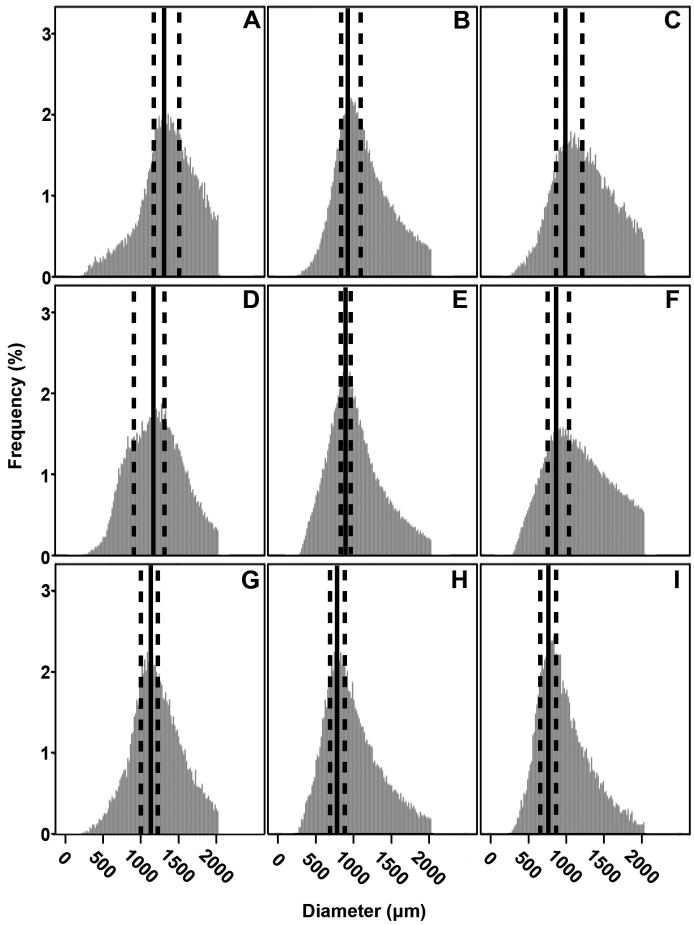

Figure 3. Size distribution of *glaA::GFP* (A,D,G), *aguA::GFP* (B,E,H) and *faeA::GFP* (C,F,I) micro-colonies that were or were not transferred from repressing CM (A-C) to inducing MM (D-F) or inducing CM (G-I). Solid black lines flanked by dashed black lines represent the modes and their 95 % confidence intervals for the micro-colony populations, respectively.

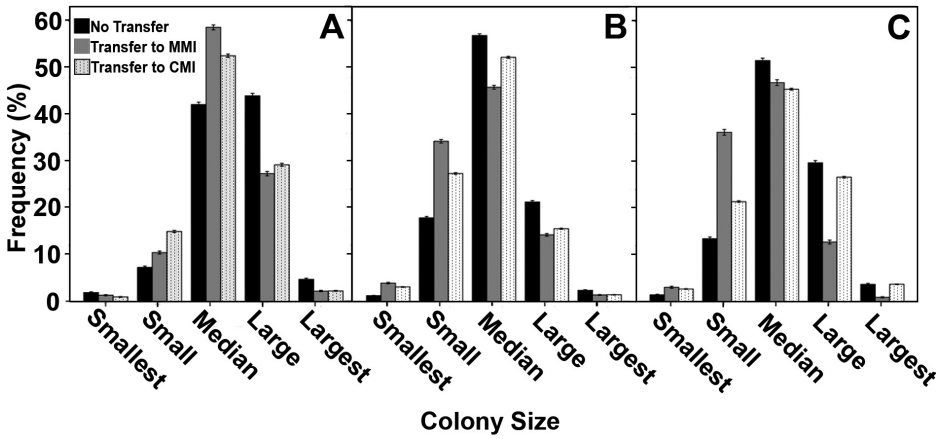

Figure 4. Proportions of size categories of *glaA::GFP* (A), *aguA::GFP* (B) and *faeA::GFP* (C) micro-colonies that were not or were transferred from repressing CM to inducing MM or CM. Error bars represent 95 % confidence intervals.

Relation between micro-colony size and expression of genes encoding secreted proteins

The relation between micro-colony diameter and GFP fluorescence intensity was studied in the reporter strains *glaA::GFP*, *aguA::GFP* and *faeA::GFP*. As expected, non-induced strains only showed low fluorescence (data not shown). Total fluorescence intensity of reporter strains transferred to inducing medium increased quadratically with increasing diameter (Supplemental Figure 1). Consequently, fluorescence intensity per micro-colony volume (FV^{-1}) per diameter was best fitted by a hyperbolic function (Figure 6A, C, E, Supplemental Figure 7A, C, E). In this function, FV^{-1} is maximal at the vertical asymptote x_b of the hyperbolic function, while with increasing diameter x, FV^{-1} decreases. Maximal FV^{-1} of *glaA::GFP, aguA::GFP* and *faeA::GFP* micro-colonies that had been induced in MM was found at a diameter of 288, 211 and 26 µm, respectively (Table 1). When strains were induced in CM maximal FV^{-1} was found at a diameter of 169 and 216 µm for *aguA::GFP* and *faeA::GFP,* respectively. Conservative selection parameters derived from micro-colony profiles could lead to micro-colonies being omitted from analysis unintentionally. Therefore, the diameter where FV^{-1} is maximal could be smaller in reality. This goes particularly for the CMM induced *glaA::GFP* strain as α is equal to 0, making $_b$ an arbitrary number intersecting γ with a minimum concurrent with the smallest recorded diameter. A hyperbolic function also has a horizontal asymptote (γ) that approaches a steady state. For *glaA::GFP, aguA::GFP* and *faeA::GFP* induced in MM this steady state was reached at 1556, 1381 and 954 µm, respectively. The horizontal asymptote was reached at a diameter of 1254 and 1532 when *aguA::GFP and faeA::GFP* were induced in CM (Table 1). Slopes did not converge to a steady state in the case of CMM-induced *glaA::GFP*.

Table 1. Values of relevant parameters from models describing the relation between micro-colony size and expression of *gfp* from the *glaA, aguA* and *faeA* promoters. Numbers between brackets indicate the 95% confidence interval. x_b is micro-colony diameter where fluorescence intensity per volume (FV^{-1}) is maximal, x_γ represents the diameter where FV^{-1} remains constant when diameter increases, r_γ is the predicted radius of the fluorescent band at this steady state and r_γ / 0.5 x_γ represents the percentage of the radius of the micro-colony that shows fluorescence.

Inducing medium	Promoter	x_b (µm)	x_γ (µm)	r_γ (µm)	r_γ / 0.5 x_γ (%)
MM	*glaA*	288 (±8)	1556 (-80/+109)	156 (±0.8)	21.5 (±0.1)
	aguA	211 (±11)	1381 (-11/+15)	89 (±0.2)	8.7 (±0.02)
	faeA	26 (±0.3)	954 (-1.7/+2.7)	13 (± 0.2)	2.7 (±0.02)
CM	*glaA*	-	-	-	-
	aguA	169 (±3)	1254 (±3)	37 (±0.3)	1 (±0.01)
	faeA	230 (±12)	1532 (±4)	31 (±2)	2 (±0.01)

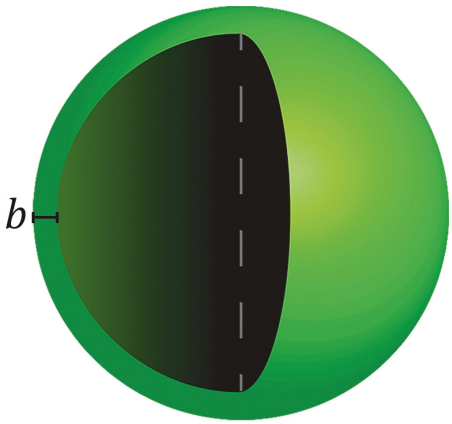

Figure 5. Schematic drawing of a model micro-colony as a perfect sphere. *b* denotes the width of the fluorescent shell.

Increase in fluorescence intensity in the region between x_b and x_γ cannot be explained solely by increase in micro-colony volume when parameter α in EQ2 is shown not to be zero. This suggests that fluorescence is not evenly distributed over micro-colony volume but occurs in a concentric peripheral shell (Figure 5). Indeed, z-stacks of confocal laser scanning microscopy images showed a fluorescent concentric zone with a radius of 149, 128 and 63 µm for *glaA::GFP, aguA::GFP* and *faeA::GFP* when induced in MM and 69, 102 and 118 µm when induced in CM, respectively (Table 2, Figure 8). To determine whether the width of the concentric shells depends on micro-colony size, fluorescence intensity per surface area was modeled (Figure 6B, D, F; Figure 7B, D, F). In the case of MM, change in width was < 10%. Modelling inferred that in the case of MM expression of *faeA* occurs in a relatively small concentric shell with

a constant radius of 13 µm. In contrast, *glaA* is expressed in a concentric outer shell that increases in width from 144 to 157 µm as micro-colonies increase in diameter from 288 to 1556 µm. On the other hand, *aguA* was predicted to be expressed in a concentric outer shell with a width decreasing from 105 µm to 92 µm in the 210 to 1381 µm size range of micro-colonies. When reporter strains were induced in CM, *glaA* was predicted to be expressed in a concentric outer shell that increases with a square root relationship. On the other hand, *aguA* was predicted to be expressed in a concentric outer shell that has a width decreasing from 85 µm to 37 µm in the 169 to 1245 µm size range of micro-colonies. Moreover, *faeA* was predicted to be expressed in a concentric outer shell with a width decreasing from 85 µm to 31 µm in the 230 to 1532 µm size range of micro-colonies. Together, upon growth of the micro-colonies, the volume of the expression zone increases. At the same time its relative radius and volume decreases compared to the total diameter and volume of the pellet.

Table 2. Radius of the expression zones of *glaA-*, *aguA-*, and *faeA*-driven *gfp* expression as shown by confocal microscopy. Numbers between brackets indicate the 95% confidence interval.

Inducing medium	Promoter	Fluorescent radius (µm)	Colony Diameter (µm)	Fluorescent radius (%)
MM	*glaA*	149 (±19)	1673 (±197)	17,8 (-4/+5)
	aguA	128 (±18)	1332 (±256)	19,2 (-5/+8)
	faeA	63 (±17)	1795 (±155)	7 (-2,4/+2,8)
CM	*glaA*	69 (±7)	1025 (±22)	6,7 (±0,6)
	aguA	102 (±16)	1045 (±43)	9,8 (±1,5)
	faeA	118 (±13)	1065 (±27)	11,1 (±1,2)

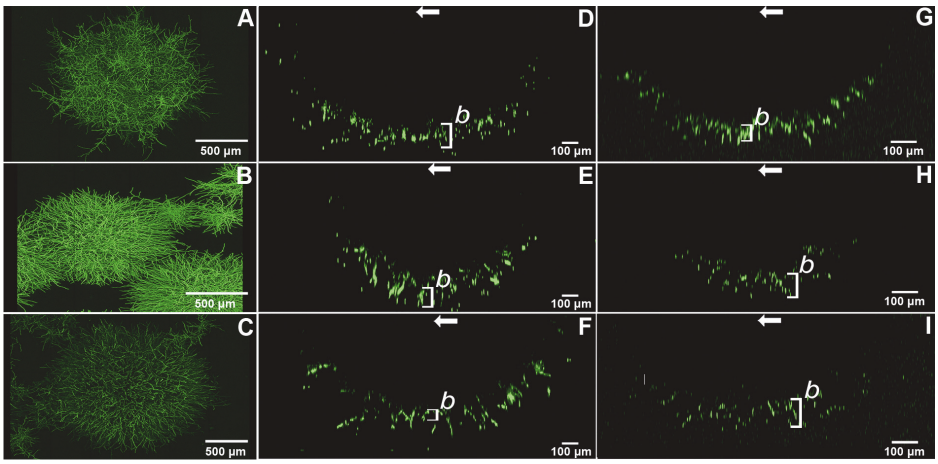

Figure 8. Maximum intensity Z projections of confocal scanning images of the center of micro-colonies (A,B,C) and their respective YZ profiles from the center (D-I) of *glaA::GFP* (D,G), *aguA::GFP* (E,H) and *faeA::GFP* (F,I) that were induced in either MM (D,E,F) or CM (G,H,I). Arrows mark the center of the colony. Bars marked *b* denote the mean thickness of the fluorescent shell.

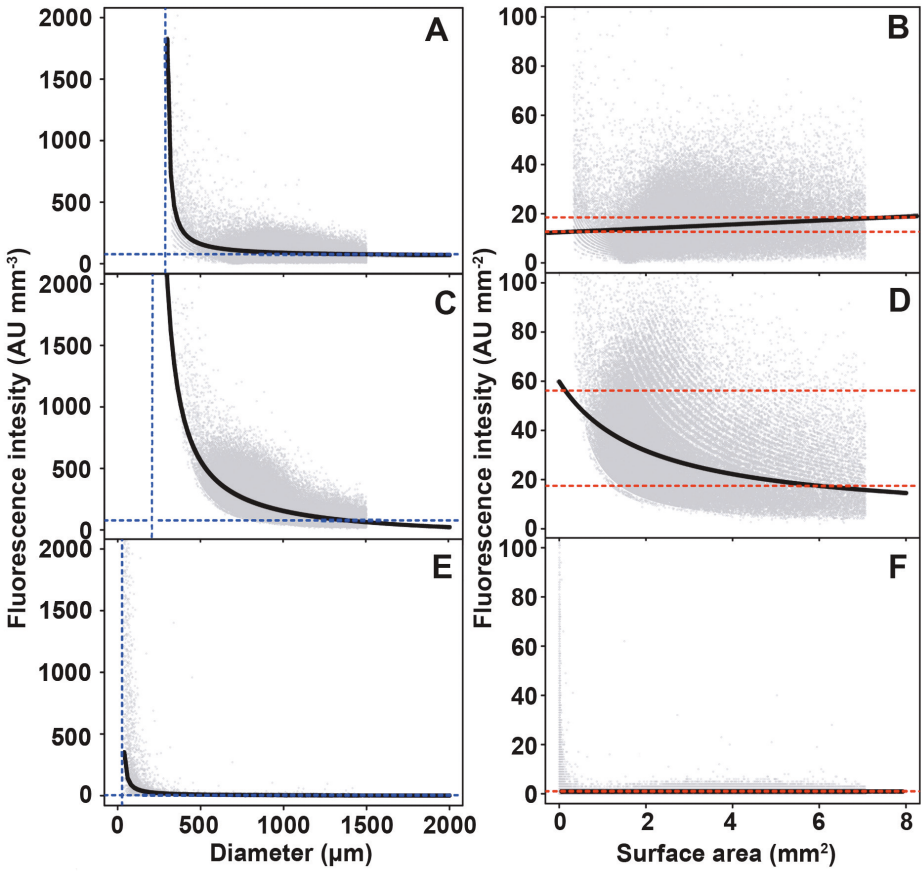

Figure 6. Relation between fluorescence per volume and pellet diameter (A,C,E) and fluorescence per surface area and surface area (B,D,F) of strains expressing *gfp* from the *glaA* (A, B), *aguA* (C, D) and *faeA* (E, F) promoter. Gray circles represent individual micro-colonies. The solid line represents the best fit as determined by quantile regression of the median. Horizonal blue dashed lines represent FV^{-1} at steady state and vertical blue dashed lines represent the diameter where fluorescence intensity is maximal. The red dashed lines (D-F) represent minimal and maximal fluorescence intensity mm^{-2} as predicted by EQ3.

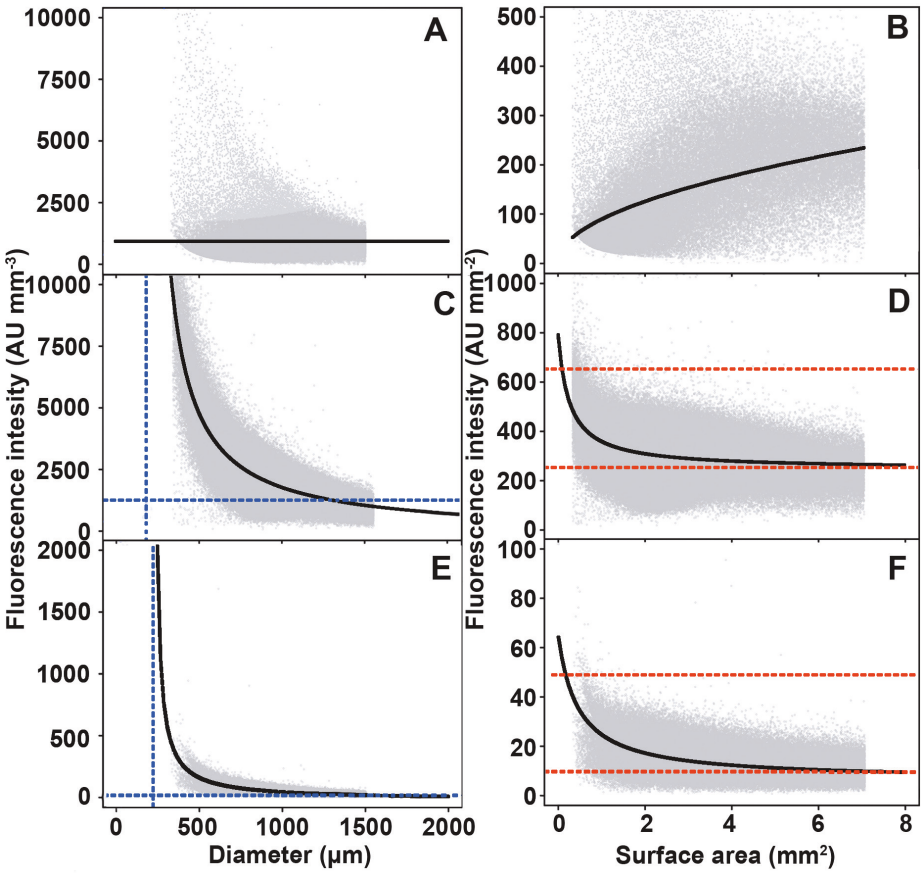

Figure 7. Relation between fluorescence per volume and pellet diameter (A,C,E) and fluorescence per surface area and surface area (B,D,F) of strains expressing *gfp* from the *glaA* (A, B), *aguA* (C, D) and *faeA* (E, F) promoter after transfer to inducing complete medium. Gray circles represent individual micro-colonies. Light gray squares represent individual micro-colonies discarded from the analysis using clustering with Gaussian mixture modelling. The solid line represents the best fit as determined by quantile regression of the median. Horizontal blue dashed lines represent FV^{-1} at steady state and vertical blue dashed lines represent the diameter where fluorescence intensity is maximal. The red dashed lines (D-F) represent minimal and maximal fluorescence intensity mm^{-2} as predicted by EQ3.

DISCUSSION

Morphology of the mycelium in liquid cultures such as bioreactors depends on various factors including inoculum size, surface composition of the inoculum, medium composition, pH of the medium and mixing conditions[1,7,11]. In addition, presence of molecules such as chelating agents or anionic polymers may affect morphology. Here, it was shown that transfer from a complete medium to a minimal (i.e. defined) medium or to a complete medium, in particular when also changing the carbon source, affects the morphology of the mycelium. To our knowledge this is the first time this has been quantified. Future studies will assess the underlying mechanisms and whether fragmentation can be used to optimize productivity in liquid cultures.

So far, it had not been established how secretion of proteins relates to the morphology of the mycelium. Driouch et al.[11] showed that 400-µm-wide micro-colonies express the glucoamylase gene *glaA* throughout the mycelium while expression in mm-sized micro-colonies is only observed at the outer periphery. However, this study made use of the presence or absence of titanate micro-particles to control morphology. Therefore, changes in spatial gene expression may have been the result of the addition of the micro-particles. This study made use of the inherent heterogeneity of microcolony morphology within a liquid shaken culture, as opposed to comparing two cultures with different medium composition. Expression of *glaA, faeA* and *aguA* per micro-colony volume generally decreased with increasing micro-colony size in a hyperbolic way eventually approaching a steady state where expression per micro-colony volume is constant. The hyperbolic relationship between colony diameter and fluorescence per volume is explained by expression taking place in a concentric shell at the periphery of the micro-colony (Figure 5). Expression in such a concentric shell was confirmed by fluorescence microscopy. When compared to confocal microscopy data models were reasonably accurate in predicting the fluorescent radius of *glaA* and *aguA* after transfer to inducing minimal medium; predicting the fluorescent radius of *glaA::GFP* within its 95% confidence interval and underpredicting the fluorescent radius for *aguA::GFP* by 16%. Yet, the expression zone of *faeA* deviated by 72%. For reporter strains transferred to inducing complete medium the fluorescent radius of the *glaA::GFP* strain could not be predicted because there were only 3% entirely fluorescent colonies in the culture. The fluorescent radius of *aguA::GFP* and *faeA::GFP* was under-predicted by 64% and 74%, respectively. Underprediction of the expression zone of *faeA* may be caused by the relatively low expression of this gene, while underprediction of expression zones may also be caused by selection of micro-colonies in the confocal analysis that had not yet reached their steady state (i.e. they were too small). Particle analysis is therefore the preferred tool to assess expression zones because it takes into account all micro-colonies sizes in a culture instead of analyzing single micro-colonies.

GFP expression was also found at the outer part of macro-colonies[13]. In contrast, the spatial expression in micro-colonies resulting from the *aguA* and *faeA* promoters was not in accordance with those in macro-colonies[13]. These genes were found to be expressed throughout the macro-colony and the colony center, respectively. The different spatial expression patterns in micro- and macro-colonies may be explained by inducer penetration. Micro-colonies in liquid shaken cultures are 3D structures. Penetration of the inducer into the center of the colony may be difficult. In contrast, macro-colonies grown on solid medium are near 2D and therefore the inducer in the underlying medium has access to all zones of the colony. However, this does not explain why the radius of the fluorescent concentric shell of the *faeA::GFP* strain is relatively small when compared to the *aguA::GFP* strain despite the fact that both genes are induced by xylose. Possibly, these genes respond to different concentrations of the inducer.

The fact that expression of the genes encoding secreted proteins occurs at the periphery of the micro-colony in a shell with a relatively constant width means that the volume of this shell decreases relatively to the total volume of the micro-colony when the micro-colony becomes larger. Thus, cultures with uniform small micro-colonies (diameters at x_b) would be more productive than cultures with uniform large micro-colonies (diameters at x_γ) assuming similar biomass is formed per volume culture medium. Glucoamylase, α-glucuronidase and feruloyl esterase production would then be 54, 56 and 91 % less efficient at steady state diameters compared to micro-colonies with diameters at x_b (i.e. diameter where expression per volume unit is maximal) if grown in minimal medium. In inducing complete medium, α-glucuronidase and feruloyl esterase production would be 61 and 65 % less efficient at steady state diameters compared to micro-colonies with diameters at x_b.

MATERIALS AND METHODS

Strains and culture conditions

Aspergillus niger strains N402, UU-A005.4, AR9#2 and AV11#3 were used in this study (Table 3). The former strain was used as a control, while the latter three strains (called *faeA::GFP*, *glaA::GFP* and *aguA::GFP* in this chapter) express *gfp* from the *faeA*, *glaA* and *aguA* promoter, respectively. Strains were grown at 30 °C on minimal medium (MM[13]) with 25 mM xylose and 1.5% agarose (MMXA). MMXA cultures were grown for three days, after which conidia were harvested using saline-Tween (0.8% NaCl and 0.005% Tween-80). 250 ml liquid cultures were inoculated with $1.25*10^9$ freshly harvested conidia and grown at 200 rpm and 30 °C in 1 L Erlenmeyer flasks in complete medium (CM) (MM containing 0.5% yeast extract and 0.2% enzymatically hydrolyzed casein)

supplemented with either 25 mM glucose (CMG; repressing *aguA* and *faeA* expression) or 25 mM xylose (CMX; repressing *glaA* expression). Mycelium was harvested after 16 h and washed twice with PBS. Ten g of biomass (wet weight) was transferred to CMX or MM with 25 mM xylose (MMX) (inducing *aguA* and *faeA*) or to CM or MM with 25 mM maltose (CMM, MMM) (inducing *glaA* expression). Biomass of cultures prior to transfer and after 4 h of growth in inducing medium was quantified after freeze drying.

Table 3. Strains used in this study.

Strain	Parental Strain	Genotype	Reference
N402	CBS 120.49	*cspA1, amdS*$^{-}$	16
AR9#2	AB4.1 (derivative of N402)	pyrG^{+}, sGFP(S65T) under regulation of the *A. niger glaA* promoter	12
AV11#3	AB4.1 (derivative of N402)	pyrG^{+}, sGFP(S65T) under regulation of the *A. niger aguA* promoter	12
UU-A 005.4	NW249 (derivative of N402)	Nic-, leu- arg-, pyrG^{+}, sGFP(S65T) under regulation of the *A. niger faeA* promoter	12

Flow cytometry

Mycelium of liquid shaken cultures (biological triplicates) was fixed overnight at 4 °C in 4% paraformaldehyde in PBS, washed twice with PBS and taken up in 50 ml PBS supplemented with 150 mM glycine to quench autofluorescence. Diameter and fluorescence were analyzed using a BioSorter (Union Biometrica, Boston, MA, USA) equipped with a 2 mm nozzle (fluidics and optics core assembly (FOCA) 2000) and using a 488 nm solid state laser. At least 10000 particles per replica were analyzed for size and fluorescence measurements. Diameter of micro-colonies was determined from the time of flight (TOF) using $x = \frac{-b \pm \sqrt{b^2 - 4a \cdot -TOF}}{2a}$ (a=0,00215762, b=5,433189436). This formula was inferred from the TOF of beads with diameters of 42 µm, 250 µm and 500 µm. Micro-colony volume (V) was calculated from the diameter assuming spherical morphology. To analyze the relation between micro-colony morphology and gene expression, particles with a TOF <165 and >13005 arbitrary units (AU) were removed from the dataset because they are outside the object size range of the FOCA (i.e. <30 µm and >1500 µm). In addition, particles were removed with fluorescence peaks <80 AU. Peaks >80 AU but with a fluorescence width <2000 AU were also removed. Finally, particles were removed that had an integrated density <25 AU for the green, red and yellow channels. The integrated green value (in AU) from the BioSorter was taken as a measure for fluorescence intensity (F). Micro-colonies of the non-fluorescent wild-type strain N402 were selected similarly but in this case particles were removed that had fluorescence peaks <100 AU. Additionally, integrated density of fluorescent signals was no longer a selection variable.

Differences in size distributions were quantified using two-sample Kolmogorov-Smirnov tests. Particles were distributed into five size categories based on diameter. Micro-colonies with diameters <2.5th, >2.5th <25th ,>25th <75th, >75th <97.5th and >97.5th percentile were designated smallest, small, median, large and largest, respectively. Differences in ratio between categories were analyzed using chi-square tests. Change of diameter after transfer to different media was described by resampling with 1000-fold replacement. For each resample the mode was recorded as the central tendency. The median of the modes and the 2.5th and 97.5th percentile provided an approximation of the mode and its confidence intervals. For biomass quantification, micro-colonies were not fixed, filtered using coffee filters, transferred to 50 ml falcon tubes and freeze-dried. Differences in dry weight were analyzed using Kruskal–Wallis H test.

Modelling GFP fluorescence relative to micro-colony diameter

Median fluorescence intensity data of micro-colonies that had been transferred to inducing medium were modelled with the R package quantreg[14] using non-linear quantile regression. Data pertaining to the fluorescence intensity per unit of volume (mm^3) were best described by the non-linear hyperbolic functions EQ1 and EQ2.

EQ1 $$FV^{-1} = \frac{a}{x - b} + c$$

EQ2 $$\frac{dF}{dV} = \frac{\alpha}{V} + \gamma$$

In these equations, F denotes fluorescence intensity and V micro-colony volume (mm^3). In EQ1, x denotes micro-colony diameter (µm), α represents the maximal fluorescence intensity per volume, c denotes the arbitrary intercept of this function and b represents the location of the vertical asymptote (i.e. the diameter x_b of the micro-colony where fluorescence intensity per volume is maximal). In EQ2, α denotes the maximal fluorescence intensity per volume, while γ represents a steady state between increase in fluorescence intensity and increase in volume. The x coordinate x_γ of the intersect of EQ1 and γ gives the diameter of micro-colonies that have reached this steady state.

Parameters x_b and x_γ were transformed to their corresponding surface areas, S_b and S_γ (mm^2) and used as input in EQ3 to determine if fluorescence intensity increases proportionally to micro-colony size. Using S_b and S_γ as input for S, EQ3 gives the minimal ($FS^{-1}{}_b$) and maximal ($FS^{-1}{}_\gamma$) observed fluorescence intensity per unit of surface area. EQ3.1 was used for *glaA::GFP*, while EQ3.2 was used for *aguA::GFP* and *faeA::GFP*.

EQ3.1 $$FS^{-1}{}_{(b;\gamma)} = gS_{(b;\gamma)} + i$$

EQ3.2 $$FS^{-1}{}_{(b;\gamma)} = \frac{g_0}{S_{(b;\gamma)} - l} + i$$

in which S denotes surface area in mm^2, while i represents the intercept, g_0 the slope and l the arbitrary location of the vertical asymptote. In this equation, any difference between $FS^{-1}{}_{\gamma}$ and $FS^{-1}{}_{b}$ in fluorescence per surface area is due to changing fluorescence intensity within the micro-colony, change of the radius of a hypothesized fluorescent shell, and/or the change in volume of this fluorescent shell due to increase in micro-colony size. EQ4 was used to rule out that the difference between $FS^{-1}{}_{\gamma}$ and $FS^{-1}{}_{b}$ is explained by the change in volume of the fluorescent shell. In this equation the change of fluorescence intensity per surface area is given by the derivative of FS^{-1}: $f'(S)$.

EQ4 $$\Delta FS^{-1}{}_{b\gamma} = \int_{S_b}^{S_\gamma} f'(S) - \int_{V_{s_b}}^{V_{s_\gamma}} f'(S_v)$$

where V_{s_b} and V_{s_γ} represent the minimal and maximal volume of the fluorescent shell. For derivative $f'(S_v)$ the variable S has been substituted by $\frac{S^{\frac{3}{2}} - (S - S_b)^{\frac{3}{2}}}{6\sqrt{\pi}}$ to describe the volume of the fluorescent shell. When $\Delta FS^{-1}{}_{b_\gamma}$ does not equal 0, fluorescence intensity mm^{-2} can only be explained by change in width of the fluorescent shell in the z-axis or its fluorescence intensity. The surface and volume independent change of fluorescence intensity of the fluorescent shell is revealed by EQ5.1 and EQ5.2, which were used for *glaA::GFP* and *aguA::GFP* and *faeA::GFP,* respectively.

EQ5.1 $$F_i = \int_{S_b}^{S_\gamma} \frac{\Delta FS^{-1}{}_{b\gamma}}{S_\gamma - S_b} S - \frac{\Delta FS^{-1}{}_{b\gamma}}{S_\gamma - S_b} S_b$$

and

EQ5.2 $$F_i = \int_{S_b}^{S_\gamma} \frac{\frac{(FS^{-1} - FS^{-1}{}_b)}{(S - S_b)} \frac{(S - S_\gamma)}{(FS^{-1} - FS^{-1}{}_\gamma)}}{\frac{(\sqrt{g} + i + \Delta FS^{-1}{}_{b\gamma} - FS^{-1}{}_\gamma)}{(\sqrt{g} + l - S_b)} \frac{(\sqrt{g} + l - S_\gamma)}{(\sqrt{g} + i - FS^{-1}{}_b)}} - FS^{-1}{}_b$$

When F_i is substituted in EQ6, the hypothesized increase of fluorescent radius z is found.

$$V_s = \frac{F_i + \int_{S_b}^{S_\gamma} FS^{-1}{}_{(b:\gamma)}}{m}$$

$$V_{s_b} = \frac{\int_{S_b}^{S_\gamma} FS^{-1}{}_{(b:\gamma)}}{m}$$

EQ6

$$V_s \cdot 10^9 = \frac{1}{6}\pi(x^3 - (x - x_b)^3)$$

$$V_{s_b} \cdot 10^9 = \frac{1}{6}\pi(x^3 - (x - x_b)^3)$$

$$\mathrm{z} = \frac{x_b \cdot x_{V_s}}{2x_{V_{s_b}}} - 0.5x_{V_{s_b}}$$

Where m is the relation between fluorescence intensity and volume of the fluorescent shell. $FS^{-1}{}_{(\gamma)}$ or $FS^{-1}{}_{(b)}$ should be input in EQ6 for fluorescent shells that decrease or increase in width with surface area, respectively. The relative fluorescent radius of a micro-colony is then given by EQ7.

EQ7

$$I_x = \frac{0.5x_{V_{s_b}} + z}{0.5x}$$

Finally, the percentage of fluorescent micro-colony volume at the steady state is given by EQ8, in which V_0 is the total volume of the micro-colony and V_1 is the non-fluorescent volume.

$$V_0 = \frac{1}{6}\pi(x)^3$$

EQ8

$$V_1 = \frac{1}{6}\pi((x - I_x \cdot x)^3)$$

$$I_v = \frac{V_0 - V_1}{V_0}$$

When micro-colonies were transferred to CM, equations described above remained valid for the *aguA::GFP* and *faeA::GFP* strains. However, the *glaA::GFP* strain was now best described by a horizontal line at γ, with b as an arbitrary intersect of this line.

Confocal laser scanning microscopy

Fluorescence of GFP was localized in micro-colonies using a DMI 6000 CS AFC confocal microscope (Leica, Mannheim, Germany). Micro-colonies were fixed and washed (see above), transferred into a glass bottom dish (Cellview™, Greiner Bio-One, Frickenhausen, Germany, PS, 35/10 MM) and embedded in 1% low melting point agarose at 45 °C. Micro-colonies were imaged at 20x magnification (HC PL FLUOTAR L 20x/0,40 DRY). GFP was excited by white light laser at 472 nm using 32-40 % laser intensity. Fluorescent light emission was detected with hybrid detectors in the range of 490-525 nm. Pinhole size was 1 Airy unit. In all cases, z-stacks were made of imaged micro-colonies using 100 slices with a slice thickness of 5.4-10 µm. Image analysis was performed with Fiji[15].

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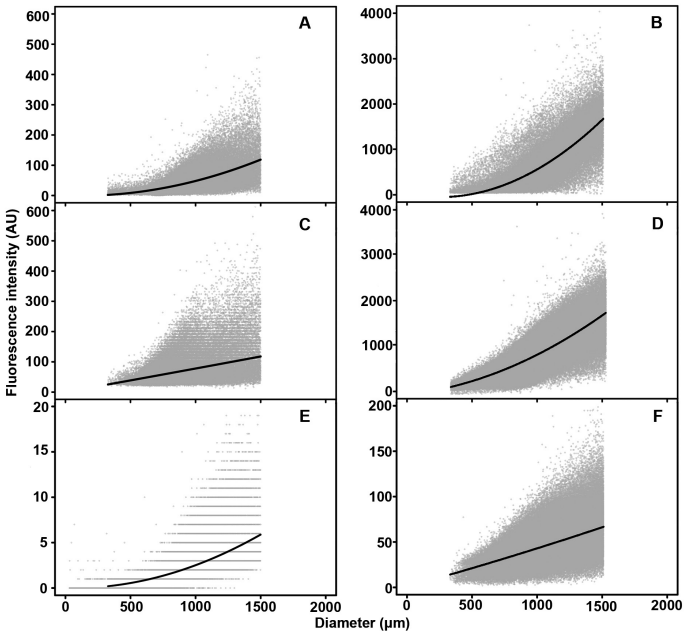

Supplementary Figure 1. Relation between fluorescence intensity and diameter of micro-colonies of strains expressing *gfp* from the *glaA* (A,B), *aguA* (C,D) and *faeA* (E,F) promoter that had been transferred to inducing minimal medium (A,C,E) or complete medium (B,D,F). Gray circles represent individual micro-colonies. The solid line represents the best fit as determined by quantile regression of the median.

CHAPTER 3

Functional specialization of hyphae in *Aspergillus oryzae* colonies

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ABSTRACT

Hyphae at the outer part of colonies of *Aspergillus niger* and *Aspergillus oryzae* are heterogeneous with respect to transcriptional and translational activity. This heterogeneity is maintained by Woronin body mediated closure of septal pores that block inter-hyphal mixing of cytoplasm. Indeed, heterogeneity between hyphae is abolished in Δ*hexA* strains that lack Woronin bodies. The sub-population of hyphae with high transcriptional and translational activity secretes enzymes that degrade the substrate resulting in breakdown products that serve as nutrients. The role of hyphae with low transcriptional and translational activity was not yet known. Here, it was shown that this subpopulation is more resistant to environmental stress in *A. oryzae*, in particular to temperature stress, when compared to hyphae with high transcriptional and translational activity. In contrast, all hyphae of the Δ*hexA* strain of *A. oryzae* were sensitive to heat stress explained by the reduced heterogeneity in this strain. Together, it was shown that different subpopulations of hypha secrete proteins and resist heat stress at different levels, showing the complexity of a fungal mycelium.

INTRODUCTION

Hyphae of filamentous fungi grow at their tips and branch sub-apically giving rise to a network of hyphae called mycelium. A vegetative fungal mycelium is heterogeneous with respect to gene expression, growth and secretion[1]. For instance, only part of the hyphae at the periphery of a mycelium of *Aspergillus niger* secrete glucoamylase[2]. This is due to heterogeneous expression of its encoding gene *glaA* in this part of the colony[3,4]. In fact, two populations of hyphae can be distinguished; those that highly and those that lowly express this gene[3]. Hyphae with high *glaA* expression also highly express other genes encoding secreted proteins. Moreover, they have a high rRNA content and highly express the glyceraldehyde-3-phosphate dehydrogenase gene *gpdA*[5]. From these studies it was concluded that two populations of hyphae can be distinguished at the outer part of the vegetative mycelium; those with a "high" and those with a "low" transcriptional and translational activity. Low activity would be sufficient to support growth but high activity would be needed to support secretion of high amounts of protein.

Hyphae of fungi belonging to the Ascomycota and the Basidiomycota are compartmentalized by porous septa. The diameter of the septal pore varies between 50 and 500 nm allowing passage of cytosol and even organelles[6-9]. The septal pores of ascomycetes can be closed by peroxisome-like organelles called Woronin bodies to prevent excessive cytoplasmic bleeding after hyphal injury[10-14]. These organelles also reversibly close septa of intact growing hyphae of *Aspergillus oryzae*[15] and *A. niger*[16,17]. The Woronin bodies of *Aspergillus nidulans* and *Aspergillus fumigatus* are located near the septal pore but are also found in other parts of the hypha[14,18]. In the case of *A. fumigatus* they are tethered via the Lah protein to the septal pore[19]. The lumen of Woronin bodies is filled with hexagonal rods of the HexA protein[10,20]. Deletion of *hexA* in *Neurospora crassa*, *Magnaporthe grisea, A. oryzae* and *A. niger* results in the absence of Woronin bodies. This abolishes closure of septa after hyphal damage[10-13] and in intact growing hyphae[15-17]. The latter leads to reduced heterogeneity in composition and activity between neighboring hyphae[15-17].

So far, it has been shown that the sub-population of hyphae that has high transcriptional and translational activity highly secrete proteins. A role of the lowly active hyphae was not yet clear. In this study it is shown that these hyphae of *A. oryzae* are more resistant to environmental stress, in particular to heat. Together, it is concluded that sub-populations of hyphae at the outer part of *A. oryzae* colonies have different functions.

RESULTS

Environmental conditions affect septal plugging in A. niger and A. oryzae

The effect of environmental conditions on plugging of the apical septum of leading hyphae of *A. niger* and *A. oryzae* was assessed. To this end, wild-type strains N402 and RIB40 were grown for 2 days at 30 °C, after which they were either or not exposed to temperature stress, pH stress, hypo- or hypertonic conditions, 25 μg ml^{-1} phleomycin or 4 mg ml^{-1} hygromycin. Exposure to these antibiotics mimic a confrontation with streptomycetes that produce these antibiotics in soil. Alternatively, strains were starved for two days for carbon or nitrogen. Significant changes in plugging incidence of the most apical septa were found when *A. oryzae* and *A. niger* were exposed to 45 °C for 1.5 h (Figure 1). This temperature stress increased plugging incidence from 60 to 75 % and 31 to 83 % in the case of *A. niger* and *A. oryzae*, respectively. Additionally, plugging incidence of apical septa in *A. niger* increased to 75 % under hypertonic conditions and decreased to 45 % when exposed to high pH. In the case of *A. oryzae,* apical plugging incidence increased to 48, 86 and 48 % under low pH conditions, C-starvation and N-starvation, respectively. Together, these data show that environmental conditions affect septal closure of *A. niger* and *A. oryzae*. Yet, their response to such conditions is different.

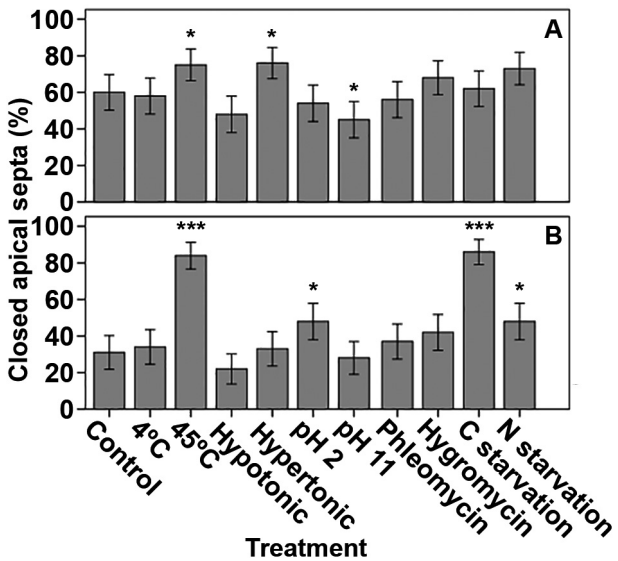

Figure 1: Percentage of closed apical septa of growing hyphae at the periphery of *A. niger* N402 (A) and *A. oryzae* RIB40 (B) colonies that had been subjected to different stress conditions. Error bars indicate 95 % confidence intervals. Asterisks (* $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$) indicate significant differences between control conditions and treatment conditions.

Growth of A. niger and A. oryzae ΔhexA strains can be affected by environmental conditions

The diameter of 24-h-old colonies of control and Δ*hexA* strains of *A. niger* (N402 and N402ΔhexA, respectively) and *A. oryzae* (RB#153.1 and RB#149.1, respectively) was measured after growing at standard conditions or after carbon starvation. Colony diameter was 2 mm for all strains at standard growth conditions. This was also the case for the *A. niger* and *A. oryzae* control strains that had been grown under carbon limitation. In contrast, colony diameter of *A. niger* and *A. oryzae* Δ*hexA* strains was 2.4 mm and 0.6 mm, respectively, after 24 h of carbon starvation. This shows that growth of the *A. oryzae* Δ*hexA* strain but not the wild-type strain is affected by carbon starvation. In the next step, growth of the carbon limited colonies was prolonged for 1.5 h under carbon limitation, while colonies that had been grown at standard growth conditions were either or not exposed for 1.5 h at 45 °C or hypertonic conditions. In all cases, this was followed by incubation for 24 h at standard growth conditions. Hyphae of the wild-type 49.5 h-old *A. niger* colonies had extended by 2.3 mm, irrespective whether they had been exposed to stress (Figure 2). The *A. niger* Δ*hexA* strain showed similar growth except for hyphae that had been exposed to 45 °C for 1.5 h. These hyphae had only extended by 1 mm. No significant differences were observed in the case of *A. oryzae,* irrespective of strain or heat treatment (Figure 2). Together, this shows that *A. oryzae* can restore its growth rate after exposure to stress, if affected at all, irrespective of the presence of *hexA*. In contrast, sensitivity of *A. niger* Δ*hexA* cannot be restored by growing at standard growth conditions.

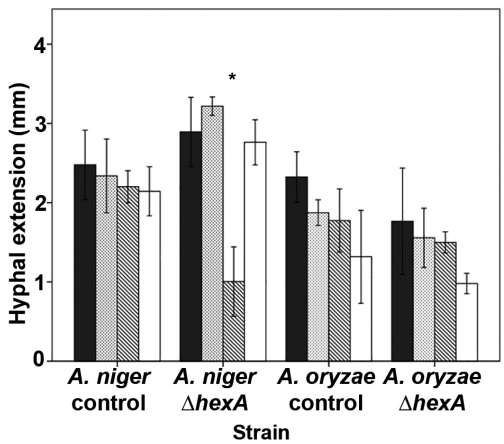

Figure 2: Hyphal extension (mm) of 24-h-old control (N402, RB#153.1) and Δ*hexA* (N402Δ*AnhexA*, RB#149.1) strains that had either or not (black bars) been exposed to hypertonic conditions (dotted bars) or 45 °C (diagonal stiped bars) for 1.5 h and subsequently allowed to recover for 24 h under non-stress conditions. White bars denote colonies that had been exposed to C-starvation for 25.5 h, after which growth was prolonged for 24 h under standard growth conditions. Error bars indicate standard deviations. Asterisk ($p \leq 0.05$) indicates significant differences from control conditions within strains.

Hyphal heterogeneity promotes adaptability of the colony to stress conditions

A. oryzae was shown to recover from heat stress by growing for 24 h at standard growth conditions (see above). As a next step, growth of hyphae and *glaA*-driven GFP fluorescence was measured immediately after heat shock. This was done with the control (RB#140.1) and the Δ*hexA* (RB#141.3) strains that express *gfp* from the glucoamylase *glaA* promoter. Expression of *glaA* was induced by transfer to maltose containing medium. This was followed by either or not exposing the colonies at 45 °C for 1.5 h. Expression of *glaA* within hyphae at the periphery of colonies of the *A. oryzae* control and Δ*hexA* strain were best described by a bimodal distribution when grown under control conditions. The fraction of high fluorescent hyphae was 50 % and 7.5 % for the control and Δ*hexA* strain, respectively. After heat shock, heterogeneity was reduced in the *A. oryzae* control strain and abolished in the Δ*hexA* strain (Figure 3).

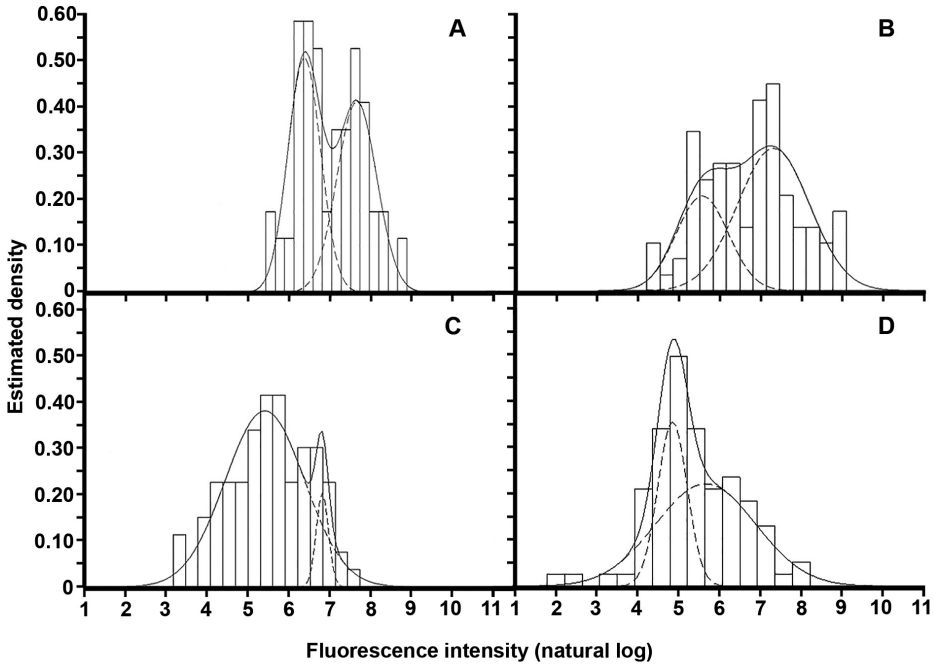

Figure 3: Estimated density plots based on normalized natural logarithm transformed fluorescence intensity data of hyphae of RB#140.1 (A,B) and RB#141.3 (C,D) that had either (B,D) or not (A,C) been exposed to 45 °C for 90 minutes. The former strain has Woronin bodies, while the latter does not.

Mean fluorescence intensity of hyphae at the colony periphery had reduced 90 minutes after heat-shock from 958 to 844 and 262 to 234 arbitrary units for the *A. oryzae* control and Δ*hexA* strain, respectively. No reduction in fluorescence intensity was observed for colonies exposed to control conditions. Low fluorescent hyphae

of the control and the Δ*hexA* strain that had not been exposed to 45 °C had grown 39 and 67 μm h^{-1}, respectively, while the high GFP expressing hyphae had extended 63 and 82 μm h^{-1} (Figure 4). Growth of heat stress exposed hyphae that showed low GFP fluorescence was reduced to 18 and 7 μm h^{-1} in the case of the control strain and the Δ*hexA* strain, respectively. In contrast, extension of hyphae that were highly fluorescent was reduced to 2 μm h^{-1} for both strains. Together, hyphae of the control *A. oryzae* strain that highly express *glaA* are more sensitive to heat stress than hyphae lowly expressing *glaA*. Furthermore, both lowly and highly expressing hyphae of the Δ*hexA* strain showed faster growth under control conditions, while they were more sensitive to temperature stress. This implies a trade-off between fast growth and adaptability to environmental stress.

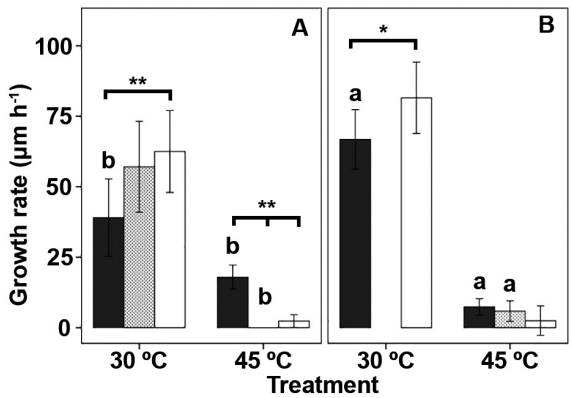

Figure 4: Highly (black bars), intermediate (white dotted bars) and lowly (white bars) *glaA* expressing hyphae of the *A. oryzae* control strain RB#140.1 (A) and the Δ*hexA* strain RB#141.3 (B) grown at 30 °C or 45 °C. Statistics did not indicate an intermediate population of fluorescent hyphae in the case of the Δ*hexA* strain grown at 30 °C. Error bars represent 95% confidence intervals. Lower case a and b indicate significant differences in growth rate for fluorescence intensity levels between strains. Asterisks ((* $p \leq 0.05$, ** $p \leq 0.01$) and lines indicate significant differences in growth rate for hyphae with different fluorescence intensity levels within treatments. All growth rates of hyphae with different fluorescence intensity levels are significantly different between treatments

DISCUSSION

A sub-population of hyphae with low transcriptional and translational activity and a subpopulation of hyphae with high such activities are found at the outer part of colonies of *A. oryzae* and *A. niger*[3,16,17]. The highly active hyphae secrete proteins, while the lowly active hyphae are more resistant to environmental stress, in particular to high temperature stress.

Woronin bodies maintain the heterogeneity of hyphae at the outer part of the *Aspergillus* colony[15]. As a consequence, GFP fluorescence intensity distribution of

hyphae of the *A. oryzae* Δ*hexA* strain is unimodal. Here, a bimodal GFP fluorescence intensity distribution was found. Yet, participation of the highly active hyphae was only 7.5 %, while this was 50% in the case of the wild-type. Thus, our data also support the role of Woronin bodies in maintenance of hyphal heterogeneity.

Part of the septa of vegetative hyphae of the basidiomycetes *Schizophyllum commune* and *Rhizoctonia solani* and the ascomycetes *A. oryzae* and *A. niger* are closed[15-17,21-23]. Incidence of septal closure in vegetative growing hyphae of *S. commune* depends on environmental conditions. Heat shock, hypertonic shock, and presence of antibiotics promote septal plugging, while low glucose reduces plugging incidence[22]. Such an effect was not observed in *A. oryzae*[15]. Here, we did show an effect of environmental conditions on septal plugging both for *A. niger* and *A. oryzae.* This is due to the use of a larger sample size and adapted statistical analysis. Plugging incidence of the apical septa of leading hyphae of *A. oryzae* was affected by heat treatment, low pH conditions, C-starvation and N-starvation. On the other hand, plugging incidence in *A. niger* was affected by exposure to heat, high pH, and hypertonic conditions. Together, these data indicate that closure of septa is not a stochastic process in *Aspergillus* but a result of environmental conditions. Results also show that *A. oryzae* and *A. niger* respond differently to these conditions. This may be caused by the limits of the environmental conditions that allow growth of these aspergilli[24]. For instance, *A. niger* can still grow at pH 1.5, while growth of *A. oryzae* only occurs above pH 4.

A. niger and *A. oryzae* strains with Woronin bodies were not affected in growth during carbon starvation. In contrast, growth of the *A. oryzae* Δ*hexA* strain was severely affected. Hyphal extension of the *A. niger* and *A. oryzae* Δ*hexA* strains was also affected by heat treatment. This effect was only found directly after the treatment in the case of *A. oryzae* Δ*hexA*, while in the case of the *A. niger* Δ*hexA* strain the effect of temperature was even observed after 24 h of recovery. It may well be that septal closure mediated hyphal heterogeneity is also instrumental in other environmental stresses. Deletion of a gene encoding the Woronin body associated transmembrane protein TmpL in *Alternaria brassicicola* and *A. fumigatus* results in hypersensitivity to oxidative stress[25]. Moreover, a Δ*HEXA* strain of *Magnaporthe grisea* is not able to survive N-starvation[26]. In addition, *hexA* is upregulated in *Trichoderma atroviride* stressed by the organophosphate pesticide dichlorvos[27].

Lack of Woronin bodies not only abolishes hyphal heterogeneity, it also increases growth rate. Tegelaar and Wösten[28;Chapter 4] found that wild-type *A. niger* hyphae grow at 84 % of the growth rate of the *A. niger* Δ*hexA* strain. Here, a similar effect was shown for *A. oryzae*. Hyphae with high transcriptional and translational activity grew 23 % slower than the Δ*hexA* strain, while this was even 42% in the case of lowly active hyphae. Our data thus suggest that fast growth comes with a reduced ability

to respond to environmental stress. Together, it is concluded that the outer part of a wild-type *A. oryzae* colony consists of hyphae that are specialized in release of nutrients from the substrate by secreting proteins and hyphae that have higher stress resistance. These two types of hyphae ensure efficient colonization of substrates with changing environmental conditions.

MATERIAL AND METHODS

Strains, media and spore isolation

Strains used in this study (Table 1) were grown on minimal medium (MM)[29] with 200 mM xylose or 25 mM maltose as a carbon source or on CD + Met medium[30] supplemented with 1 % glucose. Spores were harvested from 3-day-old cultures that had been grown on complete medium (MM with 0.2 % tryptone, 0.1 % casamino acids, 0.1 % yeast extract, 0.05 % yeast ribonucleic acids, and 1 % glucose) using saline Tween (ST, 0.9 % NaCl (w/v), 0.05 % (v/v) Tween-20).

Table 1. Strains used in this study.

Strain	Parental strain	Genotype	Reference
A. oryzae RIB40		Wild-type	39
A. niger N402	NRRL 3	Δ*cspA1*	40
A. niger N402Δ*hexA*	N402	Δ*cspA1* Δ*hexA hygR+*	This study
A. niger RB65.2	N402Δ*hexA*	Δ*cspA1* Δ*hexA hygR+ AnhexA phleoR+*	This study
A. oryzae RB#140.1	NSRKu70-1-1AS	Δ*ku70 glaA::sGFP niaD+*	15
A. oryzae RB#141.3	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1 glaA::sGFP niaD+*	15
A. oryzae RB#153.1	NSRKu70-1-1AS	Δ*ku70 niaD*$^{+}$	This study
A. oryzae RB#149.1	NSRK-ΔHx5	Δ*ku70* Δ*Aohex1 niaD*$^{+}$	This study

Construction of A. oryzae strains

NSRKu70-1-1AS and NSRK-ΔHx5 were transformed with pNR10 harboring the *niaD* gene[31]. The resulting strains RB#153.1 and RB#149.1 are prototrophic for nitrate.

Construction of A. niger strains

To inactivate *hexA* (An07g04570) of *A. niger,* its 0.9 kb upstream and 1.0 kb downstream flanking regions were amplified with primer pairs BN090 and BN091 and BN088 and

BN089 (Table 2), respectively, using genomic DNA of strain N402 as a template. The up- and down-stream sequences were inserted by BP recombination into pDONR™ P4-P1R and pDONR™ P2R-P3 (Invitrogen, www.invitrogen.com), respectively, generating the 5' and 3' entry clone plasmids pDONR_P-HexA and pDONR_T-HexA. The hygromycin resistance cassette (*hygR*) was amplified with primers hygroFW and hygroREV (Table 2) using pAN7.1[32] as a template and inserted into pDONR™ P1-P2R (Invitrogen) by BP recombination generating the center entry clone plasmid pDONR_hygR. The 5', 3', and center entry constructs were subjected to LR Clonase in the presence of pDEST R4-R3 (destination vector) to obtain the final plasmid pΔ*AnHexA*. The DNA fragment including the *hexA* flanking regions interspersed by the hygromycin resistance cassette was amplified by PCR using primers BN090 and BN089 and plasmid pΔ*AnHexA* as a template and was introduced into *A. niger* strain N402, generating strain N402Δ*hexA*. Disruption of the *hexA* gene was confirmed by Southern blotting using genomic DNA that had been digested with BamHI and KpnI.

To complement the N402Δ*hexA* strain, the *hexA* gene was amplified by PCR with primer pair AnhexA-FW-NotI / AnhexA-REV-PstI. The PCR product was digested with restriction enzymes NotI and PstI and introduced in plasmid pRB206[33] that had been cut with the same enzymes. This resulted in plasmid pB065 that contains the *hexA* gene and a phleomycin resistance cassette. pB065 was transformed to the Δ*hexA* strain. Complementation of *hexA* was assessed by chromosomal DNA extraction and PCR with primer pair ORF-hexA-FW / ORF-hexA-REV. Chromosomal DNA from N402 and *ΔhexA* strains was used as a control. PCR was positive for transformants RB65.2, RB65.3, RB65.4 and negative for the *ΔhexA* strain. Sequencing of the PCR bands confirmed *hexA* identity. To confirm phenotypic complementation, septal plugging of apical septa was assessed by laser microdissection. When the complemented strain RB#65.2 was grown under control conditions, 12 % of apical septa were closed when cut in the apical compartment; thus confirming the strain was complemented.

Table 2. Primers used in this study. Underlined sequences indicate *attB* recombination sequences.

Primer name	Sequence
BN088	GGGGACAGCTTTCTTGTACAAAGTGGGCCCACACATCCACCTTCCC
BN089	GGGGACAACTTTGTATAATAAAGTTGACAAGGACACCTCCAGCTCTTC
BN090	GGGGACAACTTTGTATAGAAAAGTTGCAGCCCAAGTGGAGAGCGACAAG
BN091	GGGGACTGCTTTTTTGTACAAACTTGCTTGCGAGAAGGAGGGGAATCAA
hygroFW	GGGGACAAGTTTGTACAAAAAAGCAGGCTAGGATTTCGGCACGG
hygroREV	GGGGACCACTTTGTACAAGAAAGCTGGGTTGTGGAGTGGGCGCTTACAC
AnhexA-FW-NotI	TAGCTATAGCGGCCGCAGTTGATCTAGCGCGTGAACG
AnhexA-REV-PstI	TCGCTATACTGCAGTTACGACGGCACGAAACGGC
ORF-hexA-FW	ATGGGTTACTACGACGACGACG
ORF-hexA-REV	CATCCTCGAAGGCCTCACGG

Transformation of Aspergillus

Protoplasts of *A. niger* and *A. oryzae* were generated as described[34] and transformed using polyethylene glycol[35]. Nitrate prototrophic (*niaD*$^{+}$) strains of *A. oryzae* were selected on MMS medium (minimal medium pH 6.0, 0.95 M sucrose and 1.5 % agar). Transformants of *A. niger* were selected on MMS medium containing 100 µg ml^{-1} hygromycin or 25 µg ml^{-1} phleomycin (Invivogen; www.invivogen.com).

Quantification of septal plugging

Aspergillus strains were grown under water saturating conditions in 35 mm glass bottom dishes (MatTek Corporation, Ashland, MA, USA, P35G-1.5-20-C)[15]. To this end, 30 µl 60 °C CD + Met medium with 1 % glucose and 1 % agarose (CDMGA) was pipetted into the glass bottom dish that had been pre-warmed at 50 °C. A total of 250 spores in 0.5 µl saline Tween was placed in the middle of an 18 mm cover slip and were left to dry. Cover slips were placed spore-side down on the non-solidified CDMGA. When CDMGA had solidified, 2 ml of liquid CD + Met medium (CDMG) was added on top of the culture. Temperature stress was imposed by incubating 2-day-old colonies at 4 °C or 45 °C. For pH stress, CDMG of 2-day-old cultures was replaced by CDMG with a pH of 2 or 11 adjusted with HCl or NaOH, respectively. For hypo- and hypertonic conditions, liquid CDMG of 2-day-old cultures was replaced with demi water or 1 M $MgSO_4$, respectively. Cultures that were C- or N-starved were exposed to C- or N-limiting conditions for 2 days starting at the time of inoculation. To this end, CDMGA was used with 0.2% glucose instead of 1 % glucose or CDMGA without methionine, respectively. The liquid medium on top of the culture did not contain carbon- or nitrogen source. For antibiotics treatment, CDMG and CDMGA were supplemented with either 25 µg ml^{-1} phleomycin or 4 mg ml^{-1} hygromycin. The second compartment (counting from the tip) was ruptured by laser dissection using the laser pressure catapulting function (LPC) of the P.A.L.M. laser dissecting microscope (Carl Zeiss AG; Oberkochen, Germany). To this end, 60-70 % of the power of the pulsed UV-laser was used. Dissecting the second compartment enabled us to assess the plugging state of the apical septum by monitoring the flow-out of cytoplasm from the apical to the compartment out of the damaged compartment[15]. Each experiment was repeated five times for 100 apical septa in total.

Recovery of hyphal extension after exposure to stress conditions

Control and Δ*hexA* strains of *A. niger* and *A. oryzae* were grown in glass bottom dishes as in the previous section. After 24 h of growth, colonies were either or not exposed to 45 °C or hypertonic conditions for 1.5 h. For C-starvation, colonies were grown from the moment of inoculation in CDMGA with 0.2% glucose instead of 1 %

glucose. The liquid medium on top of the culture did not contain carbon source. To recover, liquid medium was replaced by fresh CDMG and left to grow for another 24 h. Hyphal extension within biological triplicates was monitored using P.A.L.M. software and the microscope of the P.A.L.M. laser dissection set up (Carl Zeiss AG; Oberkochen, Germany).

Hyphal extension during heat treatment

Lumox® film (25 µm thickness; Greiner BioOne, Frickenhausen, Germany) was cut into 18 mm diameter circles and 0.5 µl spore solution containing 50,000 spores of *A. oryzae* strain RB#140.1 or RB#141.3 was pipetted in the middle of the hydrophobic side of the lumox foil and allowed to dry. Lumox® film was then placed, hydrophobic side down, on a polycarbonate (PC) membrane on MM with 1.5 % agarose and supplemented with 200 mM xylose (MMXA). After 2 days of growth at 30 °C, the Lumox® film with the adhering fungal colony was transferred, colony side down, to 500 µl MM supplemented with 25 mM maltose (MMM) in a Cellview™ cell culture dish (Greiner Bio-One PS, 35/10 MM, 627861) for 4 hours at 30 °C. Colonies were then exposed to either 30 °C or 45 °C for 90 minutes. The cell culture dishes were then placed under the P.A.L.M. laser dissecting microscope (Carl Zeiss AG; Oberkochen, Germany) using a Plan-Neofluar 40x/0.6 objective and a CCD camera (AxioCam ICc 1, Carl Zeiss AG, Oberkochen, Germany). Fluorescent, growing, leading hyphae were selected, imaged and their growth was recorded for 90 minutes. Experiments were done in triplicate for each strain and treatment conditions with 30 selected hyphae per replicate.

Statistics

Differences in radial growth between control and treatment conditions were assessed using ANOVA with two-sided Dunnet's post-hoc tests or Kruskall-Wallis test followed by pairwise comparisons. Differences in hyphal extension between strains were assessed using ANOVA with Bonferroni post-hoc corrections. A $\chi 2$ test was used to assess differences between septal plugging proportions during stress conditions. To assess the relation between fluorescence intensity and growth speed, either or not under stress conditions, a test for bimodality a described by Frankland and Zumbo (2009)[36] and as described by Vinck et al. (2005)[3]. When populations could be described as bimodal, low fluorescence expressing hyphae were defined as those with a total corrected cellular fluorescence (CTCF)[37] $\leq \bar{x}_1$ + its upper 95 % confidence interval (CI), intermediate fluorescence expressing hyphae as $> \bar{x}_1$ + its upper 95 % CI and $< \bar{x}_2$ - its lower 95 % CI and high fluorescence expressing hyphae as $\geq \bar{x}_2$ - its lower 95 % CI. Impact of fluorescent hypha location and clustering on hyphal fluorescence

intensity was assessed using multinomial logistic regression and Gaussian Mixture Clustering using the R package mclust[38] followed by Spearman's rank correlation, respectively. Growth speeds were bootstrapped 1000 times and a three-way ANOVA was carried out. Differences in fluorescence intensity were analyzed by repeated measures ANOVA or Kruskall-Wallis test. All analyses were carried out in IBM SPSS 24 (IBM Corp. Released 2016. IBM SPSS Statistics for Windows, Version 24.0. Armonk, NY: IBM Corp.).

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CHAPTER 4

Functional distinction of hyphal compartments

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This Chapter is based on Tegelaar M and Wösten HAB. 2017. Functional distinction of hyphal compartments. Sci. Rep. 7:6039

ABSTRACT

Hyphae of higher fungi grow at their tips and are compartmentalized by porous septa that enable inter-compartmental cytoplasmic streaming. Woronin bodies discontinue cytoplasmic streaming by plugging the septal pores. Here, it was assessed whether apical compartments of *Aspergillus niger* sustain their own growth or whether their growth depends on subapical compartments. Hyphae of wildtype and the Δ*hexA* strain, lacking Woronin bodies, had a similar morphology and growth rate. A total of 58 % and 17 % of the hyphae continued growing, respectively, after dissecting the 2nd compartment. Extension rate of the apical compartments that continued growing was not affected, even when the carbon or nitrogen source was limiting. Thus, apical compartments are self-sustaining in growth. It was also shown that the first 8 subapical compartments of the wildtype, but not of the Δ*hexA* strain, function as a backup system for growth by forming new branches when their apical neighboring compartment has been damaged. This backup system is pivotal in nature because of the life style of fungi to continuously explore their surrounding substrate that may prove hostile.

INTRODUCTION

Mycelia of filamentous fungi consist of interconnected hyphae that grow at their apices and that branch subapically. Hyphae of fungi belonging to the Ascomycota and the Basidiomycota are compartmentalized by septa. These cross-walls have a central pore of 50-500 nm[1-3] that allows inter-compartmental and inter-hyphal cytoplasmic mixing. However, it was recently shown that mixing in *Aspergillus* is constrained by Woronin bodies that plug the septal pores[4,5]. These peroxisome-derived organelles plug about 50 % of the most apical septa. Plugging incidence increases sub-apically resulting in 100 % closure after the 9th compartment. From these results it was concluded that hyphal compartments transform from a unicellular to a multicellular system[5]. Plugging of septal pores thus abolishes indiscriminate mixing of cytoplasm, but selective transport is possible via transporters in the septal plasma membrane[6].

The multicellular nature of the sub-apical part of hyphae explains why zones of a colony are heterogeneous with respect to gene expression, growth, and secretion[7-18]. Even neighboring hyphae can be heterogeneous in cytoplasmic composition[4,7,8,14,15,18-24]. This heterogeneity is abolished in a Δ*hexA* strain[4]. This strain lacks Woronin bodies because it does not produce the main protein component of these peroxisome-like organelles[25] and, as a consequence, septal plugging is rarely observed[4,26].

The fact that the apical compartments of *Aspergillus* hyphae form a unicellular system would agree with the concept of the peripheral growth zone defining the number of compartments that are needed to maintain the maximum hyphal growth rate[27]. This zone was described to consist of 11, 14, 6, and 13 compartments in the case of *Aspergillus niger, Aspergillus wentii, Aspergillus nidulans,* and *Penicillium chrysogenum*, respectively. However, here it was shown that apical compartments of *A. niger* are self-sustaining in growth thus disproving the concept of the peripheral growth zone. The subapical compartments do have a function by acting as a back-up system for growth when an apical compartment is damaged.

RESULTS

Apical compartments maintain their growth after laser-dissection

Light microscopy showed that the hyphal growth rate of the *A. niger* wild-type and Δ*hexA* strains was similar with a mean extension rate of 99 ± 5 and 93 ± 8 μm h^{-1}, respectively. Length (358 ± 62 and 350 ± 64) and diameter (5.8 ± 0.2 and 6.0 ± 0.3) of the apical compartments of wild type and Δ*hexA* were also similar.

Hyphae were laser dissected within the 2nd, 3rd, 4th, and 11th compartment. Mean hyphal growth rate of both strains was not reduced when the filaments had been dissected in the 11th compartment. In contrast, it was reduced by 39, 22, and 23 % after dissecting the 2nd, 3rd, and 4th compartment of wild-type hyphae, respectively. These values were higher for Δ*hexA* with 82, 47, and 49 %, respectively.

A total of 42, 20, 22, and 9 % of wild-type hyphae stopped growing, respectively, when they had been dissected in the 2nd, 3rd, 4th, or 11th compartment (Table 1). The apical septum had been open in all hyphae that had stopped growing after dissection of the 2nd compartment. Conversely, wild-type hyphae that remained growing had either a closed (20%) or open (80%) apical septum at the moment of dissection. The percentage of Δ*hexA* hyphae halting growth after dissecting the 4th or 11th compartment was similar to that of wild-type. In contrast, the percentage was higher when the 2nd or 3rd compartment was dissected. These data show that Woronin bodies protect hyphae from stopping growth after a subapical compartment is damaged.

Growth rate of wild-type and Δ*hexA* hyphae that remained growing after cutting in the 2nd, 3rd, 4th, or 11th compartment was not affected (Table 1). Similar results were obtained when the C- or N-source was excluded from the liquid medium (Data not shown; for growth conditions see Material and Methods). These data show that reduction of the mean growth rate of dissected hyphae is solely due to the fraction of hyphae that stop growing after this damaging event.

Predictors for continued growth of dissected hyphae

Growth rate of wild-type and the Δ*hexA* hyphae was normally distributed (Figure 1). The relatively slow and fast-growing hyphae within these normal distributions did not show differences in the incidence of continued growth after dissection in the second compartment (Data not shown). Thus, no causality was shown between continued growth and growth rate of the hyphae. The initial volume of wild-type apical compartments that continued or stopped growing was not significantly different (Table 2) and therefore was not a predictor of continued hyphal growth. In contrast, residual cytoplasmic volume in the apical compartment did differ after dissecting the 2nd compartment. Similar results were obtained with Δ*hexA*. However,

Table 1. Proportion of wild-type and Δ*hexA* hyphae that continue growing after laser dissection and their mean growth rate (± 95% confidence intervals) before and after laser dissection.

		Intact compartment(s)				
		1	2	3	10	Control
wt hyphae that continue growing	Proportion	58 %	80 %	78 %	91 %	100 %
	Growth rate before dissection	97 ± 5	100 ± 3	77 ± 3	88 ± 5	87 ± 2
	Growth rate after dissection	104 ± 5	97 ± 3	74 ± 4	87 ± 4	86 ± 3
Δ*hexA* hyphae that continue growing	Proportion	17 %	53 %	59 %	100 %	100 %
	Growth rate before dissection	111 ± 12	113 ± 6	105 ± 5	116 ± 6	92 ± 2
	Growth rate after dissection	103 ± 17	112 ± 15	88 ± 8	112 ± 8	91 ± 3

the cytoplasmic volume that was lost was 5.6- and 2.6-fold higher in the case Δ*hexA* hyphae that had stopped or continued growing after laser dissection, respectively, when compared to their wild-type equivalents (Table 2). Binary logistic regression showed that the length of the apical compartment is also a predictor for continued growth after laser ablation (Supplemental Text 1).

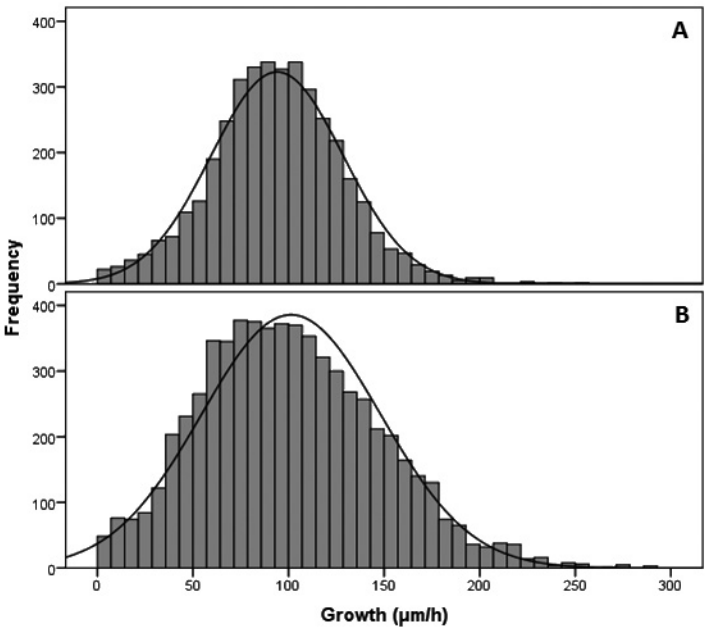

Figure 1. Distribution of growth rate of wild-type and Δ*hexA* hyphae of *A. niger*.

Hyphae were selected that had their apical septum at a distance < or > 400 µm from the apex. These hyphae were dissected at 400 µm from the tip. 11 % and 43 % of the wild-type and Δ*hexA* hyphae with a septum < 400 µm from the apex (i.e. these hyphae were cut in the 2nd compartment) stopped growing. In contrast, all wild type and Δ*hexA* hyphae with a septum > 400 µm from the apex (i.e. these hyphae were cut in the 1st compartment) stopped growing. Cutting within the first compartment at 400 µm from the apex resulted in a loss of 49 % and 68 % of the cytoplasm for Δ*hexA* and wild-type hyphae, respectively, while 46 % and 32 % was lost when the dissection site at 400 µm from the tip was within the second compartment. These results show that the presence of a septum between the apex and the site of dissection, independent of the state of the septum, is the best predictor whether a hypha continues its growth after ablation.

Table 2. Mean length, width, volume, and growth rate (± 95% confidence intervals) of apical compartments of wild-type and Δ*hexA* before and after laser dissection in the 2nd compartment.

		Apical length (µm)	Hyphal width (µm)	Growth rate (µm/h) Before	Growth rate (µm/h) After cutting	Apical volume (pl) Before	Apical volume (pl) After cutting	Volume lost (pl)	Volume remaining (%)
wt	Compartments that stopped growing	293 ± 61	6.3 ± 0.4	92 ± 7	0 ± 0	6.4 ± 1.7	3.9 ± 1.8	2.4 ± 1.7	62 ± 21
	Compartments that continued growing	363 ± 67	5.7 ± 0.2	97 ± 5	112 ± 6	9.2 ± 1.3	8.1 ± 1.4	1.1 ± 0.6	86 ± 7
Δ*hexA*	Compartments that stopped growing	287 ± 47	5.9 ± 0.3	108 ± 7	6 ± 4	8.4 ± 1.8	2.3 ± 1	6.2 ± 1.5	29 ± 12
	Compartments that continued growing	524 ± 151	6.2 ± 0.9	119 ± 14	127 ± 21	16 ± 8.4	10 ± 7.8	6.3 ± 0.9	50 ± 26

Disruption of the Spitzenkörper is linked to the fate of hyphal fragments

The Spitzenkörper of *A. niger* FG7 is fluorescently labelled due to an eGFP::SncA fusion protein[28]. Surface area, length, and fluorescence intensity of the Spitzenkörper in the apical compartment was determined before and after dissecting the 2nd compartment. Surface area, length, and fluorescence intensity of the Spitzenkörper of hyphae that stopped growing after dissection were smaller when compared to those of hyphae that remained growing (Figure 2; Table 3). These differences were not observed prior to dissection. These data show a relation between continued growth after dissection of the 2nd compartment and integrity of the Spitzenkörper.

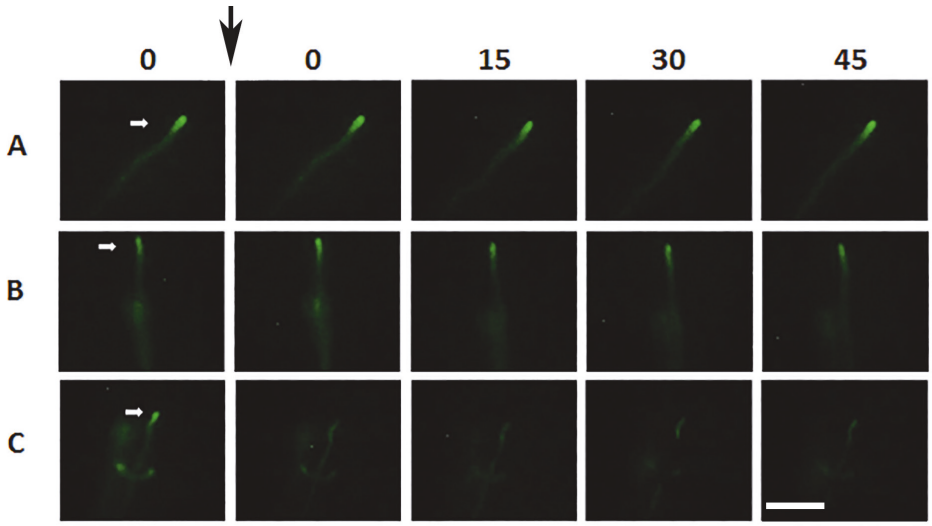

Figure 2. Fluorescently labelled Spitzenkörper before and after dissection in the 2nd compartment in a control hypha (A), a hypha continuing (B) and a hypha stopping growth (C) after dissection. Numbers indicate the time after laser dissection. White arrows denote the tip for which fluorescence was recorded. The black arrow indicates the moment of cutting. Bar represents 25 µm.

Table 3. Length, surface area, and fluorescent intensity (± 95% confidence intervals) of the Spitzenkörper before and after laser dissection in the second compartment.

	Before cutting			After cutting		
	Length (µm)	**Surface (µm²)**	**Fluorescent Intensity (AU)**	**Length (µm)**	**Surface (µm)**	**Fluorescent Intensity (AU)**
Continuing Hyphae	9.2 ± 1.3	42 ± 7	1205 ± 123	7.2 ± 0.9	32 ± 4	1039 ± 70
Stopping Hyphae	6.2 ± 3.8	37 ± 22	1145 ± 297	3.8 ± 2.2	22 ± 14	766 ± 212

Subapical compartments reinitiate growth after ablation of their apical neighboring compartment

Branching incidence was assessed for compartments 2, 5, 8, and 10 of wildtype and Δ*hexA* hyphae that had either or not been cut in the apical neighboring compartment. Branching in subapical compartments was hardly (< 2 % for the 2nd compartment), if at all (for the 5th, 8th, and 10th compartment), observed when Δ*hexA* hyphae had been dissected in their neighboring apical compartment. In contrast, dissection of compartment 1 of wild-type hyphae increased branching frequency of compartment 2 from 30 % to 93 % within a 4 h period ($p \leq 0,05$). Similarly, dissection of compartment 4 or 7 increased branching incidence from 29 % to 71 % and from 2 % to 31 %, respectively. Branching incidence of compartment 10 did not increase significantly (0 % versus 4.5 % without and with cutting) when hyphae were dissected in compartment 9. Branching incidence in compartments 2 and 5 was statistically higher than branching incidence in compartments 7 and 9 for both cut and uncut wild-type hyphae. Together, these data show that sub-apical compartments act as a back-up for growth when apical compartments are damaged. This back-up is not functional in the absence of Woronin bodies. Moreover, the back-up capacity of hyphal compartments diminishes when compartments are further away from the hyphal tip.

DISCUSSION

Septate fungal hyphae like those of *A. niger* have been reported to require a minimal length of 1200 µm[29] or 11 intact compartments[27] to allow the maximum growth rate. However, it was shown showed that apical compartments (mean length 354 µm) sustain their own maximum growth rate. This discrepancy is probably explained by the methodology adopted. Lhoas[29] and Trinci[27] damaged multiple hyphae with a metal blade and growth rate was expressed as the increase in colony diameter. In contrast, in this study single hyphae were dissected with a laser and mean growth rate was determined, taking into account the heterogeneity between the leading hyphae at the edge of the colony. In the case of the wild-type 42 % of the hyphae stopped growing after dissecting the second compartment. These hyphae reduced the average growth rate of the whole population of hyphae. At the same time, average growth rate was not affected of those hyphae that remained growing after dissecting the second compartment. This shows that apical compartments do not depend on subapical compartments to sustain their growth.

The autonomy of apical compartments raised the question what the function is of subapical compartments. Subapical compartments may have a feeding function under nutrient deprivation, which was suggested from the finding that glucose is transported from the center to the periphery of the *A. niger* colony[6]. Yet, in our experimental set up apical compartments were still autonomous when N- and

C-sources were limited. It was shown that subapical compartments function as a back-up system. All hyphae stopped growing when apical compartments were dissected. This was accompanied by an increased branching incidence of the second compartment. These branches formed new exploring hyphae. Similarly, branching incidence was increased in compartment 5 and 8 after dissection of compartments 4 and 7, respectively. Branching incidence of compartment 10 was low, if observed at all, after dissecting compartment 9. This implies that this compartment and other more subapical compartments have lost their capacity to reinitiate growth. The ability of subapical compartments 2-8 to restore hyphal growth after their apical neighboring compartment has been damaged is pivotal considering the fungal life style. Mycelia continuously extend by colonizing their surrounding substrate. Apical compartments of exploring hyphae are the first to be confronted with microbes or other organisms within the non-explored substrate that can damage or feed on hyphae. Subapical branching combined with extension at an angle of the axis of the damaged hyphae provides a way to escape these organisms.

Woronin bodies can close septal pores, thus preventing cytoplasmic bleeding after hyphal damage[25,30-32]. In addition, these organelles maintain inter-hyphal and inter-compartmental heterogeneity of cytoplasmic composition[4]. Here, it was shown that Woronin bodies are not involved in the growth rate of hyphae before or after ablation but two additional functions were found for these organelles. First, branching in the sub-apical compartment was not observed in Δ*hexA* hyphae after ablation of the apical neighboring compartment. This shows that Woronin bodies are essential for the sub-apical back-up system of growth. Second, Woronin bodies make hyphae more resilient to loss of cytoplasm. Half of wild-type hyphae were calculated to remain growing when 61% of the cytoplasmic volume in the apical compartment is retained, while 76 % was needed in the case of Δ*hexA* hyphae. The resilience of the wild-type to cope with loss of cytoplasm is likely due to its ability to plug the septal pore, thus increasing the chance of the apical compartment to regain turgor. Experimental evidence indicates that not only loss of cytoplasm but also (partial) disintegration of the Spitzenkörper may result in halting of growth. Although in many cases occurrence of these processes will correlate, this is not necessarily the case. For instance, a strong but short outflow of cytoplasm may cause disintegration of the Spitzenkörper but would retain a sufficient amount of cytosol.

The observation that hyphae of *A. niger* always stop growing when the apical compartment is damaged implies that positioning of a septum close to the tip reduces the chance of abolished growth simply because the length available for a fatal damage would be less. In fact, fungi may increase septal incidence (i.e. reduce compartment length) to cope with stress. Indeed, length of apical compartments

of *A. niger* is reduced at temperatures above 30 °C, coinciding with an increased branching incidence[33].

Together, evidence is provided for the first time that apical hyphal compartments of *A. niger* are autonomous with respect to growth. The subapical compartments are however metabolically active consuming a major part of resources. This drain of resources could be prevented by controlled autolysis of subapical compartments. This, however, would also result in the absence of the sub-apical back-up system of hyphal growth that is used when apical compartments become damaged.

MATERIALS AND METHODS

Strains and growth conditions

Strains N402[34], Δ*hexA*[26] and FG7[28] of *A. niger* were grown at 30 °C in water-saturated air at 700 lux white light (Osram Lumilux L36w/840, Osram, Munich, Germany). Spores were harvested in 10 ml 0.9 % NaCl (w/v), 0.05 % (v/v) Tween-20 from 7-day-old cultures grown in 9 cm Petri dishes on complete medium (CM) consisting of minimal medium (0.6 % $NaNO_3$, 0.15 % KH_2PO_4, 0.05 % KCl, 0.05 % $MgSO_4$ $7H_2O$, 0.2 ml^{-1} Vishniac solution[35]) with 0.2 % tryptone, 0.1 % casamino acids, 0.1 % yeast extract, 0.05 % yeast ribonucleic acids, 1.5 % agarose, and 25 mM maltose. These spores were used to inoculate glass bottom dishes (MatTek Corporation, Ashland, MA, USA, P35G-1.5-20-C). To this end, the dishes and medium were pre-warmed to 50 °C and 60 °C, respectively. 0.5 µL spore solution (50,000 spores) was placed on a glass coverslip (18 mm in diameter and 0.16-0.19 mm thick) and left to dry. Pre-warmed CDMMA (30 µL) consisting of CD + Met medium (0.3 % $NaNO_3$, 0.2 % KCl, 0.1 % KH_2PO_4, 0.05 % $MgSO_4.7H_2O$, 0.002 % $FeSO_4.7H_2O$, 0.0015 % methionine, pH 5.5[36] with 1 % agarose and 25 mM maltose was added on top of the glass bottom dish and immediately covered with the coverslip with the spores facing the medium. After the 118 µm thick layer of medium had solidified, 2 ml liquid CDMM was pipetted on top of the coverslip. CDMMA with 0.2 % maltose and CDMM medium without a carbon source or CDMMA and CDMM without methionine were used to expose hyphae to C- or N-limiting conditions.

Microscopy

Hyphae were dissected using a PALM Microbeam system linked to an Axiovert 200 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) and a 3CCD color camera (HV-D30, Hitachi Kokusai Electric Inc., Tokyo, Japan). The glass bottom dish cultures were incubated at the microscope stage for 90 min at 25 °C before dissection. Hyphal growth rate within the peripheral zone of the colony was recorded every 5 min during a 45 min period, after which half of the hyphae were dissected in

the 2nd, 3rd, 4th, or 11th compartment using the laser pressure catapulting function of the PALM MicroBeam system (laser power 55 %, focus 59 %). At the moment of dissection it was assessed whether septal pores were open or closed. Septa were classified as open when cytosol was leaking through the septal pore upon dissection[4]. Hyphal growth was recorded every 5 min during a 45 min period immediately after dissection, non-dissected hyphae serving as control. Width and length of the compartment pre- and post-dissection were also recorded, as well as translocation (µm) of vacuoles after cutting. From these parameters the post-dissection volume of the apical compartment was calculated using $V_e = V_0 - V_1$ where V_0 equals $\frac{1}{2}\pi\left(\frac{2}{3}{d_0}^3 + {d_0}^2\left(l - \frac{1}{2}d_0\right)\right)$ and V_1 equals $\frac{1}{2}\pi\left(\frac{2}{3}{d_1}^3 + {d_1}^2\left(l - \frac{1}{2}d_1\right)\right)$ in the case volume was calculated based on reduction of hyphal diameter or V_1 equals $\frac{1}{2}{d_0}^2\pi t_1$ when volume was calculated based on translocation of vacuoles (Figure 3).

Fluorescence was recorded using a HV-D30 camera with settings for white balance 5600 K; gain +8 dB; contour correction high; shutter speed 1/50; digital gain +6 dB; gamma on; contrast off; knee automatic. Total corrected cellular fluorescence was calculated with ImageJ. The surface of the Spitzenkörper was estimated by using the ImageJ threshold function using a brightness of 27.

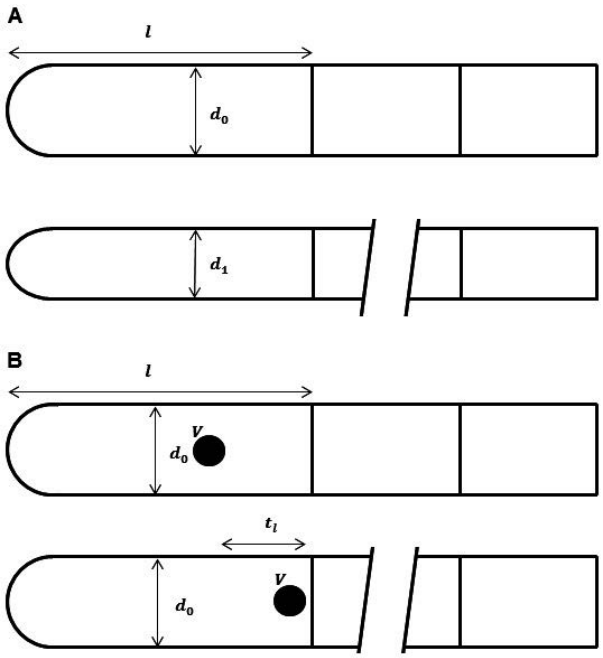

Figure 3. Parameters used to calculate loss of cytosolic volume after dissection of a neighboring compartment. d_0 and d_1 denote the diameter of the compartment before and after dissection, respectively. l is the total length of the compartment, while l_t represents the distance vacuoles traveled after dissection.

Statistics

Experiments were performed using biological duplicates (assessment of growth rate) or triplicates (plugging state, loss of volume, and renewed subapical growth). A one-way ANOVA with a Sidak post-hoc test or Kruskall-Wallis test with subsequent Wilcoxson rank sum tests was carried out to determine whether growth rate differed between hyphae with a different number of intact compartments. A χ^2 test was performed with post-hoc z-tests and a Bonferroni correction for multiple comparisons to assess differences in plugging incidence between septa. The effect of plugging on hyphal growth was evaluated using one-way ANOVA and a Sidak post-hoc test, differences in hyphal growth before and after cutting were determined using paired sample t-tests. Differences in volume loss were statistically analyzed using two-way ANOVA, followed by correlation and regression analyses. Relative values pertaining to volume of apical compartments before and after cutting were analyzed using a paired sample t–test with and without logit transformations. Analysis of bimodality was performed as described[20]. Renewed growth from compartments located subapically from compartments damaged by laser dissection was analyzed by a χ^2 test with post-hoc z-tests and a Bonferroni correction for multiple comparisons.

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Supplemental Text 1

Binary logistic regression predicted that 50 % of the ΔhexA hyphae would continue growing when 76 % of the volume remained in the apical compartment after dissecting the 2nd compartment. A remaining cytoplasmic volume ≥ 11.2 pl and ≤ 4.7 pl predicted that all ΔhexA hyphae would continue or stop growing, respectively. In contrast, a remaining volume > 2.7 pl predicted that 100 % of the wild-type hyphae would continue growing. Length of wild-type and Δ*hexA* apical compartments was also a predictor of continued growth with an accuracy of 88 % as determined by binary logistic regression. Both data sets predicted that for every 10 µm increase in length of the apical compartment the likelihood of stopping after dissecting the second compartment decreased by 7.5 %. All hyphae were predicted to continue growing with a compartment length of 77 µm for the wild-type and 629 µm for the Δ*hexA* strain.

CHAPTER 5

Fungal apical compartments are self-sustaining in growth

Martin Tegelaar, George P.A. van der Lans and Han A. B. Wösten.

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ABSTRACT

Hyphae of mucoromycetes are non-septate, while hyphae of ascomycetes and basidiomycetes are compartmentalized by porous septa. Initially, the pores of septa of the ascomycetes *Aspergillus niger* and *Aspergillus oryzae* are open, while incidence of septal closure increases towards the older (i.e. the subapical) parts of the hyphae. Thus, hyphal compartments transform from a unicellular to a multicellular system. Unicellularity would allow sub-apical compartments to support growth of the apical compartment and supports the concept of the peripheral growth zone. This zone defines the number of compartments that are needed for a hypha to maintain its growth rate. The peripheral growth zones of the ascomycetes *A. niger* and *Penicillium chrysogenum* would consist of 11 and 13 intact compartments and 8660 µm for the non-septate hyphae of the mucoromycete *Rhizopus stolonifer.* However, it was recently demonstrated that apical compartments of *A. niger* are self-sustaining. This was shown by assessing the growth rate of individual hyphae before and after hyphal dissection in the second compartment. Using the same methodology, it is demonstrated that growth rate of single apical compartments of *P. chrysogenum* and the basidiomycete *Schizophyllum commune* as well as the 500-µm-apical region of *R. stolonifer* are also self-sustaining in growth. In contrast, single 2nd compartments (obtained by dissection of the first and third compartment) of septate fungi or the region between 500 and 1000 µm from the tip of *R. stolonifer* were severely impacted in their growth rate. Together, it is concluded that the peripheral growth zone of the researched septate fungi is restricted to a single rather than multiple compartments and that also the peripheral growth zone of *R. stolonifer* is much shorter than previously reported.

INTRODUCTION

Filamentous fungi form mycelia that consist of a network of hyphae. These hyphae extend at their apices and branch subapically. Hyphae of mucoromycetes are generally non-compartmentalized, while those of ascomycetes and basidiomycetes are divided by septa. These septa have a 50-500 nm wide central pore flanked by invaginations of the cell wall lined with plasma membrane[1-3]. By plugging the pores, intercompartmental and interhyphal cytoplasmic mixing is interrupted[4]. Intact growing hyphae of *Aspergillus oryzae*[5] and *Aspergillus niger* Chapter 3 plug their pores with Woronin bodies, while hyphae of the basidiomycete *Schizophyllum commune* close their pores with the septal pore cap[6]. Environmental conditions impact incidence of septal closure in *S. commune*[7], *A. oryzae* and *A. niger* Chapter 3. Low glucose levels reduce plugging incidence in *S. commune*, while presence of antibiotics, heat shock, and hypertonic shock promote septal closure[7]. Plugging incidence of septa of *A. oryzae* is affected by heat treatment, low pH conditions, C-starvation and N-starvation, while that of *A. niger* is affected by exposure to heat, high pH, and hypertonic conditions Chapter 3. Incidence of septal closure in *Aspergillus* also increases in time. Half of the newly formed apical septa are open, while septa flanking the 10th compartment are always closed[5,6]. From this and the fact that septal closure abolishes bulk mixing of cytoplasm it was concluded that hyphal compartments transform from a unicellular to a multicellular system[5]. Yet, selective transport is still possible via transporters in the septal plasma membrane[8].

The fact that the apical compartments of *Aspergillus* hyphae form a unicellular system is in line with the concept of the peripheral growth zone. This zone defines the number of compartments that is needed for a hypha to maintain its growth rate[9]. The peripheral growth zones of *A. niger* and *Penicillium chrysogenum* would consist of 11 and 13 intact compartments and 8660 µm for the non-septate hyphae of *Rhizopus stolonifer.* Notably, it was recently shown that apical compartments of *A. niger* are self-sustaining[10], thus reducing its peripheral growth zone to one compartment. Both the studies of Trinci[9] and Tegelaar and Wösten[10,Chapter 4] determined the growth zone by determining the extension rate of hyphae before and after hyphal damaging. The former study assessed growth rates of colonies, and thus included hyphae that stopped growing after hyphal damage. In contrast, the study of Tegelaar and Wösten[10,Chapter 4] determined the growth rate of individual hyphae before and after dissection of the second compartment (when counting from the tip). Cutting reduced the mean growth rate of all dissected hyphae. However, extension rate was not affected when only hyphae were taken into account that continued growing after dissection. The latter explains the difference in peripheral growth zones between

the studies of Trinci[9] and Tegelaar and Wösten[10,Chapter 4]. Here, it is shown that single apical compartments of the ascomycete *P. chrysogenum* and the basidiomycete *S. commune* and the first 500 µm of hyphae of *R. stolonifer* are also self-sustaining in growth. In contrast, single 2nd compartments (obtained by dissection of the first and third compartment) of septate fungi or the region between 500 and 1000 µm from the tip of *R. stolonifer* were severely impacted in their growth rate.

RESULTS

Closure of a septum, or the presence of a septum at all, is not a prerequisite for continued growth after sub-apical hyphal damage

The width of the non-septate hyphae of *R. stolonifer* was 3.7 ± 0.4 µm when grown in MM (Table 1). Width of the hyphae of *P. chrysogenum* and *S. commune* was 3.9 ± 0.05 and 4.1 ± 0.16 µm, respectively, and the length of their apical compartments was 194 ± 6 and 293 ± 24 µm.

Growth rate of hyphae of *P. chrysogenum, S. commune* and *R. stolonifer* was 93 ± 1, 173 ± 16 and 102 ± 20 µm h^{-1}, respectively (Table 1). Mean hyphal growth rate was reduced by 8, 4, and 41 % after dissection within the second compartment of *P. chrysogenum* and *S. commune* or 500 µm from the apex in the case of *R. stolonifer* (called pseudo-apical compartment from now on) (Table 1). A total of 9, 4, and 40 % of the (pseudo-)apical compartments of *P. chrysogenum, S. commune* and *R. stolonifer* stopped growing after dissection, respectively. Growth of single apical compartments of *P. chrysogenum* and *S. commune* was not affected when only hyphae were taken into account that remained growing after cutting. Notably, even apical hyphal fragments of the coenocytic *R. stolonifer* that continued growing did not show a reduction in extension (Table 1). The apical septum of all dissected *S. commune* hyphae was open before cutting, while 21 % of the *P. chrysogenum* apical septa were closed. More specifically, 29 and 17 % of the apical septa were closed of the *P. chrysogenum* apical compartments that stopped and continued growing after dissection, respectively. Together, these findings show that closure of a septum, or the presence of a septum at all, is not a prerequisite for continued growth after dissection. Yet, the presence of a septum contributes to the survival of hyphae after injury.

On average, the (pseudo-) apical compartments that halted their growth after dissection lost 11, 51 and 69 % of their cytoplasm in the case of *P. chrysogenum, S. commune* and *R. stolonifer,* respectively. In contrast, single apical fragments that continued their growth lost 1, 13 and 27 % of their cellular volume, respectively. These data show that growth of the apical fragments of the septate fungi *P. chrysogenum* and *S. commune* are more sensitive to loss of volume when compared to *R. stolonifer.*

Table 1. Length, width, and growth rate of the first compartment of *P. chrysogenum* and *S. commune* and the apical zone of *R. stolonifer* before and after laser dissection. Mean growth rate (± 95 % confidence interval) before and after dissection is indicated of all dissected hyphae and of those hyphae that continued growing after laser dissection.

	Length (µm)	Width (µm)	Growth rate before dissection (µm h^{-1})		Growth rate after dissection (µm h^{-1})	
			all hyphae	growing hyphae after dissection	all hyphae	growing hyphae after dissection
P. chrysogenum	194 ± 6	3.9 ± 0.05	79 ± 4	79 ± 4	73 ± 4	80 ± 4
S. commune	293 ± 24	4.1 ± 0.16	173 ± 16	174 ± 15	168 ± 20	175 ± 20
R. stolonifer	500	3.7 ± 0.4	102 ± 20	74 ± 16	30 ± 10	60 ± 16

Subapical compartments assume peripheral growth after dissection of the apex.

Hyphae of *A. niger, P. chrysogenum,* and *S. commune* were dissected in the middle of the apical compartment, while *R. stolonifer* was cut at the very tip after growing in MM. Growth from the 2nd compartment, or, in the case of *R. stolonifer,* sub-apical to the damaged apex was recorded after laser dissection. These compartments of *A. niger, P. chrysogenum, S. commune* and the 500-µm-peripheral zone of *R. stolonifer* had formed lateral branches before dissection in 10, 18, 50 and 100 % of the cases, respectively. The mean extension rate of these branches was 14.8 ± 3.7, 14.9 ± 3.2 and 148.2 ± 20.6 µm h^{-1} for *A. niger, P. chrysogenum* and *S. commune*, respectively (Table 2). No growth was observed from previously formed branches in *R. stolonifer.* After laser dissection of the apex of the main hypha, previously formed lateral branches had a mean extension rate of 9.5 ± 3.8, 13.9 ± 1.6, 129.8 ± 10.6 and 43.6 ± 23.8 µm h^{-1} for *A. niger, P. chrysogenum, S. commune* and *R. stolonifer,* respectively (Table 2). Thus, laser dissection of the apical zone did not affect growth rate of branches that were previously formed from the 2nd compartment of the septate *A. niger, P. chrysogenum* and *S. commune* and stimulated growth from previously formed branches of *R. stolonifer.*

After dissection of the apical compartment, newly formed branches appeared through the apical septum in 47, 57 and 10 % of the hyphae of *A. niger, P. chrysogenum* and *S. commune*, respectively (Figure 1). These newly formed branches had a mean extension rate of 21.6 ± 2.3, 16.2 ± 1.3 and 56.3 ± 54.9 µm h^{-1} (Table 2). In contrast, newly formed lateral branches originating from the 2nd compartment had a mean extension rate of 16.1 ± 2.1, 31.2 ± 5.4 and 100 ± 14.6 µm h^{-1}. After apical injury, *R. stolonifer* hyphae placed a septum at a mean distance of 69 ± 13 µm from the apical wound whenever growth was resumed by branches that were newly formed under this new septum (Figure 1). These branches had a mean growth rate of 43.2 ± 6.8 µm h^{-1} (Table 2).

Table 2. Growth rate of existing and newly formed branches and the time period between dissection and the formation of new branches. Growth rates of lateral branches from single 2nd compartments before laser dissection was not determined (N.D.) because they were assumed to be similar to those of connected 2nd compartments. Apical and basal branches were not formed by *R. stolonifer* single pseudo-2nd compartments and basal branches were not formed for connected (pseudo-)2nd compartments (N/A).

Second compartment	**Growth rate existing branches (µm h^{-1})**		**Growth rate newly formed branches (µm h^{-1})**			**Time between dissection and formation of new branches (min)**		
	Before dissection	After dissection	Apical branch	Basal branch	Lateral branch	Apical branch	Basal branch	Lateral branch
				Aspergillus niger				
Connected	14.8 ± 3.7	9.5 ± 3.8	21.6 ± 2.3	N/A	16.1 ± 2.1	77 ± 20	N/A	59 ± 19
Single	N.D.	14.1 ± 12.4	9.1 ± 2.1	6.6 ± 1.5	10.9 ± 5.7	69 ± 44	165 ± 113	69 ± 81
				Penicillium chrysogenum				
Connected	14.9 ± 3.2	13.9 ± 1.6	16.2 ± 1.3	N/A	31.2 ± 5.4	65 ± 10	N/A	94 ± 30
Single	N.D.	27.3 ± 8.9	6.4 ± 1.9	5.7 ± 1.6	14.2 ± 9.9	60 ± 31	81 ± 19	100 ± 172
				Schizophyllum commune				
Connected	148.2 ± 20.6	129.8 ± 10.6	56.3 ± 54.9	N/A	100 ± 14.6	180 ± 0	N/A	74 ± 25
Single	N.D.	11.4 ± 23.8	17.8 ± 27.6	9.9 ± 17.6	6.5 ± 3.9	566 ± 311	346 ± 378	508 ± 179
				Rhizopus stolonifer				
Connected	0	43.6 ± 23.8	N/A	N/A	43.2 ± 6.8	N/A	N/A	20 ± 18
Single	N.D.	0	N/A	N/A	15.5 ± 14.4	N/A	N/A	321 ± 436

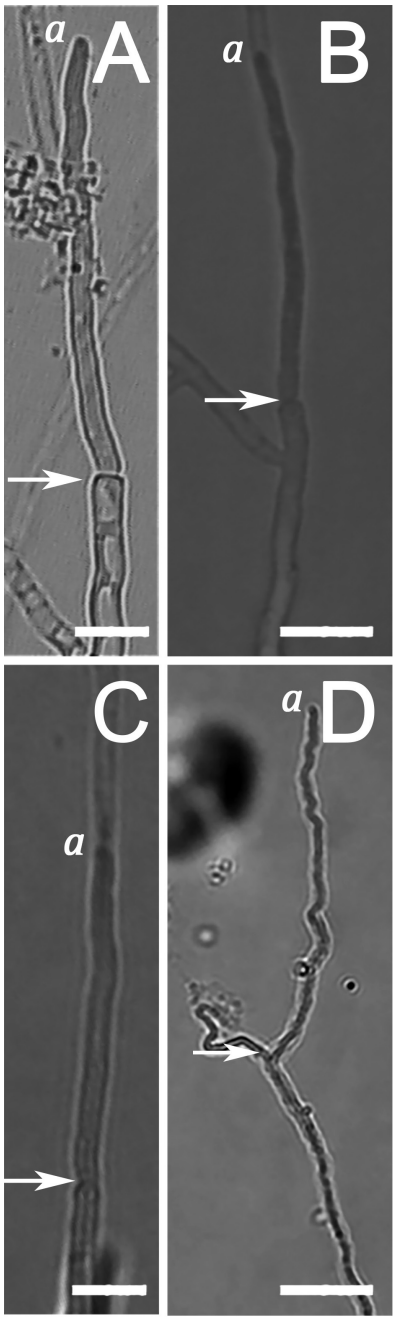

Figure 1. Growth response of the (pseudo-)second compartment of hyphae after dissection in the apical compartment. Growth from the second compartment through the apical septum of hyphae of *A. niger* (A) *S. commune* (B) and *P. chrysogenum* (C) and formation of a lateral branch in *R. stolonifer* (D). Arrows denote the location of the apical septa and α denotes the location of the new apex. Scale bars represent 20 µm.

Growth from single (pseudo-)2nd compartments was recorded after dissection of hyphae both within the (pseudo-)apical and (pseudo-)3rd compartments. To this end, *R. stolonifer* was cut 500 and 1000 µm from the apex. After dissection, the mean extension rate of existing branches was 14.1 ± 12.4, 27.3 ± 8.9, and 11.4 ± 23.8 µm h^{-1} for *A. niger*, *P. chrysogenum* and *S. commune*, respectively (Table 2). No growth was observed from previously formed branches in *R. stolonifer*. Newly formed branches that grew through the apical septum of the 2nd compartments had a mean extension rate of 9.1 ± 2.1, 6.4 ± 1.9 and 17.8 ± 27.6 µm h^{-1} (Table 2) and were formed in 69, 60 and 45 % of the 2nd compartments of *A. niger, P. chrysogenum* and *S. commune*, respectively. Newly formed branches that grew through the basal septum of single 2nd compartments (Figure 2) had a mean extension rate of 4.8 ± 1.7, 2.4 ± 1.2 and 9.9 ± 17.6 µm h^{-1} [1] (Table 2) and were formed in 43, 37 and 36 % of the 2nd compartments of *A. niger, P. chrysogenum* and *S. commune*, respectively. In total, 79, 70 and 55 % of all single 2nd compartments resumed growth by forming a branch through either apical, basal, or both septa for *A. niger, P. chrysogenum* and *S. commune*, respectively (Figure 2). Lateral branches were newly formed in 8, 2 and 50 % of the cases in *A. niger, P. chrysogenum* and *S. commune*, respectively. In the case of *R. stolonifer*, only lateral branches were formed from single 2nd compartments. These branches were observed in 17.2 % of the single pseudo-2nd compartments and had a mean extension rate of 15.5 ± 14.4 µm h^{-1} (Table 2). A third of these branches were formed within 60 minutes after laser dissection. Half of these single 2nd compartments were formed after 240 minutes. Formation of arbitrarily located branches from single (pseudo-)2nd compartments was not related to the formation of apical, basal, lateral or presence of previously formed lateral branches, or to cytoplasmic volume of these compartments in either fungus. Together, data shows that growth of existing lateral branches originating from single 2nd compartments of *S. commune* was reduced 13-fold while those of *A. niger* and *P. chrysogenum* maintained their growth. Growth of lateral branches of pseudo-2nd compartments of *R. stolonifer* (that all did not grow before dissection; see above) was not reactivated upon dissection. In all cases, growth of newly formed branches from single (pseudo-)2nd compartments was lower than that of intact hyphae, in particular for *S. commune* that showed an eightfold lower growth rate of newly formed branches.

Single 2nd compartments of *S. commune* were reintegrated into the mycelium in 36 % of cases by hyphal fusion. This reintegration happened between 297 and 528 minutes after dissection. Median growth rate from these reintegrated compartments was reduced by 72 % compared to the branch that reintegrated the compartment (88.4 and 24.6 µm h^{-1}, respectively). Time between fusion of branches with single 2nd compartments and subsequent continued growth from

the reintegrated single compartments was 109 ± 93 minutes. Growth rate from the reintegrating branch was no predictor of the time needed until recovery of growth. Reintegrated 2nd compartments of *S. commune* showed no polarity, meaning that these compartments showed no propensity for either apical, lateral or basal growth (Figure 2E-I).

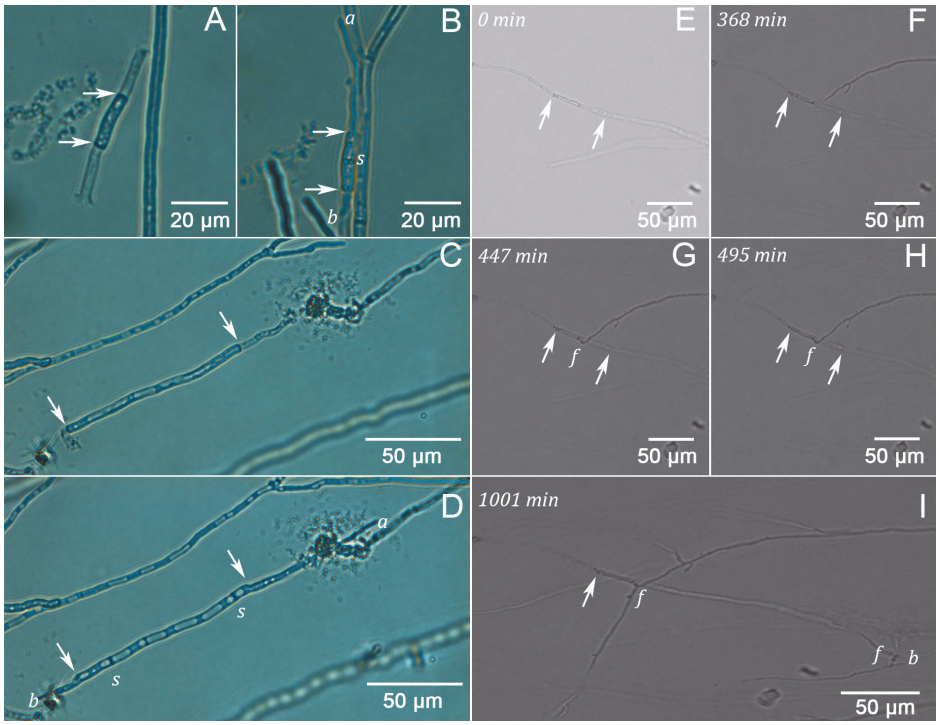

Figure 2. Single second compartments of *P. chrysogenum* (A,B), *A. niger* (C,D) and *S. commune* (E-I) 60 (A,C), 240 (B,D) and 0-1001 (E-I) min after laser dissection. Arrows denote the location of septa and *a* and *b* denote the location of the apex of hyphae formed through the apical of basal septum, respectively. Fusion (*f*) between the single second compartment and the mycelium of *S. commune* is observed 447 min after laser dissection (G). Growth subsequently restarts through the basal septum of the single second compartment (H). New growth from the single second compartment is capable of forming anastomoses. Fusion initiating hyphae continue their growth in the direction they were growing pre-fusion (I).

Predictors for continued growth of dissected hyphae

Compartmental volume is a predictor of continued growth of the first compartment of *A. niger* after dissecting the second compartment [Chapter 4]. Here, it was assessed whether growth rate, compartmental volume and/or the amount of cytoplasmic loss are predictors of continued growth after hyphal dissection of *P. chrysogenum* and *R. stolonifer*. *S. commune* was not included since only 4 % of its hyphae stopped

growing after hyphal dissection within the first compartment (see above) hampering statistical analysis of potential predictors of continued growth. Growth rate of the *P. chrysogenum* and *R. stolonifer* hyphae was distributed unimodally (Figure 3). The relatively slow- or fast-growth rates within these unimodal distributions were not predictive of continued growth after dissection of the second compartment of these fungi (Data not shown). The initial volume of *P. chrysogenum* apical compartments that continued or stopped growing was 2.6 ± 0.2 or 2 ± 0.3 pl (Table 3). This and the fact that relative residual cytoplasmic volume in the apical compartment after dissecting the 2nd compartment was similar between stopping or continuing apical compartments (Table 3) shows that volume of the first compartment is a predictor of continued growth in the case of *P. chrysogenum*. Differences in absolute or relative cytoplasmic volumes of pseudo-apical compartments before or after laser dissection of the pseudo-2nd compartment of *R. stolonifer* were not found between hyphae that stopped or continued their growth. The same was found for single (pseudo-)2nd compartments of *P. chrysogenum* and *R. stolonifer* and also for *A. niger* (Table 3). Together, these results show that cytoplasmic volume is a predictor of continued growth of apical compartments of *A. niger* Chapter 4 and *P. chrysogenum*.

Table 3. Volumes of single (pseudo-)apical or single (pseudo-)2nd compartments before and after laser dissection of (pseudo-)2nd compartments and (pseudo-) apical and (pseudo-) 3rd compartments, respectively. [a] Data from Tegelaar and Wösten, 2017.

	Volume Before laser dissection (pl)	**Volume After laser dissection (pl)**
Aspergillus niger		
Stopping apical compartment [a]	6.4 ± 1.7	3.9 ± 1.8
Continuing apical compartment [a]	9.2 ± 1.3	8.1 ± 1.4
Stopping second compartment	0.9 ± 0.3	0.9 ± 0.3
Continuing second compartment	0.9 ± 0.1	0.9 ± 0.1
Penicillium chrysogenum		
Stopping apical compartment	2 ± 0.3	1.8 ± 0.4
Continuing apical compartment	2.6 ± 0.2	2.5 ± 0.2
Stopping second compartment	0.6 ± 0.1	0.6 ± 0.1
Continuing second compartment	0.6 ± 0.2	0.6 ± 0.2
Rhizopus stolonifer		
Stopping pseudo-apical compartment	6.1 ± 2.4	2 ± 2.8
Continuing pseudo-apical compartment	4.8 ± 1.1	3 ± 0.8
Stopping pseudo-second compartment	5.1 ± 1.1	2.6 ± 0.9
Continuing pseudo-second compartment	6.4 ± 3.8	2.2 ± 1.7

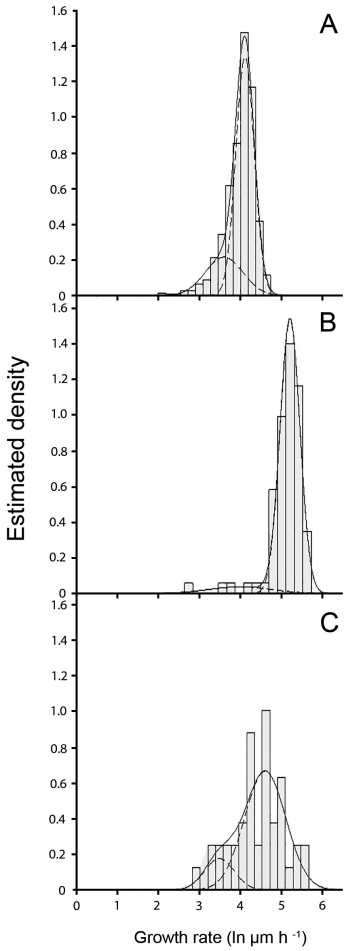

Figure 3. Distribution of growth rate of peripheral leading hyphae of *P. chrysogenum* (A), *S. commune* (B) and *R. stolonifer* (C).

DISCUSSION

Fungal hyphae have been reported to require a minimal length or minimum number of compartments to maintain their growth rate[9]. For instance, the peripheral growth zones of *A. niger* and *P. chrysogenum* would consist of 11 and 13 intact compartments and 8660 µm for the non-septate hyphae of *R. stolonifer.* However, it was shown that the apical compartment of *A. niger* can maintain its own growth rate[10,Chapter 4] Thus, sub-apical compartments are not needed to support growth of the apical compartments of *A. niger* hyphae but were rather shown to function as back-up to maintain peripheral growth in the case of apical injury. Here, it was shown that

(pseudo-)sub-apical compartments are also not needed to support growth of (pseudo-)apical compartments of the basidiomycete *S. commune,* the ascomycete *P. chrysogenum* and the mucoromycete *R. stolonifer.* Like *A. niger*, these sub-apical compartments function as a back-up system in the case of apical injury.

A total of 9, 4, and 40 % of the (pseudo-)apical compartments of *P. chrysogenum, S. commune* and *R. stolonifer* stopped growing after dissection, respectively. The *P. chrysogenum* hyphae that continued growing after dissection in the second compartment lost only 1 % of their cytoplasm, while losses of 13 and 27 % were found for *S. commune* and *R. stolonifer*, respectively. Moreover, in congruence with Tegelaar and Wösten[10,Chapter 4] a closed state of the apical septum in *P. chrysogenum* and *S. commune* was found not to be a significant contributor to the successful continuation of apical growth after dissection of the second compartment. In fact, *R. stolonifer* does not have septa in its vegetative hyphae but those hyphae that continued growth after dissection still maintained their initial growth rate.

The property of single (pseudo-)apical compartments to continue their growth raised the question if (pseudo-)2nd compartments also have this potential. This was assessed by cutting in both the first and third compartment or, in the case of *R. stolonifer* by cutting 500 and 1000 µm from the apex. Growth from single (pseudo-)2nd compartments took place from existing or newly formed branches. The branches were formed laterally, or grew through the apical or basal septum. Apical growth of the branches was about 4-fold (*R. stolonifer*), 13-fold (*P. chrysogenum*), 11-fold (*A. niger* [Chapter 4]) and 10-fold (*S. commune*) lower when compared to single (pseudo-) apical compartments. This lower growth rate may be caused by decreased turgor pressure[11] or loss of Ca^{2+} potential[12], secretory vesicles[13] or proteins[14]. In addition, the medium surrounding the detached (pseudo-)2nd compartments may be reduced in nutrients when compared to the medium the apical compartments are exposed to.

The fact that pseudo-apical and even single pseudo-second compartments of *R. stolonifer* can continue growing after being isolated from the rest of the mycelium is remarkable. Intuitively, one would assume that the pseudo-apical and pseudo-second compartments would lose their cytoplasm after dissection. However, total loss of cytoplasm occurs with low incidence, if at all. This may be explained by the capillary force of the hyphae and by organelles like vacuoles that block the opening(s) of the dissected compartments. Clearly, the presence of septa promotes the incidence of hyphal survival after dissection. However, the finding that growth of pseudo-apical septa is still observed after loss of 27 % of the cytoplasm suggests that *R. stolonifer* has evolved mechanisms to maintain growth after loss of a relatively high percentage of cytoplasm. Such mechanisms seem to be absent in ascomycetes and basidiomycetes and would partly compensate for the absence of septa in the aseptate fungi.

MATERIAL AND METHODS

Strains and growth conditions

Strains N402 of *A. niger*[15] and *P. chrysogenum* Wisconsin 54-1255[16] were grown at 30 °C in water-saturated air in the dark. Strains *R. stolonifer* CBS 112376 and *S. commune* H4-8 (matA43 matB41; FGSC 9210[17]) were grown at 25 °C and 50 % humidity in ~750 lux white light (C65 100mA 5730, NS12 spectrum, Valoya, Helsinki, Finland). *R. stolonifer* plates were closed by cellophane. Spores of *A. niger* and *P. chrysogenum* were harvested in 10 ml 0.9 % NaCl (w/v), 0.05 % (v/v) Tween-20 from 7-day-old cultures grown in 9 cm Petri dishes on complete medium (CM). This medium consisted of minimal medium (MM; 0.6 % $NaNO_3$, 0.15 % KH_2PO_4, 0.05 % KCl, 0.0 5% $MgSO_4$ $7H_2O$, 0.2 ml l^{-1} Vishniac solution[18], pH 6.0) supplemented with 0.5 % yeast extract, 0.2 % casamino acids, 25 mM maltose and was solidified with 1.5 % agarose. Spores of *R. stolonifer* were grown as described above, but harvested after 3 days. *S. commune* was grown on polycarbonate membranes (Whatman Cyclopore™, 76 mm diameter 0.1 µm pore size, Osmonics; GE Water Technologies) placed on *S. commune* minimal medium (SCMM; per liter, 22 g glucose-monohydrate, 1.5 g L-asparagine-monohydrate, 0.12 mg thiamine, 1 g K_2HPO_4, 0.46 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 5 mg $FeCl_3 \cdot 6H_2O$ and 1 ml Trace elements (per liter: 0.06 g HBO_3, 0.04 g $(NH_4)_6Mo_7O_{24} \cdot 4H2O$, 0.2 g $CuSO_4 \cdot 5H_2O$, 2 g $ZnSO_4 \cdot 7H_2O$, 0.1 g $MnSO_4 \cdot 4H_2O$, 0.4 g $CoCl_2 \cdot 6H_2O$, 1.2 g $Ca(NO_3)_2 \cdot 4H_2O$))[19] solidified with 1.5 % agarose. After 3 days the *S. commune* mycelium was macerated in 50 ml SCMM for one minute at high speed with a Waring Blender. The homogenated mycelium was transferred to a 50 ml Greiner tube and was incubated for 24 h at 25 °C in the dark. The spores and micro-colonies obtained in these ways were used to inoculate glass bottom dishes (Cellview™ cell culture dishes, PS, 35/10 mm, Greiner Bio-One, Frickenhausen, Germany). To this end, the dishes and medium were pre-warmed to 60 °C. 0.5 µL spore solution (50,000 spores), or one *S. commune* micro-colony, was placed on a glass coverslip (18 mm in diameter and 0.16–0.19 mm thick) and left to dry. Spores were allowed to dry completely, while micro-colonies were allowed to dry until no more medium was visible around the micro-colony. 30 µL pre-warmed MMA (MM supplemented with 25mM maltose and 1 % agarose) or SCMMA (SCMM with 1 % agarose), was added to the middle of the glass bottom dish's glass bottom, and immediately brought in contact with coverslip-adhered *A. niger, P. chrysogenum* and *R. stolonifer* spores or *S. commune* micro-colonies, respectively. After the layer of medium had solidified, 2 ml of corresponding liquid medium (i.e. (SC)MM supplemented with 25 mM maltose) was pipetted on top of the coverslip. Laser dissection was performed after 48 h of growth.

Microscopy

Hyphae were dissected using a PALM Microbeam system (laser power 35 %, focus 57 %) linked to an Observer.Z1 inverted microscope (Carl Zeiss AG, Oberkochen, Germany) and a CCD color camera (AxioCam ICc 1, Carl Zeiss AG, Oberkochen, Germany). Growth rate of hyphae and branches was recorded every 5 min during a 15 min period prior to dissection. In addition, it was assessed whether septal pores, if present, were open or closed at the moment of dissection. Septa were classified as open when cytosol was leaking through the septal pore upon dissection[5]. Hyphal growth after dissection was recorded every 5 min for 45 min for *P. chrysogenum* and *S. commune* hyphae cut in the 2nd compartment. Growth from hyphae cut in the apex or in both apex and 3rd compartment in *A. niger* and *P. chrysogenum* or its equivalent in *R. stolonifer* was recorded every hour during a 4 h period after dissection, with non-dissected hyphae serving as control. Single 2nd compartments of *S. commune* were followed for >7h using the PALM RoboSoftware time-lapse function. Width and length of compartment pre- and post-dissection were also recorded, as well as translocation (µm) of vacuoles after cutting. From these parameters the post-dissection volume of the apical compartment was calculated[10].

Statistics

Experiments were performed using at least biological triplicates, each with at least 7 technical replicates. Single 2nd compartments of *S. commune* that were followed using time-lapse had 11 biological replicates and no technical replicates. T-tests or Mann-Whitney U tests were carried out to determine differences in growth rate between treatment and control apical compartments. Differences in hyphal growth or volume before and after cutting were determined using paired sample t-tests, followed by correlation and regression analyses. Analysis of bimodality was performed as described[20].

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CHAPTER 6

Summary and General Discussion

INTRODUCTION

The fungal kingdom is characterized by a large diversity of organisms that are ubiquitous in nature and that colonize a wide variety of niches[1,2]. Fungi have a profound impact on life on earth including mankind. These organisms are involved in recycling nutrients and establish mutual beneficial and pathogenic interactions[3-6]. They are also for instance a food source[7], are used to produce food[8-10], and have potential as bio-based materials to replace plastics, fabrics, and polluting construction materials[11-13]. Moreover, they are widely used as factories for enzymes, pharmaceuticals and other small molecules[14]. Because of the impact of fungi in nature and for human society they are studied to improve and control their beneficial and damaging traits, respectively.

Fungi can grow yeast-like or by means of filaments called hyphae. In the latter case they form a mycelium that consists of a network of hyphae that grow at their tips and that branch subapically. The filamentous mode of growth provides a certain mobility to explore new substrates. Moreover, it enables penetration of substrates and allows efficient uptake of nutrients by providing a large surface area[15]. Filamentous fungi have also been shown to be superior in secretion of proteins when compared to yeasts[16].

Fungal colonies have been shown to be heterogeneous with respect to growth, metabolism, morphology and gene expression[17-21]. For instance, total RNA content and expression of the 18S *rRNA, actin, glaA*, and *faeA* gene in fungal pellets was shown to be 45 times higher in the peripheral zone when compared to the central zone[22]. Heterogeneity was also observed between zones of cm-wide macro-colonies of *Aspergillus niger*. This is illustrated by the fact that 40% of all genes are differentially expressed between five concentric zones of *A. niger* colonies, while 3 % of the genes are exclusively expressed in one of these zones[21,23,24]. Furthermore, at least three morphologically different zones can be distinguished. Towards the center, mycelial thickness increases and different hyphal layers within the different zones can be observed[24]. Heterogeneity can even be observed within a zone of a colony. A sub-population of hyphae at the outer part *A. niger* colonies secrete more proteins[25]. These hyphae also have a higher transcriptional and translational activity[26,27] but have a similar growth rate compared to the sub-population of hyphae that secrete less protein. Notably, heterogeneity between hyphae could not be observed in *Aspergillus oryzae*[28] and *A. niger*[29,30] after inactivation of the *hexA* gene. This was explained by the fact that these strains can no longer block inter-compartmental and inter-hyphal streaming by the absence of peroxisome-derived organelles that plug the septal pores. Plugging of these pores prevents mixing of cytosol, thereby

maintaining hyphal heterogeneity[28-31]. In this Thesis I studied in more detail the identity of micro-colonies in liquid cultures and the identity of individual hyphae and compartments within fungal colonies.

FUNGAL HETEROGENEITY IS INFLUENCED BY COLONY MORPHOLOGY

Pellets or micro-colonies represent one of the fungal morphologies, the formation of which is determined by the genotype of the fungus as well as its culturing conditions[32]. In **Chapter 2** it is shown that expression of the amylolytic glucoamylase gene *glaA*[33,34] and the xylanolytic genes α- glucuronidase *aguA*[35] and feruloyl esterase *faeA*[36] occurs in a concentric peripheral zone in micro-colonies of *A. niger*. When grown in minimal medium, these genes were predicted to be expressed in a concentric peripheral zone with a radius of 156, 89 and 13 µm for *glaA*, *aguA* and *faeA*, respectively. The existence of the concentric peripheral expression zones was confirmed by confocal microscopy. These results imply that small micro-colonies have higher protein expression per unit of volume. Previous studies also indicate that small micro-colonies secrete more protein than large micro-colonies[20,45]. However, those studies made use of different medium compositions to obtain micro-colonies with different size. Thus, it could not be excluded that the observed effect was due to the composition of the medium and not to the size of the micro-colonies. By making use of the heterogeneity in micro-colony size within a culture, results of **Chapter 2** unambiguously show expression per volume for these genes is increased in small colonies. In particular, the most productive micro-colonies per unit of biomass would be those that do not exceed the radius of the peripheral expression shell. Micro-colony diameter in bioreactors can be directed to such dimensions by adding microparticles, additives like glycerol and calcium chloride, or nucleating agents. Alternatively, pH, agitation rate, the type of carbon source and inoculum size impact micro-colony diameter[21,37-46]. Secretion of small metabolites is also affected by mycelium morphology. For instance, reduction in diameter of *Penicillium chrysogenum* pellets led to a doubling in final penicillin concentration[41].

ORIGIN OF FUNGAL HETEROGENEITY

Hyphae of fungi belonging to the Ascomycota and the Basidiomycota are compartmentalized by septa. These cross-walls have a central pore of 50–500 nm[47-49] that allow inter-compartmental and inter-hyphal cytoplasmic mixing. Yet, Woronin bodies constrain cytoplasmic mixing in *Aspergillus* by plugging the septal pores[28].

Plugging incidence is low in compartments close to the hyphal tip but increases when the distance to the tip becomes larger. As a consequence, the fungal mycelium converts from a unicellular to a multicellular system[31]. Septal pores within growing hyphae of basidiomycetes can also be closed. In this case, the septal pore cap is involved in plugging[50]. Notably, closure of septal pores was induced by environmental conditions like hypertonic conditions, elevated temperature, presence of ethanol or the antibiotic nourseothricin. The environmental cues inducing Woronin-body-induced septal plugging in *Aspergillus* was induced in **Chapter 3**. Like *S. commune*, increased septal plugging was observed in *A. niger* and *A. oryzae* hyphae that were exposed to elevated temperature. Plugging incidence also increased when *A. oryzae* was exposed to carbon starvation, or when *A. niger* was exposed to hypertonic conditions or nitrogen starvation. Interestingly, induction of plugging did not lead to increased heterogeneity in glucoamylase expression in apical compartments of *A. oryzae*. Rather the opposite was true. Plugging was quantified by rupturing apical compartments. It may be that Woronin bodies merely locate very close to the septal pore under temperature stress, but do not actually plug them, allowing for continued streaming of cytoplasm. Additionally, increased temperature leads to increase fluidity of membranes, possibly enabling Woronin bodies to pass through the septal pore.

Deletion of the *hexA* gene encoding the HexA protein results in absence of Woronin bodies and as such abolishes closure of septa by these organelles leading to increased homogeneity in *A. oryzae*[28]. In **Chapter 3** it was found that the same *A. oryzae* Δ*hexA* strain that was used by Bleichrodt et al. (2012)[28] still displayed heterogeneity, albeit much reduced when compared to the wild-type. This heterogeneity was abolished after exposure to increased temperatures, indicating that heterogeneity not only results from closure of septal pores by Woronin bodies, but also from an additional, temperature sensitive, mechanism with low plugging efficiency. This may be due to proteins that aggregate at, and may occlude, the septal pore[51,52]. Additionally, elevated temperatures lead to increased membrane fluidity, indicating a possible role for non-specific organelles in the plugging of the septal pore.

Transcriptional and translational heterogeneity was related to protein secretion. The sub-population with high transcriptional and translational activity was found in the secretor type of hyphae[26]. Notably, **Chapter 3** shows that the subpopulation of hyphae with low such activities are more resistant to environmental stress, in particular to heat. These results indicate that the outer part of *Aspergillus* colonies consist of both secretor and survivor hyphae. Such functional differentiation is expected to increase fitness of the pioneering hyphae that continuously explore new territories by growing away from the colony center.

FUNCTIONAL DISTINCTION OF HYPHAL COMPARTMENTS

Septate fungi have been reported to need a minimum number of compartments to maintain their growth rate[53]. For instance, hyphae of the ascomycetes *A. niger* and *P. chrysogenum* would need at least 11 and 13 compartments to maintain maximal growth rate, respectively. On the other hand, the coenocytic hyphae of the mucoromycete *Rhizopus stolonifer* would require a minimum length of 8660 µm. These findings led to the concept of the peripheral growth zone; the number of compartments or the length of a hypha needed to obtain maximal growth rate[53]. **Chapter 4** and **Chapter 5** revisited this concept by cutting hyphae with a laser at different positions from the tip and by monitoring growth rate of individual hyphae. Previously, cutting was done with a scalpel and average growth rate was determined of all hyphae at the periphery of the colony. Notably, single apical compartments of *A. niger* (**Chapter 4**) and *P. chrysogenum* (**Chapter 5**) were shown to be able to sustain their own growth, thus effectively reducing the peripheral growth zone of these fungi to a single compartment only. A similar result was obtained with the basidiomycete *S. commune* (**Chapter 5**). Even 500 µm apical fragments of the coenocytic hyphae of *R. stolonifer* were able to maintain maximum growth rate. Thus, also in this case the peripheral growth zone was adjusted downward. In line with this, single apical compartments of a Δ*hexA* strain (a pseudo-coenocytic strain) that continued their growth after dissection in the second compartment did so with a similar extension rate as before dissection (**Chapter 4**).

Survival of single apical compartments of *A. niger* (**Chapter 4**) and *P. chrysogenum* (**Chapter 5**) could be predicted by relative remaining volume in the apical compartment after laser dissection. In addition, disruption of the Spitzenkörper in *A. niger* predicted a (temporary) halting of growth. Yet, presence of a septum between the apex and the site of dissection was the best predictor of continued growth in both the *A. niger* N402 and Δ*hexA* strain (**Chapter 4**). A total of 58, 91, 96, and 60 % of the apical compartments of *A. niger, P. chrysogenum*, *S. commune* and the 500-µm apical zone of *R. stolonifer* continued growing after dissection, respectively. These apical (pseudo-)compartments that maintained their growth lost 14, 1, 13 and 27 % of their cellular volume, respectively (**Chapter 4, 5**). Results also show that *R. stolonifer* is less sensitive to loss of cytoplasm to continue growth after injury. This property may have evolved as a consequence of the absence of septa. The mechanisms underlying survival after loss of cytoplasm in *R. stolonifer* are not yet known.

Dissection of apical compartments led to increased branching from subapical compartments of *A. niger, P. chrysogenum*, *S. commune* and *R. stolonifer* (**Chapter 4, 5**). These branches generally had a lower growth rate, compared to apical growth.

This indicates that subapical compartments provide a back-up system when apical compartments are injured. These sub-apical compartments are not equipped to maintain their own growth rate when separated from their respective mycelium. Single 2nd compartments of *P. chrysogenum* and *S. commune* and the 500 µm long subapical zone of *R. stolonifer* (re-)initiated growth at lower proportions after dissection when compared to their respective apical compartments. In contrast, single 2nd compartments of *A. niger* reinitiated growth at larger proportions than their single apical compartments. Additionally, whenever apical growth was (re-) initiated from single (pseudo-)2nd compartments it was reduced 11, 13, 10 and 4-fold when compared to single (pseudo-)apical compartments for *A. niger, P. chrysogenum*, *S. commune* and *R. stolonifera, respectively* (**Chapter 4, 5**). This reduction in viability may be caused by the depletion of nutrients inside, or in the direct environment of, the hyphal fragments.

CLOSING THOUGHTS

Organisms that live in a heterogeneous environment must constantly adapt their behavior to survive. A mycelium could be defined as a clonal population of growing hyphal tips. Such a clonal population can employ two main strategies with regards to a changing environment. First, the environment could be sensed by (phenotypically uniform) individuals that immediately switch their phenotype to the one most suited to their environment. Alternatively, phenotypically diverse individuals colonize the substrate, accepting some may not be suited for their current environment but may be well adapted to a potential change in environment, i.e. bet-hedging. When environments change frequently the former strategy is preferred, while the latter strategy is preferred under homogeneous conditions[54]. Indeed, *Aspergillus* colonies grown under homogeneous conditions send out exploring hyphae that are heterogeneous in composition and activity[21-31,This thesis]. Since heterogeneity in hyphal composition and activity, promotes adaptability and survival when a colony becomes exposed to stress conditions like the researched high osmolarity, high temperature or low nitrogen conditions, an induced switch to a homogeneous mycelium will be difficult to accomplish. Indeed, increased homogeneity is observed in the *A. oryzae* Δ*hexA* mutant strain and increased sensitivity to high temperatures is observed in the *A. niger* Δ*hexA* mutant strain (**Chapter 3**). When stress-susceptible apical compartments encounter a lethal environmental condition, it does not mean the end for a hypha. Subapical compartments have been shown to assume peripheral growth after loss of the apex and even single (pseudo-)2nd and (pseudo-)apical compartments can reinitiate growth in absence of contact with the rest of the mycelium.

The perception that deletion of the *hexA* gene, and with it the loss of functionality from Woronin bodies, is the sole cause that leads to homogeneity is questioned due to results gathered in **Chapters 3, 4** and **5**. It is clear from these chapters an additional, temperature dependent, method of septal pore or hyphal occlusion is present in fungi. This may be caused by proteins that aggregate at the septal pore[67,68] or by organelles like vacuoles that accidentally plug the septal pores.

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6

APPENDIX

Nederlandse samenvatting
Curriculum Vitae
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Acknowledgements

NEDERLANDSE SAMENVATTING

INLEIDING

Het rijk der schimmels wordt gekenmerkt door een grote diversiteit aan soorten welke alomtegenwoordig zijn en een grote verscheidenheid aan niches koloniseren. Schimmels hebben een grote invloed op al het leven op aarde, waaronder de mensheid. Deze organismen zijn betrokken bij het recyclen van voedingsstoffen en brengen wederzijdse voordelige of pathogene interacties tot stand. Schimmels kunnen ook een voedselbron of –producent zijn en hebben potentieel als biologische materialen die kunststoffen, dierlijk leer of vervuilende bouwmaterialen vervangen. Bovendien worden ze gebruikt als biologische fabrieken voor enzymen en farmaceutische producten. Vanwege de grote impact die schimmels hebben op de natuur en de menselijke samenleving moeten ze worden bestudeerd om hun nuttige en schadelijke eigenschappen respectievelijk te verbeteren en te kunnen beheersen.

Schimmels kunnen gistachtig groeien of door middel van filamenten genaamd hyfen. In het laatste geval vormen ze een mycelium dat bestaat uit een netwerk van hyfen die aan hun uiteinden groeien en zich onder te top vertakken. De filamenteuze wijze van groei biedt schimmels een zekere mobiliteit om zo nieuwe substraten te koloniseren en zijn superieur met betrekking tot de opname van voedingsstoffen en uitscheiding van eiwitten.

Er is aangetoond dat filamenteuze schimmelkolonies heterogeen zijn met betrekking tot groei, metabolisme, morfologie en genexpressie. Heterogeniteit werd waargenomen tussen en binnen zones van centimeter-brede kolonies van *Aspergillus niger.* Een subpopulatie hyfen in het buitenste deel van *A. niger* kolonies scheiden bijvoorbeeld relatief meer eiwitten uit. Deze hyfen hebben ook een hogere transcriptionele en translationele activiteit maar hebben een vergelijkbare groeisnelheid in vergelijking met de subpopulatie van hyfen die minder eiwitten uitscheiden. Heterogeniteit tussen hyfen wordt niet meer waargenomen in *Aspergillus oryzae* en *A. niger* na inactivatie van het *hexA*-gen. Dit werd verklaard door het feit dat deze stammen niet langer de cytoplasmatische stroming tussen compartimenten of hyfen blokkeren doordat de van peroxisomen afgeleide organellen, genaamd Woronin lichamen, de septumporiën niet langer konden afdichten. In dit proefschrift heb ik de identiteit van micro-kolonies en de identiteit van afzonderlijke hyfen en compartimenten in schimmelkolonies nader bestudeerd.

HETEROGENITEIT BINNEN SCHIMMELS WORDT BEÏNVLOED DOOR MORFOLOGIE VAN SCHIMMELKOLONIES

Micro-kolonies, ook wel pellets genoemd, zijn een van de mogelijke schimmelmorfologieën. In **Hoofdstuk 2** wordt aangetoond dat expressie van het amylolytische glucoamylase-gen *glaA* en de xylanolytische genen α-glucuronidase *aguA* en feruloylesterase *faeA* plaatsvindt in een concentrische perifere zone in micro-kolonies van *A. niger*. Wanneer micro-kolonies worden opgekweekt in minimaal medium, werd voorspeld dat deze genen tot expressie werden gebracht in een concentrische perifere zone met een straal van 156, 89 en 13 µm voor respectievelijk *glaA*, *aguA* en *faeA*. Het bestaan van de concentrische perifere expressiezones werd bevestigd door confocale microscopie. Deze resultaten impliceren dat kleine micro-kolonies hogere eiwitexpressie per volume-eenheid hebben. Deze resultaten tonen voor het eerst een ondubbelzinnig verband aan tussen morfologie van micro-kolonies en genexpressie. Met deze resultaten wordt gesteld dat de meest productieve micro-kolonies per eenheid biomassa die zijn die de straal van de perifere expressiezone niet overschrijden.

OORSPRONG VAN HETEROGENITEIT IN SCHIMMELS

Hyfen van schimmels die behoren tot de Ascomycota en de Basidiomycota worden opgedeeld in compartimenten door septa. Deze dwarswanden hebben een centrale porie van 50 - 500 nm welke menging van cytoplasma tussen hyfen en compartimenten mogelijk maken. Woronin-lichamen kunnen menging van cytoplasma in *Aspergillus* voorkomen door de septumporiën af te dichten. Als gevolg van de afsluiting van septumporiën verandert het schimmelmycelium van een eencellig naar een meercellig systeem. In **Hoofdstuk 3** werden de milieufactoren die septumporieafdichting door Woronin-lichamen in *Aspergillus* induceren onderzocht. In *A. niger* en *A. oryzae* hyfen die werden blootgesteld aan verhoogde temperatuur nam de proportie afgesloten apicale septumporiën toe. Zo ook wanneer *A. oryzae* werd blootgesteld aan medium met koolstofgebrek en wanneer *A. niger* werd blootgesteld aan hypertonische omstandigheden of medium met stikstofgebrek. Interessant is dat inductie van septumporieafdichting niet leidde tot verhoogde heterogeniteit in glucoamylase-expressie in apicale compartimenten van *A.* oryzae. Echter, het tegenovergestelde was waar.

Deletie van het *hexA*-gen dat codeert voor het HexA-eiwit resulteert in afwezigheid van Woronin-lichamen en heft als zodanig de afsluiting van septumporiën door deze organellen op, wat in eerder onderzoek leidde tot verhoogde homogeniteit in *A.*

oryzae. In **Hoofdstuk 3** werd gevonden dat dezelfde *A. oryzae* Δ*hexA*-stam die in dit onderzoek werd gebruikt nog steeds heterogeniteit vertoonde, zij het veel minder in vergelijking met het wildtype. Deze heterogeniteit werd opgeheven na blootstelling aan verhoogde temperaturen, wat aangeeft dat heterogeniteit niet alleen het gevolg is van het sluiten van septumporiën door Woronin-lichamen, maar ook van een extra, temperatuurgevoelig, mechanisme met een lage septumporieafsluitingsefficiëntie.

De subpopulatie van hyfen die eiwitten uitscheiden hebben een hoge transcriptionele en translationele activiteit. In **Hoofdstuk 3** is te zien dat de subpopulatie van hyfen met lage transcriptionele en translationele activiteit beter bestand is tegen omgevingsstress, zoals hitte. Deze resultaten geven aan dat het buitenste deel van *Aspergillus*-kolonies bestaat uit zowel secretor- als persistortype hyfen. Verwacht wordt dat een dergelijke functionele differentiatie de *fitness* van de koloniserende hyfen, die continu nieuwe en veranderende omgevingen tegenkomen, zal vergroten.

FUNCTIONEEL ONDERSCHEID VAN HYFE-COMPARTIMENTEN

Van schimmels met septa is gerapporteerd dat ze een minimaal aantal compartimenten nodig hebben om hun groeisnelheid te handhaven. Hyfen van de ascomyceten *A. niger* en *P. chrysogenum* zouden bijvoorbeeld ten minste 11 en 13 compartimenten nodig hebben om hun maximale groeisnelheid te handhaven. Aan de andere kant zouden de coenocytische hyfen van de mucoromyceet *Rhizopus stolonifer* een minimale lengte van 8660 µm nodig hebben om de maximale groeisnelheid te handhaven. Deze bevindingen hebben geleid tot het concept van de perifere groeizone; het aantal compartimenten of de lengte van een hyfe die nodig is om een maximale groeisnelheid te verkrijgen. **Hoofdstuk 4** en **Hoofdstuk 5** testten dit concept door hyfen met een laser op verschillende posities van de punt te snijden en door de groeisnelheid van individuele hyfen te volgen. Voorheen werd snijden gedaan met een scalpel en werd de gemiddelde groeisnelheid bepaald van alle hyfen aan de periferie van de kolonie. Opvallend is dat afzonderlijke apicale compartimenten van *A. niger* (**Hoofdstuk 4**) en *P. chrysogenum* (**Hoofdstuk 5**) in staat zijn hun eigen groei te handhaven, waardoor de perifere groeizone van deze schimmels effectief tot slechts één compartiment wordt gereduceerd. Een soortgelijk resultaat werd verkregen met de basidiomyceet *S. commune* (**Hoofdstuk 5**). Zelfs 500 µm lange apicale fragmenten van de coenocytische hyfen van *R. stolonifer* konden de maximale groeisnelheid handhaven. Dus ook in dit geval werd de eerder gerapporteerde perifere groeizone naar beneden aangepast. Overeenkomstig met dit gegeven is gebleken dat afzonderlijke apicale compartimenten van een Δ*hexA*-stam (een pseudo-coenocytische stam) ook hun groeisnelheid konden handhaven na dissectie in het tweede compartiment (**Hoofdstuk 4**).

Overleving van apicale compartimenten van A. niger (Hoofdstuk 4) en P. *chrysogenum* (**Hoofdstuk 5**) kon worden voorspeld middels het relatieve resterende volume in het apicale compartiment na laserdissectie. Bovendien voorspelde verstoring van de Spitzenkörper in *A. niger* een (tijdelijke) stopzetting van de groei. Toch was de aanwezigheid van een septum tussen de tip en de plaats van dissectie de beste voorspeller van voortdurende groei in zowel de *A. niger* N402 als de Δ*hexA*-stam (**Hoofdstuk 4**). Een totaal van 58, 91, 96 en 60% van de apicale compartimenten van *A. niger*, *P. chrysogenum*, *S. commune* en de 500 µm apicale zone van *R. stolonifer* groeiden respectievelijk na dissectie door. Deze apicale (pseudo-) compartimenten die hun groei handhaven, verloren respectievelijk 14, 1, 13 en 27% van hun cellulair volume (**Hoofdstuk 4, 5**). Resultaten tonen dus aan dat *R. stolonifer* minder gevoelig is voor verlies van cytoplasma met betrekking tot handhaving van groei na verwonding.

Dissectie van apicale compartimenten leidde tot verhoogde vertakkingen vanuit subapicale compartimenten van *A. niger*, *P. chrysogenum*, *S. commune* en *R. stolonifer* (**Hoofdstuk 4, 5**). Deze vertakkingen hadden over het algemeen een lagere groeisnelheid vergeleken met apicale groei. Dit geeft aan dat subapicale compartimenten een back-upsysteem bieden wanneer apicale compartimenten gewond raken. Deze subapicale compartimenten zijn niet uitgerust om hun eigen groeisnelheid te behouden wanneer ze worden gescheiden van het mycelium. Afzonderlijke 2e compartimenten van *P. chrysogenum* en *S. commune* en de 500 µm lange subapicale zone van *R. stolonifer* (her)initieerden groei met lagere groeisnelheid na dissectie in vergelijking met hun respectievelijke apicale compartimenten. Deze vermindering van groei kan worden veroorzaakt door de uitputting van voedingsstoffen in of in de directe omgeving van de hyfefragmenten.

CURRICULUM VITAE

Martin Tegelaar was born the 27th of September 1987 in Rotterdam, The Netherlands. He followed his secondary education at the Emmauscollege in Rotterdam and received his HAVO and VWO diploma in 2004 and 2006, respectively. In 2006, he also started his Bachelor Biology at Utrecht University. This was followed by a Master Environmental Biology with a specialization in Fungal Biology in 2010. His major internship was done in a shared project between the Fungal Physiology group of prof dr. R.P. de Vries at the Central Bureau of Fungal Cultures (now Westerdijk Institute) and the Microbiology group of prof. dr. H.A.B. Wösten at Utrecht University. He worked on the characterization of the L-rhamnose pathway in *Aspergillus niger* under direct supervision of dr. B.S. Gruben. His minor research internship was once again carried out in the Microbiology group of prof. dr. H.A.B. Wösten, this time under direct supervision of dr. L.G. Lugones. Here, he worked on the chemotropic growth towards wood and volatiles displayed by *Schizophyllum commune*. After graduating in 2013, Martin worked for a short time as a junior researcher for the project "Biodiversity restoration in grasslands; understanding the role of plant traits and their plasticity in N:P stoichiometry and competition" followed by another short period as Teaching and Research Assistant in the Microbiology group of prof. dr. H.A.B. Wösten. In 2014, he started his PhD project that was combined with a teaching position in that same group. Martin studied fungal heterogeneity with respect to growth and gene expression. His project was funded by the Netherlands Organisation for Scientific Research research project 823.02.015. Currently, Martin works as a Postdoc in the Microbiology group of prof. dr. H.A.B. Wösten at Utrecht University. Here, he works on altering and improving the conductive properties of fungal mycelia to make them more suitable for use as sustainable building materials.

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LIST OF PUBLICATIONS

Tegelaar M and Wösten HAB. 2017. Functional distinction of hyphal compartments. Sci. Rep. 7:6039.

Teertstra WR, Tegelaar M, Dijksterhuis J, Golovina EA, Ohm RA and Wösten HAB. 2017. Maturation of conidia on conidiophores of *Aspergillus niger*. Fungal Genet. Biol. 98: 61-70.

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Tegelaar M, van der Lans GPA and Wösten HAB. 2020. Apical but not sub-apical hyphal compartments are self-sustaining in growth. Antonie van Leeuwenhoek. doi:10.1007/s10482-020-01383-9.

Tegelaar M, Aerts D, Teertstra WR and Wösten HAB. 2020. Spatial induction of genes encoding secreted proteins in micro-colonies of *Aspergillus niger.* Sci. Rep. 10:1536.

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