



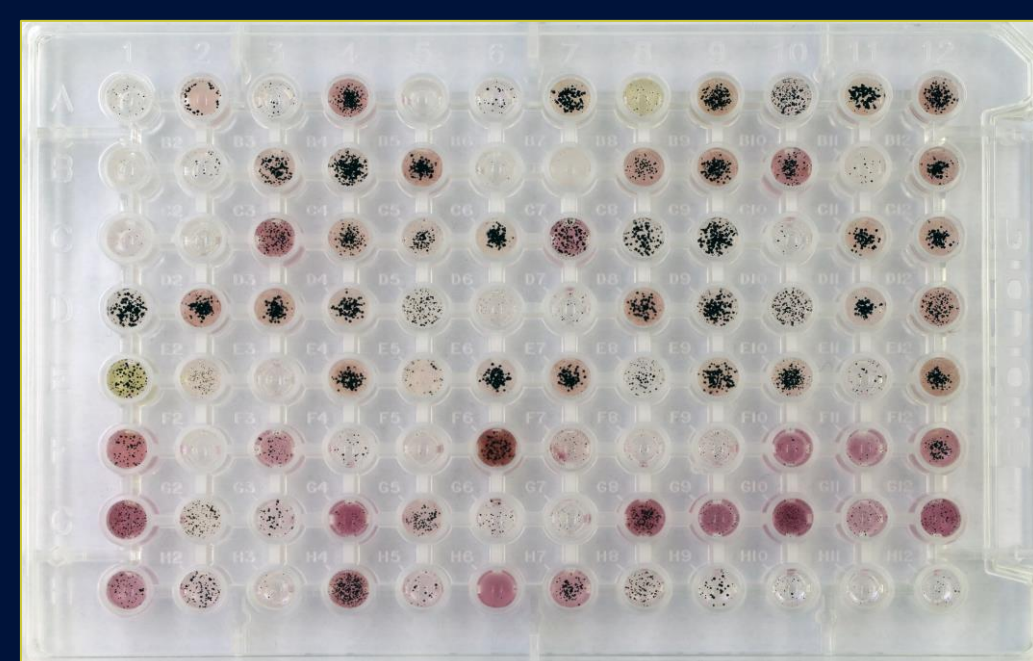
REPAINT – THE ARTIFICIAL INTELLIGENCE ALGORITHM FOR COMPREHENSIVE PHENOTYPING OF CONIDIAL FUNGI

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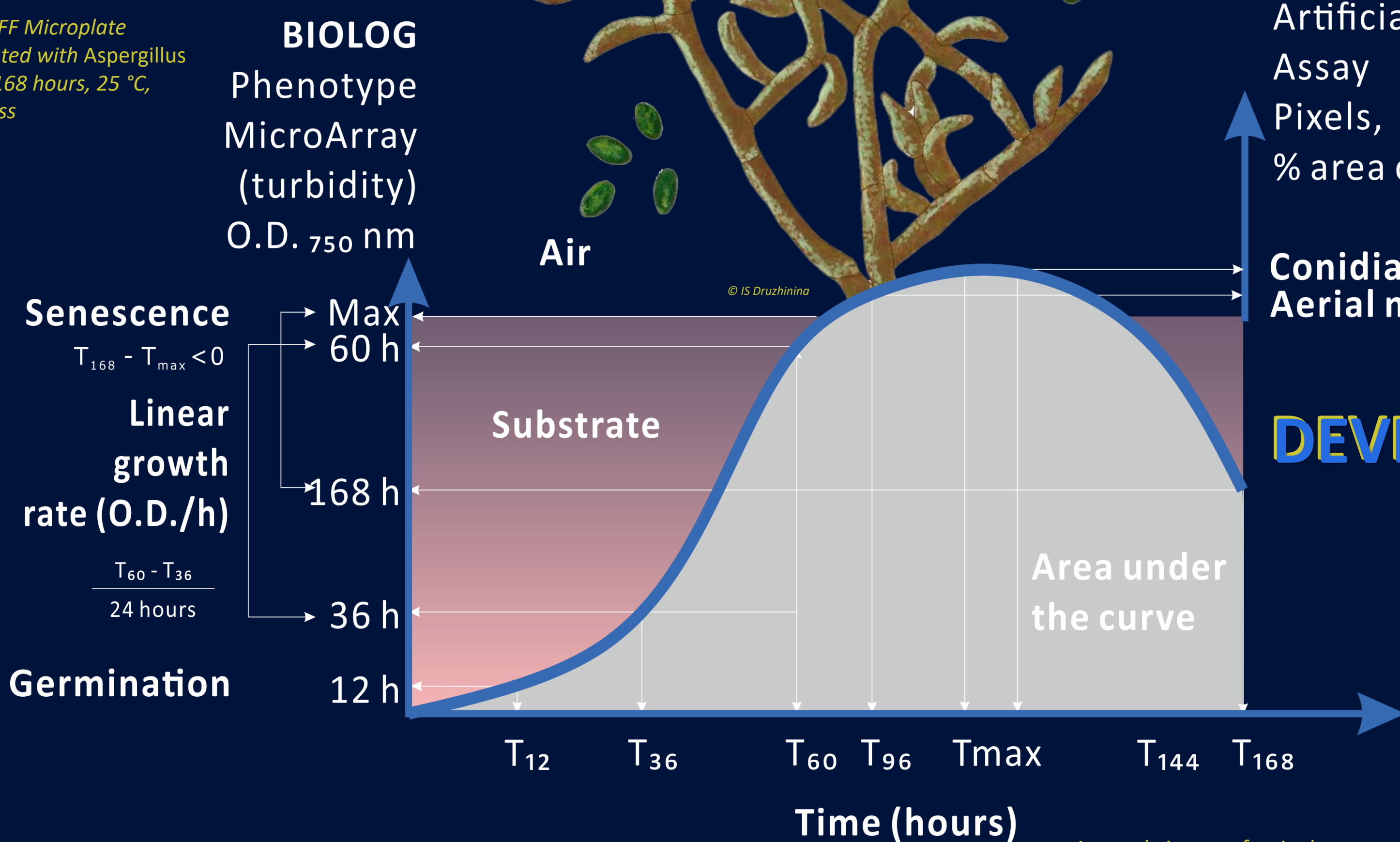
In fungi, the genetic distances assessed from the genomes are huge, and the two seemingly similar molds belonging to one genus can share only the three quarters of their genes, i.e., differ as humans from fish. The understanding of these genomic variants requires the consideration of fungal phenotypes. However, *in situ* studies of fungal biology are impeded by the identification ambiguity, their diminutive bodies, which develop inside a substrate and have irregular life cycles and exceptional metabolic and ecological plasticity. Therefore, the interpretation of the genomic traits frequently solely depends on *in vitro* phenotyping, and the lack of quantitative measures can represent a bottleneck for understanding different phenotypes.

In this study, we aim to develop a high-throughput comprehensive phenotyping system for conidial fungi in pure culture. We consider that phenotypes should be assessed (i) at several stages of the life cycle (the development), (ii) in versatile nutritional conditions, (iii) exposed to a variety of abiotic stressors, and (iv) be quantitative.

The commonly used Biolog FF MicroPlates containing 95 carbon sources and water offer a convenient platform for such phenotyping. These microplates can be inoculated with spore suspensions either in a semisolid Phytogel or water. In addition, FF MicroPlates can be supplemented with xenobiotics or stress-generating compounds (e.g., ROS-generating chemicals or osmolytes) and exposed to different spectra of electro-magnetic radiation, temperature, or other conditions.



Biolog FF Microplate inoculated with *Aspergillus niger*, 168 hours, 25 °C, darkness



BACKGROUND

Fungal growth curve in the Biolog FF Microplate and ecophysiological parameters assessed by BIOLOG and REPAINT assays

REPAINT

Reproduction potential
Artificial Intelligence
Assay
Pixels,
% area covered

Conidiation
Aerial mycelium

DEVELOPMENT

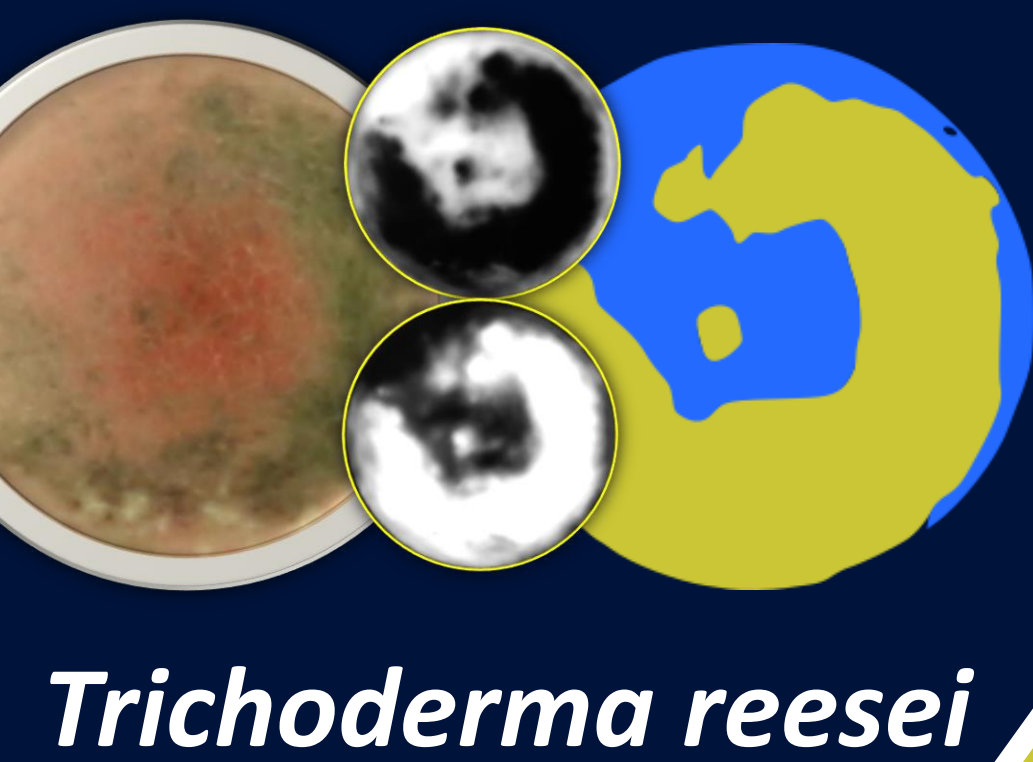
REPRODUCTIVE POTENTIAL ARTIFICIAL INTELLIGENCE ASSAY

The goal of the image analysis algorithm is to inspect the high resolution images of Biolog FF Microplates inoculated with spore suspensions of tested fungi following the standard protocol for the Biolog Phenotype Microarrays (see Druzhinina et al., 2006).

Prior to well analysis, the individual positions of the wells are determined by finding the well plate structure in the image. The detected wells are cropped and resized to a fixed 256x256 pixels to become invariant against changes of image size. To separate regions covered by conidia or hyphae, we use a machine learning algorithm based on a U-Net (Ronneberger et al., 2015) with an InceptionResNetV2 backbone (Szegedy et al., 2017). The network is trained on 60 images of well plates and their corresponding ground-truth annotations, created by the developers.

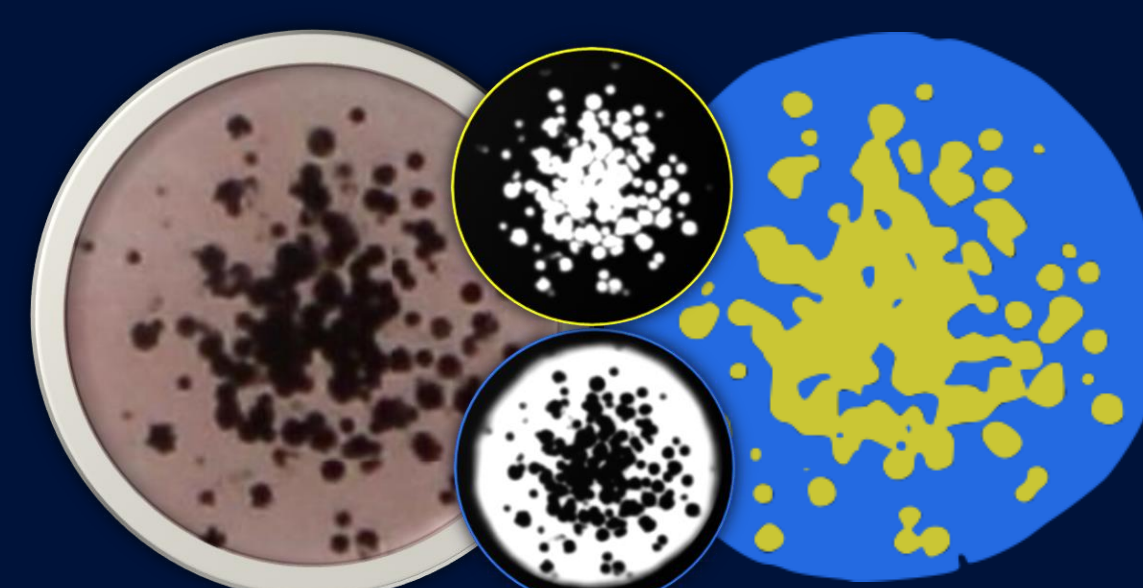
The images contain well plates in several stages of fungal life cycle (development) for five different model species: *Trichoderma reesei* (Hypocreales), *Metarhizium robertsii* (Hypocreales), *Neurospora crassa* (Sordariales), *Aspergillus niger* (Eurotiales), and *Penicillium* sp. (Eurotiales). During analysis, the algorithm estimates for each pixel whether it is covered by hyphae or conidia, without differentiating between fungal taxa. Based on the results, the percentage of the area covered in conidia and hyphae are calculated. These percentages are evaluated over five different annuli of each well.

SELECTED CARBON SOURCES

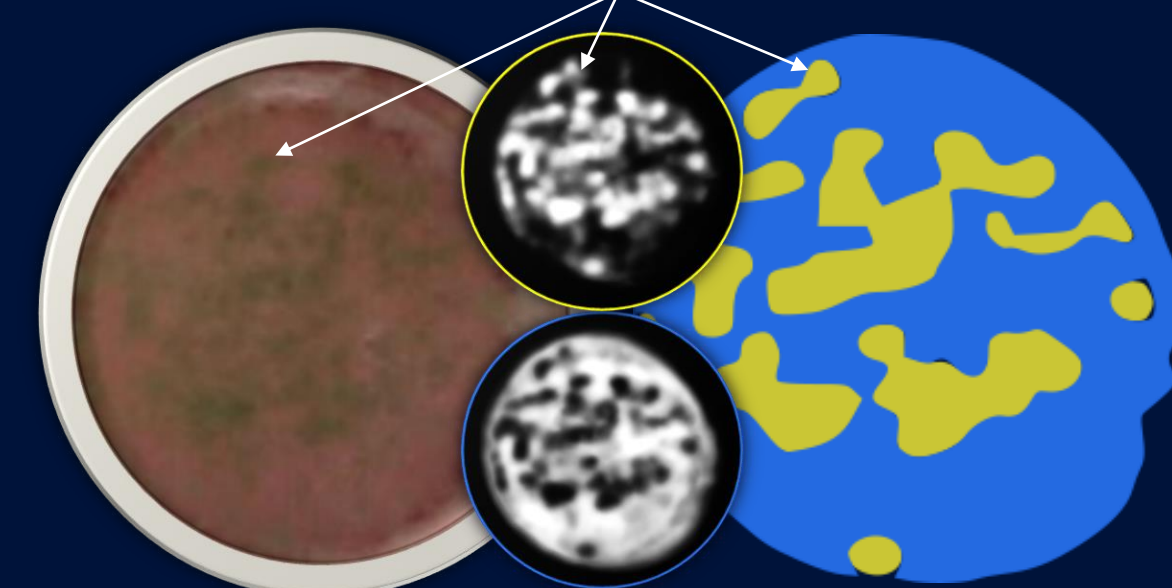


Trichoderma reesei

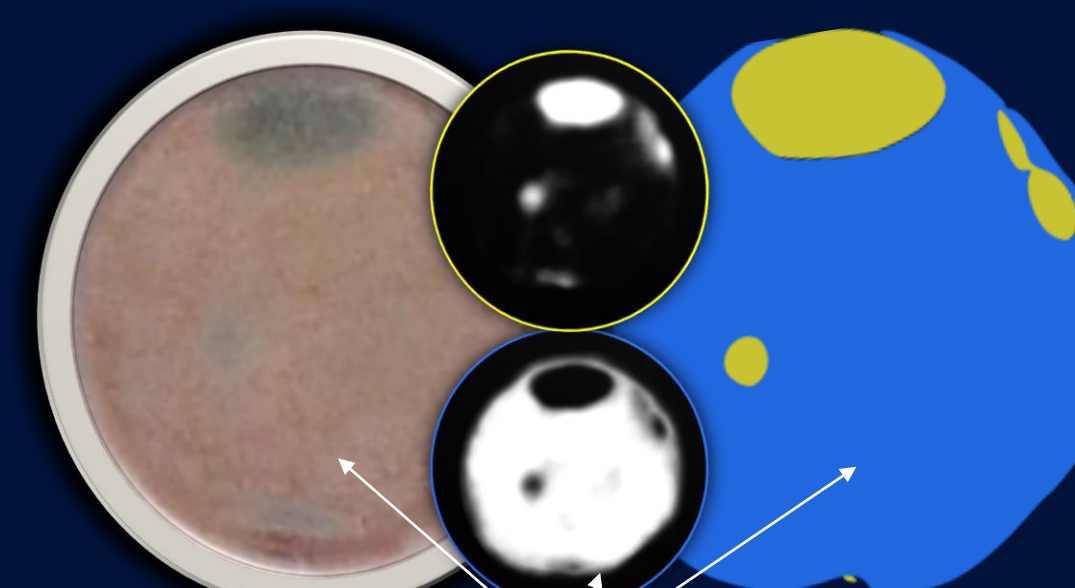
Aspergillus niger



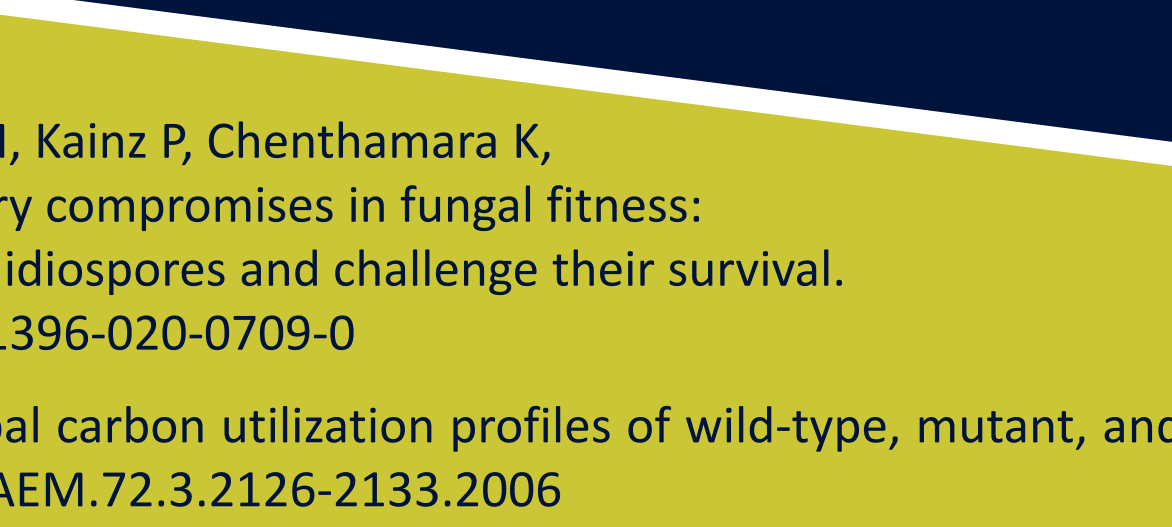
conidia



Penicillium sp.



Metarhizium robertsii



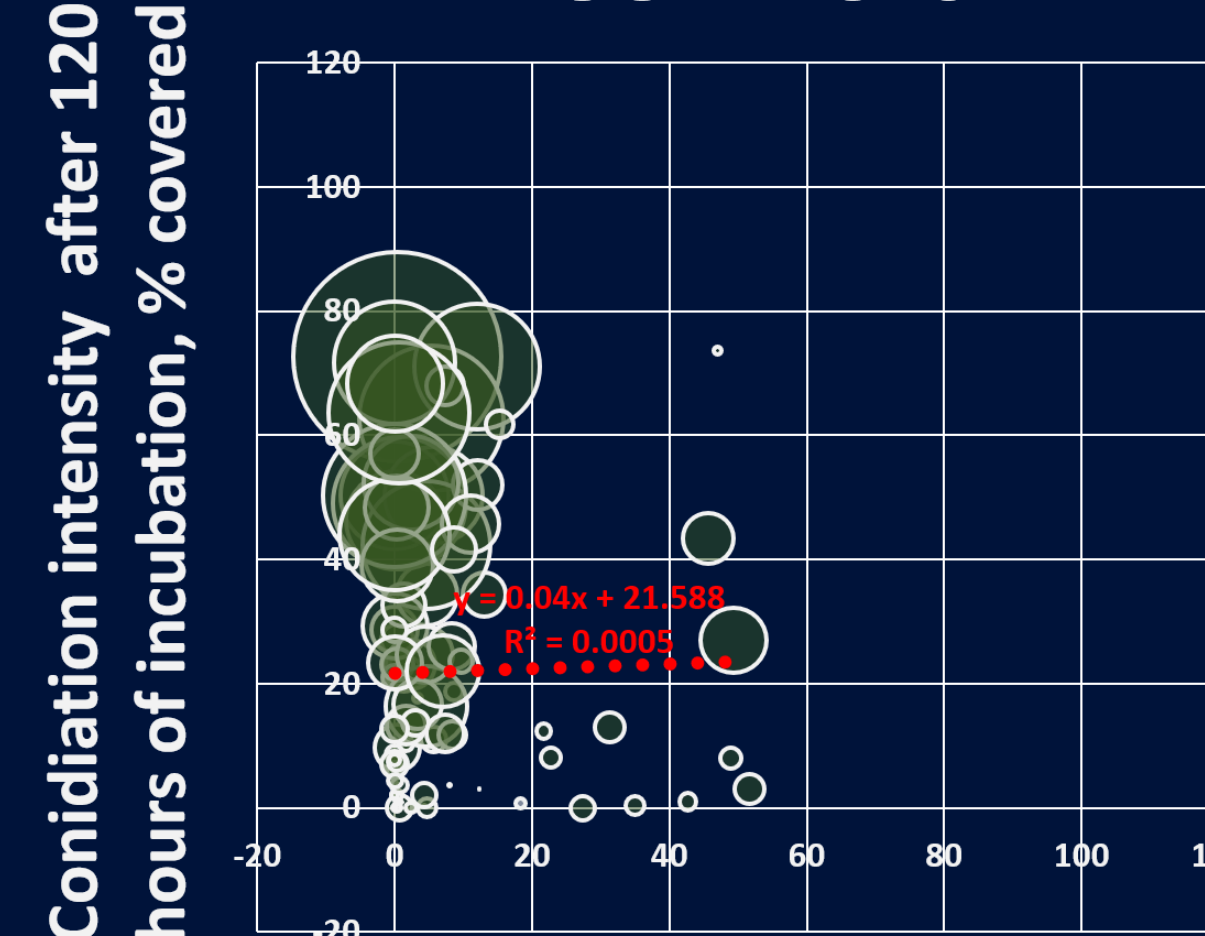
hyphae

PILOT RESULTS BASED ON THE ANALYSIS OF 35 STRAINS OF TRICHODERMA

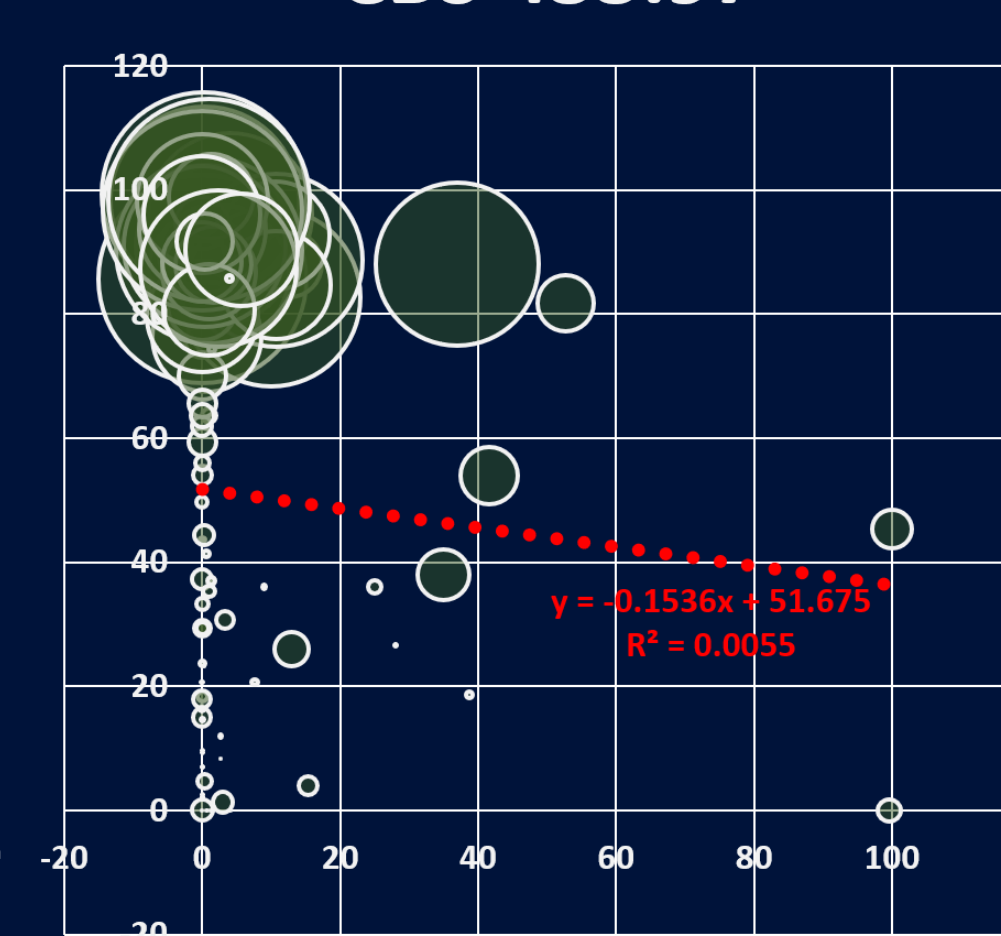
R STRATEGY

THE "CONIDIAL" OR "CLONAL" STRAINS: IF THE FUNGUS CAN GROW AT LEAST A BIT, IT TRIES TO PRODUCE SPORES AT THE FIRST INSTANCE. MOST AERIAL HYPHAE ARE CONIDIOPHORES. THESE STRAINS LIKELY LIVE IN AN UNSTABLE ENVIRONMENT

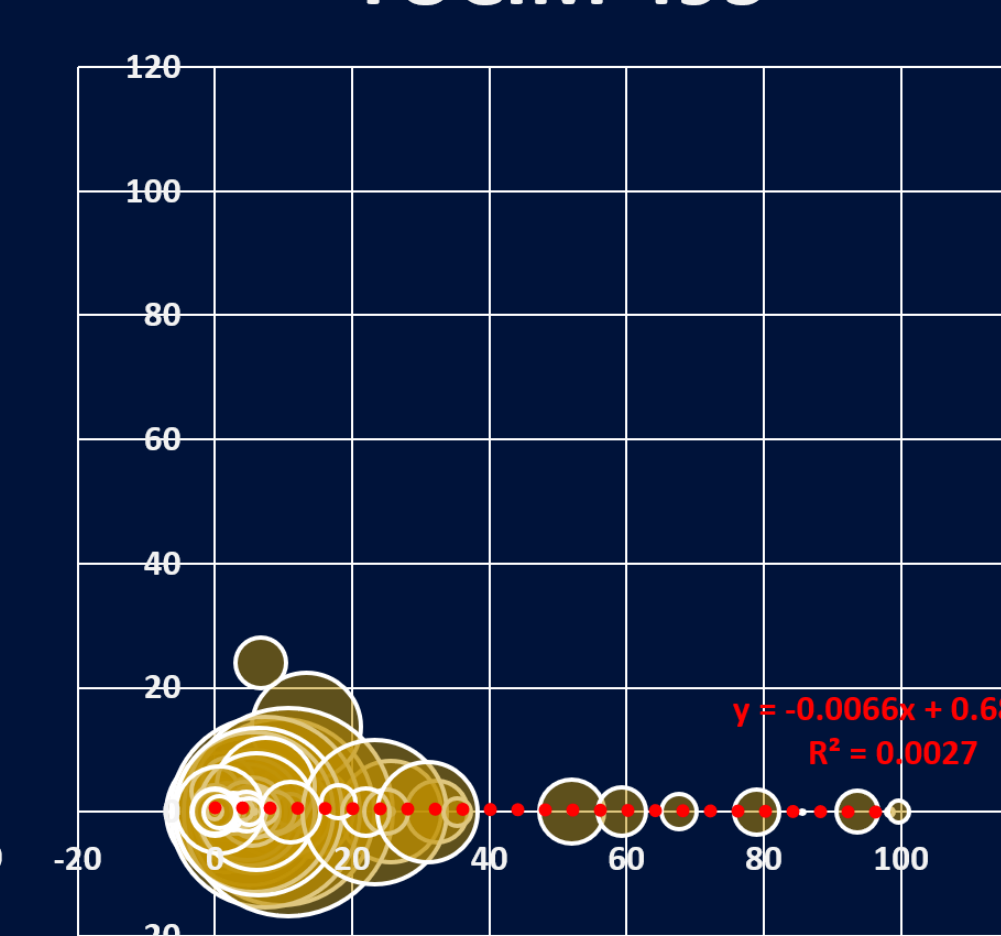
T. pleuroticola TUCIM 1540



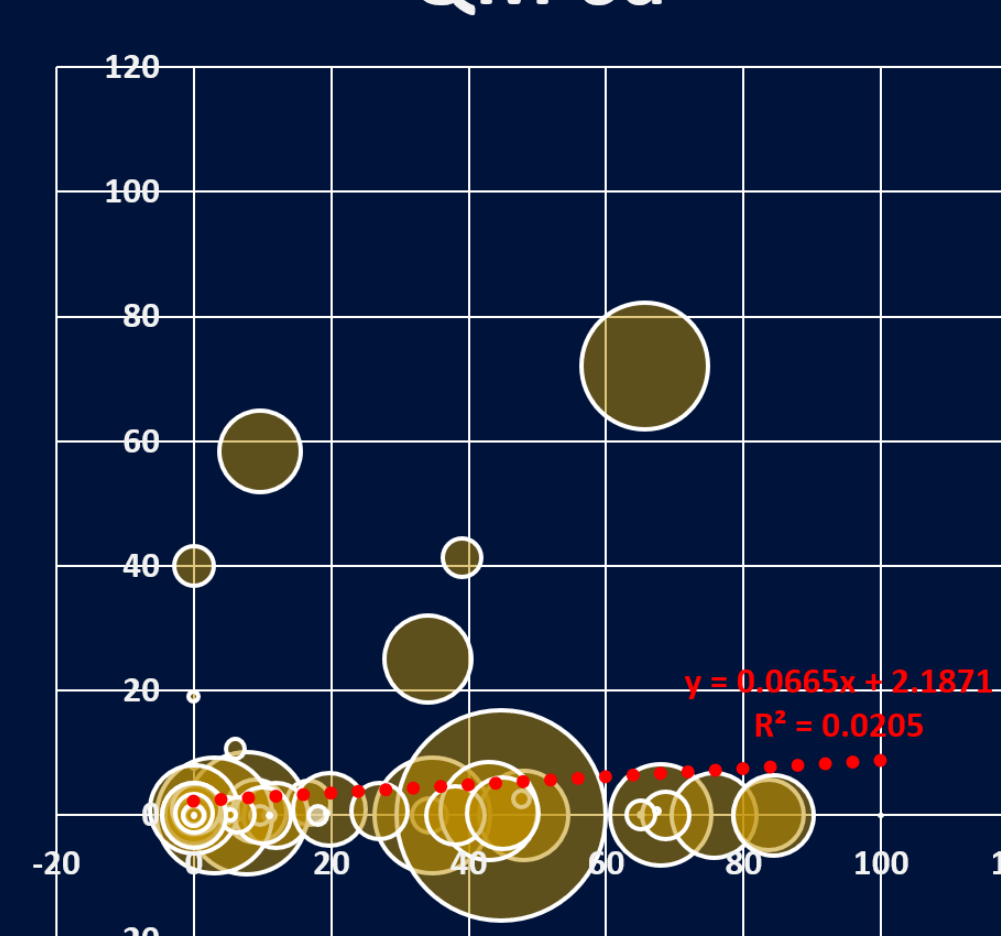
T. asperellum CBS 433.97



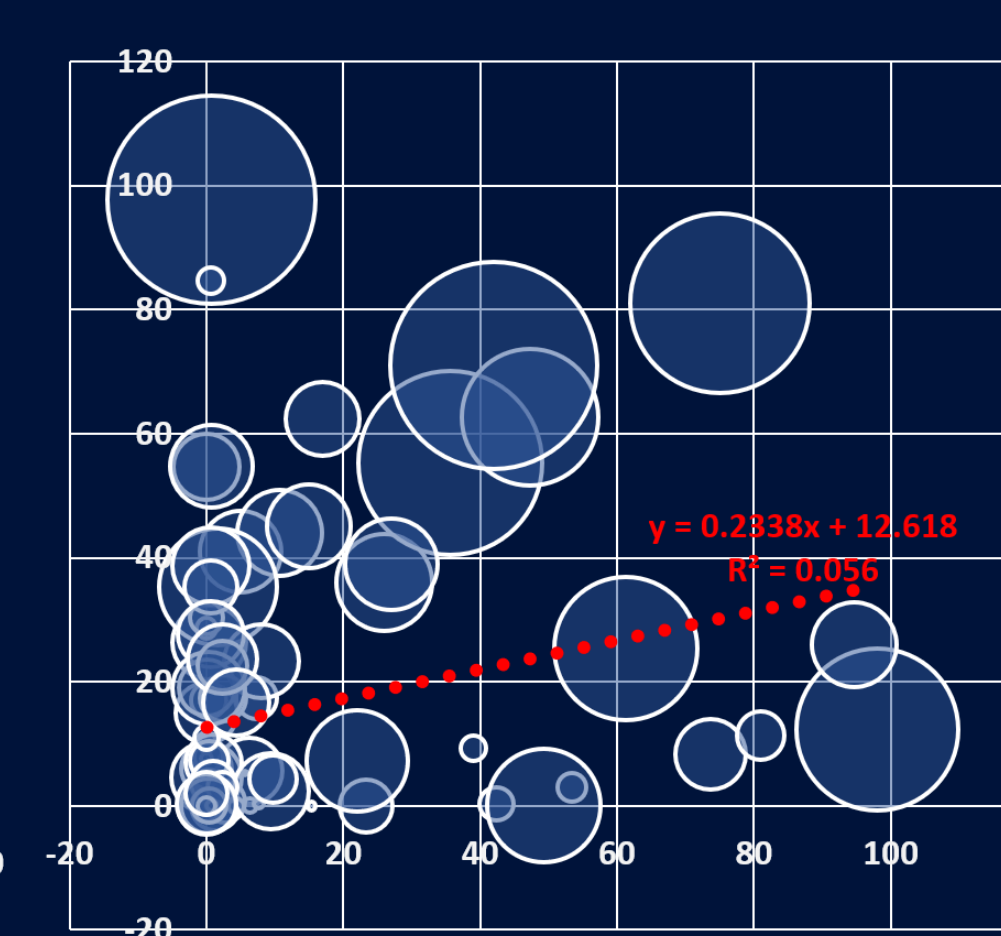
T. minutisporum TUCIM 495



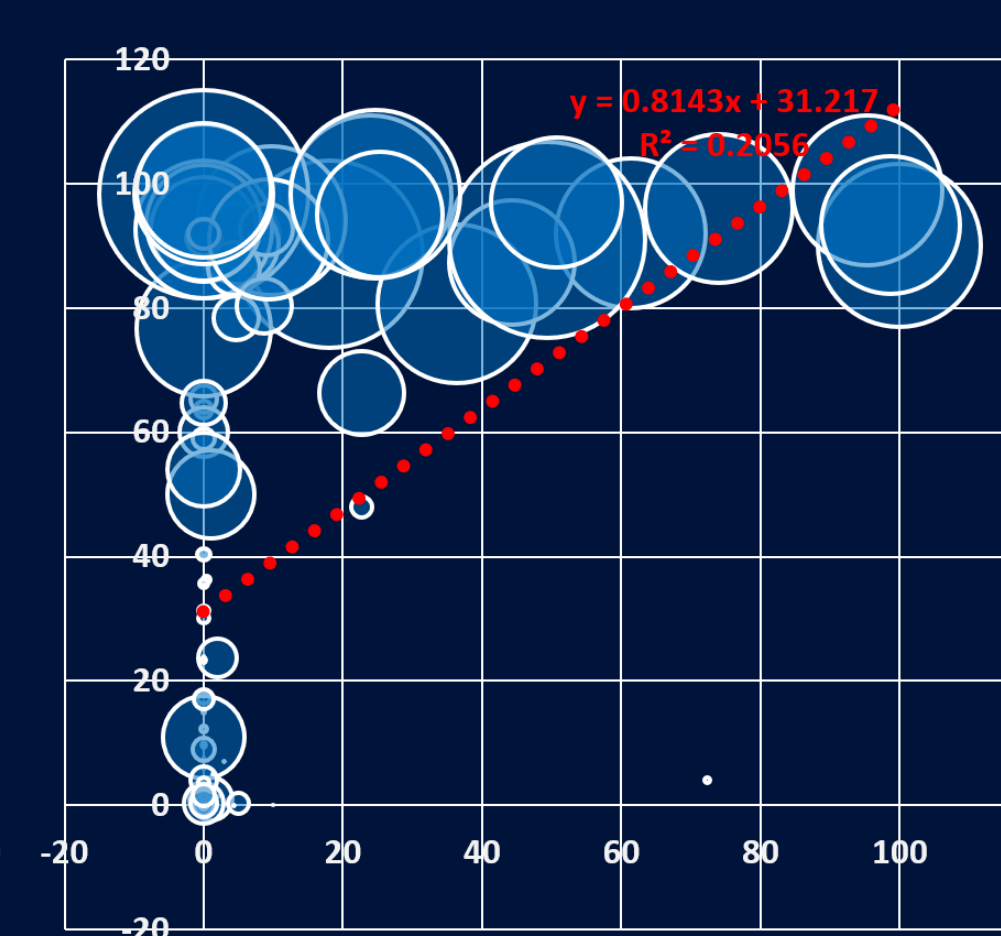
T. reesei QM 6a



T. harzianum CBS 226.95



T. virens Gv 29-8

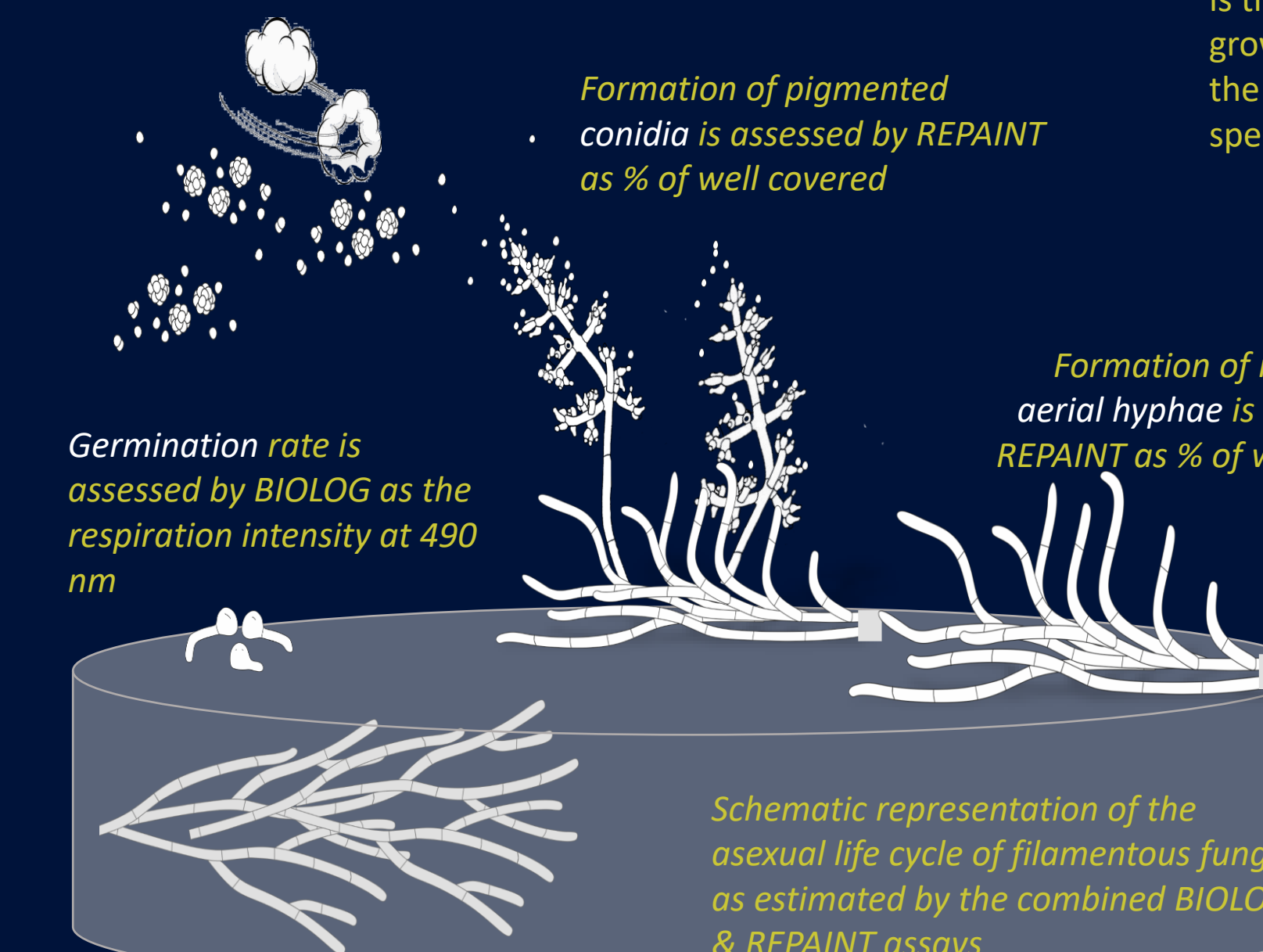


Linear growth rate on individual carbs, O.D. 750 nm per day

The largest circle is the fastest growth (100% for the given species)

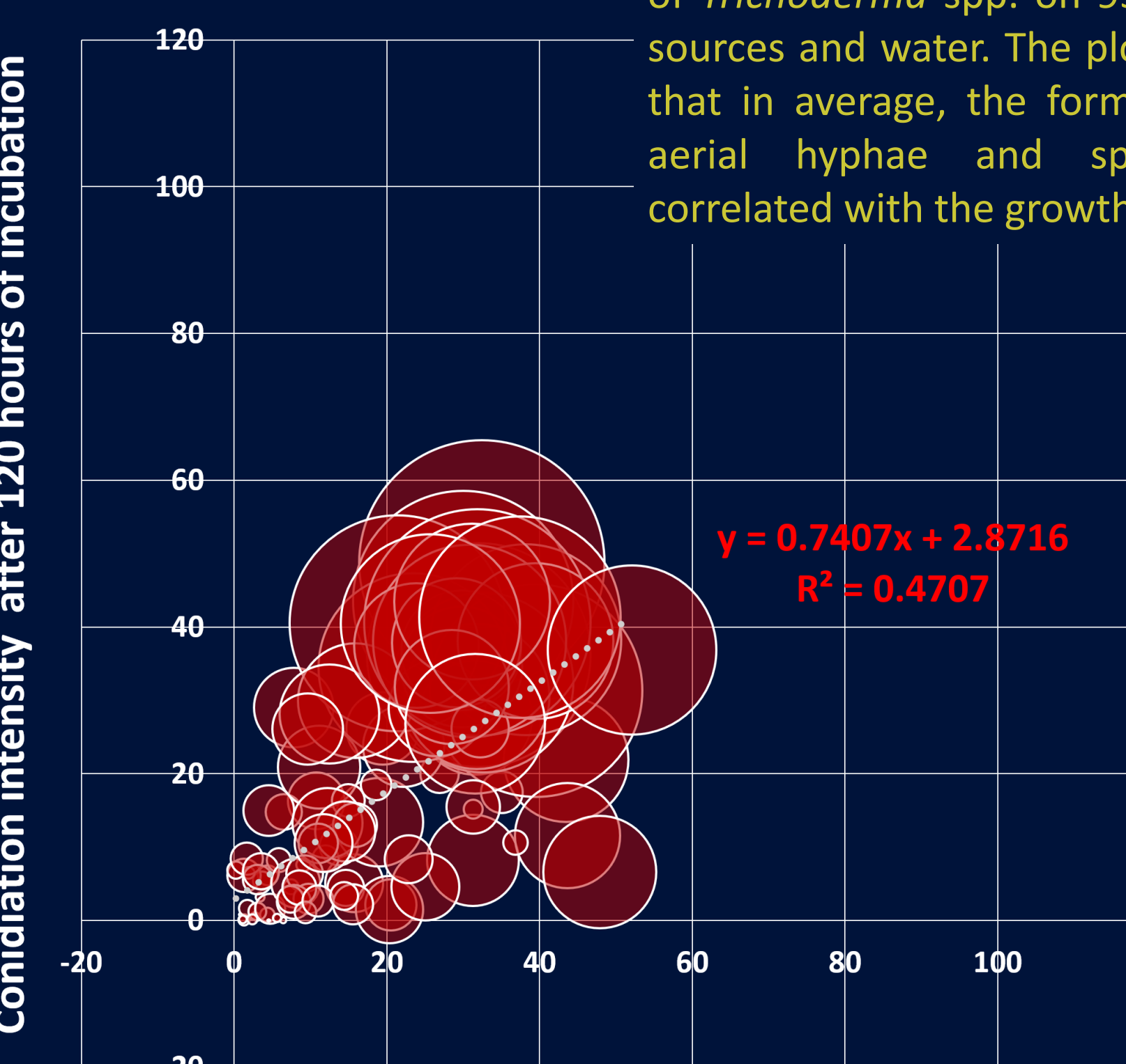
Development of aerial hyphae after 96 hours of incubation, % covered

The development of fungi in microplates can be divided into at least four stages and quantified (Cai et al., 2020). First, the spore germination is best reflected by the turbidity values at 490 nm corresponding to the intensity of the red formazan dye formed when the idonitrotetrazolium violet incorporated into FF MicroPlates is reduced by succinate dehydrogenase (citric acid cycle). Second, the development of feeding hyphae is assessed by the turbidity values at 750 nm at multiple time points. Then, the reproductive potential of a fungus (aerial stages) is assessed by analyzing high-resolution digital images of FF MicroPlates. For this purpose, we developed REPAINT – the REproduction Potential Artificial INTeelligence assay. Specifically, the image of every well of the microplate is automatically converted to the numerical values corresponding to the per cents covered by hyphae or conidia (the third and fourth stages of the life cycle). In this presentation, we report the results for several model fungi and show how Biolog and REPAINT can be used to reveal different developmental strategies within one fungal genus, how specific carbon sources influence conidiation of most fungi, and how the dynamics of spore germination is determined by abiotic and genetic factors and is not influenced by available nutrients.



Submerged growth, feeding hyphae in the substrate, (the growth rate is assessed by BIOLOG as turbidity at 750 nm)

Conidiation intensity after 120 hours of incubation



The genus-average development of *Trichoderma* spp. on 95 carbon sources and water. The plot shows that in average, the formation of aerial hyphae and spores is correlated with the growth rate.

Development of aerial hyphae after 96 hours of incubation

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