

Diffusive Signalling in *Aspergillus* Germination Inhibition

└ Introduction

└ The *Aspergillus* genus

└ Introduction

INTRODUCTION

The *Aspergillus* genus

- Well studied species of fungi with relevance in molecular factories, food spoilers and pathogens.
- Their main method of reproduction is through asexual spores (conidia).
- This study focuses on *A. niger*, but a lot of the phenomena apply to *A. fumigatus*, *A. flavus*, *A. oryzae*, *A. nidulans*, *A. terreus* and others.



1. The system of interest is the germination of conidia, the asexual spores of moulds from the genus *Aspergillus*. Most of the data is related to *Aspergillus niger*, but some occasional gaps in the data are filled with findings in research about other species of *Aspergilli*, due to their similarity.

Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Introduction
- └ Hypothesis
- └ Introduction

INTRODUCTION

Hypothesis

- Upon inoculation in an aqueous medium, a germination inhibitor diffuses away from the conidium.
- Experiments have highlighted **1-octen-3-ol** as a likely candidate [3, 4, 5]
 - Unknown heat-labile peptides may also act as inhibitors [6]
- Once diffusion is complete at the spore surface below a certain threshold, the conidium breaks dormancy and enters a swelling phase.
- It has been observed that in an externally non-inhibited spore swelling begins after about 4 hours from inoculation



1. The hypothesis that this research is testing is whether there is a system of inhibition that works via diffusive signalling and prevents spores from germinating prematurely. Upon inoculation, each spore is assumed to contain a limited amount of inhibitor, which gradually diffuses away.
2. 1-octen-3-ol has long been a prime suspect in this, but recent findings show contradictory results about its role and rather hint on the role of heat-labile proteins.
3. Normally a single spore begins swelling at about 4 hours from its inoculation, which indicates its break of dormancy. That means that up to this time, the inhibitor must have diffused away below a critical threshold to allow germination.

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└ Introduction

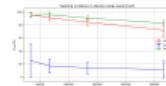
└ Hypothesis

└ Introduction

INTRODUCTION

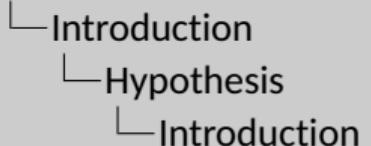
Hypothesis

- An increased density of spores drives the local inhibitor concentration high, reducing germination probability.
 - Densities above 10^5 spores/mL exhibit germination inhibition (approx. 20% less spores germinate) [5, 7].
- This could be due to a reduced concentration gradient between the spore and the medium.



- Interestingly, when the spore density is increased, the amount of germinated spores reduce. The absolute number of germinated spores depends on the inducing carbon source in the medium, but it is noticeable that the density increase from thirty thousand to three hundred thousand spores/mL causes a drop of about 20% in germinated spores.
- This means that, likely, the saturation of inhibitor in the medium creates a reduced concentration difference between the spore and the medium and slows down diffusion.

Diffusive Signalling in *Aspergillus* Germination Inhibition



INTRODUCTION

Experiment overview

Single-spore experiments

- Goal: estimate the properties of the inhibitor based on its diffusion time
 - use knowledge of existing molecules;
 - fit sensible parameters.



Multi-spore experiments

- Goal: given a constant rate of inhibitor release, replicate increasing inhibition independent on spore density.
 - Is it only the general density that matters?
 - or also the local clustering due to spore aggregation?



1. The experiments that my research is dealing with look at the problem from two sides. First of all, using diffusion simulations and analytical formulas I am trying to narrow down the properties of the inhibitor using the knowledge about diffusion time and existing molecule properties.
2. Second of all, once specific diffusion parameters for a single spore are defined, it is observed how an increased density of the sources drives the overall concentration up. This can be either looked at more globally, by using a uniformly spaced grid of spores, or more locally, by looking at dense clusters of aggregated spores that form a porous medium.

Diffusive Signalling in Aspergillus Germination Inhibition

└ Model Assumptions

└ General assumptions

└ Model Assumptions

MODEL ASSUMPTIONS

General approach

- The experiments consist of:
 - time-dependent numerical solutions of the diffusion equation over a discrete lattice
 - analytical solutions of the concentration at the spore (when applicable and available) - used for verification and calibration of the numerical scheme
- The consistency of the numerical solutions over several discretisation scales is tested:
 - Spore scale (mid-resolution)
 - Super-spore scale (low resolution)
 - Cell wall scale (high resolution)
- So far, most of the experiments carried out are at the spore scale.



1. I am tackling the research question by performing numerical simulations but also deriving analytical solutions in parallel. Comparing the results helps me understand the underlying mechanisms better and verify the functioning of the methods.
2. I am also looking at multiple scales of discretisation of the numerical scheme, depending on the level of detail I want to obtain. In the first set of experiments, the size of a spore is taken as a discretisation step, viewing a single spore as a node on the lattice. A more coarse-grained view interprets the spore as a negligibly small source that drives the concentration at a lattice site up, without being an obstacle for diffusion. At the other extreme, a fine-grain resolution discretises the spore at a cell-wall scale, which allows us to capture more geometry-related effects in close spore packings.

Diffusive Signalling in *Aspergillus* Germination Inhibition

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MODEL ASSUMPTIONS

Mid-resolution simulation space

Discrete lattice

- N lattice subdivisions
- Discrete blocks of size $\Delta x = \Delta y = \Delta z = 5\text{ }\mu\text{m}$ (5 spore diameter)
- Lattice constant $\Delta x = 5\text{ }\mu\text{m}$
- Assuming water as extracellular medium

- Spore: single block in the centre of the lattice, inhibitor source
- Volume $V = 125\text{ }\mu\text{m}^3$
 - Surface area $A = 150\text{ }\mu\text{m}^2$

- 2D lattice: or thin layer of medium between 2 impermeable sheets (e.g. water film)
- Top and bottom side of spore are sealed

- 3D lattice: more natural scenario, but heavier for computation



1. The spore-scale experiments abstract the spore as a cubic volume that bounds the aqueous medium, discretised with equivalent spatial steps. Some experiments look at 2D diffusion systems, in which it is assumed that the spore is clamped between two impermeable layers (as if in a thin water film). Most of the experiments look at a more natural scenario, which is a 3D lattice, in which diffusion can happen freely in all directions.

Diffusive Signalling in Aspergillus Germination Inhibition

Model Assumptions

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Model Assumptions

MODEL ASSUMPTIONS

Inhibitor properties

- Assumed to have known molecular properties of 1-octen-3-ol:
 - Molar mass: $M_w = 126.21 \text{ g/mol}$
 - Density: $\rho_{18^\circ\text{C}} = 0.85 \text{ g/cm}^3$
 - Octanol-water partition coefficient: $K_{ow} \approx 216.23$



Figure: 1-octen-3-ol

- Assumed to have similar diffusion properties as glucose:
 - Diffusion constant in water at temperature $T = 293.15\text{K}$: $gD^{18^\circ\text{C}} = 6 \times 10^{-10} \text{ cm}^2/\text{s}[d]$ (in agar only 5% lower)[v1]
 - Diffusion constant in a cellulose film: $D \approx 1.7 \times 10^{-10} \text{ cm}^2/\text{s}[x]$
 - Permeation constant in an artificial lipid bilayer: $gP^{25^\circ\text{C}} = 1.5 \times 10^{-10} \text{ cm}^2/\text{s}[z]$ (avg from [d] and [t])



Figure: D-Glucose

- The documented threshold for 1-octen-3-ol inhibition is $10 \text{ mM} \approx 0.01 \text{ mol/l}$ [y]
- Once below this level, it is assumed swelling begins immediately

- In the first experiments, 1-octen-3-ol is assumed as an inhibitor. Since the available information is not sufficient to paint a complete picture of its diffusion properties, some assumptions are made about its diffusivity based on molecules of a similar size, like glucose. As a preliminary reference for its inhibition threshold, a value of 1 mM found in literature is used. So when the concentration drops below this (at around 4 h), it is assumed that the swelling process begins immediately.

Diffusive Signalling in Aspergillus Germination Inhibition

Model Assumptions

General assumptions

Model Assumptions

MODEL ASSUMPTIONS

Inhibitor concentration

- At $t = 0$, all the inhibitor is contained in the cell wall, behind a barrier with a diffusion coefficient, D_i
- Cell wall thickness $d_w = 130 \text{ nm}$ (*A. fumigatus*) [1]
- Total cell wall volume $V_w = 4 \cdot 35 \mu\text{m}^2 \cdot 0.13 \mu\text{m} = 19.5 \mu\text{m}^3$
- Under homogeneous distribution, standard temperature and pressure, 1-octen-3-ol has a concentration of

$$c_{max} = \frac{P_i}{d_w} = \frac{0.837 \text{ g}/\text{mol}}{19.5 \cdot 10^{-9} \text{ m}^2/\text{s}} \approx 6.52 \text{ mol/L}$$

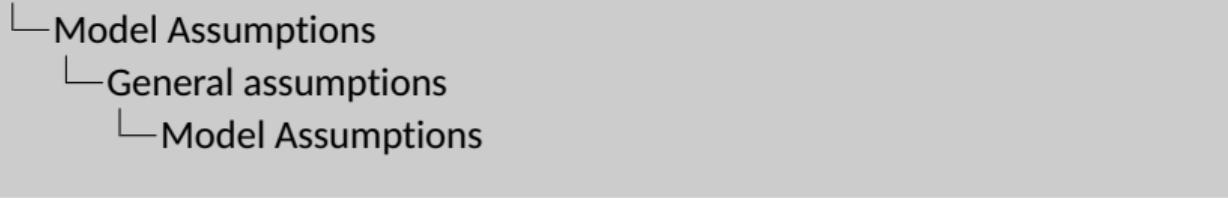
- Given the fraction of the cell wall volume occupying the entire cell volume, the maximum possible concentration is:

$$c_{max} = \frac{P_i V_w}{d_w V_c} \approx 6.52 \text{ mol/L} \cdot 0.156 = 1.018 \text{ mol/L}$$



- Another assumption based on literature is that 1-octen-3-ol is synthesised by cell membrane proteins and is contained in the inner layers of the conidial cell wall. This sets some very rough limits on its initial concentration and defines the type of barriers that it needs to permeate to diffuse out of the spore, namely the hydrophobin rodlet layer and the melanin layer of the outer cell wall.

Diffusive Signalling in *Aspergillus* Germination Inhibition



Name	Address	Address		Name	Address	Address		Name	Address
		Primary	Secondary			Primary	Secondary		
John Doe	123 Main St, Anytown, USA	123 Main St	Anytown, USA	Jane Doe	456 Elm St, Anytown, USA	456 Elm St	Anytown, USA	Bob Smith	789 Oak St, Anytown, USA
John Doe	123 Main St, Anytown, USA	123 Main St	Anytown, USA	Jane Doe	456 Elm St, Anytown, USA	456 Elm St	Anytown, USA	Bob Smith	789 Oak St, Anytown, USA
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1. There is a lot of unknown information about the inhibitor and the barriers that I am gradually filling in and updating, but the values presented previously serve as placeholders for the model parameters that I will show next.

Diffusive Signalling in Aspergillus Germination Inhibition

└ Model Assumptions

└ General assumptions

└ Model Assumptions

MODEL ASSUMPTIONS

Inhibitor diffusion

- Initial concentrations on lattice $c(x, t = 0)$:

- At spore: $c(x, t = 0) = c_0 \equiv c_{\text{spore}}$
- Elsewhere: $c(x, t = 0) = 0$
- Assumed non-replenishable during process

- Two modes of diffusion:

- direct diffusion at the interface between spore and medium
- slow release through a semi-permeable barrier



1. In the spore-scale experiments, the spore is assumed to contain all the inhibitor at time zero. In the simplest scenario, it is tested whether diffusion in the medium is the limiting factor, by setting the diffusivity of the spore equal to that of the medium. In the second scenario, the spore cell wall has its own specific permeation constant and molecules diffuse from it much slower than they do in the medium.

Diffusive Signalling in Aspergillus Germination Inhibition

Model Assumptions

Analytical verification

Model Assumptions

MODEL ASSUMPTIONS

Analytical verification

- Direct diffusion is modelled by a delta pulse at the spore node, leading to the solution:

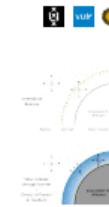
$$c(x, y, z, t) = \frac{c_{\text{tot}}}{(4\pi D t)^{3/2}} \exp\left(-\frac{|x|^2}{4Dt}\right) \quad (3)$$

- Slow release leads to a concentration at the spore

$$c_{\text{tot}}(t) = c_{\text{tot}} - \Delta c(0)e^{-rt}, \quad (4)$$

where $c_{\text{tot}} \approx 0$ [diffuses fast] and r is a decay constant:

$$\tau = \frac{V}{A P_s} \quad (5)$$



- In the normal diffusion scenario, the initial concentration can be modelled as a delta pulse on the lattice, leading to a Gaussian solution over time.
- The slow release scheme also has an analytical solution, which is under the important assumption that the outside concentration is always close to zero, and therefore the concentration drop across the barrier decreases exponentially in time. Some parameters involved in the slow release scheme are the surface-to-volume ratio and the permeation constant P_s .

Diffusive Signalling in *Aspergillus* Germination Inhibition

└ Single-spore experiments

└ Overview

└ Single-spore experiments

SINGLE-SPORE EXPERIMENTS

└ Overview

└ Lattice size of $L = 256 \times 5 \mu\text{m} = 1280 \mu\text{m}$

└ Initial concentration of $c_0 = 1.018 \text{ M}$

└ $t_{\text{end}} = 4 \text{ h}$

└ List of experiments

1. Superficial release with D of glucose in water (2D and 3D)
2. Glucose release with P_e of glucose through a lipid bilayer membrane (2D and 3D)
3. Glucose release with P_e of glucose through a CNF film (2D and 3D)
4. Glucose release with analytically estimated P_e (2D and 3D)

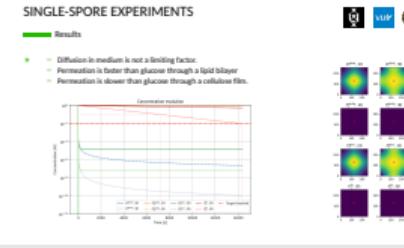
└ Fitted permeation constant is $P_e = 2.675 \times 10^{-6} \text{ cm/s}$, computed by:

$$P_e = \frac{V}{L^2} \ln \left(\frac{\Delta c(0)}{\Delta c(t)} \right) \quad (6)$$

1. The single-spore experiments compare the following scenarios, both in a 2D and a 3D system:
 - Direct diffusion of the inhibitor from the surface of the spore;
 - Permeation of a glucose-like inhibitor through a barrier similar to a lipid bilayer;
 - Permeation of a glucose-like inhibitor through a barrier similar to a cellulose nanofibril film
 - And a permeation with a constant fitted to produce a reduction to 10 mM within 4 h.
2. During these experiments, most importantly, the concentration at the spore is measured.

Diffusive Signalling in Aspergillus Germination Inhibition

- └ Single-spore experiments
 - └ Experiment results
 - └ Single-spore experiments



1. First, you can see that, as expected, diffusion happens faster in 3D systems compared to 2D systems.
2. The pink line indicates the fitted permeation constant in a 3D system, which arrives at the critical threshold in exactly 4 hours.
3. The permeation through cellulose (green lines) happens so fast that it becomes barely distinguishable from the free diffusion (blue) in large time periods.
4. The permeation through cellulose is so slow that it barely leads to a significant concentration drop within 4 h.
5. Finally, the dashed lines here indicate the analytical counterparts to the numerical schemes. The green and blue solid lines here overlap (diffusion through water and cellulose). You can see that the numerical scheme diverges from the analytical one once finite-size effects and the periodic boundary of the system start to take effect. This effect will become important in the next series of experiments.
6. The key take-away is that the actual permeation constant is somewhere in the very wide range between glucose through cellulose and glucose through a lipid bilayer membrane.

Diffusive Signalling in Aspergillus Germination Inhibition

└ Multi-spore experiments

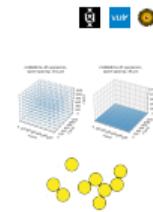
└ Overview

└ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

Overview

- Three scenarios are tested:
 - Spores are regularly distributed in a given volume.
 - Spores compact at the bottom of the medium due to gravity.
 - Clusters of spores form isolated diffusion traps for the inhibitor.



1. Since interpreting the inhibitor and barrier properties based on the fitted permeation constant is quite difficult, we take one step ahead and ask, if we assume that the fitted permeation constant is correct, does it inhibit dense spores arrangements in the way that has been observed in physical experiments?
2. In the multi-spore experiments, three different scenarios are observed, two of which have been already implemented, the third is still a work in progress.
3. First, we look at spores distributed in a grid with regular spacings within a given volume.
4. Second, we look at spores that have a more compact density as they sink to the bottom of the medium due to gravity, but still have a large overhead space for diffusion. Finally, local clusters of spores are examined for their potential to build a porous medium for diffusion, where molecules may get temporarily trapped.

Diffusive Signalling in *Aspergillus* Germination Inhibition

└ Multi-spore experiments

└ Overview

└ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

└ Uniform spore grid: assumptions

- └ Spores assumed to be arranged in a regular three-dimensional grid.
- └ Aspergillus is a spherical spore, in which the central part inhibits characteristic germination behaviour and the boundaries are irrelevant.
- └ Therefore, the grid can be considered infinite.
- └ Since the grid repeat periodically, it suffices to simulate a single spore in a volume of variable size L and a triply periodic boundary.
- └ The size L of the simulation volume corresponds to the spore-to-spore distance.

1. The uniform grid of spores is easy to simulate - simply vary the size of a periodic lattice based on the expected spacings between the spores.



Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
 - └ Overview
 - └ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

Bottom spore grid: assumptions

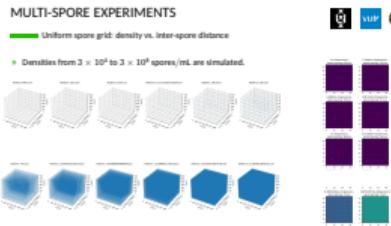
- Spores sink to the bottom of the medium due to gravity.
- Typically [1] it takes 1 h for spores to sink to the bottom of a well (150 μ L medium \rightarrow $h = 3.9$ mm of medium).
- It is assumed that while spores settle the inhibitor diffuses homogeneously like in the 2D grid scenario, so the simulations of the 2D lattice bottom array start with a concentration $c_0 = c(t=0) = 36000$.
- The lattice is periodic along the x and the y dimensions but has a Neumann boundary condition at $x = 0$ and $x = h$, (see derivative of $c(x, y, z)$ normal to the boundary).
- Spore densities from the first multi-spore experiment (5000, 10 000, 20 000), and 40 000 spores (150μ L)



1. The bottom-aligned grid of spores still makes use of periodicity in two dimensions, but has a fixed lattice height, which is based on specific medium volumes used in prior empirical experiments. It is assumed that spores need one hour to sink to the bottom, during which they have released some inhibitor approximately based on the analytical slow-release scheme.

Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
 - └ Experiment results
 - └ Multi-spore experiments



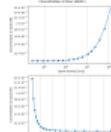
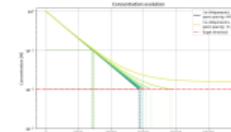
1. Here you can see how, in the uniform scheme, the distances between the spores vary according to their densities.

Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
 - └ Experiment results
 - └ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

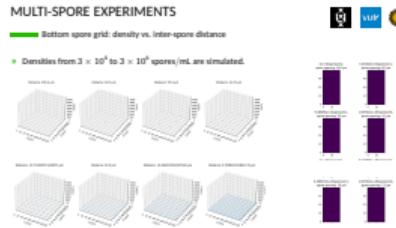
Uniform spore grid: results



1. The results show that a saturation of the inhibitor is present for large densities, but these densities are significantly higher than the ones reported in literature. For instance, the densest configuration here corresponds to a situation where there is nearly one or two spore diameters distance between neighbours.
2. It should be noted, however, that if the inhibition threshold is lowered, these observations would change.

Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
 - └ Experiment results
 - └ Multi-spore experiments



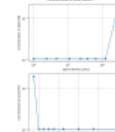
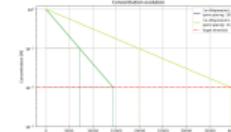
1. If the spores are only at the bottom of the lattice, their inter-spore distances would be generally be smaller than in the uniformly distributed scenario.

Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
 - └ Experiment results
 - └ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

Bottom spore grid: results



1. It turns out that the large space available for diffusion depletes the surrounding concentration right away, and a saturation is only present when the spores are literally next to each other.

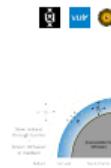
Diffusive Signalling in *Aspergillus* Germination Inhibition

- └ Multi-spore experiments
- └ Discussion
 - └ Multi-spore experiments

MULTI-SPORE EXPERIMENTS

Discussion: analytical verification

- Numerical simulations are slow and lack functional relationships.
- Analytical derivations have previously helped verify and replicate numerical schemes.
- But these have handled **direct diffusion** and **slow release** separately.
- In dense spore systems, both regimes matter (c_{out} cannot be assumed constant). But how to combine the formula?



1. One important challenge that I am trying to address now is to derive an analytical scheme which corresponds to a slow inhibitor release from multiple sources, in particular repeating infinitely with uniform distances. The formula I have derived here convolves the concentrations released at each time step with the Gaussian diffusion solution.