Fungal Biology 128 (2024) 1868–1875

Contents lists available at ScienceDirect

Fungal Biology

journal homepage: www.elsevier.com/locate/funbio

The chemotrophic behaviour of *Aspergillus niger*: Mapping hyphal filaments during chemo-sensing; the first step towards directed materials formation

Ayesha Sadaf [a], Matthias Brock [b], Carole C. Perry [a,*]

[a] *Materials and Interfaces Research Group, Interdisciplinary Biomedical Research Centre, School of Science and Technology, Nottingham Trent University, Nottingham, UK*

[b] *Fungal Genetics and Biology Group, School of Life Sciences, University of Nottingham, University Park, Nottingham, UK*

ARTICLE INFO

Handling Editor: Prof Han Wosten

ABSTRACT

In the development of fungal based materials for applications in construction through to biomedical materials and fashion, understanding how to regulate and direct growth is key for gaining control over the form of material generated. Here, we show how simple 'chemical food' cues can be used to manipulate the growth of fungal networks by taking *Aspergillus niger* as an exemplar species. Chemotrophic responses towards a range of nitrogen and carbon containing biomolecules including amino acids, sugars and sugar alcohols were quantified in terms of chemotrophic index (CI) under a range of basal media compositions (low and high concentrations of N and C sources). Growth of filamentous networks was followed using fluorescence microscopy at single time points and during growth by an AI analytical approach to explore chemo sensing behaviour of the fungus when exposed to pairs (C-C, C-N, N-N) of biomolecules simultaneously. Data suggests that the directive growth of *A. niger* can be controlled towards simple biomolecules with CI values giving a good approximation for expected growth under a range of growth conditions. This is a first step towards identifying conditions for researcher-led directed growth of hyphae to make mycelial mats with tuneable morphological, physicochemical, and mechanical characteristics.

1. Introduction

Fungi are ubiquitous, mostly filamentous microorganisms which can thrive in a range of geographical locations across the globe (Coleine et al., 2021; Sterflinger et al., 2012). These microorganisms are robust and can survive under adverse conditions of pH, temperature, light, and nutrient availability. Their survival behaviour relates to their ability to sense the surrounding environment including the location of food sources distant from the organism (Aleklett and Boddy, 2021). To understand fungal systems in diverse environmental conditions, it is important to first study their sensing abilities at the hyphal scale. This is important not only to understand their biology but also to generate tuneable materials by manipulating mycelial growth where applications as diverse as building and insulation materials, biomaterials and fashion replacements for leather will gain traction in mainstream production if we better understand growth and its relationship to the properties of the materials generated (Bagheriehnajjar et al., 2024; Haneef et al., 2017).

The most basic yet important aspect of fungal growth is their chemical sensing ability or 'chemotropism.' Chemotropism is the growth of fungi toward a particular chemical gradient generated by nutrients or signalling molecules such as peptides, enzymes, pheromones etc. Fungi including *Neurospora crassa*, *Sordaria macrospora*, *Fusarium oxysporum*, *Candida albicans*, *Rhizophydium littoreum* have been previously studied for their chemotrophic behaviour (Turrà et al., 2016). Fungi mainly exhibit chemotropic behaviour for a) nutrition b) predation c) developing pathogenicity and (d) sexual fusion (Turrà et al., 2015, 2016; Turrà and di Pietro, 2015). Chemotropic behaviour is a manifestation of the decision-making ability of the fungus. Making a choice between nutrient resources (both carbon (C) and nitrogen (N) is an interesting property to study that will pave the way to better understand fungal movement and physiology in diverse environments given the fact that these organisms are devoid of any neural network. Further, it is an important process to study in the search for simple 'tools' to engineer the form of these living materials, for the formation of useful products.

Quantifying chemotropism on solid surfaces is difficult due to (i) diffusibility/solubility issues of the test compounds and (ii) reliable and simple methods of counting/measuring (Schunke et al., 2020; Turrà et al., 2016). Hence, the present study was designed and conducted to develop an analytical approach to (i) measure the chemotropic growth of *Aspergillus niger* and (ii) to manipulate its growth for the generation of

* Corresponding author.
E-mail address: carole.perry@ntu.ac.uk (C.C. Perry).

https://doi.org/10.1016/j.funbio.2024.05.010
Received 22 May 2024; Accepted 24 May 2024
Available online 25 May 2024

biomaterials where the morphology is controlled by the chemistry of the growth medium. The biomolecules studied included simple sugars, sugar alcohols, and amino acids differing in their physicochemical properties including polarity. In the first part of the present research, we developed a linear method under a range of basal media components (modifications to the N and C sources and levels) for calculation of the chemotropic index of sugars and amino acids under a range of conditions suitable for fungal growth. In the second part, we studied whether the calculated CI values corelate with the chemo-sensing ability of *A. niger*. The agar-based system developed for the chemo sensing studies was designed to minimise interdiffusion of the chemotropic compounds and give reproducible results, which were then taken forward to be mapped and analysed by the machine learning software, Imaris. To the best of our knowledge, this software has not been used for mapping fungal filament growth. We believe our study will contribute towards understanding the chemo sensing ability of fungal systems in a more comprehensible manner and enable the directed growth of fungal based materials systems in the future.

2. Materials and methods

2.1. Strain and culture conditions

A. niger van Tieghem A1144 was obtained from the Fungal Genetic Stock Centre (Kansas, USA). The strain was grown at 28 °C on Potato Dextrose Agar (PDA) slants and stored at 4 °C. Fresh subcultures were prepared every 15 days. For chemotropic studies, the fungus was grown in minimal medium containing 5 mM of KCl, KH_2PO_4 and K_2HPO_4, 2 mM $MgSO_4$, 70 mM $NaNO_3$, pH 7.0 and Hutner's trace element solution. The medium was modified for measurement of chemotropic index and chemo sensing experiments. Initially for calculation of the chemotropic index for all the biomolecules, Hutner's minimal medium with 20 mM glucose was taken as the basal medium. This basal medium was later modified wherein for the chemotropic index of N containing compounds, $NaNO_3$ was kept at 2 mM and glucose 20 mM (nitrate was made limiting), whereas for carbon containing compounds the concentration of $NaNO_3$ was kept at 20 mM and glucose at 100 μM (glucose was made limiting). This was important for *the reliable* analysis of directional growth of the germinating conidia. For chemo-sensing experiments, initially, minimal medium agar containing 70 mM $NaNO_3$ and 10 μM glucose was used. The concentration of glucose in the chemo sensing experiments was kept at a minimum level allowing for the germination of the spores and to measure the directionality of filaments towards compounds of interest. A further set of experiments were performed using the tdTomato coding gene incorporated into the *A. niger* genome, whereby the nitrogen source was replaced with 1 mM glutamine keeping the glucose concentration unchanged as the fluorescent strain showed superior growth in the presence of glutamine compared to sodium nitrate.

2.2. Chemicals

All chemicals used in the chemotropic and chemo sensing assays, sugars; glucose, galactose, xylose, fructose; sugar alcohols sorbitol, glycerol, myoinositol and xylitol and amino acids were procured from Sigma Aldrich (United Kingdom) and used as supplied. All the sugars and amino acid were taken in their D-form and L-form respectively. The agar used was agar–agar for microbiology (CAS no. 9002-18-0, quality level 100) from Sigma–Aldrich.

2.3. Chemotropic assay

Conidial suspensions were prepared by harvesting them freshly from PDA plates grown for 5–7 days at 28 °C. Conidia were washed in phosphate-buffered saline (PBS) and counted using a haemocytometer slide. A previously described chemotropic assay was adapted and optimised for *A. niger* (Turrà et al., 2015). Briefly, modified minimal media (as described above) with 2 % (w/v) agar was poured between two coverslips (20 × 20 mm) with glass slides of 1 mm used as spacers to give uniform thickness. A central scoring line was drawn in the middle of the agar and equidistant wells (5 mm) were cut each side of the line using a microscopic scalpel and the conidia were spread on the agar using a glass capillary tube usually used for thin layer chromatography. The test compound (50 mM, 1 μl) was added in one well and water in the other as a control and incubated at 25 °C for 12–14 h under light conditions. The number of conidial germlings pointing towards the compound *vs* the ones pointing towards the control were counted on each side of the central line and the chemotropic index was calculated according to the formula $(H_{test}- H_{control})/H_{total} \times 100)$, where H_{test} is the number of conidial germlings growing towards the test compound, $H_{control}$ is the number of germlings growing towards the control, and H_{total} is the total number of germlings counted. For each analysis, 100–200 individual germlings were counted manually using a Leica Thunder imaging microscope, Wetzlar, Germany.

2.4. Chemo-sensing experiments

For the study of chemo sensing in the presence of different N and C containing compounds, single time points and live imaging studies (for 18h) were conducted. Conidia were harvested from PDA, washed with PBS buffer, and counted using a haemocytometer slide. Minimal media was poured between coverslips of 18 × 18 mm size, 1 mm thickness, and cut in a 'Y' shaped layout with a sterilised microscopic scalpel. A well (~3.6 mm in diameter) was cut at the base of the shape with a sterile glass capillary tube and 1 μl of spore suspension (100–200 spores) was added; similar wells were cut at about 10 mm distance from the inoculation point in each of the two arms of the Y shaped structure. In each of the application wells, chemotropic compounds (1 μl, 50 mM) were added. For single time point studies, this assembly was placed on moistened filter paper in a Petri dish which was incubated for specified time points at 25 °C and was imaged using a Leica Thunder imaging microscope. For live imaging experiments, the 'Y' shaped assembly was placed in a two chambered slide (μ-Slide 2 Well; Ibidi, Martinsried, Germany) with water-soaked filter paper strips on each side to provide humidity during incubation. The slide was then placed at 25 °C (to avoid drying of agar) in a temperature-controlled chamber of the Leica Thunder imaging microscope and hyphal imaging was performed at 2 h intervals. Images were analysed by the latest version of the Imaris software, version 10.0. To understand the chemo sensing behaviour of *A. niger,* different pairs of chemotropic compounds of similar or varying CI values were taken. A positive control was run in each experiment where glucose and water were taken as the chemotropic pair. For visual monitoring of dye diffusion in the Y-shaped agar set up, two hydrophilic dyes, brilliant blue (E 133) and cochenille red (E 124) were dissolved in water at 1 % (w/v) and applied (1 μl) in the application wells. The diffusion was monitored at different time periods photographically and the dye intensity was measured by Image J.

2.5. Generation of red-fluorescent A. niger strains

To generate red-fluorescent *A. niger* strains the citric acid producing *A. niger* wild-type strain FGSC A1144 (also found as ATCC 1015; CBS 113.46; NCTC 3859a; NRRL 328) was selected as the recipient strain. As fluorescent marker, a synthetic codon optimised version of the tandem-repeat tomato gene (tdTomato; GenBank accession KP100262.1) (Geib et al., 2016) was selected. The tdTomato gene was cloned under control of 1330 bp of the promoter of the constitutively expressed glyceraldehyde-3-phosphate dehydrogenase gene gpdA from *Aspergillus nidulans*. The pyrithiamine resistance gene ptrA from *Aspergillus oryzae* was used as selection marker for fungal transformation and all fragments were assembled in the pJet1.2 cloning vector.

A. niger protoplast transformation was performed as previously

described (Geib et al., 2016) and transformants were selected by the supplementation of media with 0.1 μg ml^{-1} of pyrithiamine. Transformants were pre-selected for further purification by fluorescence monitoring of transformation plates on an iBright 750 imager (Invitrogen, Thermo Fisher Scientific). Selected transformants were re-streaked three times on minimal media containing pyrithiamine to ensure that colonies no longer contained untransformed wild-type nuclei. Strains that showed bright red fluorescence of hyphae without displaying any obvious growth phenotypes were selected for further studies.

2.6. Mapping filaments by imaris

The *A. niger* hyphal growth towards chemotropic compounds was mapped for area analysis by the AI (Artificial Intelligence) based software, Imaris; the workflow is shown with images of each stage in Supplementary Fig. 1. Each image series captured by a Leica Thunder imaging microscope was opened in Imaris. To begin, first a reference frame was placed at the centre of the inoculation well from where the hyphae originate. Then, 'surfaces' component was selected from the Imaris menu to start the surface creation wizard. To avoid automatic detection of objects by Imaris which could lead to mapping errors, a region of interest containing the directional hyphae was selected and placed on each side of the reference frame. A pixel classification pluggin tool, Labkit by Fiji (Arzt et al., 2022) was used for segmentation of the fungal surfaces. The pixel classifier was trained and re-trained for optimum foreground and background distinction of mycelial growth for optimal segmentation of the hyphal filaments. After calculation of the entire segmentation by Labkit, the results obtained by the classifier were transferred to Imaris (10.0 version) for generation of results including their statistical significance (more information about the algorithm is provided in the **Supplementary section 2.6a**).

2.6.1. Statistical significance

All experiments were performed in three biological replicates (each biological replicate performed on a different date) each with two technical repeats for all measurements. Statistical analysis was performed using Prism 10.0 software (GraphPAd Software, Inc., CA, USA) using one way ANOVA with Tukey's multiple comparison test.

3. Results

3.1. The effect of media composition on chemotropic index

Simple biomolecules such as sugars, sugar alcohols and amino acids were chosen for studying the chemotropic growth of *A. niger* using a simple approach modified from Turrà et al. (2015) (Fig. 1)

Three base media were explored to investigate the effect of nitrogen source and glucose concentration on the chemotropic index for a wide range of sugars, sugar alcohols and amino acids Table 1. The first media, similar to that in the literature (Moreno-Ruiz et al., 2020) for calculation of chemotropic index (CI) consisted of a high concentration of nitrate (70 mM) as the nitrogen (N) -source and 20 mM glucose as the carbon (C)-source. Under these conditions the amino acids leucine and proline followed by charged amino acids like aspartate, glutamate, arginine, and histidine (CI of 25, 16, 13, 13 and 10 respectively) were found to have a higher chemotropic index as compared to aromatic and other amino acids. Almost all sugars including a range of monosaccharides and disaccharides except for fucose had high CI values between 18 and 35. Sugar alcohols like xylitol and inositol had CI values of 20 and 18 respectively indicating that these can also be recognised as secondary C sources by *A. niger* for germination. Other sugar alcohols like glycerol and sorbitol did not elicit a potent chemotropic response. Since the first medium contained a high basal nitrogen concentration, we prepared two further types of media for CI calculation of amino acids and sugars separately without modifying the pH which was kept neutral (pH 7.0).

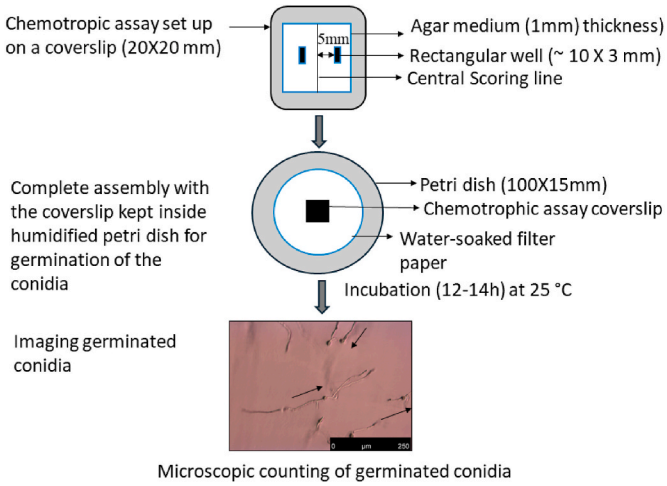

Fig. 1. Schematic representation of the set up for chemotropic assay.
The chemotropic assay was set up on a microscopic coverslip (20 × 20 mm). The coverslip was then placed on a moistened Petri dish and incubated at 25 °C for 12–14 h under light conditions for conidial germination. The conidia were counted under a microscope and the chemotropic index was calculated according to the formula $(H_{test}- H_{control})/H_{total} \times 100)$, where H_{test} is the number of conidial germlings growing towards the test compound, $H_{control}$ is the number of germlings growing towards the control, and H_{total} is the total number of germlings counted. For each analysis, 100–200 individual germlings were counted manually under a Leica Thunder imaging microscope. All experiments were performed in three biological replicates each with two technical repeats.

Table 1
Chemotropic index values of biomolecules under different media conditions against water as the control.

Sugars (Monosaccharides) (D-form)	*CI value*	*CI value*
	Media 1	Media 2
	70 mM nitrate + 20 mM glucose	20 mM nitrate + 100 μM glucose
Glucose	30 ± 0.8	35 ± 0.65
Xylose	20 ± 0.6	20 ± 0.81
Fructose	30 ± 0.87	21 ± 0.8
Galactose	18 ± 0.5	25 ± 0.56
Maltose	30 ± 0.91	21 ± 0.9
Sucrose	20 ± 0.8	15 ± 0.15
Fucose	4 ± 0.82	14 ± 0.13
Glucosamine HCl	27 ± 1.2	17 ± 0.78
Sugar Alcohols		
Sorbitol	−7.3 ± 0.34	6 ± 0.07
Glycerol	−13 ± 1	3 ± 0.03
Inositol	15 ± 0.25	10 ± 0.1
Xylitol	20 ± 0.5	12 ± 0.08
Amino acids (L-form)		**2 mM nitrate + 20 mM glucose**
Lysine	15 ± 0.35	22 ± 0.66
Leucine	20 ± 0.81	18 ± 0.36
Proline	25 ± 0.46	15 ± 0.55
Glycine	3 ± 0.023	20 ± 0.82
Glutamine	−4± 0.004	10 ± 0.33
Aspartate	16 ± 0 00.19	12 ± 0.38
Glutamate	20 ± 0.9	22 ± 0.79
Arginine	13 ± 0.17	15 ± 0.44
Histidine	10 ± 0.13	20 ± 0.75
Alanine	4 ± 0.002	20 ± 0.11
Phenylalanine	6 ± 0.18	13 ± 0.25
Tyrosine	7 ± 0.23	20 ± 0.57
Tryptophan	11 ± 0.36	18 ± 0.63

For amino acids, the nitrate concentration was reduced to 2 mM while the glucose concentration was kept constant at 20 mM and for assessment of the CI of sugars the nitrate concentration was adjusted to 20 mM while the glucose concentration was adjusted to 100 μM. For the amino acids explored, amongst the polar amino acids, lysine, histidine, glutamic acid, and tyrosine had very similar high CI values between 20 and 22 while among the non-polar ones, alanine, leucine, proline, tryptophan, and glycine had CI values that varied between 15 and 20. For experiments performed in the presence of sugars, monosaccharides like xylose, fructose, galactose had CI values of 35, 21, 25 respectively, disaccharides, sucrose, maltose also had high CI values of 15 and 21. The less common sugars like fucose and NGcHCl had CI of 14 and 17 and sugar alcohols like sorbitol, glycerol, inositol, and xylitol had lower CI values in the range of 3–12 under the given conditions. Our results show that the chemical composition of the underlying medium has a great effect on chemotrophy. E.g. sorbitol seems to act as a repellent in the presence of high glucose concentrations, but starts to attract (at low level), when glucose in the basal medium is low. Representative images of *A. niger* conidial germlings for extreme minimal and maximal chemotropic index values as well as comparison of sorbitol on three different media are shown in Supplementary Figs. 2a and 2b respectively. Therefore, the effect of high nutrient concentrations can alter the CI and play an important role in chemo attractiveness.

Based on the calculated CI values we proceeded to study directive growth when the fungus is provided with chemotropic compounds of similar or varying CI values at (a) a single time points and (b) at several time points during live imaging. Results from both experiment types are now described in next section.

3.2. Directive growth of *A. niger* during chemo-sensing

3.2.1. Imaging with calcofluor in the basal medium

Based on the results of the chemotropic index of different biomolecules we chose representative pairs of molecules: sugar:sugar, amino acid:amino acid and amino acid:sugar to study directive growth in the presence of competing compounds. Chemo-sensing experiments were performed in a basal medium at reduced levels of glucose (10 μM) with nitrate concentration maintained at 70 mM to give growth rates amenable to measurement. Calcofluor white (CFW), a fluorescent dye known to non-specifically bind to the chitin and the glucan content of the fungal cell wall (Lichius, 2022) was incorporated (1 μg/ml) to stain the fungal hyphae without disrupting the growth of the fungus. The CI values of the nitrogen and carbon compounds were recalculated under the basal medium used for chemo-sensing, Table 2. A model growth system was developed, and the area covered by the hyphae approaching chemotropic compounds was measured, initially after a 48-h growth period had elapsed.

A 'Y' shaped agar structure was made on an object slide platform and wells were made equidistant (~10 mm) from the point of inoculation by using glass capillary tubes (described in detail in the methods). In each well, a chemotropic compound was added (1 μl, 50 mM) and in the inoculation well, 1 μl of spores (~100) were added. Chemotropic pairs of similar or varying CI values were selected and the directive growth pattern was mapped using Imaris software after 48 h of growth (Supplementary Fig. 5). Fig. 2 A-C Provides a schematic of the experimental system used, a representative data set and Imaris images post processing. Fig. 3 describes quantitative results of chemo-sensing where the area covered by hyphae in response to different chemotropic agents has been plotted for material grown in the presence of the calcofluor dye.

Table 2
Chemotropic index values of biomolecules taken for chemo sensing studies.

Biomolecules (D-form of sugars and L-form of amino acids)	CI values	CI values
	Basal medium contains 10 μM glucose and 70 mM nitrate (stained with calcofluor)	Basal medium contains 10 μM glucose and 1 mM glutamine (tdTomato strain)
Aspartic acid	18 ± 0.54	
Histidine	10 ± 0.4	
Leucine	20 ±0 0.6	35 ± 0.98
Proline	28 ± 0.54	40 ± 0.1.6
Xylose	30 ± 0.85	35 ± 0.76
Sorbitol	13 ± 0.35	
Maltose	25 ± 0.87	
Xylitol		25 ± 0.58
Galactose		18 ± 00.45

For growth in the presence of the sugar xylose and the amino acid proline, a C–N pair with **CI** of **30** and **28** respectively, there was a preference for proline (77×10^5 μm^2) as opposed to xylose (63×10^5 μm^2) indicating preferential requirement for the different carbon and nitrogen sources. For the two pairs of amino acids, an N–N pair, leucine *vs* proline the growth was in accordance with the CI values for proline (**CI 28**) > leucine (**CI 20**). Proline has also been found to be an important proteinogenic acid which promotes conidial germination and could act as both C and N source for fungal growth (Ijadpanahsaravi et al., 2021; Silao et al., 2019). Leucine on the other hand, has not been found to be utilised as a carbon source by fungi with some exceptions like *A. nidulans* (Rodríguez et al., 2004). For the pair of sugars with disparate CI values this was reflected in the relative growth towards them with growth very much preferred towards maltose (93×10^5 μm^2) as opposed to sorbitol (56×10^5 μm^2). Maltose is a preferred carbon source for *A. niger* where it is shown to be consumed first followed by fructose, mannose, galactose, and xylose (Mäkelä et al., 2018). However, we want to point out that since most of the studies in the past have been done in liquid media, the possibilities of providing literature for comparison of the information we observe are sparce. The growth of glucose against water was used as a control where increased growth of the fungal hyphae towards glucose (97×10^5 μm^2) was observed as compared towards water (47 X 10^5). A diffusion experiment was conducted with two hydrophilic dyes, brilliant blue (dye 1) and cochenille dye (2) and monitor their diffusion over a period of time. The results (Supplementary Fig. 3) show that both the dyes diffuse completely within 10–14 h and reach the fungal inoculation point with minimum interdiffusion. However, since the growth of directional hyphae only occurs after 12–15 h we believe that during this time the fungus perceives the nutrient signal and grows accordingly in the direction of the applied biomolecules keeping the possibility of interdiffusion to the minimum though some level of diffusion might have occurred which led to some growth towards water (as opposed to glucose in the positive control.

We presume that the hyphae are attracted to the nutrient source since we have some initial experimental results provided in Supplementary Fig. 8 which show that there is no directionality of the hyphae in the absence of nutrient source in the application wells. Even if there is diffusion of nutrients to a certain extent, the directionality of the hyphae can only be seen when nutrients are provided in the application points. We also mapped the filament area of the quadrants which face opposite to the direction of the applied nutrient sources and the data (minimal growth) confirms that directional growth can only be seen in the quadrants which face the nutrient sources (Supplementary Fig. 4).

3.2.2. Construction of fluorescently labelled *A. niger* strain

For *in vivo* imaging of *A. niger* by simultaneously excluding the potential impacts of calcofluor white on chemotropic behaviour, the tdTomato encoding gene was introduced as a fluorescent reporter into the genome of the wild-type strain (A1144). Transformants (labelled as 4, 23, 18 and 1) expressing the fluorescent reporter were further screened for germination propensity and fluorescence intensity and strain 18 was eventually selected as optimal for further studies (Supplementary Fig. 6). The fluorescent strain was found to show more uniform growth when nitrate was replaced with glutamine. Hence for this particular strain, 1 mM glutamine was added in the basal media with 10 μM glucose (Supplementary Fig. 7). Constitutive expression of

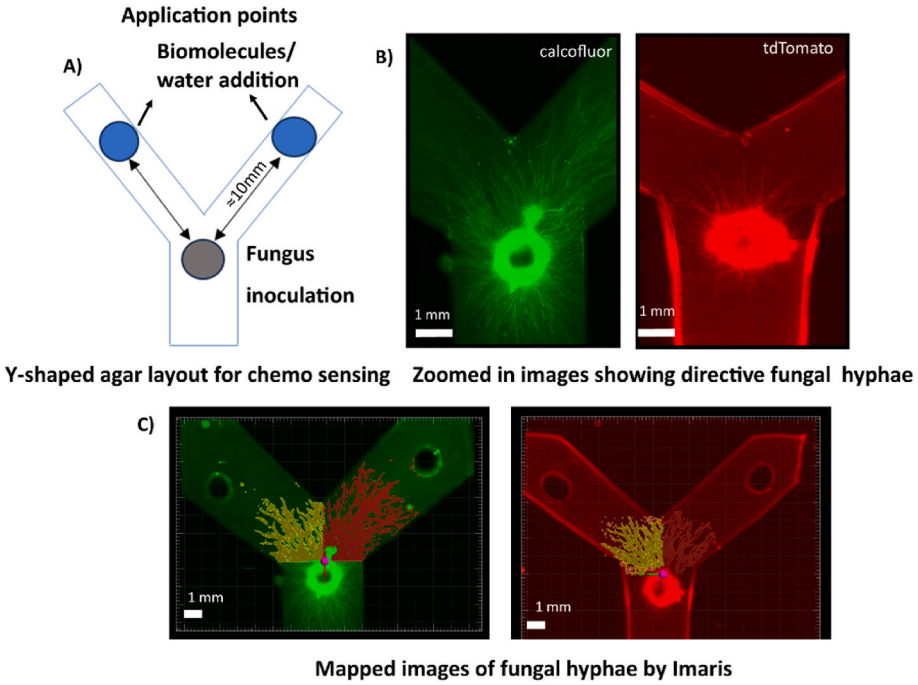

Fig. 2. Schematic representation showing (A) Y-shaped agar layout used for the chemosensing assay along with the fungal inoculation point where spores were added and the application points where the biomolecules (D-form sugars and L-form amino acids) were added (B) representative microscopic images stained with calcofluor (LHS), Hutner's minimal medium containing 70 mM nitrate and 10 μM glucose and tdTomato (RHS), Hutner's minimal medium containing 1 mM glutamine and 10 μM glucose (C) Imaris mapping of fungal hyphae with calcofluor (LHS) and tdTomato (RHS). Refer to Table 2 for the different growth conditions used for the calcofluor stained and tdTomato fungal experiments. Note: For better visualisation of fungal hyphae calcofluor white is used with a green filter.

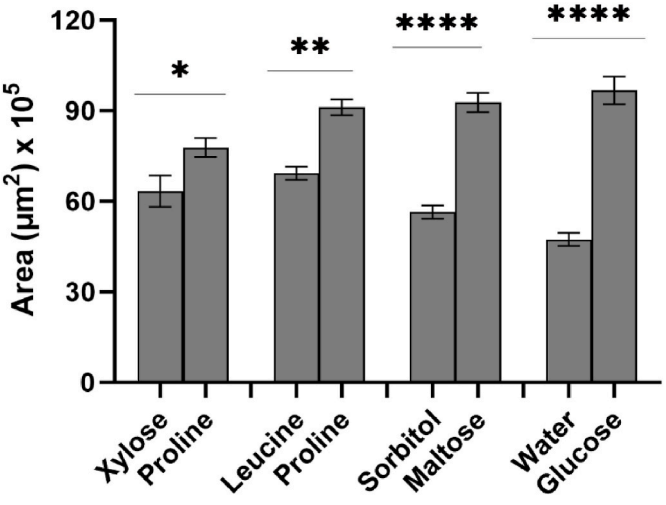

Fig. 3. Comparison of area covered by *A. niger* (A1144) hyphae towards pairs of chemotropic biomolecules after 48 h of growth with calcofluor staining. Water *vs* glucose was taken as positive control. The basal medium contained nitrate (70 mM) as nitrogen source and glucose (10 μM) as the carbon source. All the sugars and amino acids are in their D and L form respectively. Experiments were done with three biological replicates each comprising two technical repeats. Error bars correspond to ± SD * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($P \leq 0.001$).

the reporter was ensured by controlling reporter gene expression by the *gpdA* promoter, which controls expression of the glyceraldehyde-3-phosphate dehydrogenase (Archer and Xu, 2021) and is frequently used as a "housekeeping gene" in qRT-PCR studies. However, as with the expression of most essential genes, expression levels may show some variation under the applied growth conditions. Thus, while it cannot be excluded that there is some variation in gene expression under specific harsh or changing growth conditions, P*gpdA* is considered to be "always on" and fluorescence should always be trackable in the elongating filaments.

To analyse the consistency of chemotropic growth in the absence of calcofluor white, the selected td Tomato-incorporated *A. niger* strain was investigated for its chemo-sensing behaviour. The CI values of the chemotropic pairs tested were calculated for the glutamine containing medium, Table 2. A representative image of data collected during imaging together with the Imaris generated data can be found in Fig. 2C.

Fig. 4 describes the area covered by fungal hyphae in response to different chemotropic agents. For the first pair of C–N compounds, i.e., between xylose-proline (CI **35** and **40**), the area covered towards xylose was 91 × 10^5 μm^2 whereas towards proline was 61 × 10^5 μm^2 (Supplementary Fig. 9). The fungus preferred growing towards the C source under these conditions as compared to the N source, which may be easily explained by the presence of the nitrogen source glutamine incorporated into the medium. Xylose is a pentose sugar and is easily utilised by many fungi (Jeffries 1983; Seiboth and Metz, 2011). Galactose on the other hand is considered a secondary carbon source for fungi, the ability to metabolise xylose is preferred over galactose (Adnan et al., 2017; Martínez et al., 2023). The N–N pair between leucine–proline (CI **35** and **40)** showed that proline was no longer preferred as the area towards leucine and proline was similar (108 and 104 × 10^5 μm^2). For the C–C pair, xylitol-galactose was taken since this pair showed better directive growth under the modified media conditions as compared to sorbitol-maltose taken previously. Between xylitol-galactose (**CI 25** and **18**) the fungus covered an area of 103 × 10^5 μm^2 towards xylitol as compared to galactose with an area of 54 X 10^5 μm^2. Importantly, and as expected from the calcofluor labelling, the control condition comparing glucose vs water showed a strong preference towards glucose (area of 93 × 10^5 μm^2 covered) as opposed towards water (52 × 10^5 μm^2). This shows that (a) the basal medium composition strongly affects the chemotropic behaviour of the fungus, but also confirms that labelling with either calcofluor or an internally expressed fluorescent reporter allow monitoring of the chemotrophy.

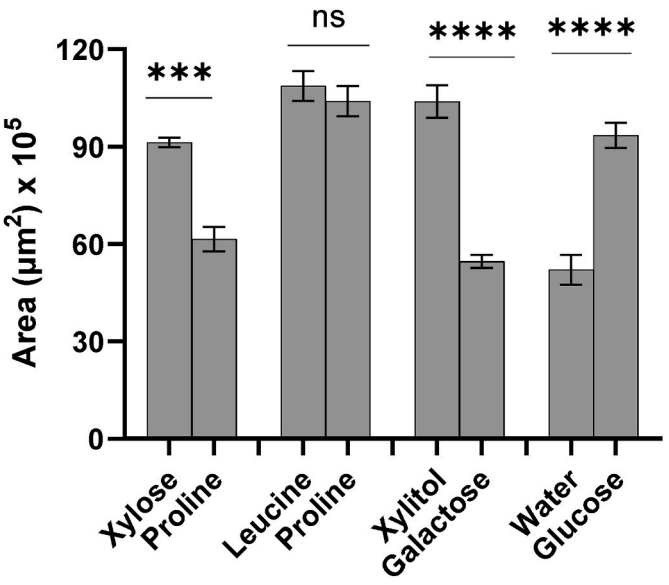

Fig. 4. Comparison of growth by tdTomato-incorporated *A. niger* hyphae towards pairs of chemotropic biomolecules after 48 h of growth. Water *vs* glucose was taken as positive control. The basal medium contained glutamine (1 mM) as nitrogen source and glucose (10 μM) as the carbon source. All the sugars and amino acids are in their D and L form respectively. Experiments were done with three biological replicates each comprising two technical repeats. Error bars = SD (n = 3) * ($p \leq 0.05$), ** ($p \leq 0.01$), and *** ($P \leq 0.001$).

Based on the single time point growth results, directive growth during the initial stages of growth was additionally studied by 'Y' shaped agar model using live imaging (Supplementary Fig. 10). After inoculation, the sample was pre-incubated for 12–14 h to allow for initial germ tube formation before moving the assembly for live imaging. This procedure assisted in assigning Z factors for better tracking of the filaments as they grew during the imaging process. Images were recorded at 2 h intervals for a period of up to 18 h post pre-incubation period meaning a total growth period of 33 h was covered. Also, for better clarity of the hyphae, the live imaging was refocussed after 5 h of initiation. Fig. 5 shows the results wherein, for the pair of C–N compounds xylose-proline (CI values of **35** and **40**), an inclination towards the C source, xylose was seen as compared to proline. This is consistent with the single time-point measurement at 48 h as shown in Fig. 4. For the N–N pair of compounds between leucine–proline, it was seen that the growth towards proline was slightly preferred as compared to leucine though both had similar CI values (**40** and **35** respectively) in the basal medium and at the 48 h time-point from single measurement showed very similar values for both amino acids.

In the C–C pair between xylitol-galactose which had CI values of **25** and **18** respectively, differences in the hyphal growth area were seen that were accordant with the single time-point measurement taken at 48 h. The area increased with increase in time towards xylitol indicating the fungus preferred this source as compared to galactose as a C-source during its period of linear growth. The positive control which had water-glucose showed higher hyphal area towards glucose, while the fungus did move towards water also but with a reduced filament area. This could be due to some diffusion in the growth medium.

Based on information given by live imaging results that provided the preferential growth direction towards chemotropic biomolecules until 33 h, we wanted to study whether the growth pattern changed after

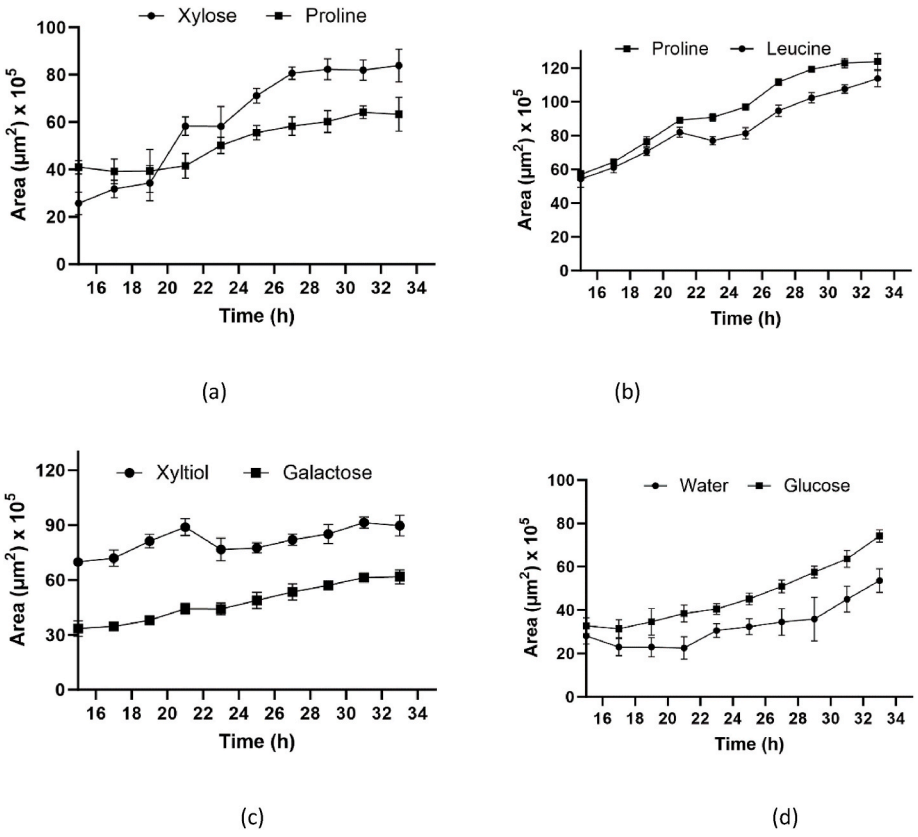

Fig. 5. Comparison of hyphal area calculated by Imaris between pair of chemotropic biomolecules in tdTomato labelled *A. niger* strain during live imaging. The basal medium contained glutamine as nitrogen source. All the sugars and amino acids are in their D and L form respectively. Experiments were done with three biological replicates each comprising two technical repeats. Error bars correspond to ± SD.

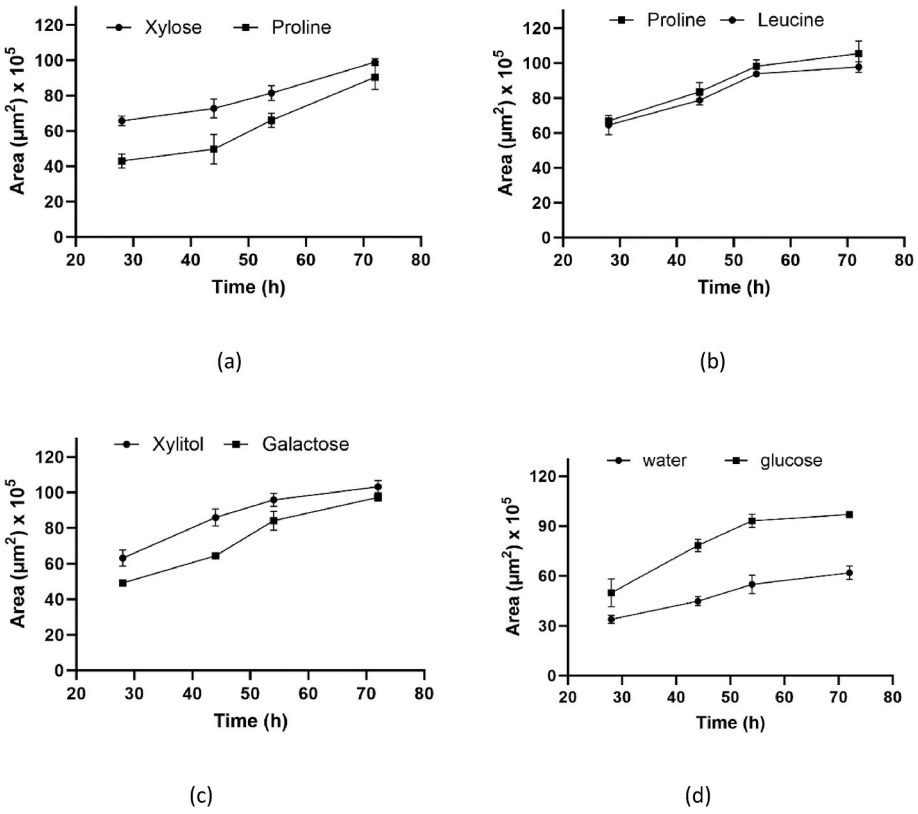

Fig. 6. Comparison of hyphal area calculated by Imaris between pair of chemotropic biomolecules in tdTtomato labelled *A. niger* strain at single time points for extended periods. The basal medium contained glutamine as nitrogen source. All the sugars and amino acids are in their D and L form respectively. Experiments were done with three biological replicates each comprising two technical repeats. Error bars correspond to ± SD.

longer intervals of time. As live imaging for time spans beyond those already applied was not feasible, we set up single time point experiments at 28, 44, 54 and 72h and measured the growth area (Supplementary Fig. 11). In the case of positive control (Fig. 6d) the growth area towards glucose showed a continuous increasing trend until 72 h (100×10^5 μm^2) as opposed to water (50×10^5 μm^2). In the case of leucine–proline (Fig. 6b), a slight increase in growth towards proline was observed until 72h as also shown by live imaging results previously. However, for xylose-proline and xylitol-galactose pairs (Fig. 6a and c), an increase in growth towards the less preferred chemotropic compound was seen after 54h i.e., towards proline and galactose in the respective pairs, indicating the possibility of the consumption of xylose and xylitol in these pairs or an adaptation towards the metabolism of the previously less preferred nutrient source.

4. Discussion

Chemotropic compounds in the immediate environment of a fungus could be a range of biomolecules like sugars, amino acids, peptides, polysaccharides which guide the growth of the hyphal tip in a specific direction (Muehlstein et al., 1988). This aspect of fungal growth is interesting yet intriguing in respect of understanding the underlying reasons and processes involved. In the past, many studies have attempted to quantify chemotropic responses and a range of assays have been developed (Moreno-Ruiz et al., 2020; Turrà and Di Pietro, 2015). In this study, we downsized an established method for quantification of chemotropic index (Turrà et al., 2015) to the millimetre scale for microscopic evaluation. Importantly, our analysis clearly showed that the basal medium used to allow initial germination and germ tube formation is an essential component in defining CI values. Thereby, the concentration and source of carbon and nitrogen applied to the basal medium resulted in modifications of the CI values. As an example, when the concentration of glucose in the basal medium was changed from a relatively high concentration of 20 mM to as low as 100 µM, the CI values for glucose, galactose and fucose increased, whereas the CI values for fructose, maltose and sucrose decreased. Furthermore, for glycerol and sorbitol, CI values obtained at high glucose concentrations were negative but changed into positive values at low glucose concentration in the basal medium. A similar effect was observed with changes in the concentration of nitrate or the addition of glutamine to the basal medium, which affected the preference for individual amino acids as attractant or the competition of sugars such as xylose versus the amino acid proline. While some studies have discussed the role of carbon and nitrogen sources as important for the overall growth of fungi such as *Serendipita indica* (Dias et al., 2020), our study clearly shows that without clear definition of the underlying growth medium composition a preference for an individual source cannot be defined.

Given the composition of the underlying basal medium, we were able to determine the chemo-sensing response of *A. niger* towards a pair of nutrient sources, which can further assist in directive growth for biomaterials formation. For this purpose, a 'Y' shaped agar model was tested to study chemo-sensing initially at single time points and for live imaging. The fungal growth area towards nutrient sources was mapped and analysed by the machine learning software, Imaris. The hyphae growing towards the nutrients were optimally segmented by the Fiji pluggin tool and trained to give the most accurate results.

While initial experiments were based on a calcofluor white labelling, we aimed to perform assays in the absence of this potential cell-wall stressing agent and generated a fluorescent strain of *A. niger* expressing the red-fluorescent reporter tdTomato. This reporter strain was not only used for single time point measurements but also for live imaging. These measurements allowed us to compare the initial preference for an individual nutrient source at early time points with those at later time points and showed that the preference may change at a later stage. The

likely explanation is due to an ongoing nutrient limitation in the underlying basal medium or the consumption of the preferred nutrient source over time. Answering this question from this microscale experiment is difficult and requires changing of the concentration of individual nutrient sources in the basal medium and of the attractant with subsequent monitoring of the CI values. However, from the data presented here it is clear that the underlying basal medium used to facilitate initial germination and growth towards a nutrient source has a great impact on the preference of the organism for the selected nutrient source and a more definitive answer towards the preference of a specific nutrient source can only be made by taking the underlying growth medium composition into account. We believe additional transcriptomic/metabolomic studies on hyphae grown towards different nutrient sources will provide us with insights concerning which pathways are followed during the consumption of nutrients along with identification of metabolic products/byproducts. The information will also help in providing a link between directional fungal growth and the underlying metabolic processes involved.

5. Conclusions

We show that growth of *A. niger* can be directed based on the compounds of interest added to the growth medium by taking their individual CI values into account. This study provides (i) tools to explore the growth of a filamentous fungus in any growth medium, including the presence of 'food' sources as well as trace elements and other factors affecting general growth, further (ii) by changing the chemical environment of the fungus, in this case of *A. niger*, directive growth can be invoked and quantified by mapping the fungal filament area. This is an initial, yet important study which provides information that could be be used to generate tuneable fungal based biomaterials that may find application as renewable source for bioactive polymers, medicine, therapeutics, and environmental interventions.

CRediT authorship contribution statement

Ayesha Sadaf: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Visualization, Writing – original draft, Writing – review & editing, Professor. **Matthias Brock:** Investigation, Resources, Writing – original draft, Writing – review & editing. **Carole C. Perry:** Conceptualization, Formal analysis, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – original draft, Writing – review & editing.

Acknowledgements

Dr Carole C. Perry and Dr Ayesha Sadaf gratefully acknowledge funding from AFOSR FA9550-20-1-0206. The authors would like to thank MTIF (Medical Technologies Innovation Facility-Clifton campus) for support with wide field imaging studies.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.funbio.2024.05.010.

References

Adnan, M., Zheng, W., Islam, W., Arif, M., Abubakar, Y.S., Wang, Z., Lu, G., 2017. Carbon catabolite repression in filamentous fungi. Int. J. Mol. Sci. 19 (1), 48.

Aleklett, K., Boddy, L., 2021. Fungal behaviour: a new frontier in behavioural ecology. Trends Ecol. Evol. 36 (9), 787–796. https://doi.org/10.1016/j.tree.2021.05.006.

Archer, M., Xu, J., 2021. Current Practices for reference gene selection in RT-qPCR of Aspergillus: Outlook and recommendations for the future. Genes 12 (2021), 960.

Arzt, M., Deschamps, J., Schmied, C., Pietzsch, T., Schmidt, D., Tomancak, P., Haase, R., Jug, F., 2022. LABKIT: labeling and segmentation toolkit for big image data. Front. Comput. Sci. 4, 10.

Bagheriehnajjar, G., Yousefpour, H., Rahimnejad, M., 2024. Environmental impacts of mycelium-based bio-composite construction materials. Int. J. Environ. Sci. Technol. https://doi.org/10.1007/s13762-023-05447-x.

Coleine, C., Stajich, J.E., de los Ríos, A., Selbmann, L., 2021. Beyond the extremes: rocks as ultimate refuge for fungi in drylands. Mycologia 113 (1), 108–133. https://doi.org/10.1080/00275514.2020.1816761.

Dias, T., Pimentel, V., Cogo, A.J.D., Costa, R., Bertolazi, A.A., Miranda, C., de Souza, S.B., Melo, J., Carolino, M., Varma, A., Eutrópio, F., Olivares, F.L., Ramos, A.C., Cruz, C., 2020. The free-living stage growth conditions of the endophytic fungus Serendipita indica may regulate its potential as plant growth promoting microbe. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.562238.

Geib, E., Gressler, M., Viediernikova, I., Hillmann, F., Jacobsen, I.D., Nietzsche, S., Hertweck, C., Brock, M., 2016. A non-canonical melanin biosynthesis pathway protects Aspergillus terreus conidia from environmental stress. Cell Chem. Biol. 23 (5), 587–597. https://doi.org/10.1016/j.chembiol.2016.03.014.

Haneef, M., Ceseracciu, L., Canale, C., Bayer, I.S., Heredia-Guerrero, J.A., Athanassiou, A., 2017. Advanced materials from fungal mycelium: fabrication and tuning of physical properties. Sci. Rep. 7 (1), 41292 https://doi.org/10.1038/srep41292.

Ijadpanahsaravi, M., Punt, M., Wösten, H.A.B., Teertstra, W.R., 2021. Minimal nutrient requirements for induction of germination of Aspergillus Niger conidia. Fungal Biol. 125 (3), 231–238. https://doi.org/10.1016/j.funbio.2020.11.004.

Jeffries, T.W., 1983. Utilization of xylose be bacteria, yeasts, and fungi. In: Pentoses and Lignin. Advances in Biochemical Engineering/Biotechnology, vol. 27. Springer, Berlin, Heidelberg.

Lichius, A., 2022. Concentration, cellular exposure and specificity of organelle selective fluorescent dyes in fungal cell biology. Fungal Biology Reviews 41, 45–51. https://doi.org/10.1016/j.fbr.2021.07.002.

Mäkelä, M.R., Aguilar-Pontes, M.V., van Rossen-Uffink, D., Peng, M., de Vries, R.P., 2018. The fungus Aspergillus Niger consumes sugars in a sequential manner that is not mediated by the carbon catabolite repressor CreA. Sci. Rep. 8 (1), 6655.

Martínez, A.A., Conboy, A., Buskirk, S.W., Marad, D.A., Lang, G.I., 2023. Long-term adaptation to galactose as a sole carbon source selects for mutations outside the canonical GAL pathway. J. Mol. Evol. 91 (1), 46–59.

Moreno-Ruiz, D., Lichius, A., Turrà, D., Di Pietro, A., Zeilinger, S., 2020. Chemotropism assays for plant symbiosis and mycoparasitism related compound screening in trichoderma atroviride. Front. Microbiol. 11 https://doi.org/10.3389/fmicb.2020.601251.

Muehlstein, L.K., Amon, J.P., Leffler, D.L., 1988. Chemotaxis in the marine fungus *Rhizophydium littoreum*. Appl. Environ. Microbiol. 54 (7), 1668–1672. https://doi.org/10.1128/aem.54.7.1668-1672.1988.

Rodríguez, J.M., Ruíz-Sala, P., Ugarte, M., Penalva, M.A., 2004. Fungal metabolic model for 3-methylcrotonyl-CoA carboxylase deficiency. J. Biol. Chem. 279 (6), 4578–4587.

Schunke, C., Pöggeler, S., Nordzieke, D.E., 2020. A 3D printed device for easy and reliable quantification of fungal chemotropic growth. Front. Microbiol. 11, 584525 https://doi.org/10.3389/fmicb.2020.584525.

Seiboth, B., Metz, B., 2011. Fungal arabinan and L-arabinose metabolism. Appl. Microbiol. Biotechnol. 89, 1665–1673.

Silao, F.G.S., Ward, M., Ryman, K., Wallström, A., Brindefalk, B., Udekwu, K., Ljungdahl, P.O., 2019. Mitochondrial proline catabolism activates Ras1/cAMP/PKA-induced filamentation in Candida albicans. PLoS Genet. 15 (2), 1–36.

Sterflinger, K., Tesei, D., Zakharova, K., 2012. Fungi in hot and cold deserts with particular reference to microcolonial fungi. Fungal Ecology 5 (4), 453–462. https://doi.org/10.1016/j.funeco.2011.12.007.

Turrà, D., Di Pietro, A., 2015. Chemotropic sensing in fungus–plant interactions. Curr. Opin. Plant Biol. 26 https://doi.org/10.1016/j.pbi.2015.07.004.

Turrà, D., El Ghalid, M., Rossi, F., Di Pietro, A., 2015. Fungal pathogen uses sex pheromone receptor for chemotropic sensing of host plant signals. Nature 527 (7579). https://doi.org/10.1038/nature15516.

Turrà, D., Nordzieke, D., Vitale, S., El Ghalid, M., Di Pietro, A., 2016. Hyphal chemotropism in fungal pathogenicity. Semin. Cell Dev. Biol. 57 https://doi.org/10.1016/j.semcdb.2016.04.020.