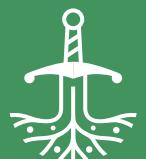


Using phenotype microarrays with filamentous fungi: potential and limitations

19.04.2023

Flavia Pinzari

Institute for Biological Systems (CNR)



EXCALIBUR



This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 817946

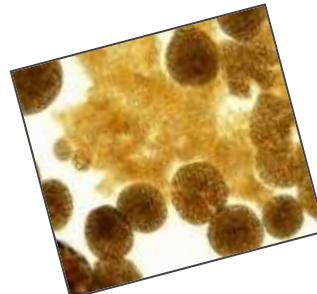
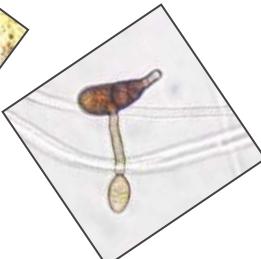
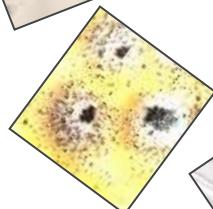




Index

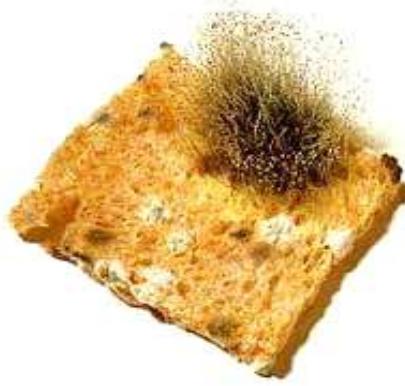


- Filamentous fungi (what they are, their life cycle, etc.)
- Why studying fungal metabolism
- How to use Phenotype microarrays with fungi (protocol, issues, problems and how to manage) + main differences with bacteria
- Examples / case studies /other potential uses
- Data analyses for fungi (some differences with bacteria)
- Future developments





- Who are the saprotrophic filamentous fungi



- The largest group of fungi
- Essential organisms in soil microbial communities
- Wide array of metabolic processes and biotransformations
- Secretion of extracellular enzymes
- Decomposition of insoluble remains of other biological entities such as lignocellulose and chitin
- Central in carbon recycling

- Why analysing their catabolic attitudes



Many saprotrophic species can also be parasitic or mutualistic



SAPROTROPHIC FUNGI





136 pp!

Fungal Diversity (2019) 97:1–136
https://doi.org/10.3837/13225-019-0043-0

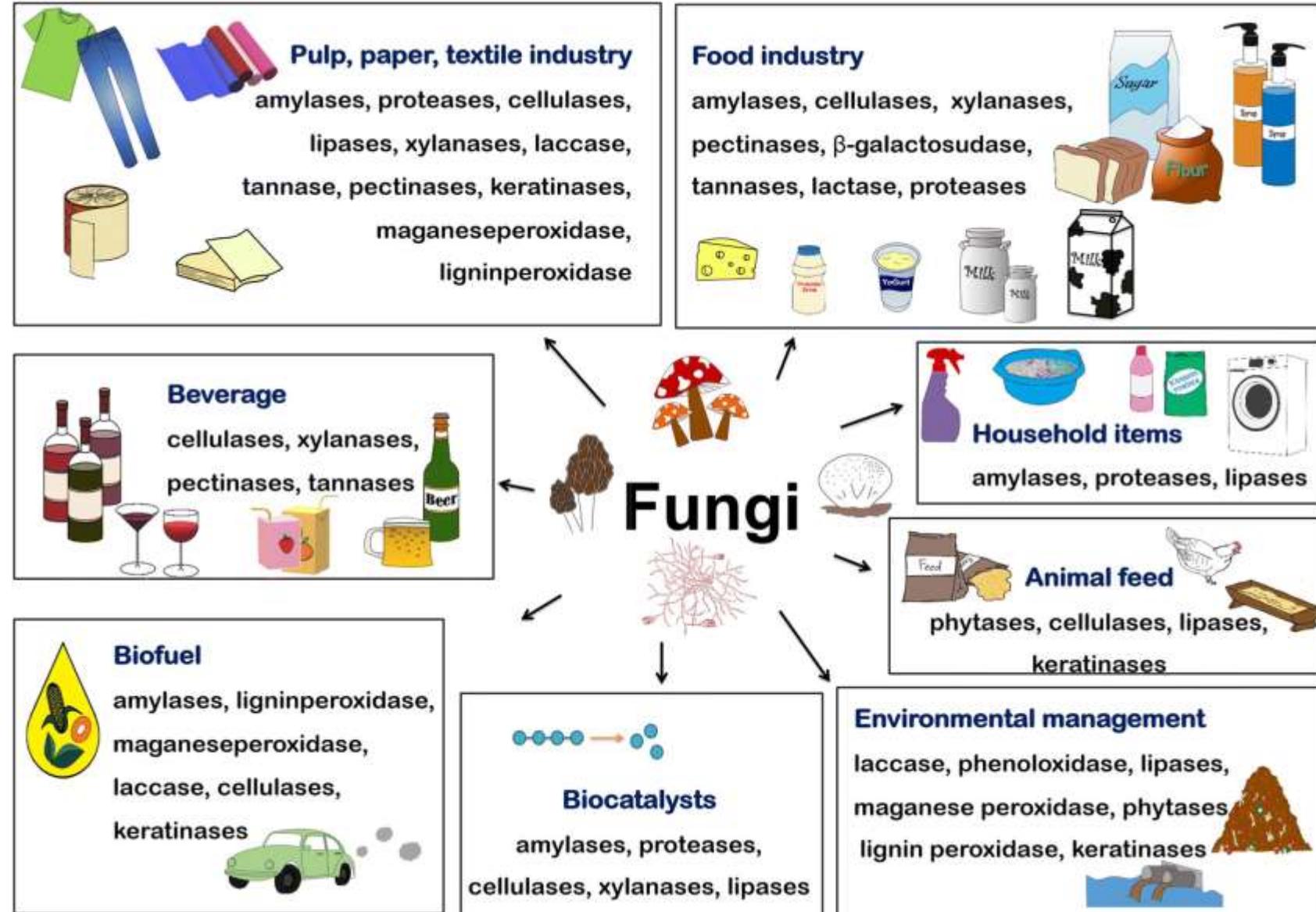
REVIEW

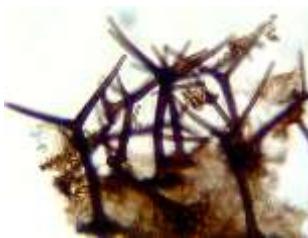
The amazing potential of fungi: 50 ways we can exploit fungi industrially

Kevin D. Hyde^{1,2,3,4,5,6} · Jianchu Xu^{7,8,9,10} · Sylvie Rapior¹¹ · Rajesh Jeewon¹⁰ · Saisamorn Lumyong^{3,11} · Allen Grace T. Niego^{1,2,12} · Pranami D. Abeywickrama^{1,2,7} · Janith V. S. Aluthmuhandiram^{1,2,7} · Reshika S. Brahmaange^{1,2,7} · Siraprasa Brooks⁹ · Amornrat Chaiyayen¹⁰ · K. W. Thilini Chethana^{1,2,7} · Putarak Chomnunt^{1,2,7} · Clara Chepkirui^{1,2} · Boonriya Chuankiat^{1,2} · Nimalk L. de Silva^{1,2,4,13} · Mingkwan Duolun^{1,4,13} · Craig Faull⁹ · Elena Gentekou⁹ · Venkata Gopulur¹⁴ · Pattrana Kakumyan^{1,2} · Dulanjalee Harischandra^{1,2,7} · Hiday Homachandran¹⁴ · Sinang Hongsanan^{15,27} · Anuruddha Karunaratna^{3,17} · Samantha C. Kerurathna¹ · Sehrion Khan¹⁰ · Jatuirong Kumla^{1,2,8} · Rujashika S. Jayawardena^{1,2} · Jian-Rui Liu¹¹ · Ningguo Liu^{1,2} · Thutsanee Luangham^{1,2,11,21} · Alan Patrick G. Macabero^{12,21} · Diana S. Marasinghe^{1,9} · Dan Meeks¹⁰ · Peter E. Mortimer^{1,20} · Peter Mutter¹⁹ · Sadia Nadir^{16,18,21} · Karaba N. Natraja¹⁶ · Sureeparn Nontachaiyapoom¹⁰ · Meghan O'Brien¹² · Watsana Pernthuz^{1,2,18} · Chayesara Phuahamsakda^{2,2} · Uma Shaanker Ramanan^{1,2} · Achala R. Rathnayaka^{2,2} · Resurreccion B. Sababe²⁹ · Birthe Sandager¹³ · Binu C. Samarakoon^{1,2} · Danushka S. Temnakkoon^{2,2} · Ramamoorthy Sri²⁸ · Wasan Siriporn^{3,11} · T. S. Suryanarayanan¹⁰ · Kanaporn Sujarit^{1,11} · Nakarin Suwanwannach^{3,13} · Thitipone Suwumwong^{1,8} · Benjarong Thonghai¹² · Narinade Thongklang² · Deping Wei^{1,2,2,19} · S. Nuwanthika Wijesinghe^{2,2} · Jake Winiski¹⁰ · Jye Yan⁷ · Erandi Yesanthri^{1,2} · Marc Stadler¹²

Received: 3 April 2019 / Accepted: 26 May 2019 / Published online: 3 July 2019
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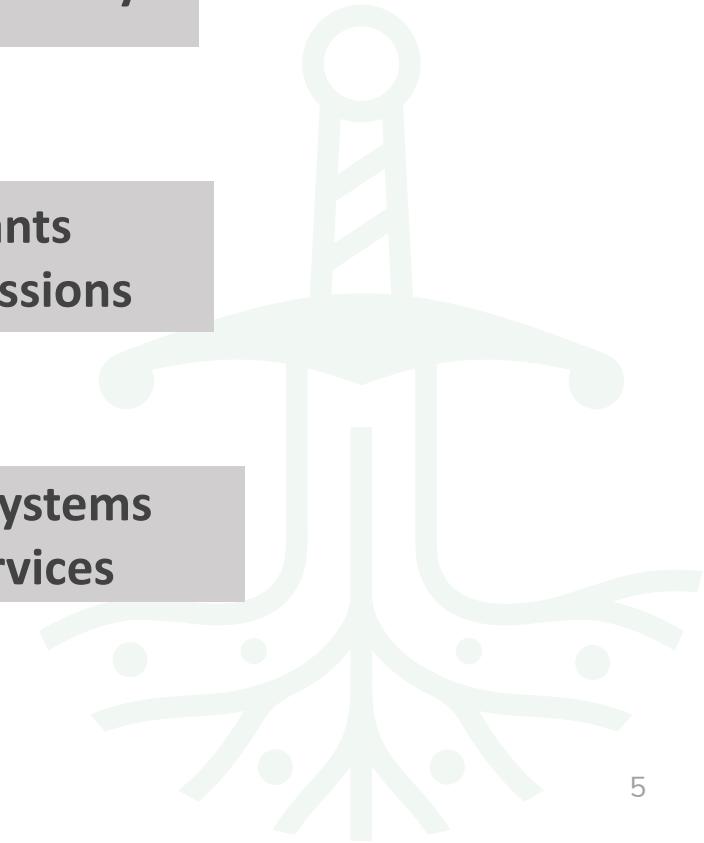
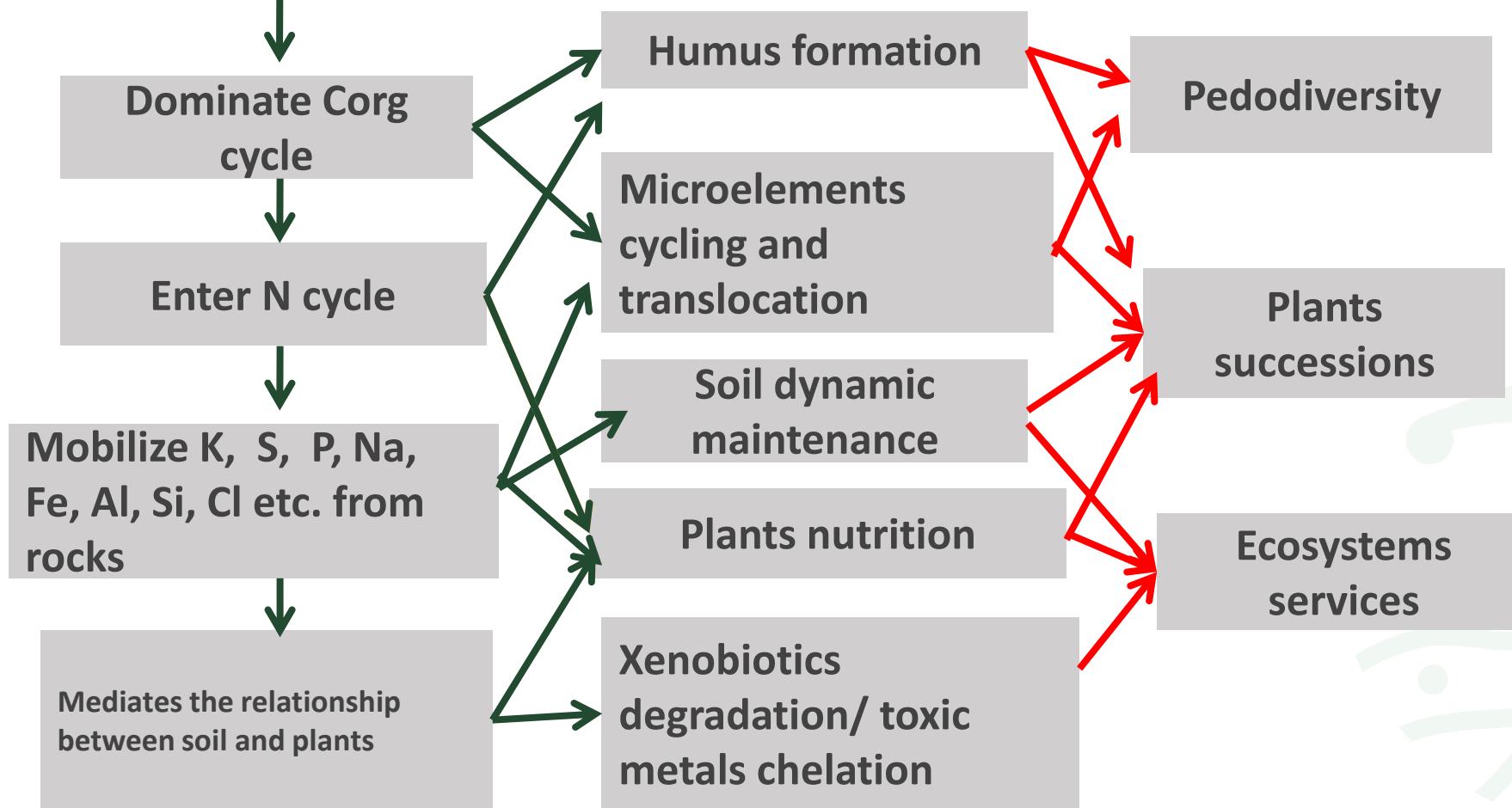
Hyde, K.D., Xu, J., Rapior, S. et al. The amazing potential of fungi: 50 ways we can exploit fungi industrially. *Fungal Diversity* 97, 1–136 (2019).



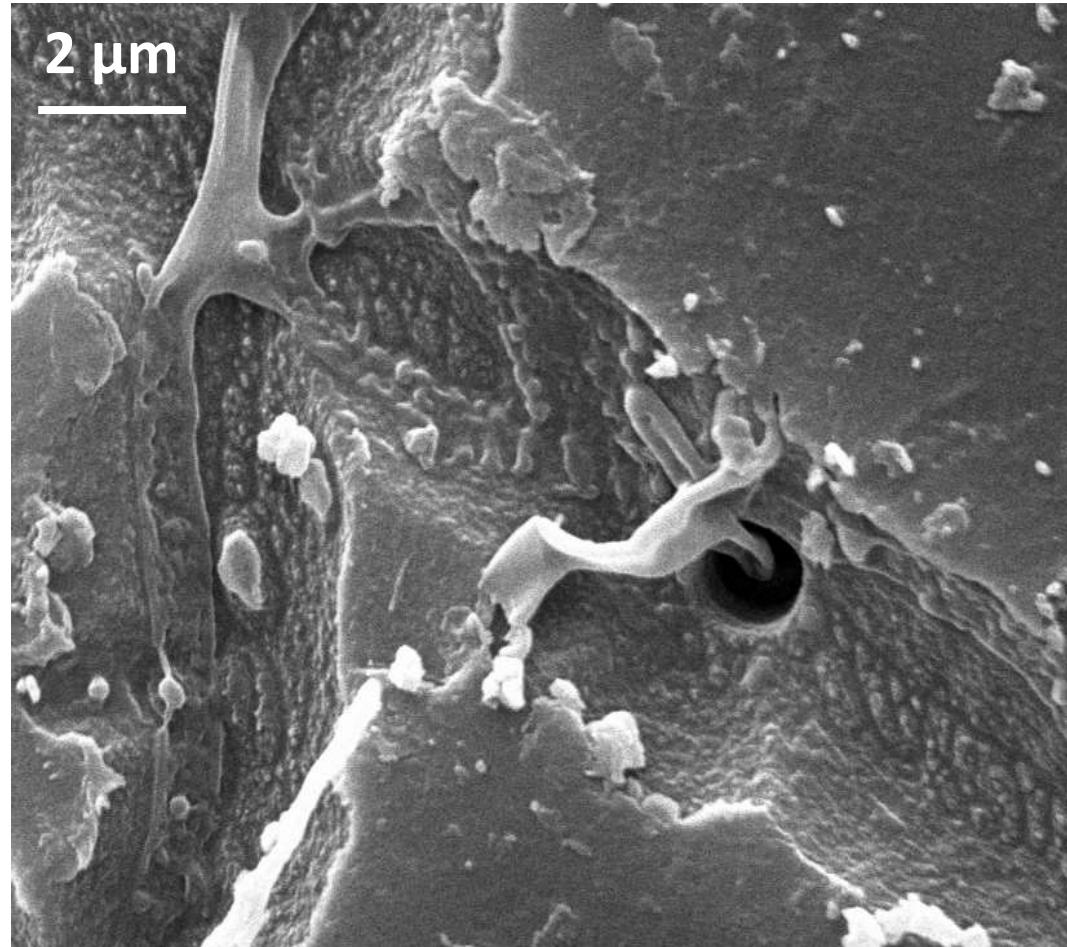


- Why analysing their catabolic attitudes

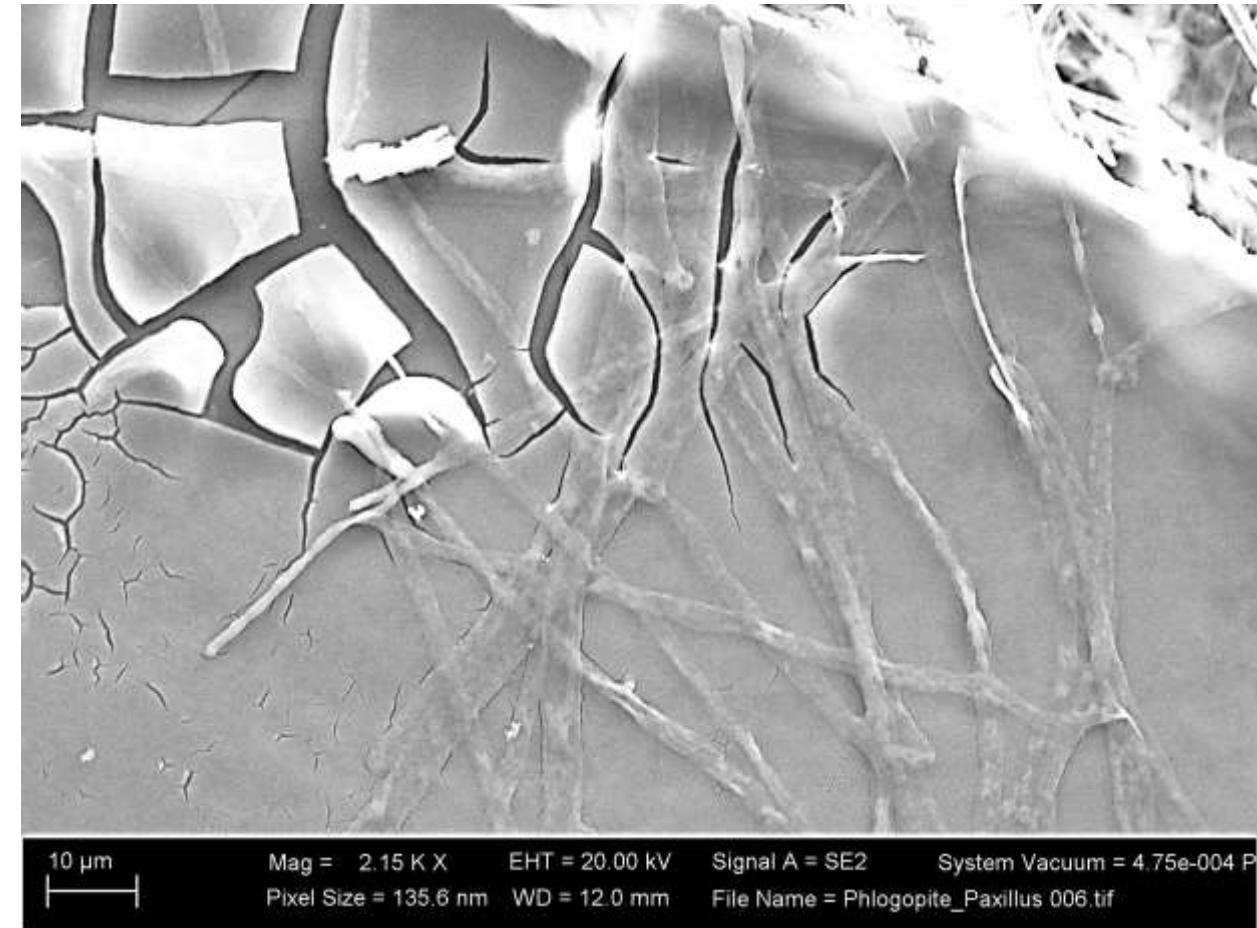
ENVIRONMENTAL ROLE OF SAPROTROPHIC FUNGI



Strong enzymes and physical interaction with substrata



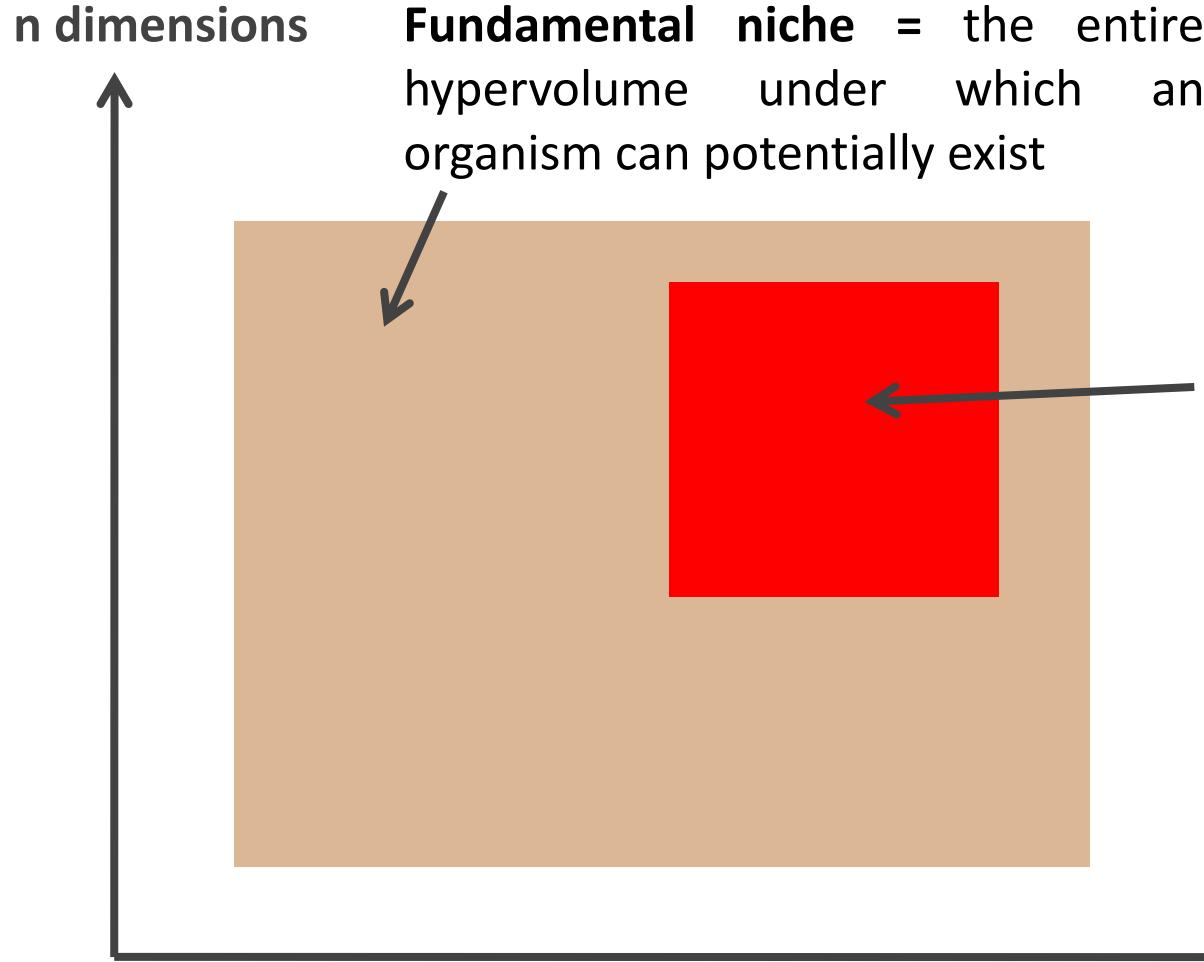
Plastic polymer



Silicate mineral



- What is a **niche** for a saprotrophic fungus



**Hutchinson
(1957)**

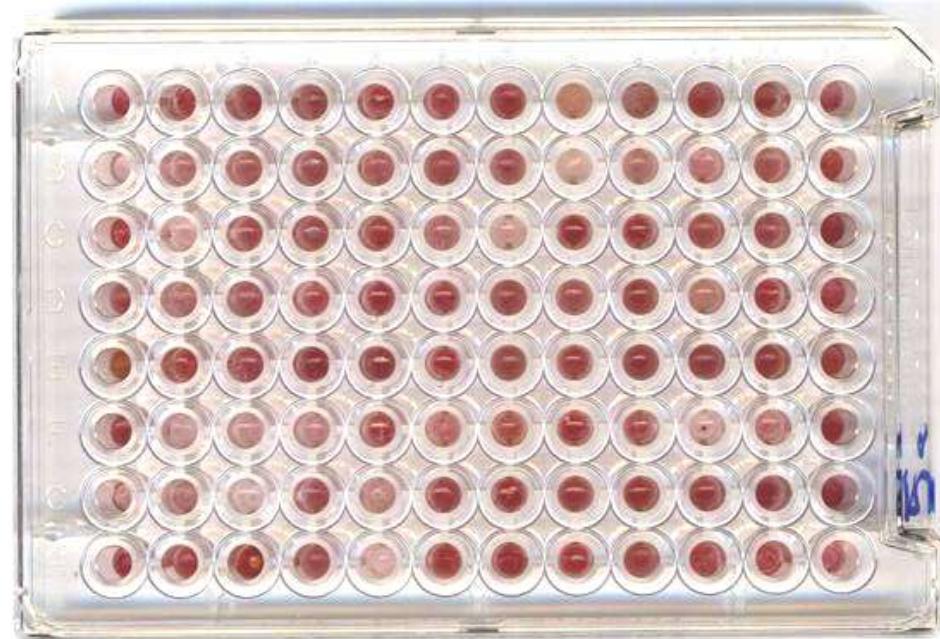


- What is a **niche** for a saprotrophic fungus

95xn
dimensions

The breadth of a species' realized or fundamental niche can be viewed in terms of the **functional attributes** of a species and its corresponding **trophic position**.

Elton (1927)



95 xn
dimensions



BIOLOG™ sells 10 panels designed to interrogate metabolic pathways along with ionic, osmotic and pH effects, and 10 panels to assess the sensitivity to various antimicrobials with different mechanisms of action. Different for fungi and bacteria.

PM1 TO PM10

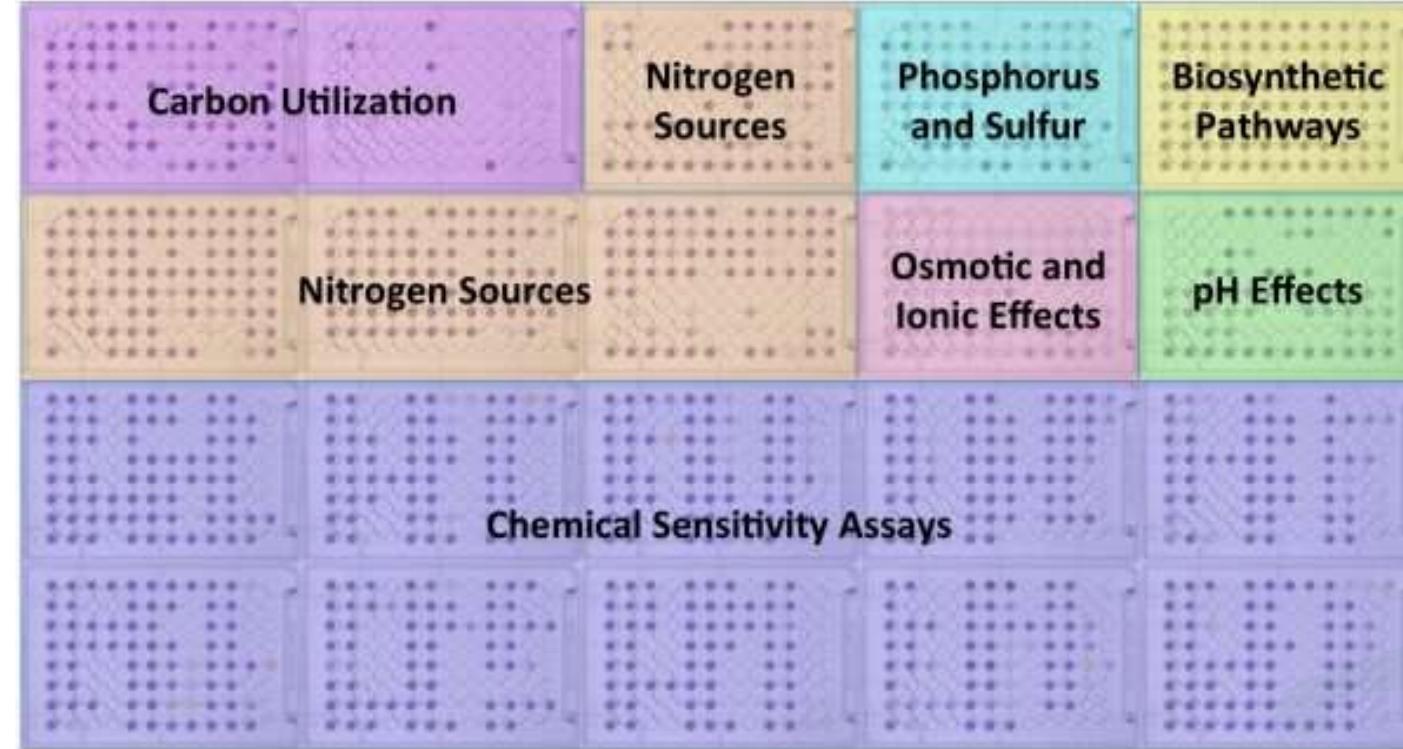
Metabolic tests for bacteria and fungi

PM11 TO PM20

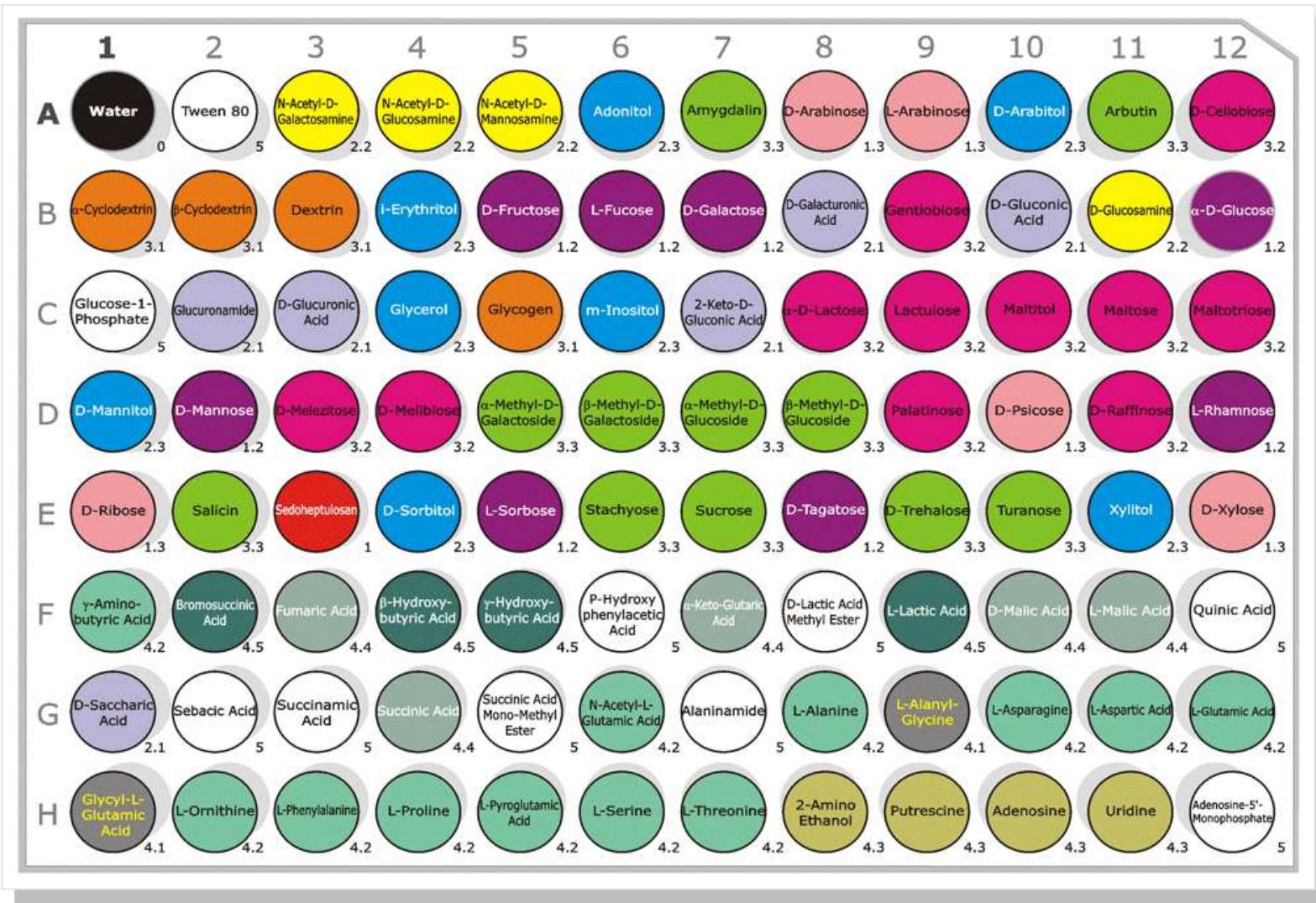
Chemical sensitivity tests for bacteria

PM21 TO PM25

Chemical sensitivity tests for fungi



<https://www.biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/>



FF plate

Heptoses

Hexoses

Pentoses

Sugar acids

Hexosamines

Polyols

Polysaccharides

Oligosaccharides

Glucosides

Peptides

L-amino acids

Biogenic and

Heterocyclic amines

TCA-cycle intermediates

Aliphatic organic acids

Atanasova L, Druzhinina IS. Review: Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. J Zhejiang Univ Sci B. 2010 Mar;11(3):151-68.



FF Microplates

A1 Water	A1 Water	Phosphate amide	E1 D-Ribose	G1 D-Saccharin Acid
A2 Tween 80			E2 Salicin	G2 Sebacic Acid
A3 N-Acetyl-D-Galactosamine	C3 D-Glucuronic Acid		E3 Sedoheptulosan	G3 Succinamic Acid
A4 N-Acetyl-D-Glucosamine	C4 Glycerol		E4 Amino acids	G4 Succinic Acid
A5 N-Acetyl-D-Mannosamine	C5 Glycogen		E5	G5 Succinic Acid Mono-Methyl Ester
A6 Adonitol	C6 m-Inositol		E6 Stachynose	G6 N-Acetyl-L-Glutamic Acid
A7 Amygdalin	C7 2-Keto-D-Gluconic Acid		E7 Sucrose	G7 Alaninamide
A8 D-Arabinose	C8 a-D-Lactose		E8 D-Tagatose	G8 L-Alanine
A9 L-Arabinose			E9 D-Trehalose	G9 L-Alanyl-Glycine
A10 D-Arabitol			E10 Turanose	G10 L-Asparagine
A11 Arbutin			E11 Xylitol	G11 L-Aspartic Acid
A12 D-Cellobiose	D-Cellobiose		E12 D-Xylose	G12 L-Glutamic Acid
B1 a- Cyclodextrin	a- Cyclodextrin		F1 g-Amino-butyric Acid	H1 Glycyl-L-Glutamic Acid
B2 b-Cyclodextrin	b-Cyclodextrin		F2 Bromosuccinic Acid	H2 L-Ornithine
B3 Dextrin	Dextrin		F3 Fumaric Acid	H3 L-Phenylalanine
B4 I-Erythritol			F4 b-Hydroxy-butyric Acid	H4 L-Proline
B5 D-Fructose			F5 g-Hydroxy-butyric Acid	H5 L-Pyroglutamic Acid
B6 L-Fucose			F6 p-Hydroxyphenyl-acetic acid	Carboxylic acids
B7 D-Galactose			F7 a-Keto-glutaric Acid	
B8 D-Galacturonic Acid			F8 D-Lactic Acid Methyl Ester	
B9 Gentibiose			F9 L-Lactic Acid	
B10 D-Gluconic Acid			F10 D-Malic Acid	
B11 D-Glucosamine			F11 L-Malic Acid	
B12 a-D-Glucose			F12 Quinic Acid	
	D2 D-Mannose			H8 2-Amino Ethanol
	D3 D-Melezitose			H9 Putrescine
	D4 D-Melibiose			H10 Adenosine
	D5 a-Methyl-D-Galactoside			H11 Uridine
	D6 b-Methyl-D-Galactoside			H12 Adenosine-5'-Monophosphate
	D7 a-Methyl-D-Glucoside			
	D8 b-Methyl-D-Glucoside			
	D9 Palatinose			
	D10 D-Psicose			
	D11 D-Raffinose			
	D12 L-Rhamnose			



Phenotype MicroArrays™ (PMs)

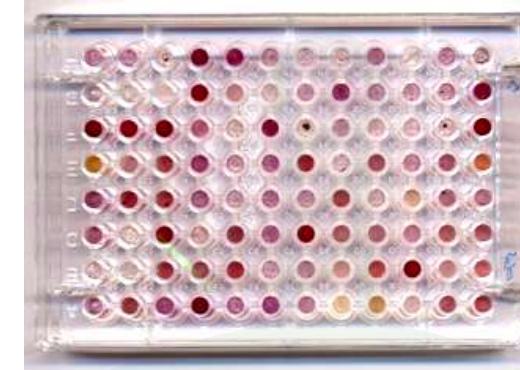
- Method based on commercial **microtiter plates** (or home made...)
- Involves **colour formation** by reduction of a tetrazolium dye + **turbidity** measurements (i.e. at 650 or 750 nm)
- Utilization of **95 separate sole carbon sources**

**redox dye + 95 different substrates/compounds
(freeze dried, reactivated with the inoculum)**

490 nm

+

650/750 nm



tetrazolium violet



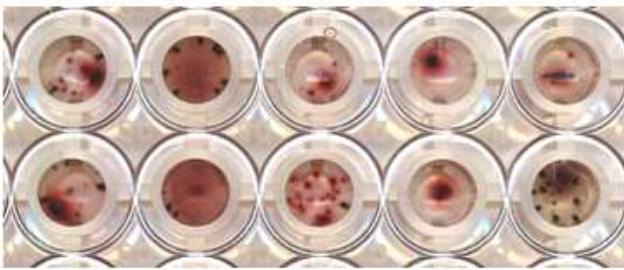
formazan

reduced by the action of succinate dehydrogenase, enabling the quantification of respiration

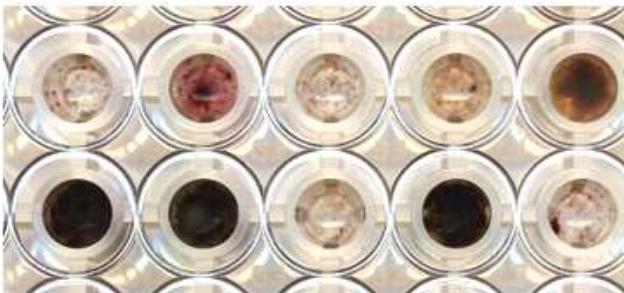
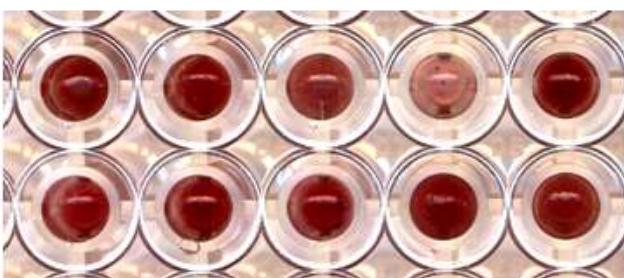
**Respiration = substrate use
(NADH formation)**

= Colour formation in the wells

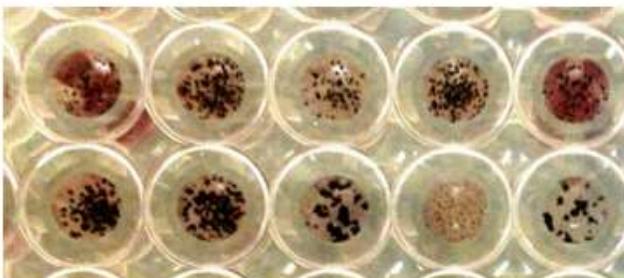
Formazan dyes are artificial chromogenic products obtained by reduction of tetrazolium salts by dehydrogenases and reductases

**a**

- Biolog™ Redox Dye **Mixes D, E, and F**
- 2,3,5-Triphenyltetrazolium chloride (**TTC**, Tetrazolium chloride)
- 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium (**INT**, Iodonitrotetrazolium chloride)
- 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (**MTT**, Thiazolyl blue tetrazolium bromide)

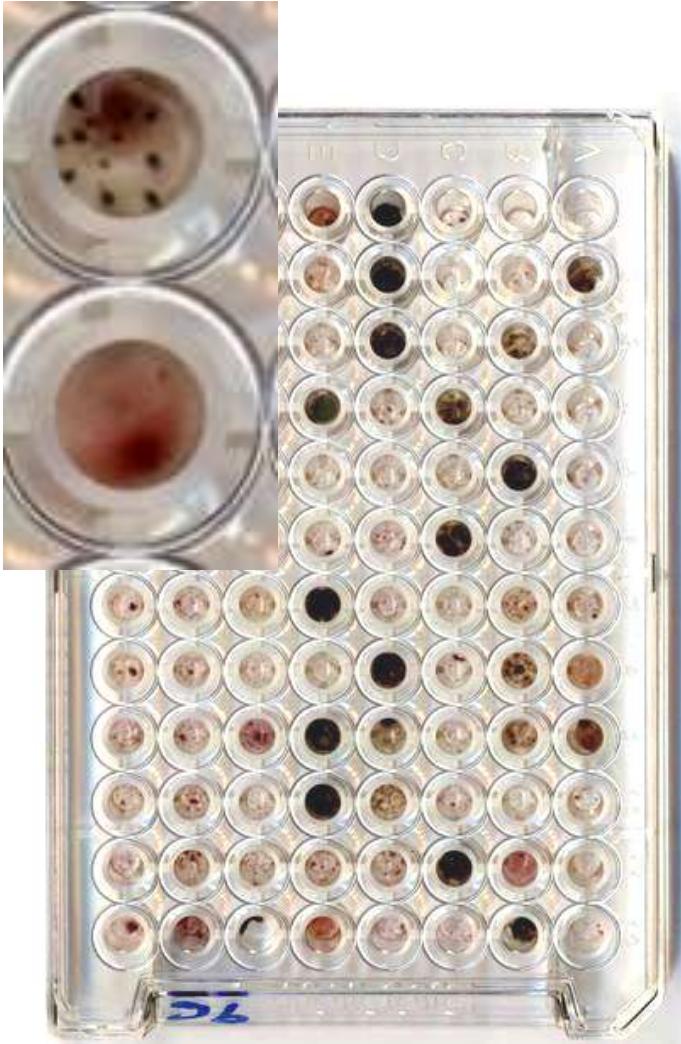
b**c**

- *Each fungal species can react differently to the dyes*
- *Toxic and nutritional effects of dyes affect the results of metabolic profiling*

d

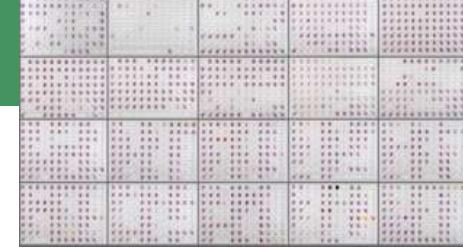
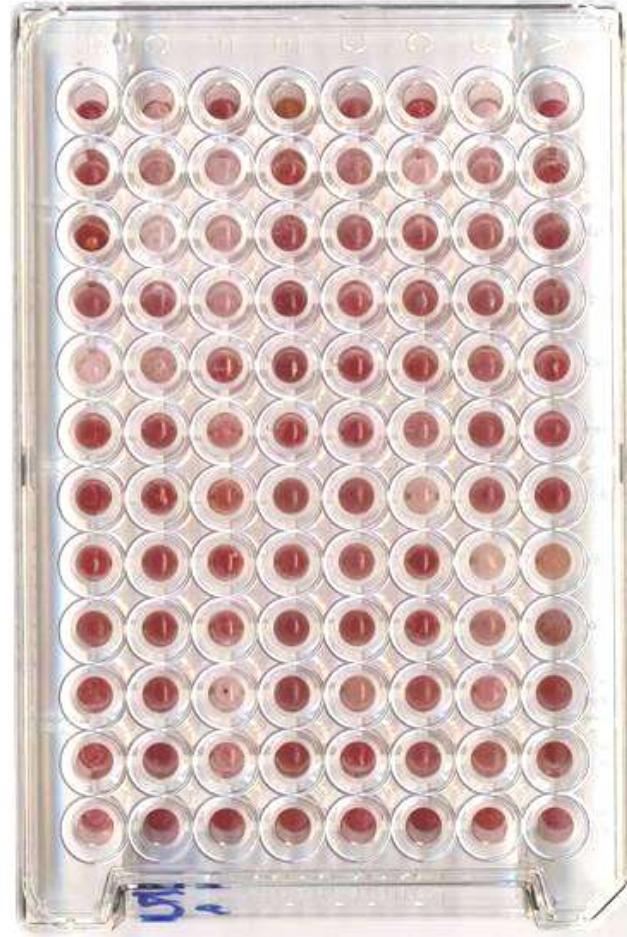


- Tips and tricks + open questions on PM use with filamentous fungi



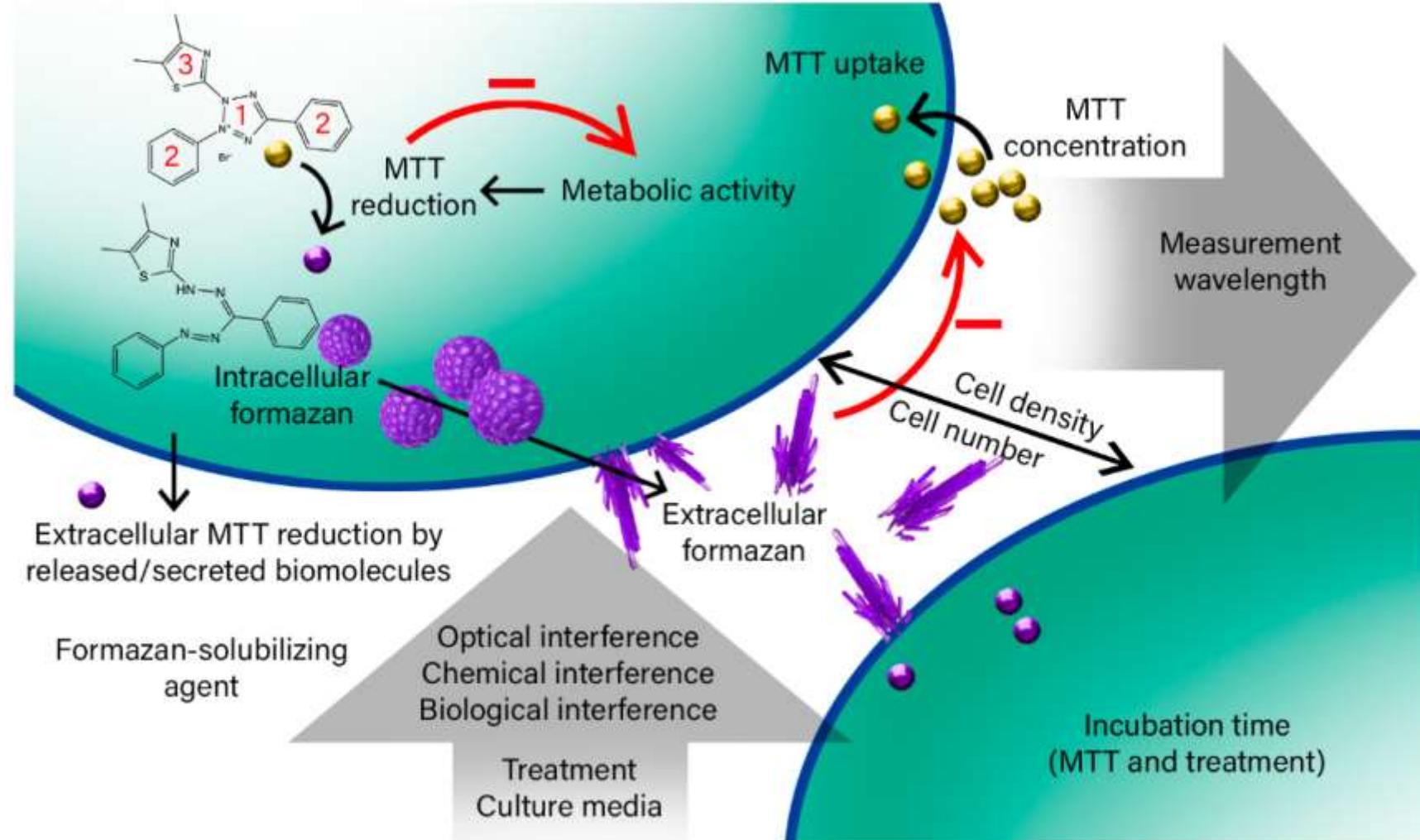
Some fungi have strongly pigmented mycelium or fruiting structures

Some fungi do not produce much colour (respiration) but produce biomass





MTT reagent (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide)



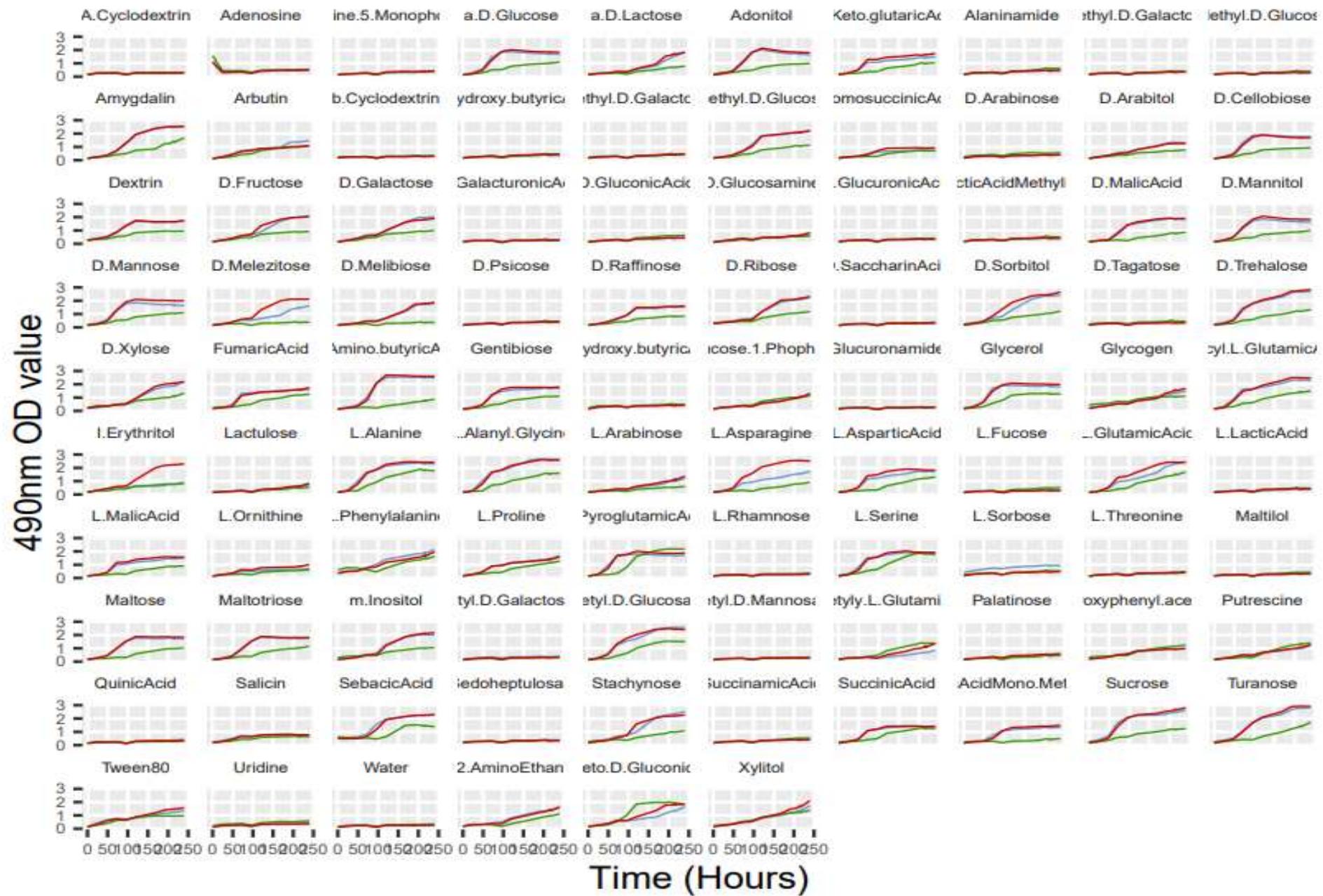
Ghasemi, M.; Turnbull, T.; Sebastian, S.; Kempson, I. The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *Int. J. Mol. Sci.* **2021**, *22*, 12827.
<https://doi.org/10.3390/ijms222312827>



Factors **affecting the final optical density (OD) measurements** in the formazan assay.

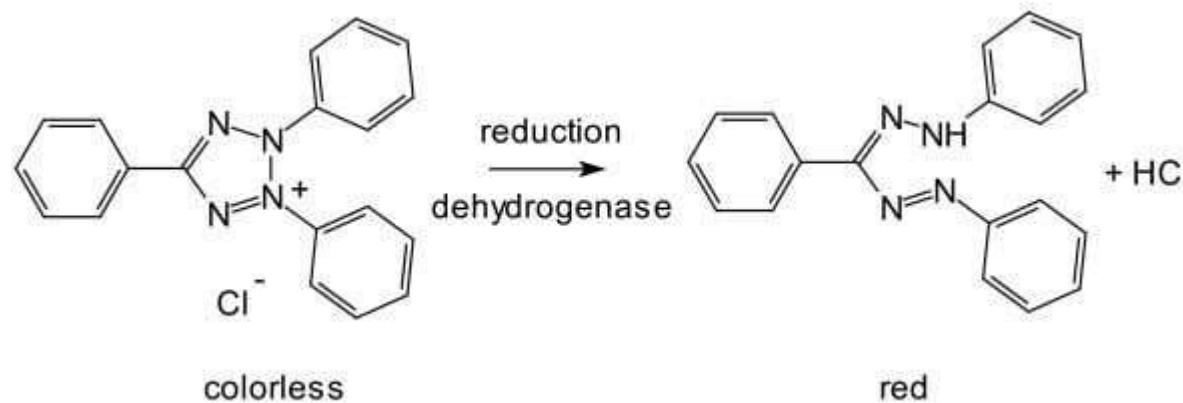
- concentration of formazan reagent and **the proportion that actually enters the cell**,
- cellular metabolic activity (which is highly dependent on a **multitude of variables** including treatments, biological effect of culture media, cell density, and impedance of cell metabolism due to **toxic effects of MTT**),
- chemical interference such as **abiotic reduction of MTT** by culture media, the tested treatment, or released cellular content,
- optical interference by all the background components, time of incubating cells with MTT reagent and/or tested treatment, and ultimately **the optical measurement**.

Ghasemi, M.; Turnbull, T.; Sebastian, S.; Kempson, I. The MTT Assay: Utility, Limitations, Pitfalls, and Interpretation in Bulk and Single-Cell Analysis. *Int. J. Mol. Sci.* **2021**, *22*, 12827. <https://doi.org/10.3390/ijms222312827>





Formazans have a **structure with 4 nitrogen (N) atoms: [-N=N-C(R)=N-NH-]** and are closely related to azo dyes with amine nitrogen (**-N=N-**) (Sen, et al. 2016). Redox dyes could be toxic to some fungal isolates.

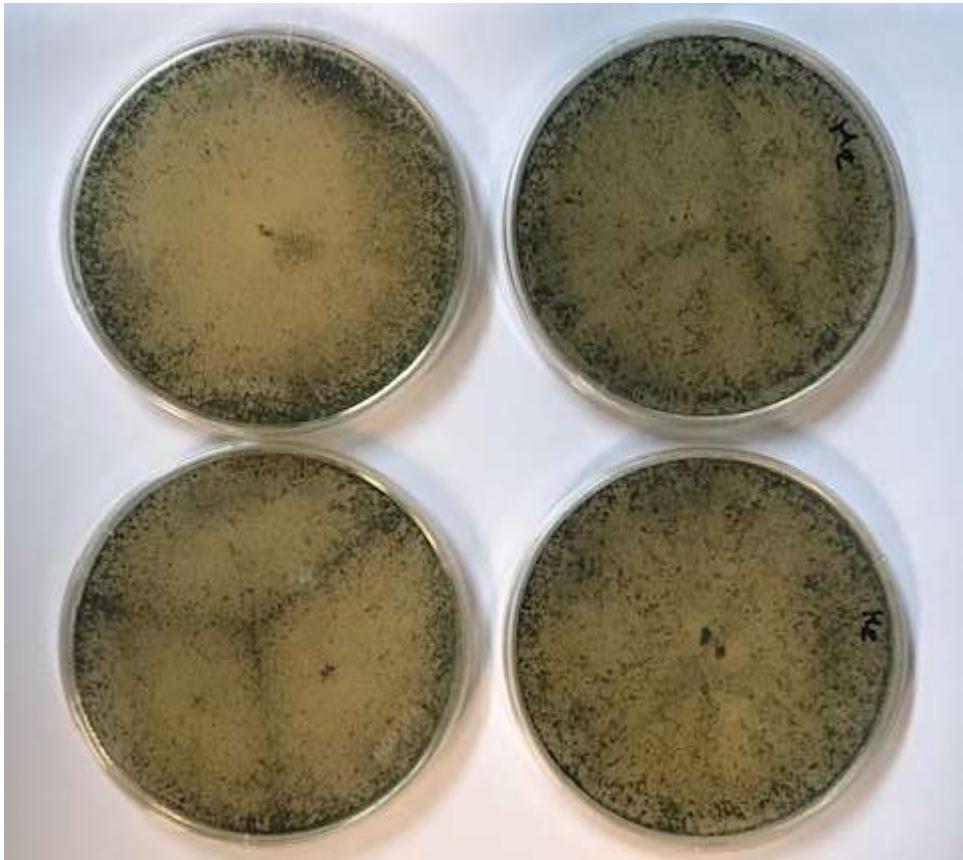


Fungi can degrade complex organic compounds with extracellular enzymes such as laccase, manganese peroxidase and lignin peroxidase, an activity applied precisely at the industrial level to **decolourise and detoxify effluents treated with azo dyes** (e.g., from textile industries) (Sen et al. 2016).

Sen SK, Raut S, Bandyopadhyay P, Raut S (2016) Fungal decolouration and degradation of azo dyes: A review. *Fungal Biol Rev* 30:112-133. doi: 10.1016/j.fbr.2016.06.003



Extremely different condition for inoculum preparation and incubation according to the fungal species/biology





Protocol for ID (different from what needed in Phenotype studies)

- 1) Grow a pure culture of a fungus on a **2% Malt Extract Agar plate** (Biolog part number 71106 for pre-poured plates) until enough conidiation is present to prepare a suspension.
- 2) **Swab** the conidia from the surface of the agar plate, and suspend to a specified density in **FF Inoculating Fluid** (Biolog part number 72106).
- 3) Pipet **100 µl of suspension** into each well of the FF MicroPlate (Biolog part number 1006).
- 4) Incubate the FF MicroPlate at **26° C for 24 – 96 hours**.
- 5) Read the MicroPlates using the Biolog MicroStation™ Reader **beginning 24 hours after inoculation**



EXCALIBUR

The Fungi identification Biolog system

Biolog Filamentous Fungi ID System

- Biolog uses a unique single dye, single color chemistry
- This chemistry can be used to measure metabolism of any carbon chemical
- Analysis of both colour development (OD490) and turbidity (OD750) provides for accurate identifications to the species level usually in two to three days
- Over 700 Species in current Database

Special Thanks to Dr John Bissett, PhD Agriculture Canada.

BIOLOG

Andre Chouankam

The video is available on Vimeo:
<https://vimeo.com/537359117>



EXCALIBUR

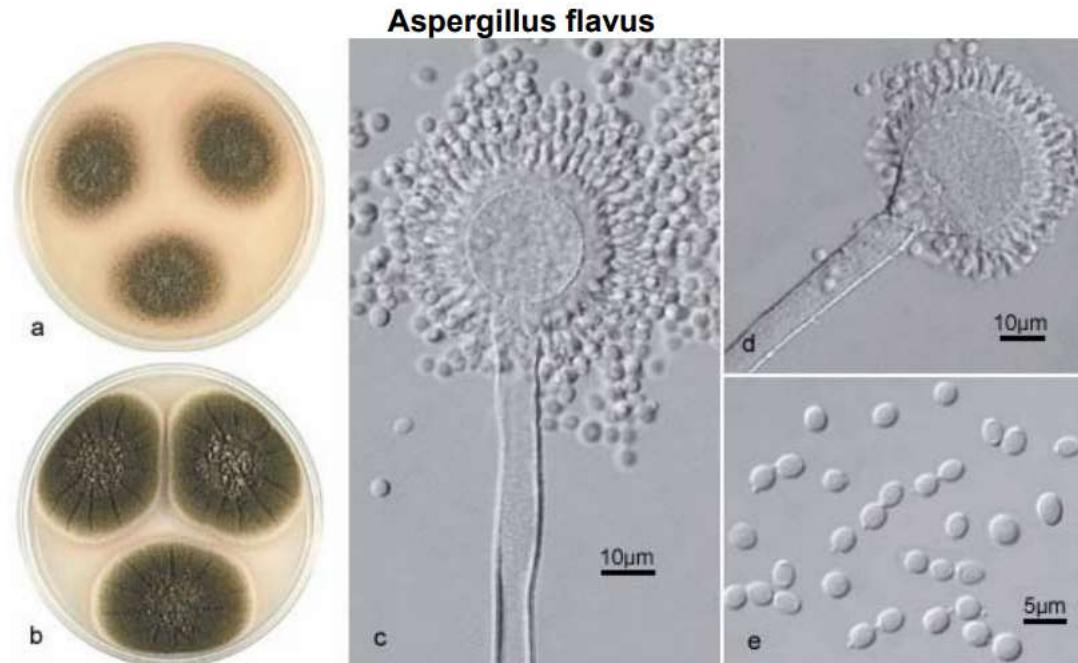
The Fungi identification Biolog system

BIOLOG

Fungi Identification Test Panel

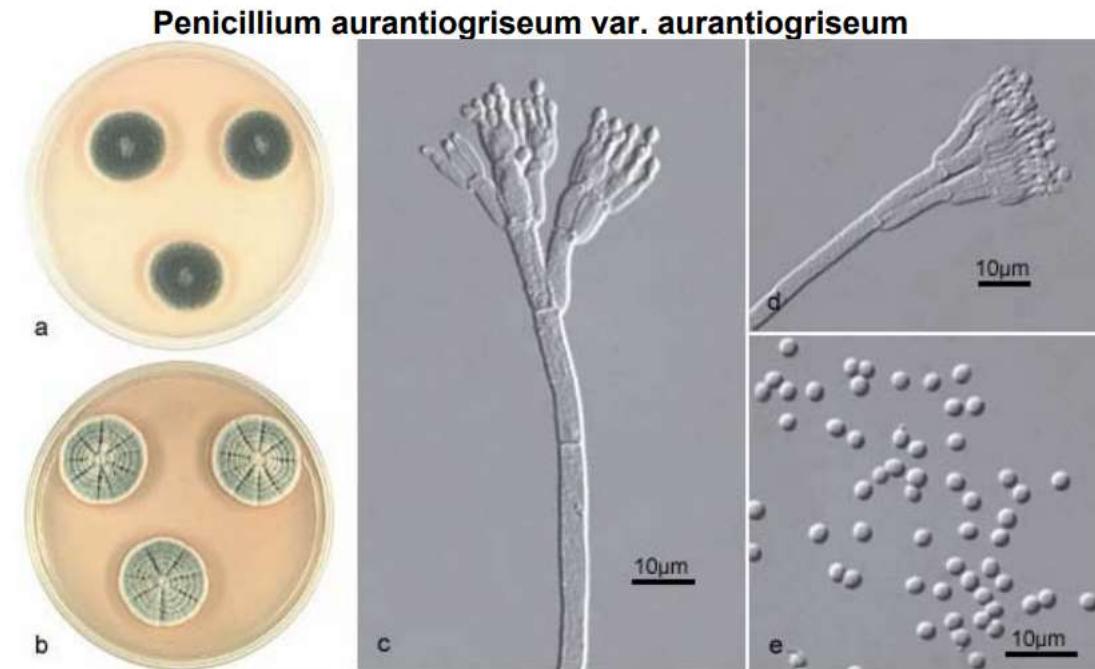
FF DATABASE PHOTO LIBRARY

An important added feature of the FF Database is a unique library of macroscopic and microscopic fungal photographs of the fungi to aid in the identification of unknown organisms. This tool can be used to confirm the identification of unknown organisms by providing a visual and morphological verification of each species identified. The following two blocks of photos and captions are examples taken directly from the database software. Please note that the macroscopic photos are full color images.



(a) MA, 7 days; (b) CYA, 7 days; (c,d) conidiophores; (e) conidia.
(a-e) CBS 282.95.

Useful photo library associated to the ID software



(a) MA, 7 days; (b) CYA, 7 days; (c,d) conidiophores; (e) conidia.
(a,b) DAOM 216724; (c-e) CBS 324.89.



Standardise?



Malt agar 2%
(18 g agar, 20 g Malt Extract
OXOID, **no peptone**, 1 L
deionised water)

Temperature 25°

Time zero very important

Incubation up to 2 weeks



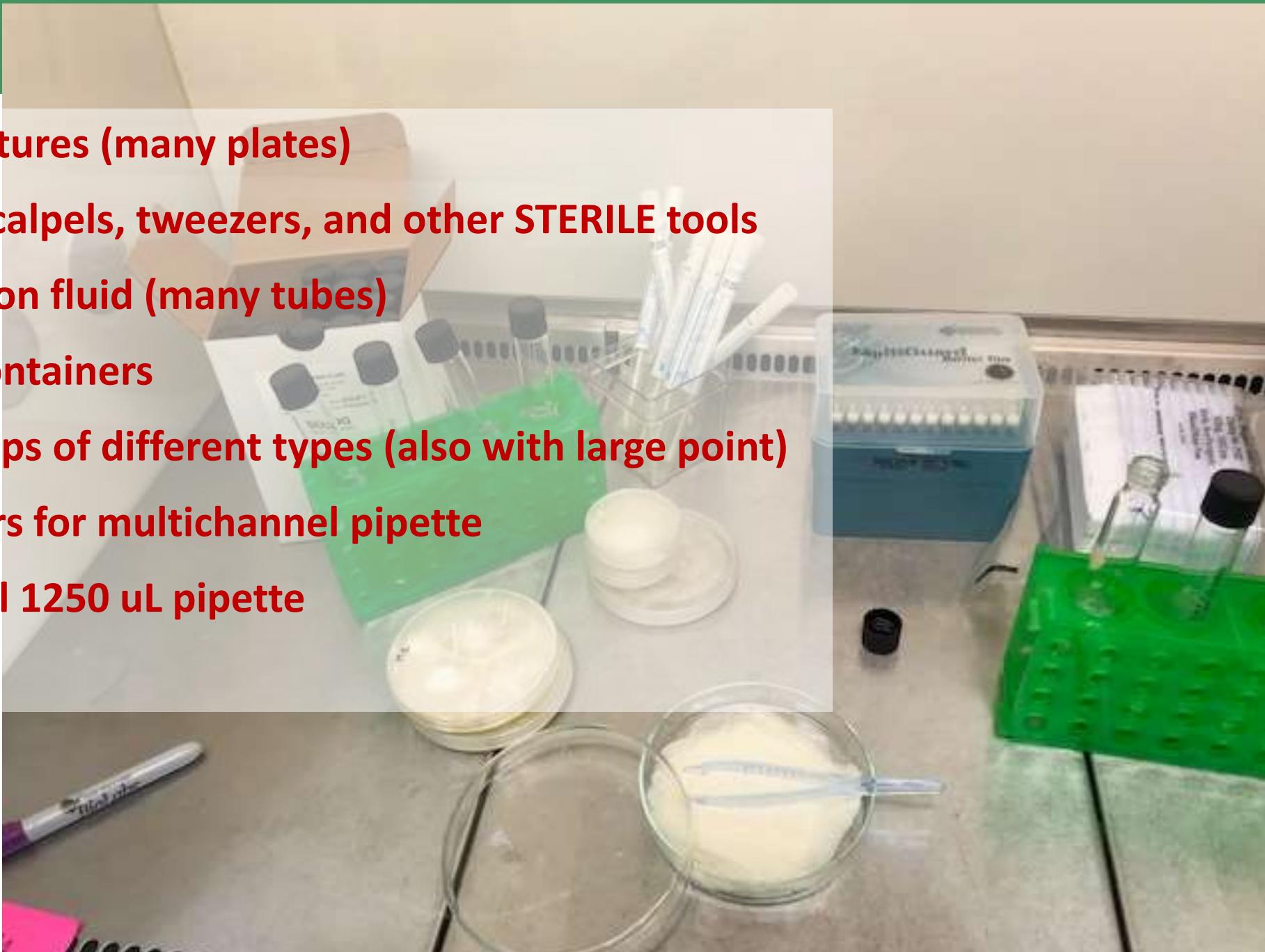
About peptone in MEA: Odds F, Hall C, Abbott A: Peptones and mycological reproducibility. Med Mycol. 1978, 16 (4): 237-246. 10.1080/00362177885380341

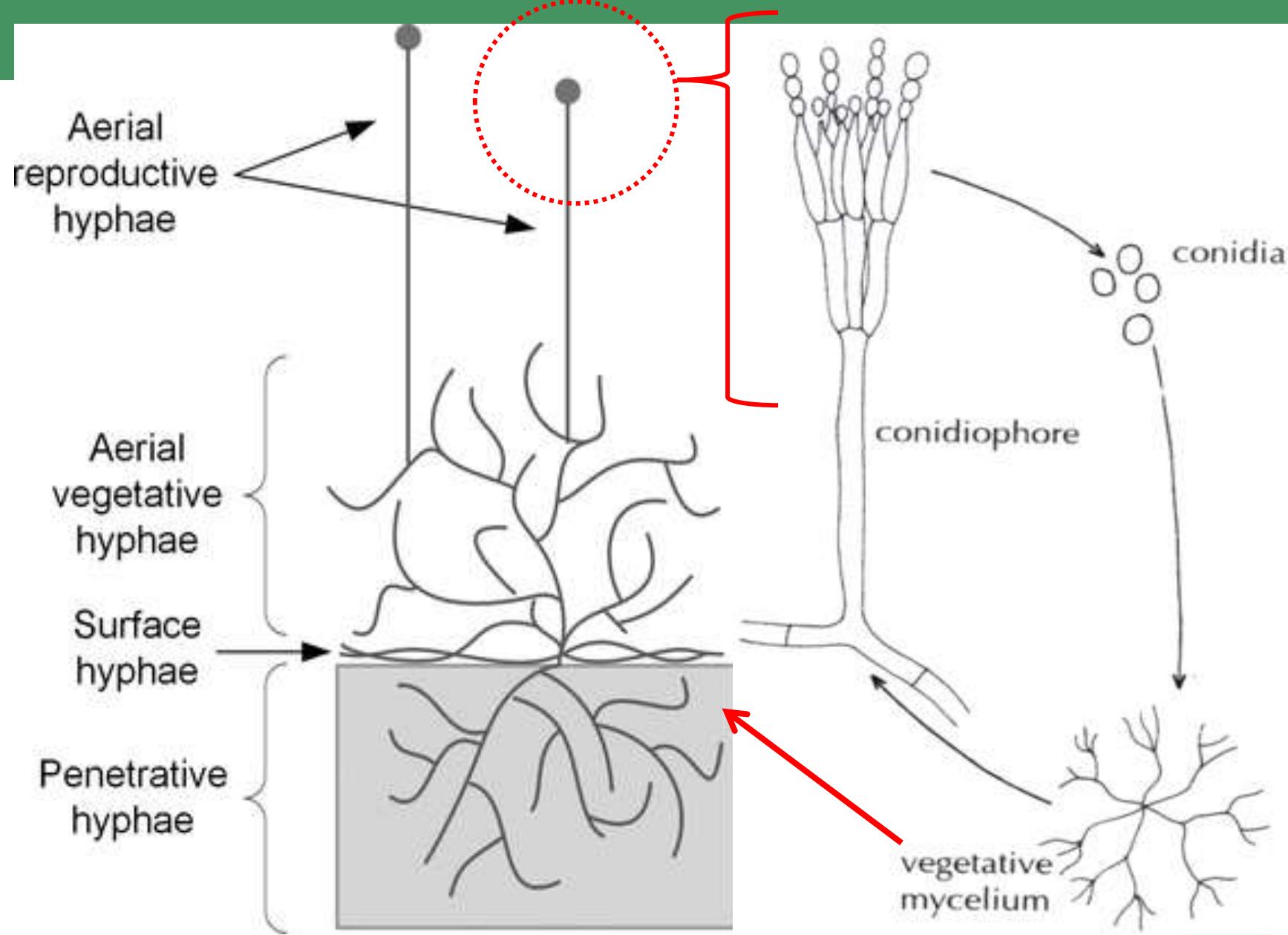


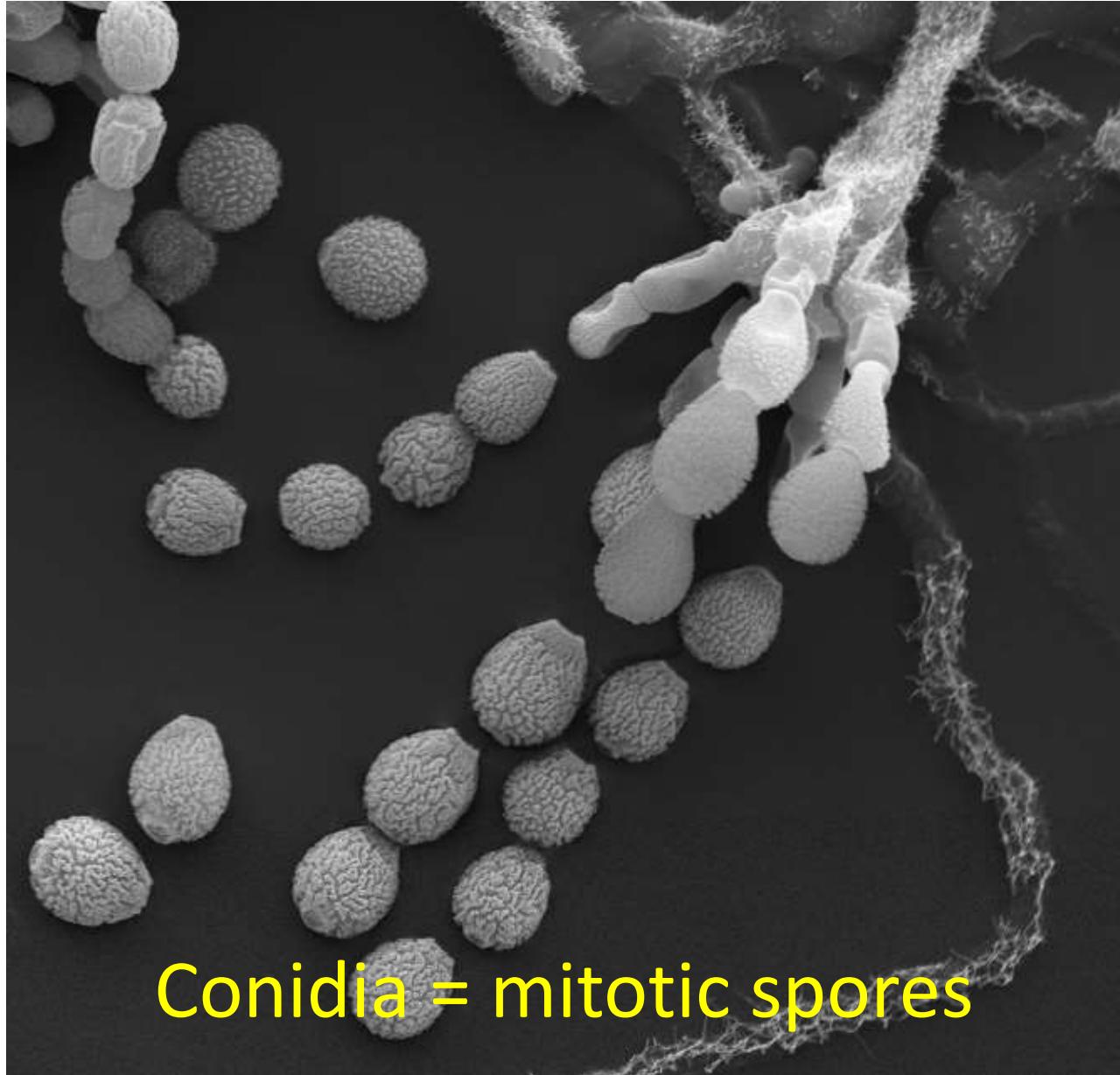
The inoculating fluids can **differ** according to arrays and substrates: PM4-IF – Tween 40, Phytigel and **D-glucose**, PM9-IF – Tween 40, Phytigel, D-glucose and additive solution containing **yeast nitrogen base**.
The FF-IF: **Tween 40** and **Phytigel** (wells already contain yeast nitrogen base)

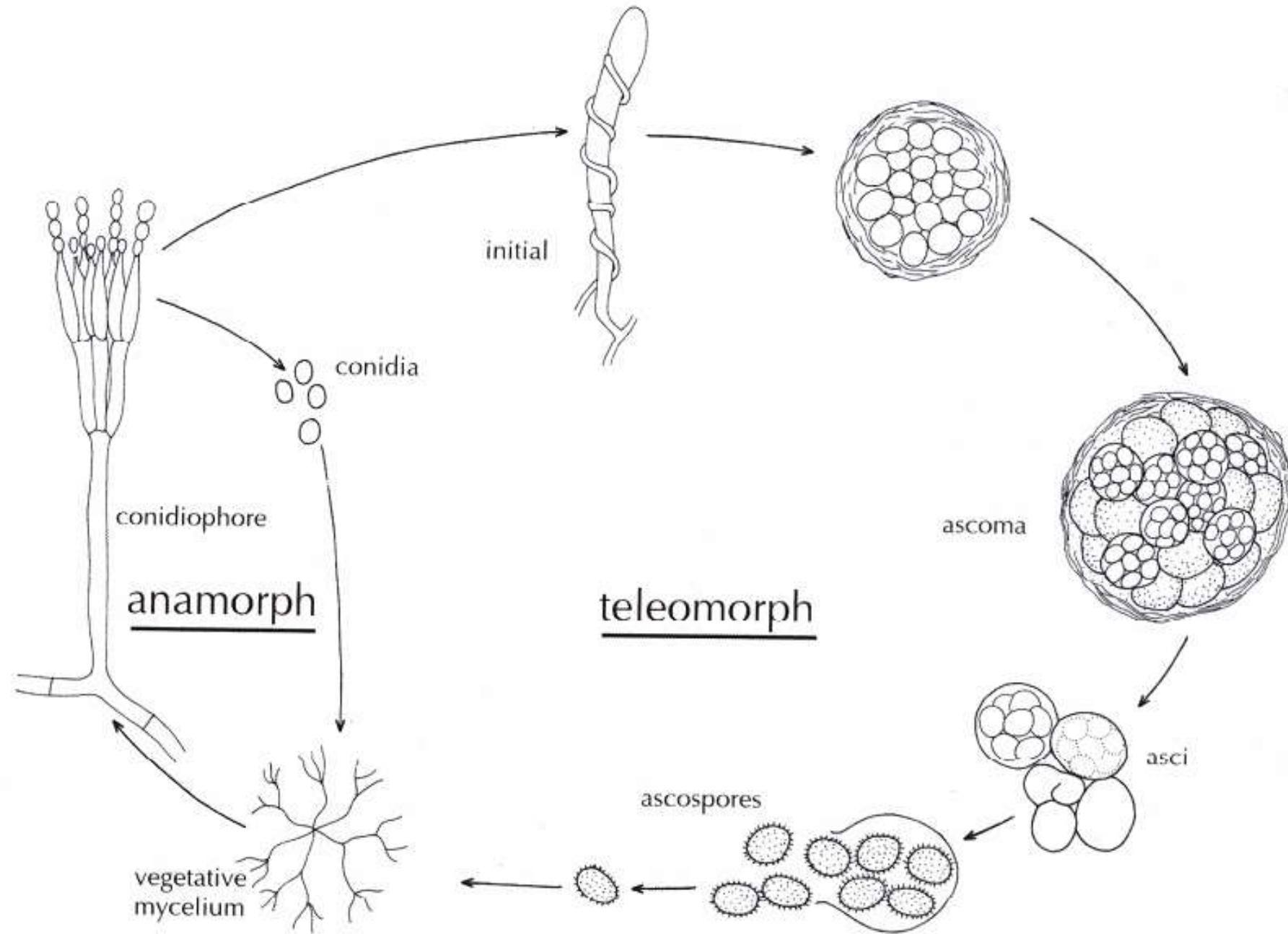


- Fresh cultures (many plates)
- Swabs, scalpels, tweezers, and other **STERILE** tools
- Inoculation fluid (many tubes)
- Sterile containers
- Pipette tips of different types (also with large point)
- Reservoirs for multichannel pipette
- 8 channel 1250 uL pipette
- racks



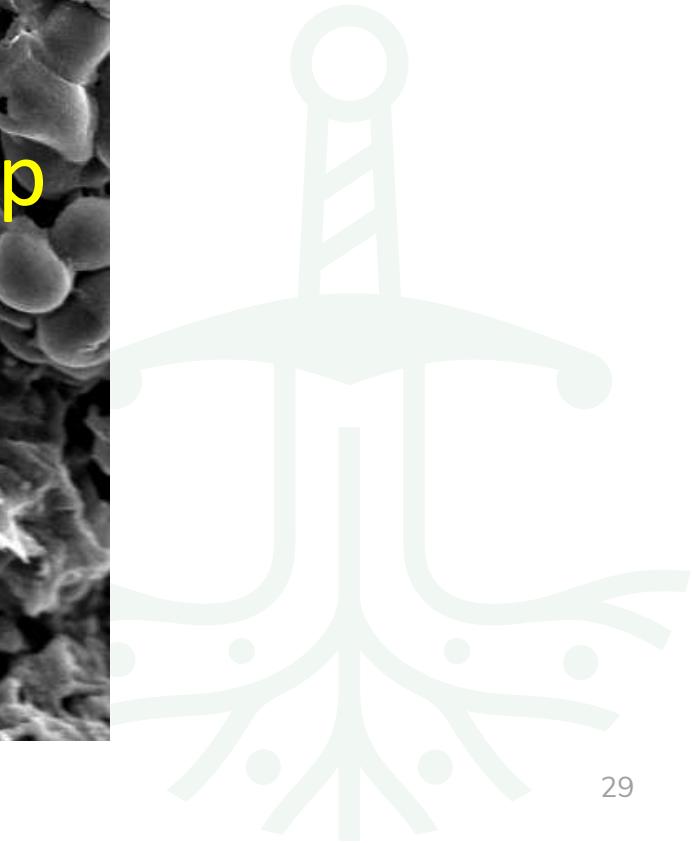
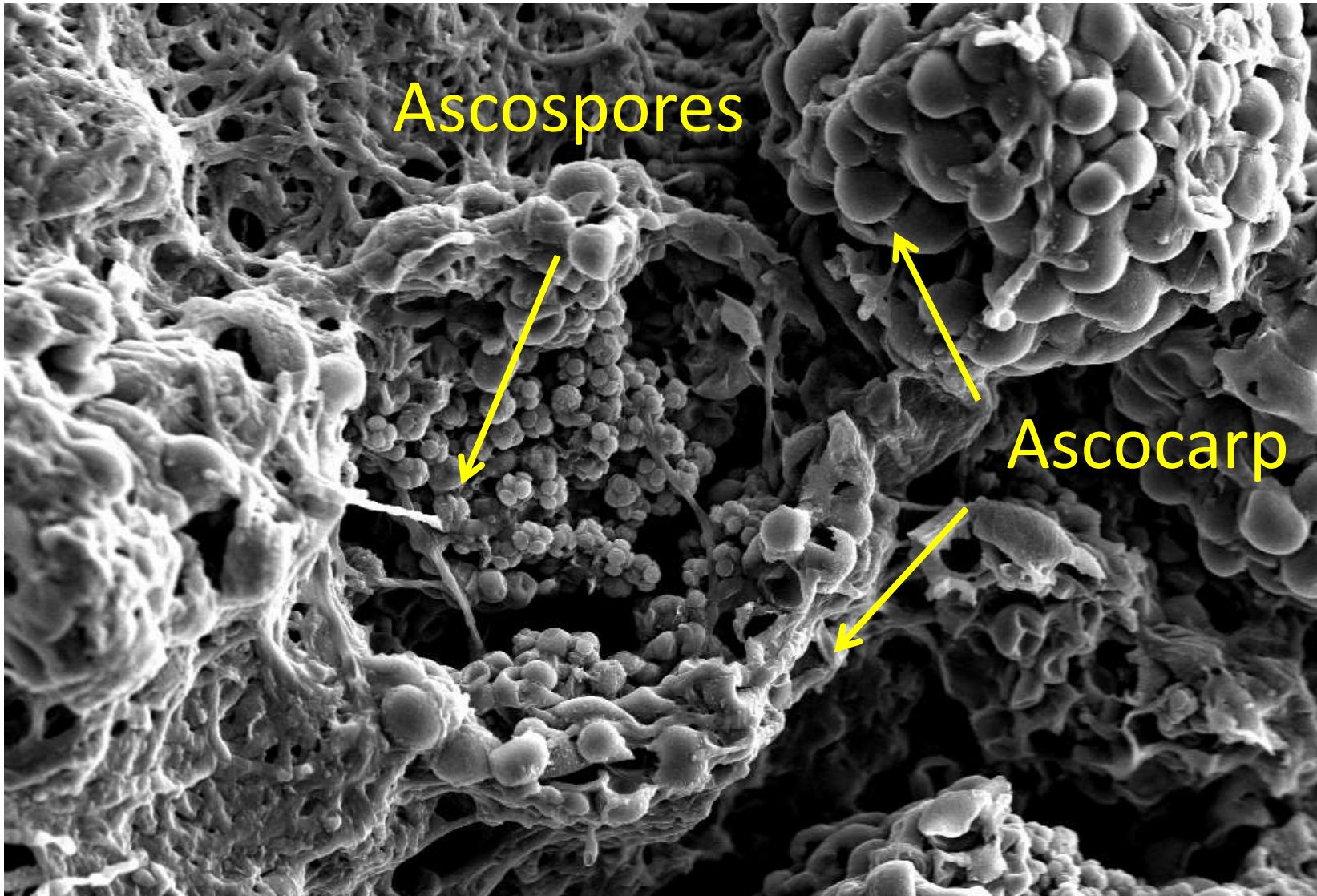




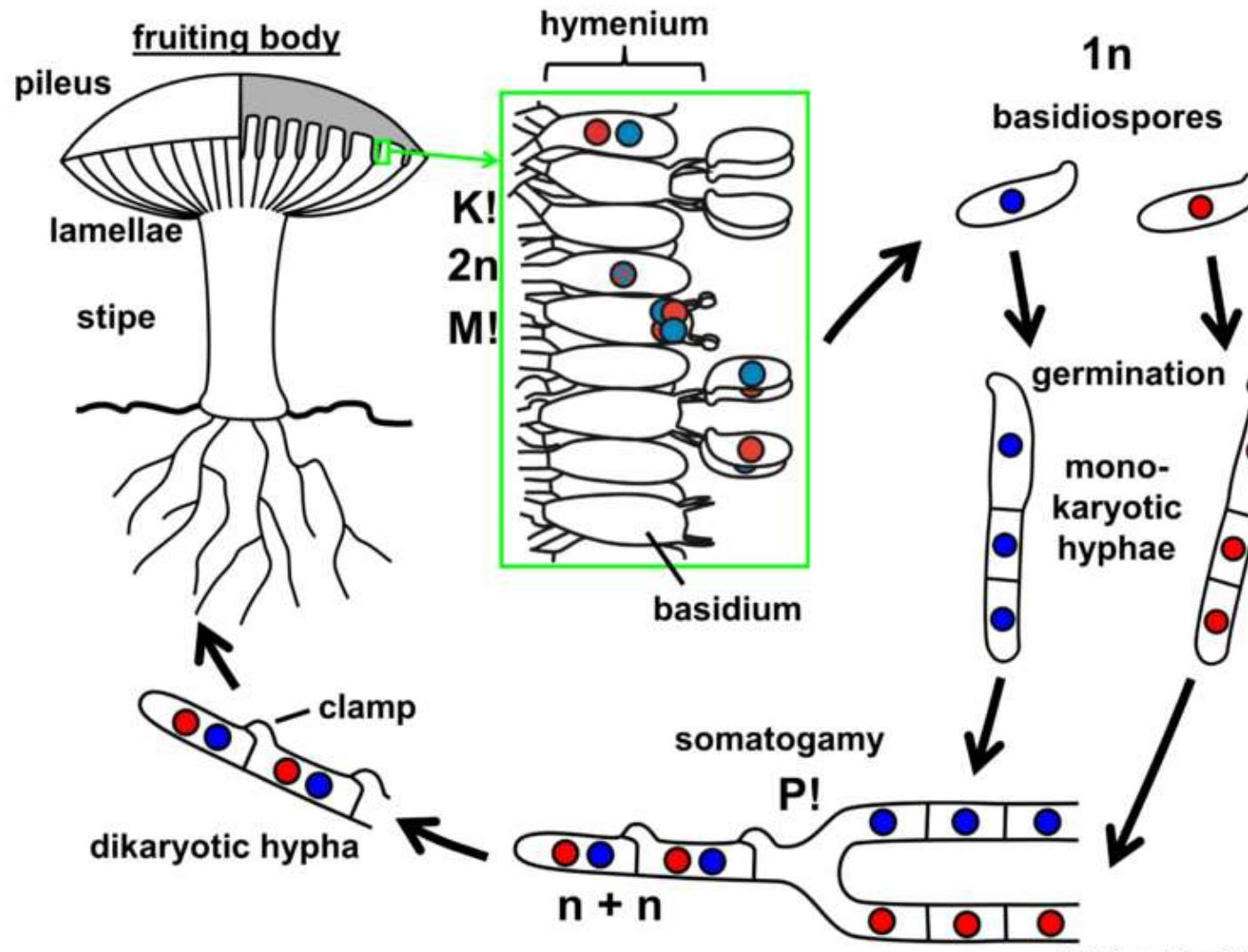




Teleomorph (sexual reproduction)







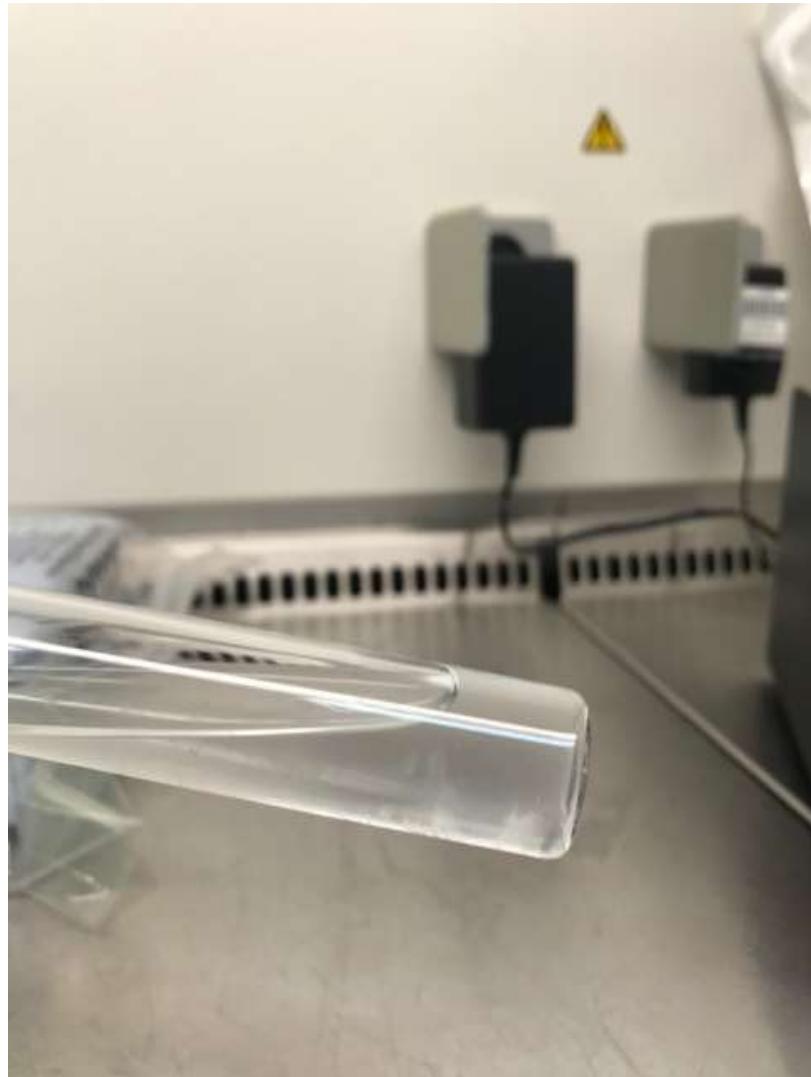
© M. Piepenbring, CC BY-SA





Liquid cultures?





No spores?
Use mycelium to perform
the inoculum

You need to disrupt
hyphae

BUT:

It can be too stressed to
give consistent results





Filtration with a sterile gauze

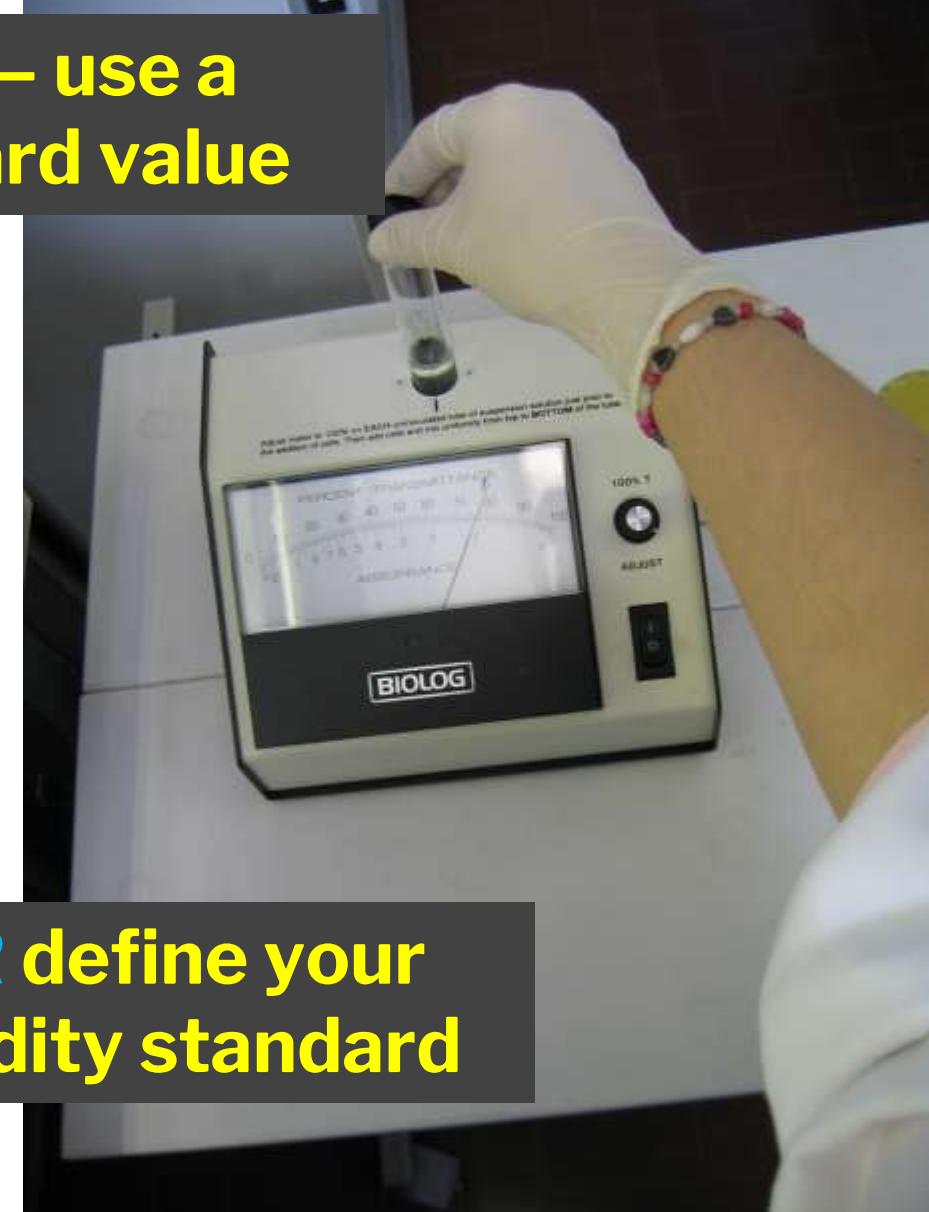




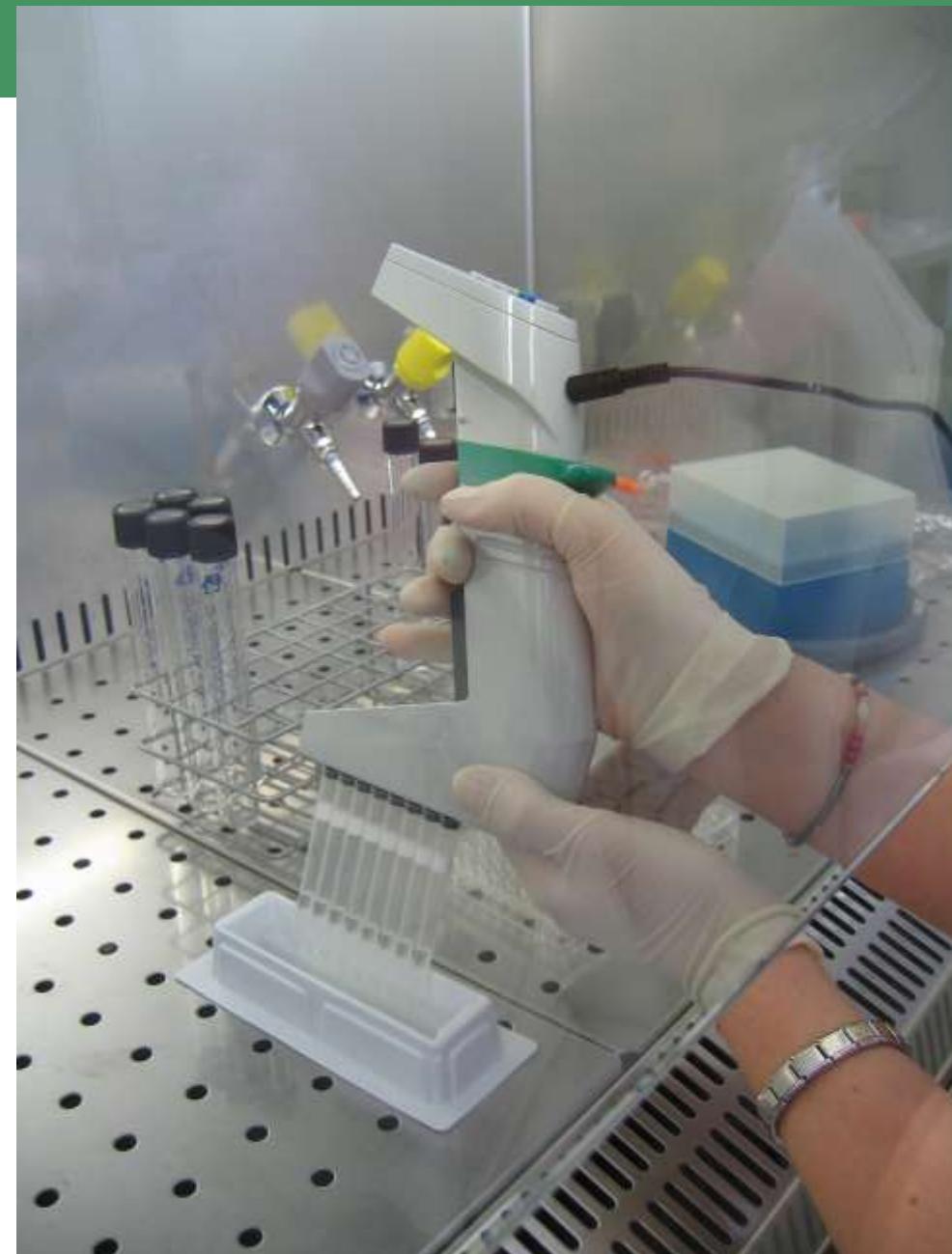
EXCALIBUR

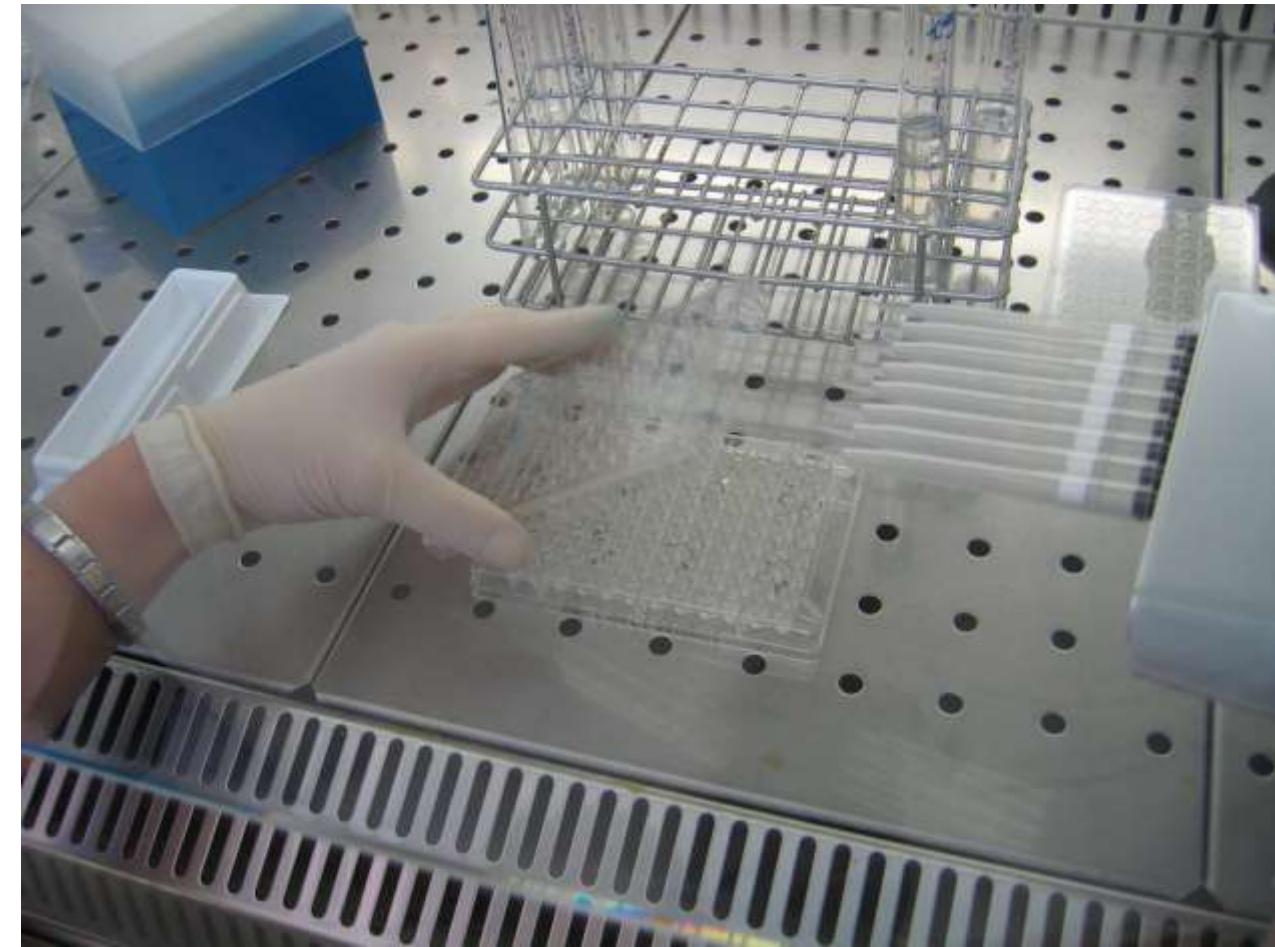
https://microbeonline.com/preparation-mcfarland-turbidity-standards/?utm_content=cmp-true

**Be consistent – use a
turbidity standard value**



**FF standard OR define your
McFarland turbidity standard**







- Tips and tricks + open questions on PM use with filamentous fungi



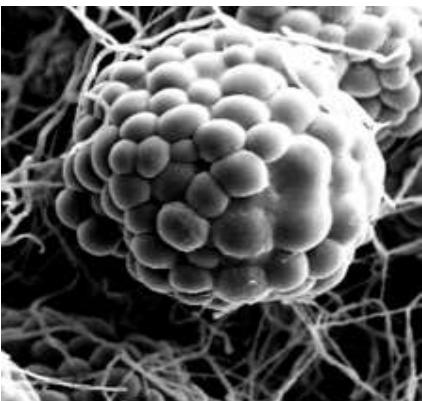
Spores

↑ Comparable
results?

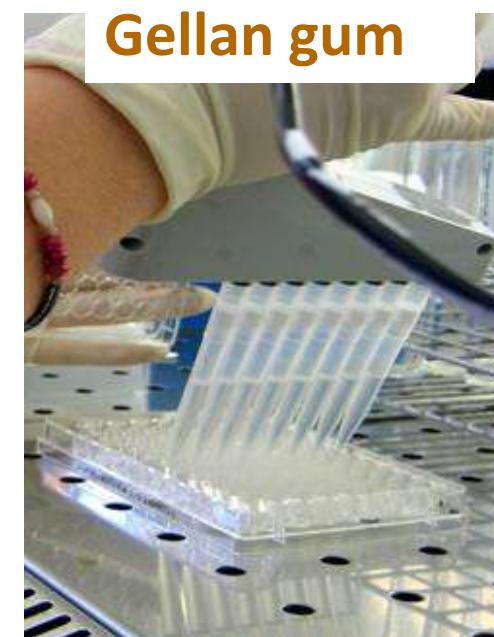


Mycelia
(Blender needed)

Filtering



Fruiting closed
structures
(Pestel needed)



Tween 80 +
Gellan gum





Impact of volatiles and interference between adjacent wells during incubation

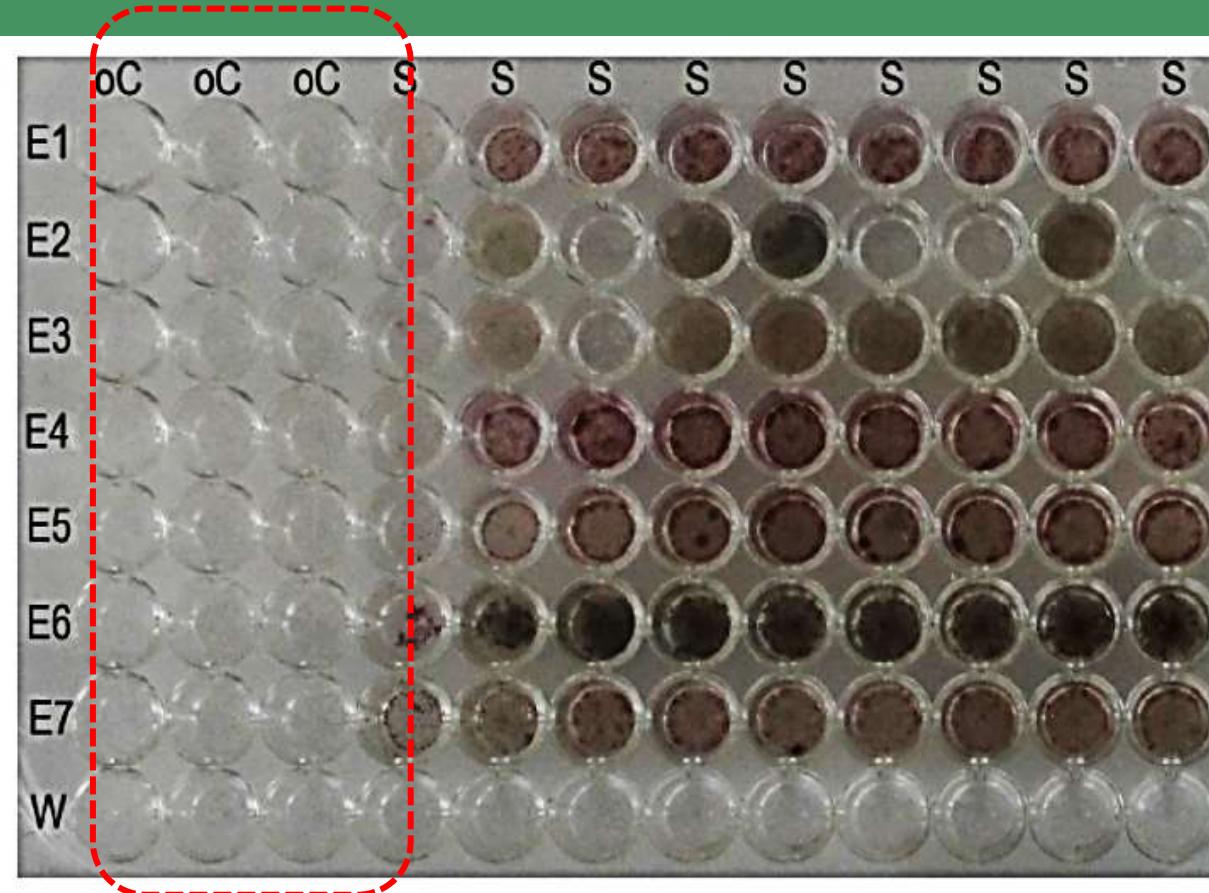
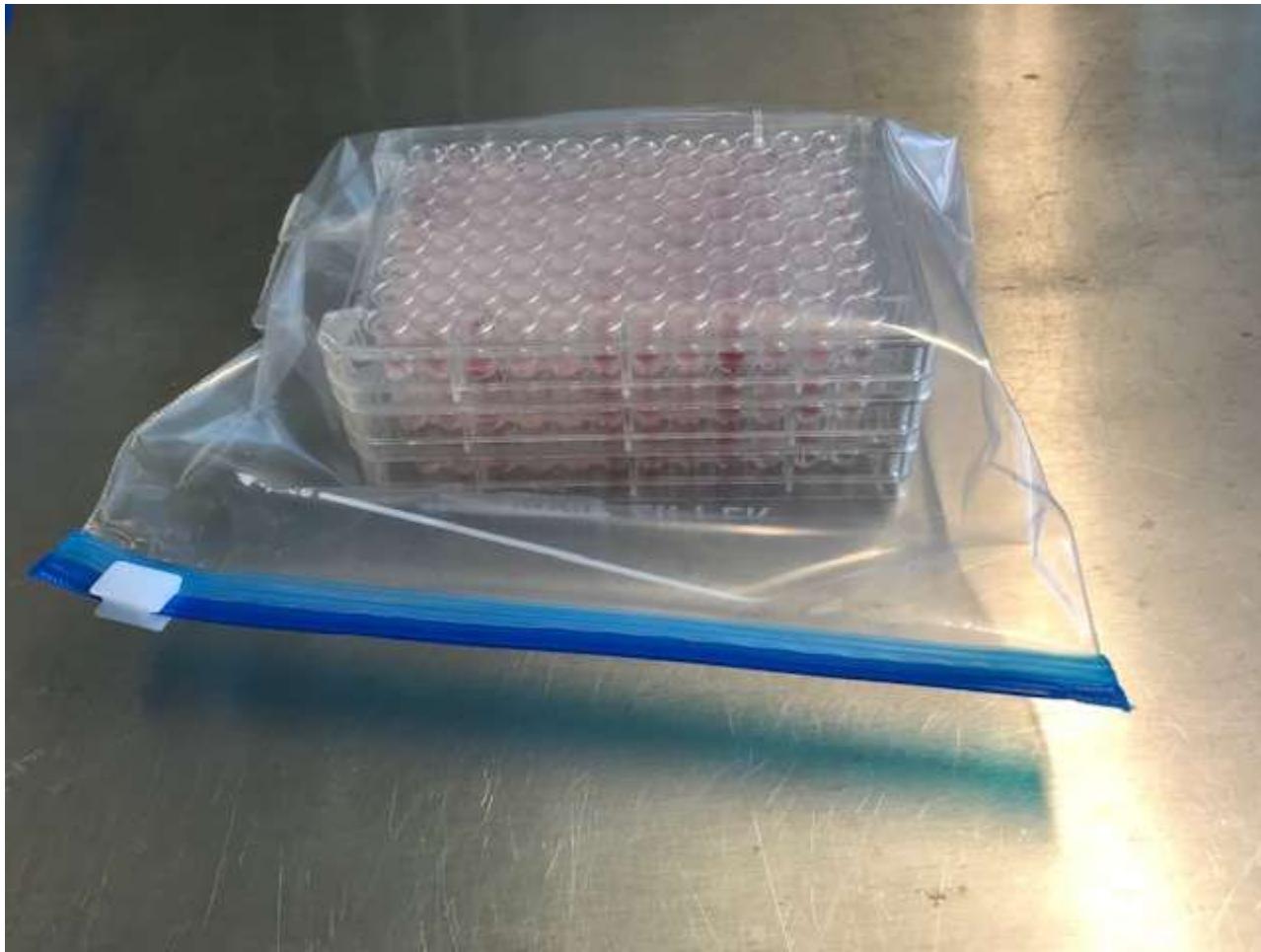


FIGURE 3 | Effect of volatile inhibitory substances on adjacent cells. All the wells of the plate were supplemented with the standard basal medium. In each row, cell suspension of one endophyte strain was added, excluding the last row one where water was added. In the first three columns, the phenolic compound o-cresol was incorporated to a final concentration of 1 g/L. Note that the fungi in the fourth and fifth columns were partially inhibited in their growth. E = endophyte; W = water; S = standard basal medium; oC = standard basal medium supplemented with o-cresol.

Blumenstein K, Macaya-Sanz D, Martín JA, Albrechtsen BR and Witzell J (2015) Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes. *Front. Microbiol.* 6:1033. doi: 10.3389/fmicb.2015.01033

Also, read paragraph «Technical Challenges with PM Method»



Always protect from dehydration (ventilated incubators are dangerous) – **BUT** avoid anoxia
AND consider the effect of VOCs





- Tips and tricks + open questions on PM use with filamentous fungi



**viscoelastic change of gellan gum solutions:
remarkable by the addition of K⁺, Na⁺, Ca²⁺, Mg²⁺.**



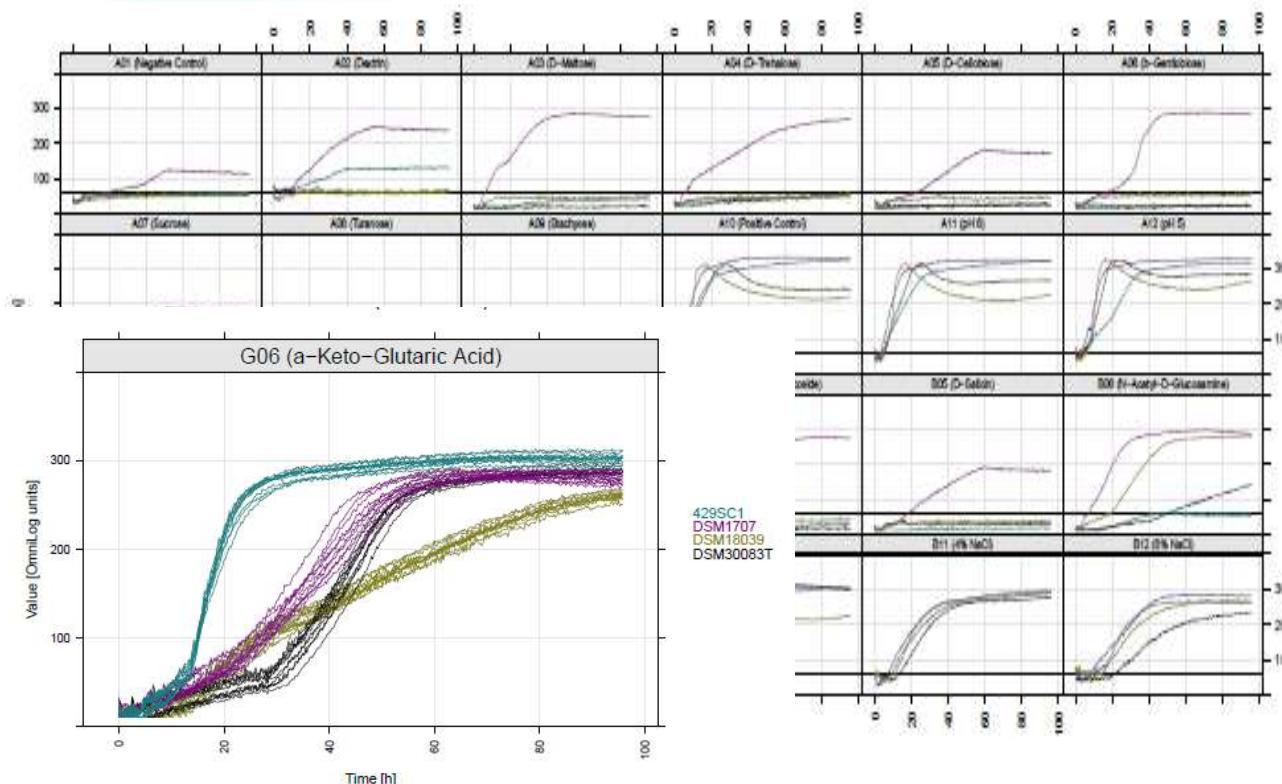
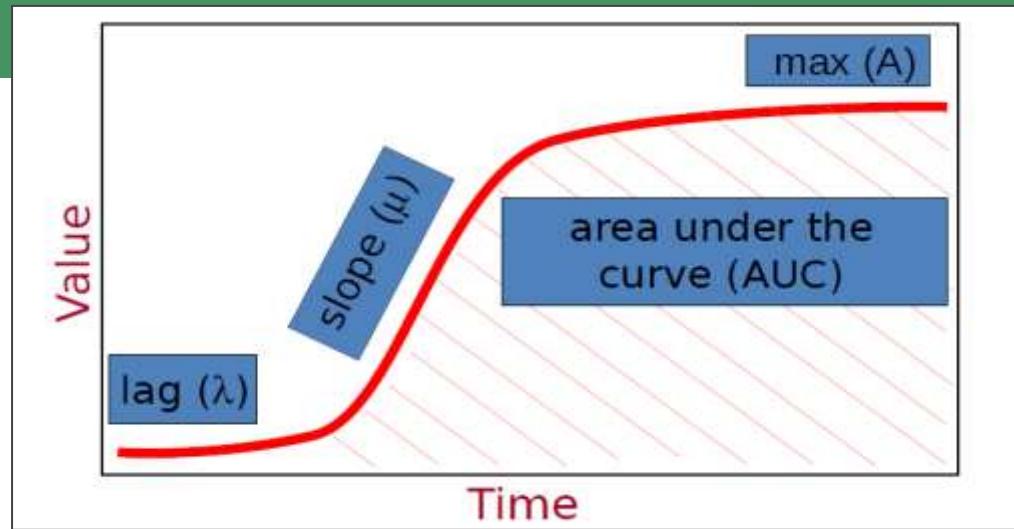
Halophilic species

They don't grow... unless...





96 wells, OD750 + OD490, PM plates, time, replicates



Biological information stored in the raw curve kinetics - can be summarized in the curve parameters **lag phase**, **steepness of slope**, maximum curve height and area under the curve (AUC).

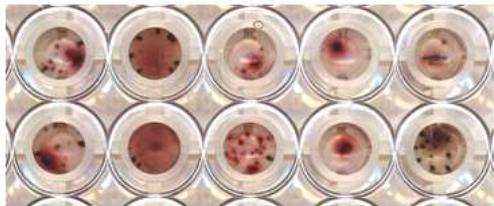
Fitting splines rather than growth models is more robust for these data (Vaas et al., 2012).



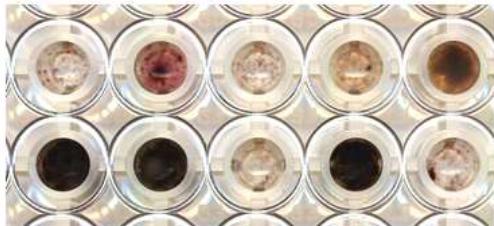
EXCALIBUR

Effect of carbon/nitrogen sources on phenotype

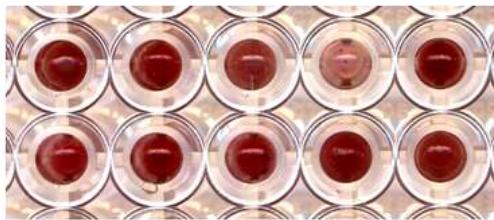
a



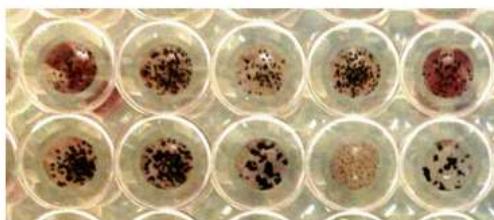
b



c

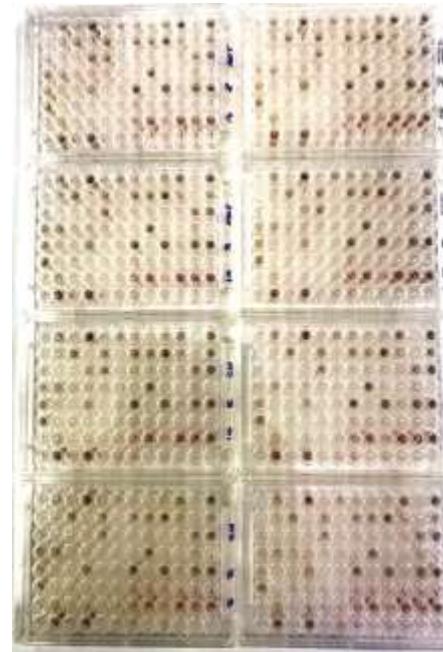


d

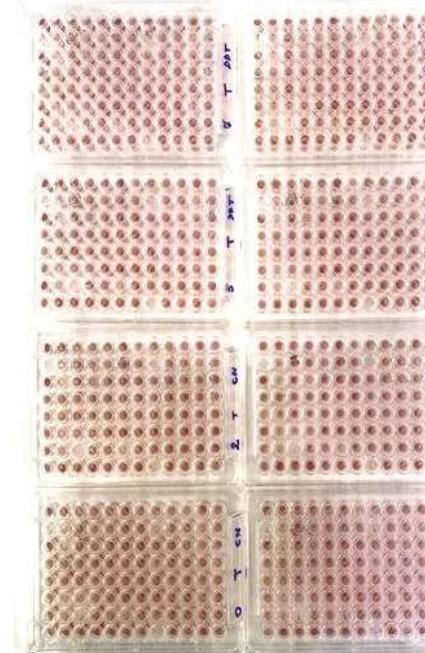


Repeatability

Rhizopus stolonifer



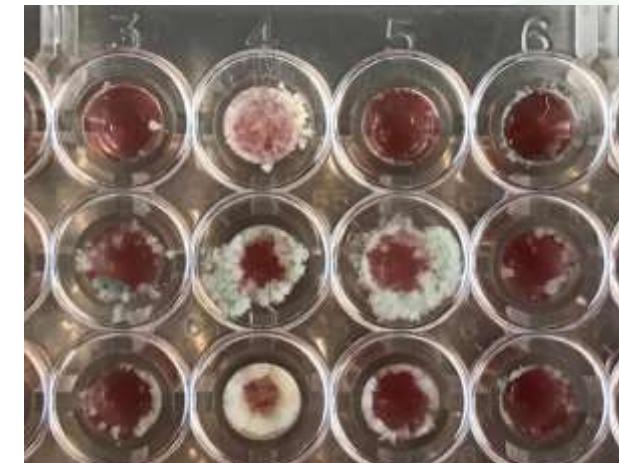
Trichoderma harzianum



Impact of carbon/nitrogen sources or chemicals on sporulation, pigmentaton or testing at different T°, light wavelenght

https://www.biolog.com/wp-content/uploads/2020/04/PM_Maps_111419.pdf

Pinzari et al. (2016) Phenotype MicroArray™ system in the study of fungal functional diversity and catabolic versatility, Research in Microbiology, 167: 710-722





Respiration (490 nm)/Biomass (750 nm) = metabolic quotient (effort/stress)

EXCALIBUR

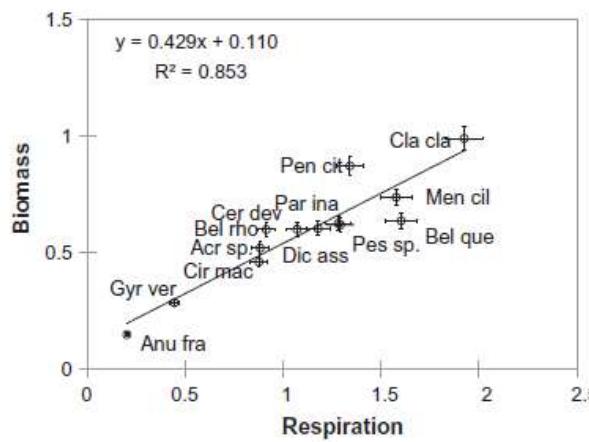
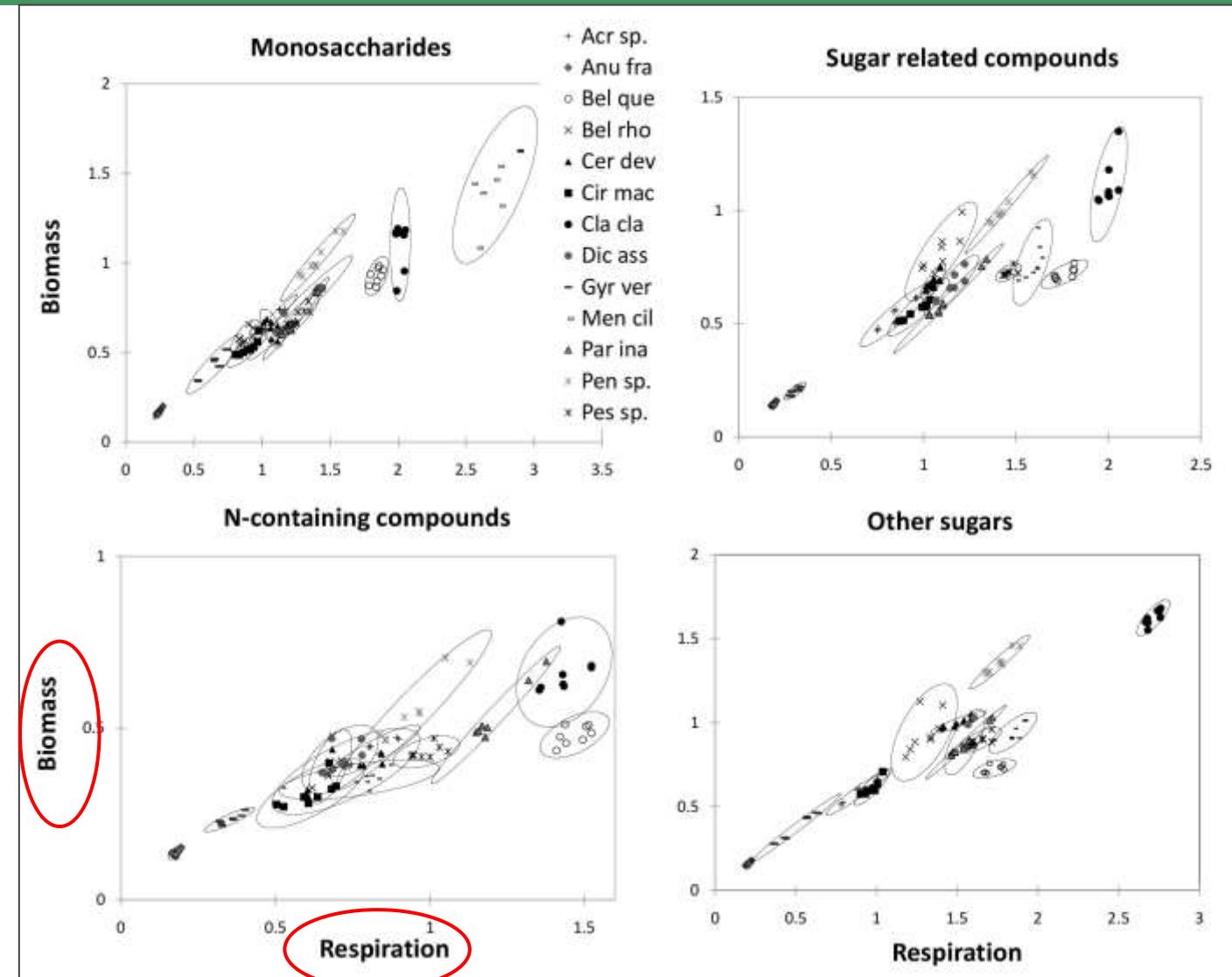


Figure 1. Relationship between OD₄₉₀ and OD₇₅₀ calculated with the averaged data obtained for the 13 litter fungi on all the 95 carbon sources present in the FF Biolog plates. Line is the best-fit regression between the two values OD₄₉₀ and OD₇₅₀ ($y = ax + b$, with $a = 0.429$, $b = 0.11$, $R^2 = 0.853$, $p < 0.001$).



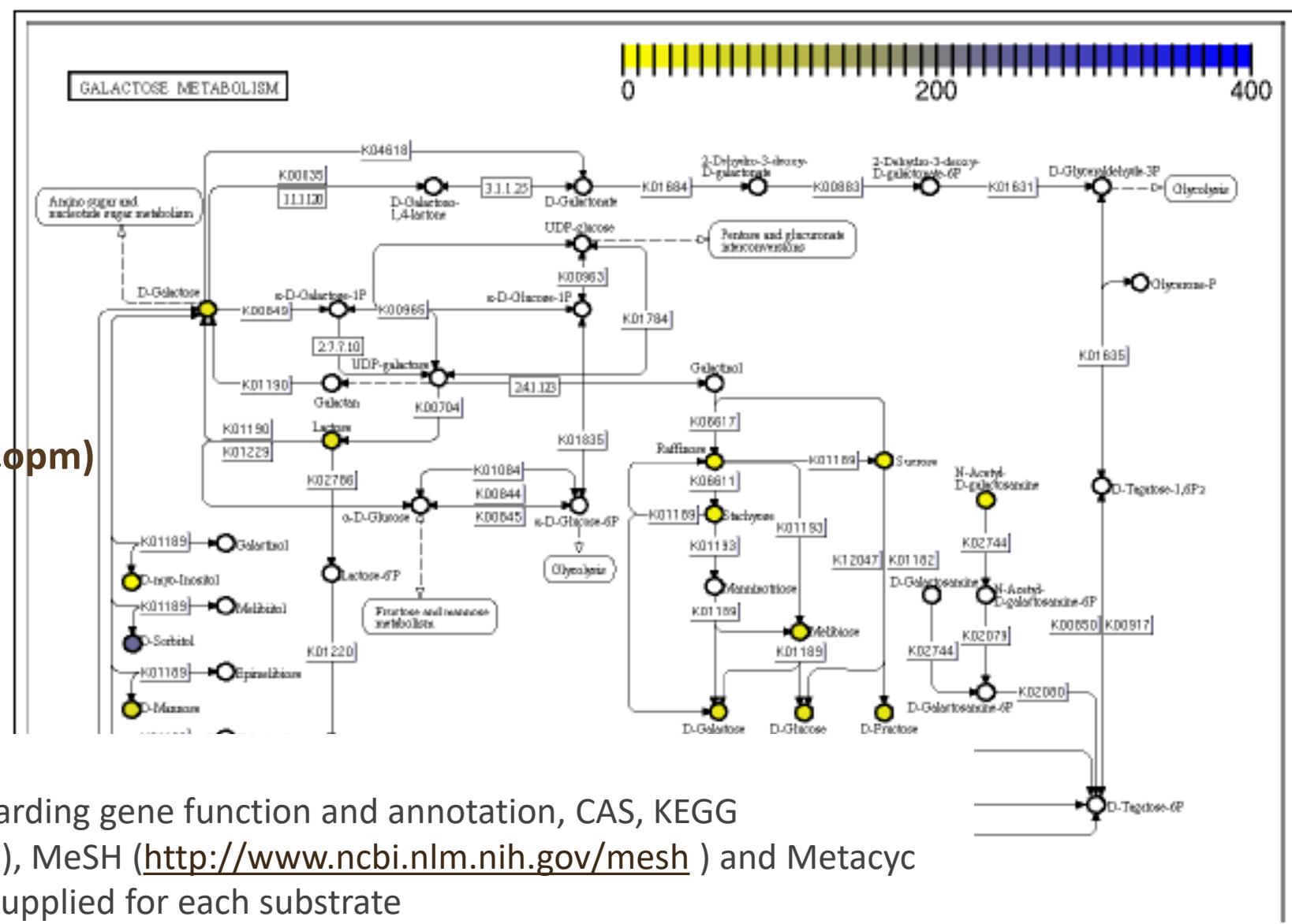
Pinzari et al. (2017) A simple method for measuring fungal metabolic quotient and comparing carbon use efficiency of different isolates: Application to Mediterranean leaf litter fungi, Plant Biosystems 151:2, 371-376



Lea A. I. Vaas et al. Bioinformatics
2013;29:1823-1824

From **opm** manual and tutorial

(<http://cran.r-project.org/package%opm>)



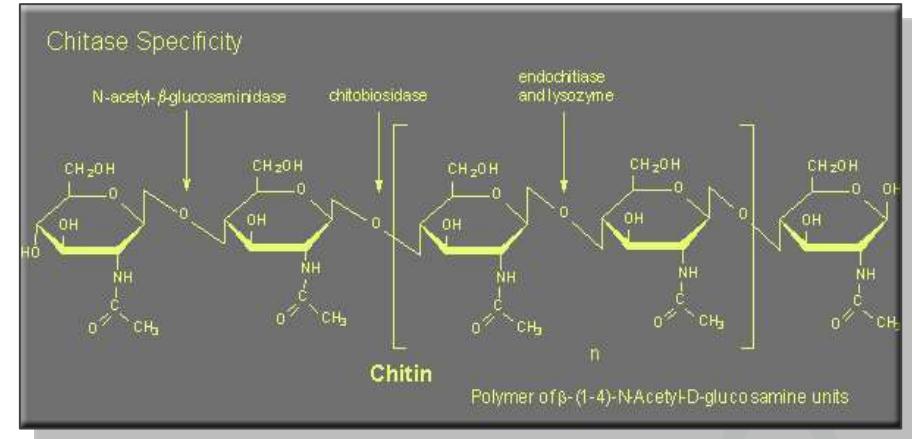
Standardized substrate names

To further explore PM data regarding gene function and annotation, CAS, KEGG (<http://www.genome.jp/kegg/>), MeSH (<http://www.ncbi.nlm.nih.gov/mesh>) and Metacyc (<http://metacyc.org/>) IDs are supplied for each substrate



regulation of enzyme production

A screening system for carbon sources enhancing *N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*)



inoculated
Biolog FF
Microplate

+ substrate for enzymatic reaction
4-nitrophenyl *N*-acetyl- β -D-glucosaminide

=> chromogenic assay
of NAGase activity
O.D. 490 nm

growth rate
measurements
O.D. 750 nm

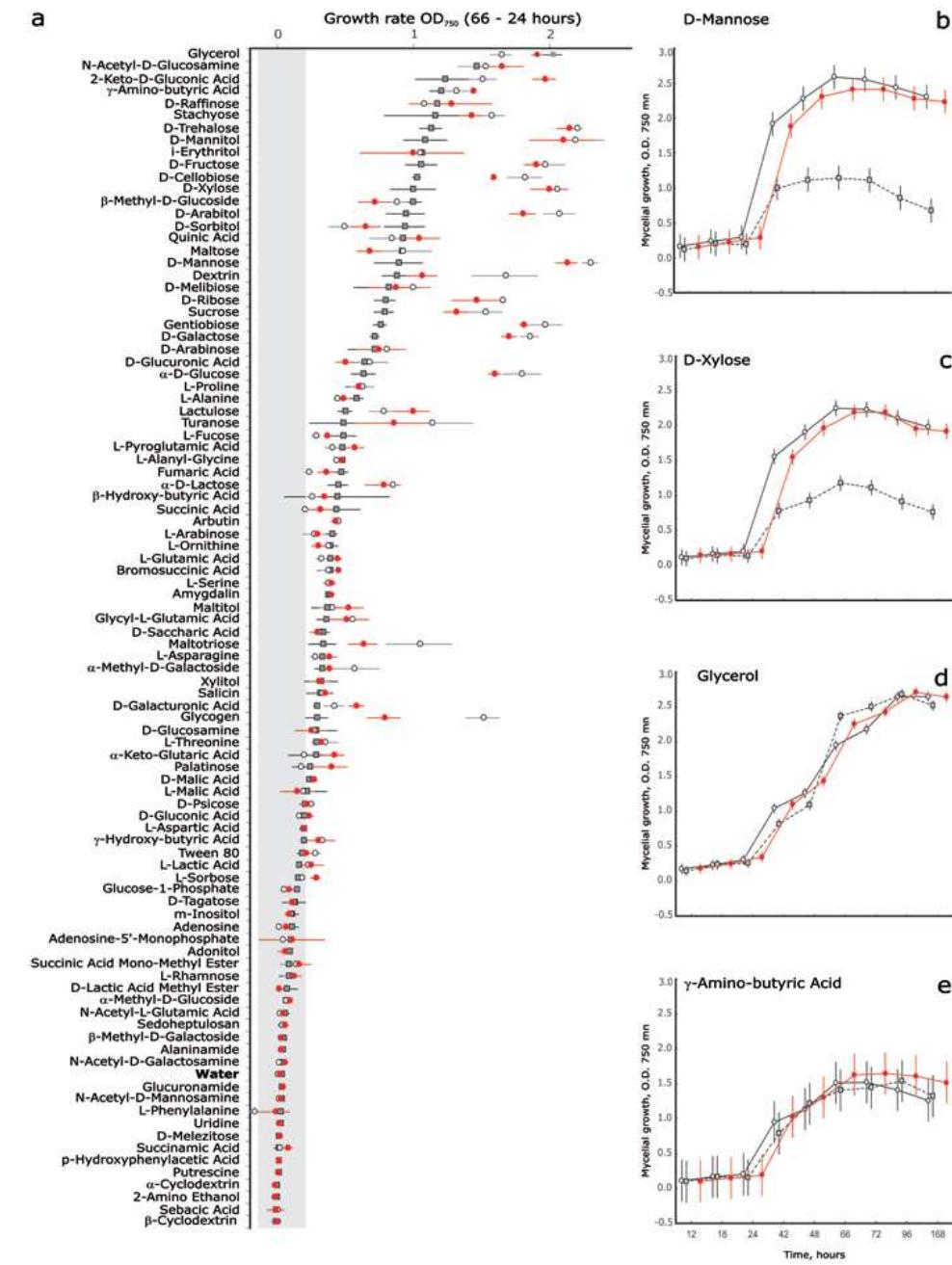
N-acetylglucosaminidase formation:

- extracellular, thus directly measurable
- chromogenic assays available (PNP, umbelliferon)
- limited enzyme multiplicity (only two genes; *nag1*, *nag2*)

assay can be targeted to a single enzyme only, using:

- reporter gene fusions (applicable in principle to all genes)
- studying single gene knock-outs

Seidl, V., Druzhinina, I. S., Kubicek, C. P. (2006) A screening system for carbon sources enhancing β -*N*-acetylglucosaminidase formation in *Hypocrea atroviridis* (*Trichoderma atroviride*), *Microbiology*, 152, 2003–2012



...photostimulation of growth

Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress

Growth rate of *H. atroviridis* on 95 carbon sources and water in darkness (squares), constant light (open circles) and an alternating 12 hours light/darkness cycle (closed, red circles) calculated as difference between OD_{750} values at 24 and 66 hours of incubation. Error bars correspond to SD at 66 hours, and were calculated from three parallel experiments. The grey shadowed area indicates the level of negative control (growth rate on water)

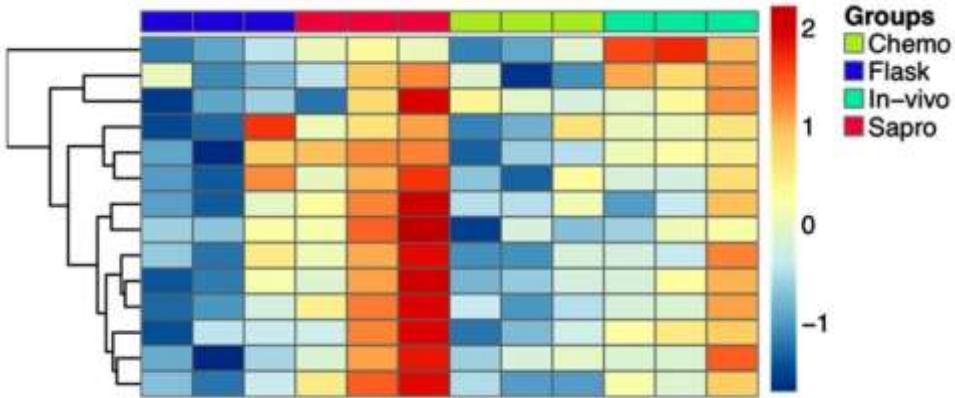
Friedl, M. A., Schmoll, M., Kubicek, C. P. and I. S. Druzhinina (2008) Photostimulation of *Hypocrea atroviridis* growth occurs due to a cross-talk of carbon metabolism, blue light receptors and response to oxidative stress, *Microbiology*; 154: 1229-1241



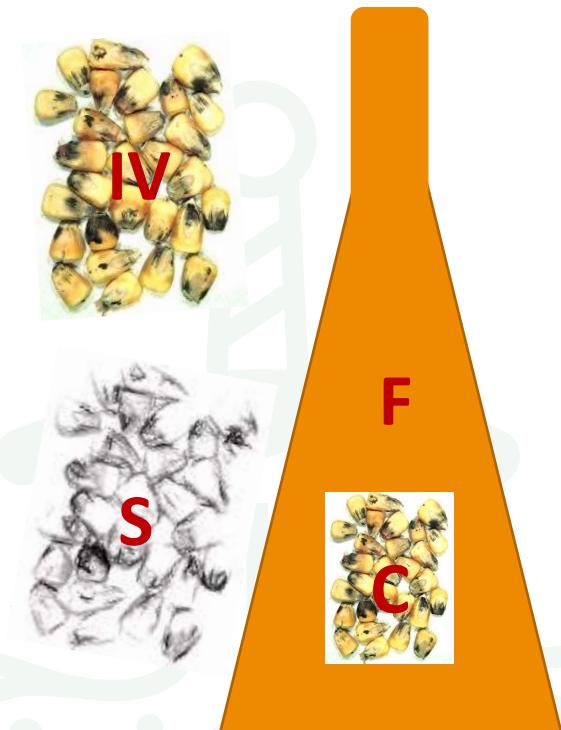
Epigenetics (nutritional memory in conidia)

Genetic and phenotypic responses within the fungus when grown under four conditions that mimic different ecological niches ranging from saprophytic growth to parasitism

The greatest transcriptional change was found between saprophytic and parasitic growth, which resulted in expression changes in over 800 genes in *A. flavus*. The fungus also responded to growth conditions, putatively by adaptive changes in conidia, resulting in differences in their ability to utilize carbon sources.



- *A.flavus* grown on CD medium (*flask*),
- flasks with CD medium containing injured maize kernels within a closed dialysis tube (*chemo*),
- grown on autoclaved maize kernels (*sapro*)
- and grown on ears in the field (*in vivo*)

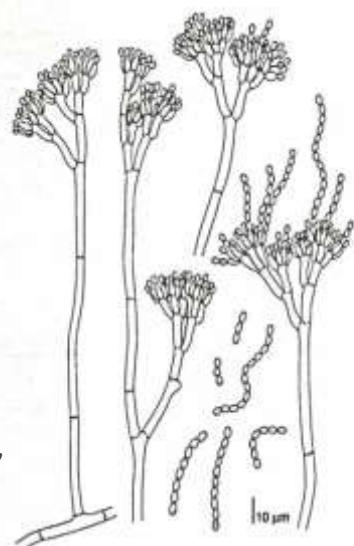
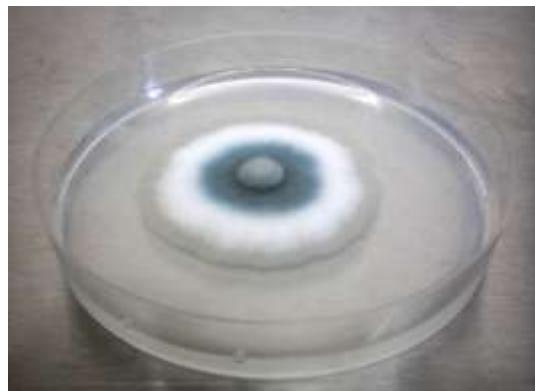


Reverberi M, Punelli M, Scala V, Scarpari M, Uva P, Mentzen WI, Dolezal AL, Woloshuk C, Pinzari Fabbri AA, Fanelli C, Payne GA. Genotypic and phenotypic versatility of *Aspergillus flavus* during maize exploitation. PLoS One. 2013 Jul 19;8(7):e68735.

Now methylation experiments disclosed new answers on environmental effect on phenotype of conidia

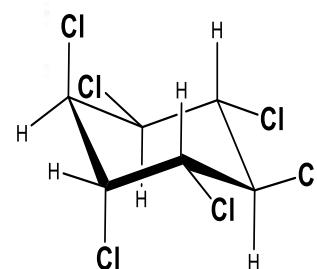
**Phenotypic effects of two organic xenobiotics,
hexachlorocyclohexane and toluene, on the saprotrophic fungus
*Penicillium griseofulvum***

- Isolation from highly HCH-polluted soil samples of different fungal species
- *Penicillium griseofulvum* was used to investigate HCH tolerance and transformation
- Batch testes were performed in liquid and solid Czapek-Dox media, amended with HCH

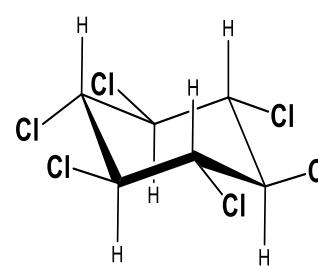


Penicillium griseofulvum Dierckx,
with conidia and conidiophores

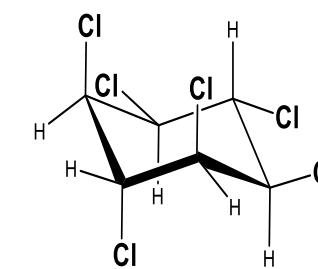
- Persistent organic pollutant
- Volatile, lipophilic, and polychlorinated
- Different isomers (including γ -HCH or Lindane, a pesticide)
- In the Stockholm Convention since 2008
- α -, β -, γ -, and δ -HCH in soils and groundwater due to Lindane use or improper disposal
- Carcinogenic and toxic effects on living organisms in ecosystems and humans



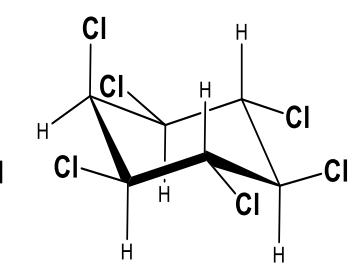
α - HCH



β - HCH

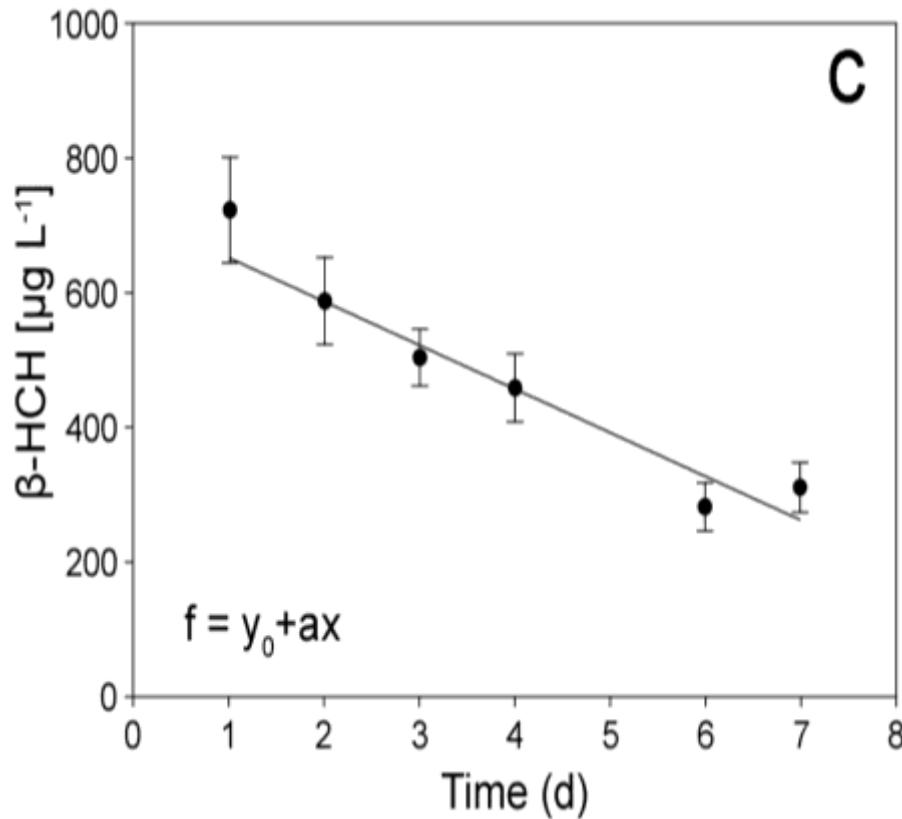


γ - HCH



δ - HCH

Penicillium griseofulvum



Gas chromatographic analysis of β -HCH concentration in the liquid Czapek-Dox medium after growth of *P. griseofulvum*.

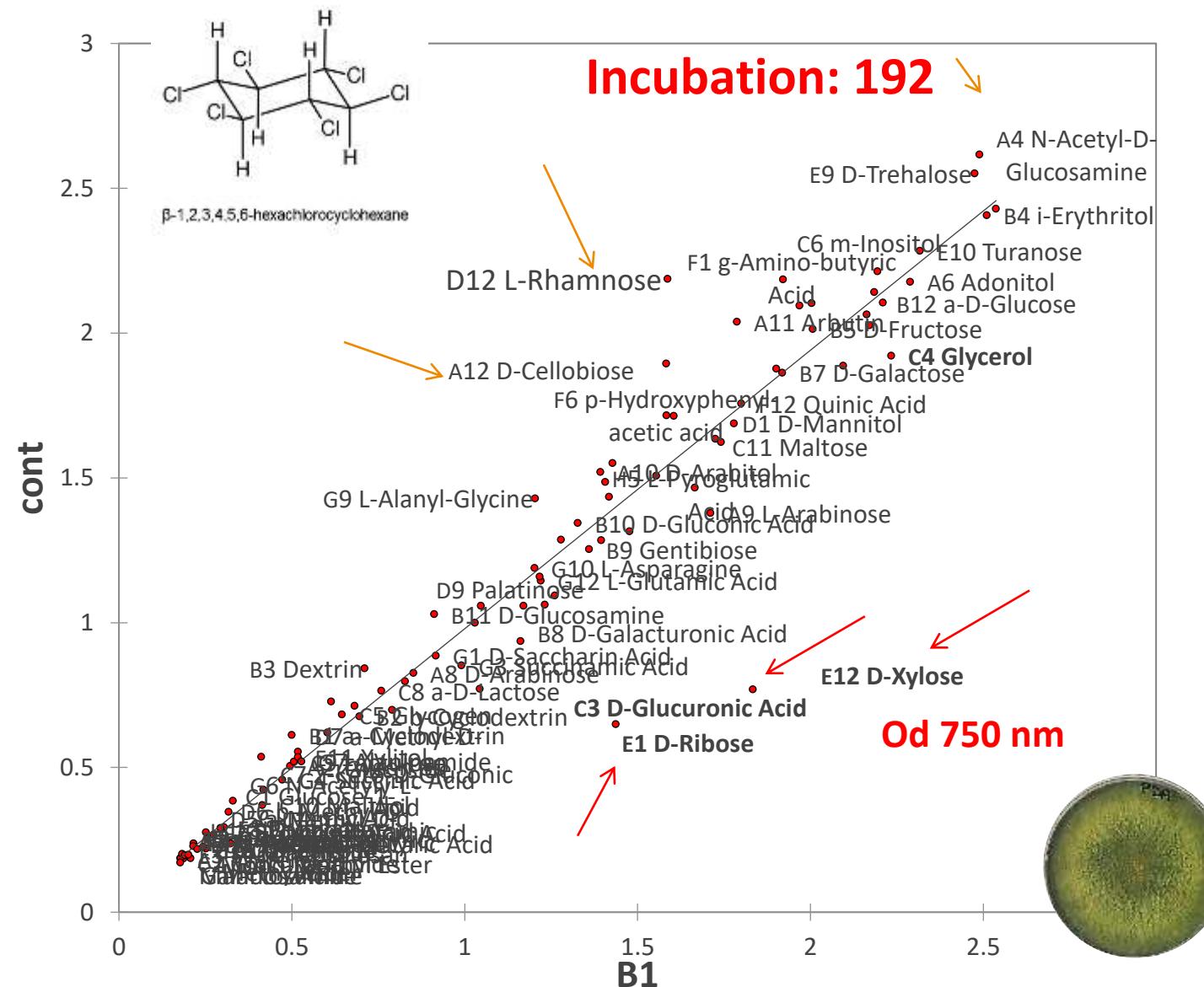
The concentration was monitored for 7 d.

Ceci, Pierro, Riccardi, Pinzari, Maggi, Persiani, Gadd, Papini (2015) Biotransformation of β -hexachlorocyclohexane by the saprotrophic soil fungus *Penicillium griseofulvum*.
Chemosphere 137: 101-107



Example of application: metabolic change at the presence of a xenobiotic compound (hexachlorocyclohexane)

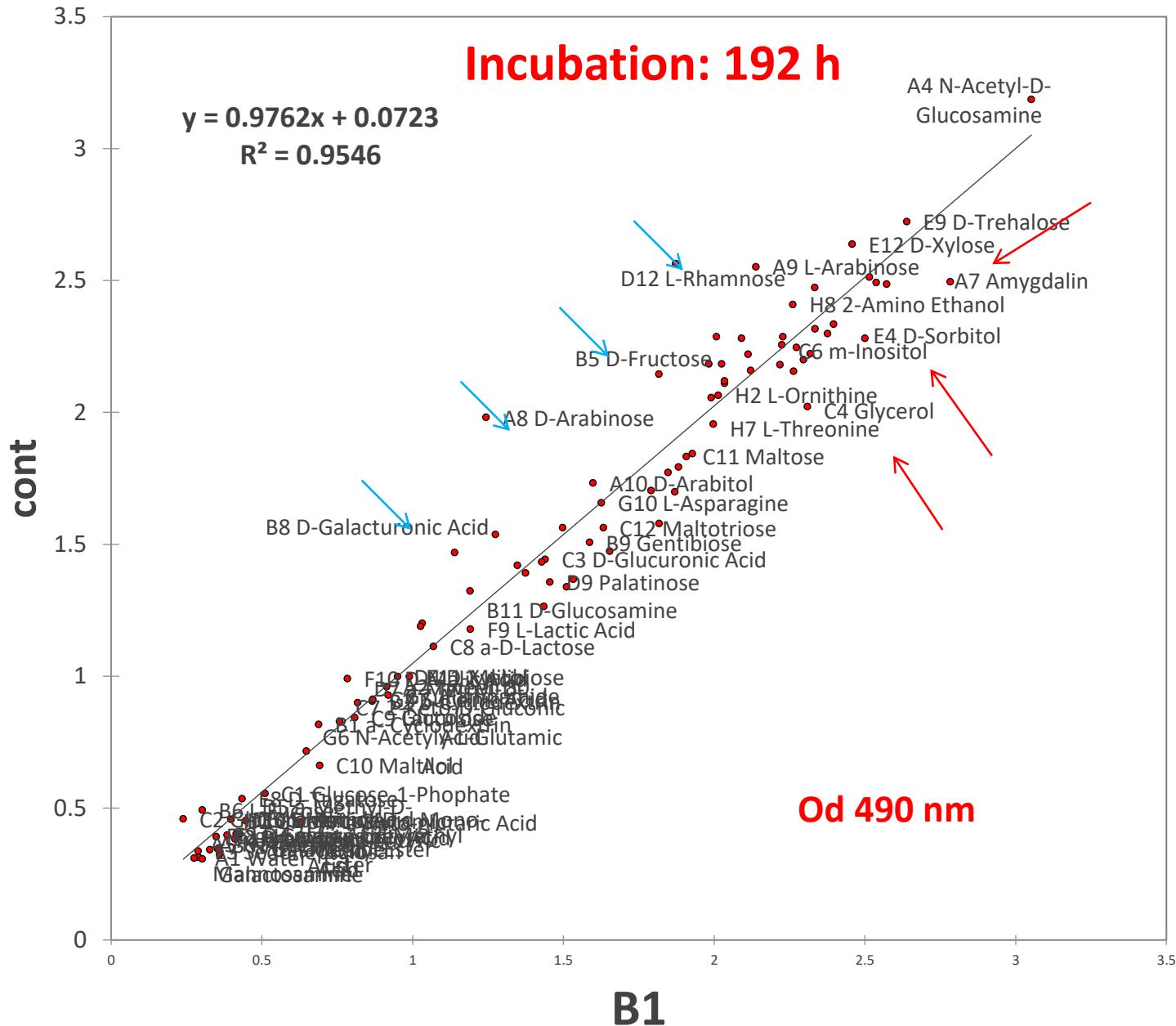
biomass





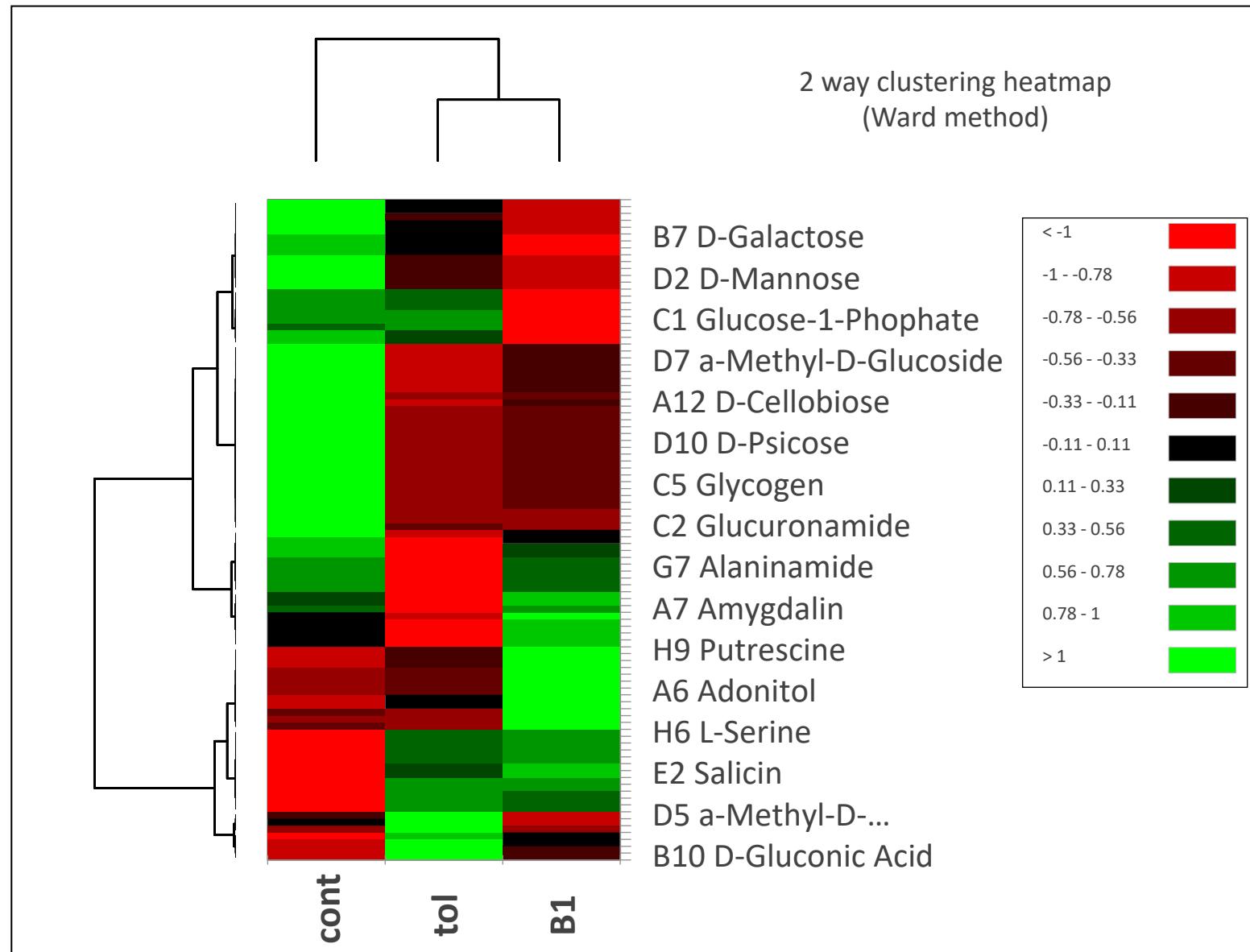
respiration

Incubation: 192 h



490 nm (respiration)

Same data analysis as
with RNA-seq datasets
(false discovery rate etc.)



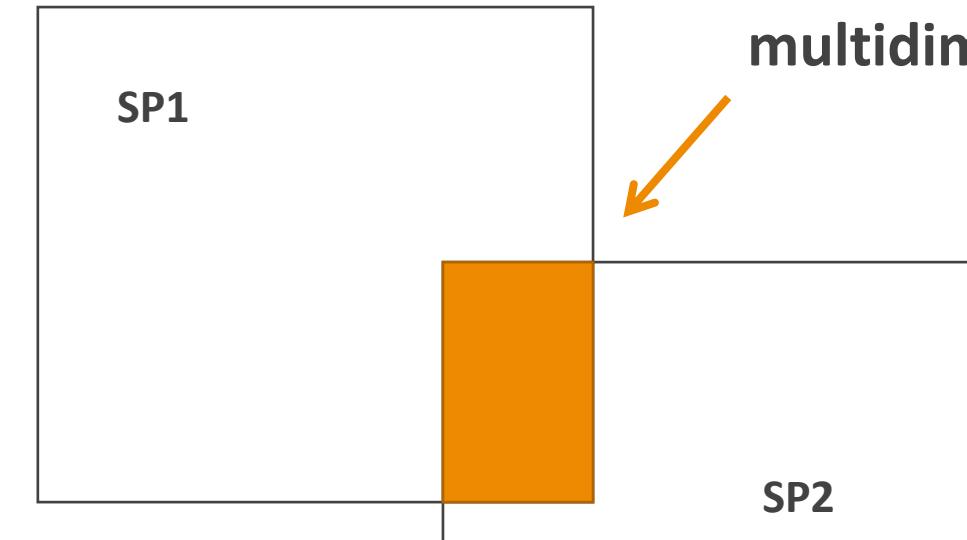


- What is the **niche overlap** and how it can be measured

n dimensions

Niche Overlap

Shared niche space of the
Hutchinsonian
multidimensional niche



n dimensions



- In vitro analysis of the effects of interactions is crucial because competition or commensalism can give rise to new compounds: i.e. enzymes, antibiotics, allopathic molecules
- or behaviors: production of resistant structures, cellular apoptosis, changes in life cycle as perfect state inhibition or stimulation, etc.
- which in turn can have definite effects on both the efficacy of the product and the variability of its properties.





Percentage Overlap

$$P_{jk} = \left[\sum_{i=1}^n (\text{minimum } p_{ij}, p_{ik}) \right] 100$$

where

P_{jk} = Percentage overlap between species j and species k

p_{ij} = Proportion resource i is of the total resources used by species j

p_{ik} = Proportion resource i is of the total resources used by species k

n = Total number of resource states

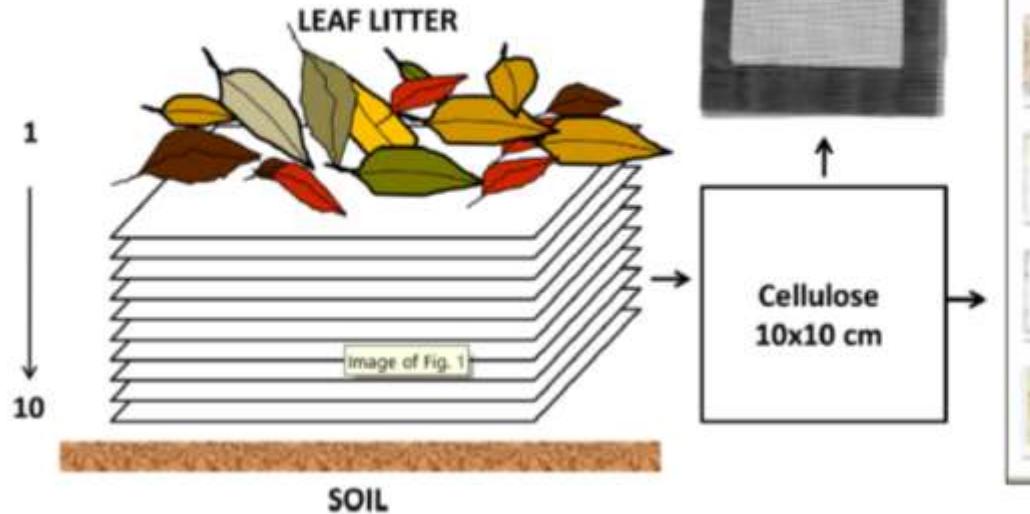
Percentage overlap is the simplest measure of niche overlap to interpret because it is a measure of the actual area of overlap of the resource utilization curves of the two species.

Lee HB, Magan N. Environmental factors and nutritional utilization patterns affect niche overlap indices between *Aspergillus ochraceus* and other spoilage fungi. Lett Appl Microbiol. 1999 Apr;28(4):300-4.

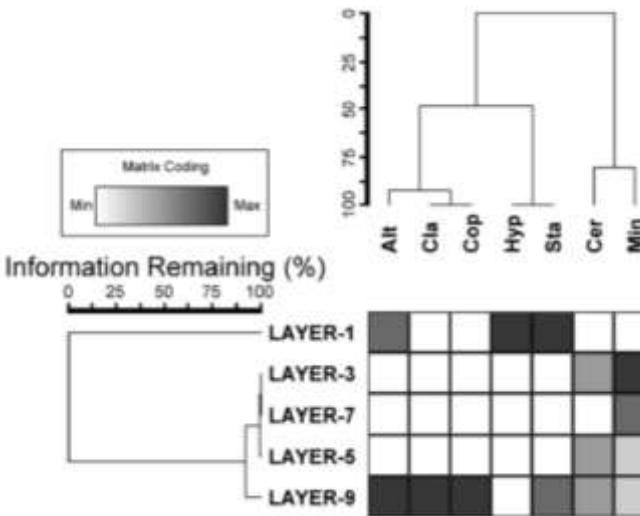
Oszust K, Cybulska J, Frąc M. How Do *Trichoderma* Genus Fungi Win a Nutritional Competition Battle against Soft Fruit Pathogens? A Report on Niche Overlap Nutritional Potentates. Int J Mol Sci. 2020 Jun 14;21(12):4235.



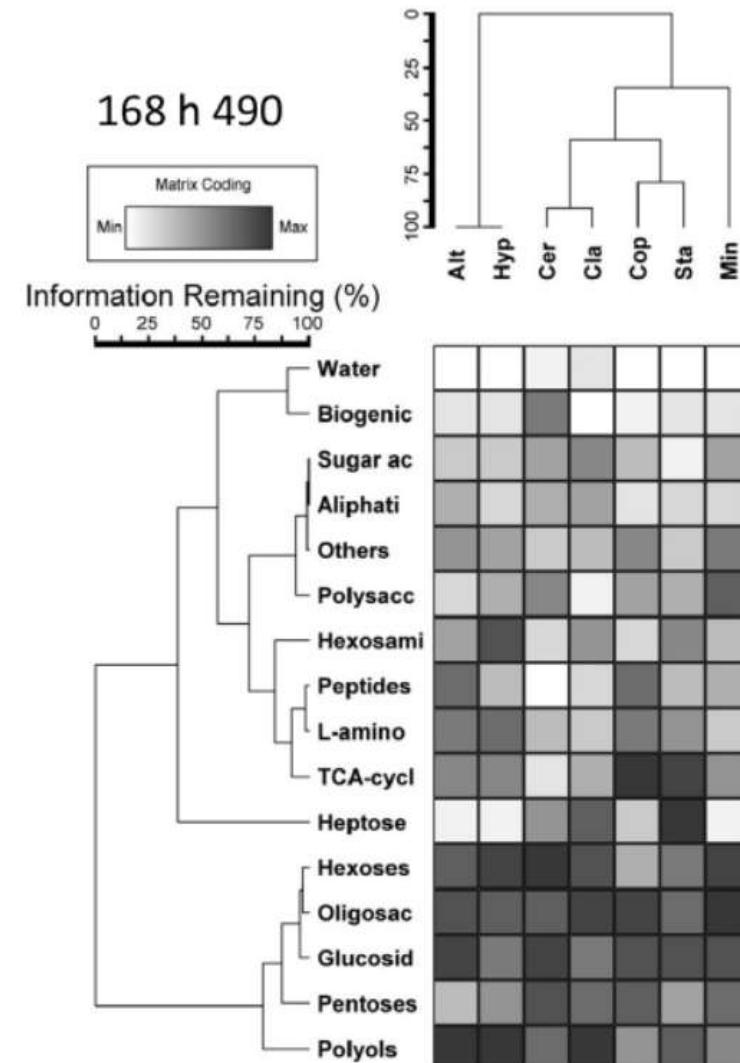
Pinzari F et al. Overlap in substrate utilisation and spatial exclusion in some microfungi which act as early cellulose colonisers in a Mediterranean environment. PEDOBIOLOGIA. 61:(2017), pp. 9-21



Spatial overlap



Trophic/diet overlap





EXCALIBUR



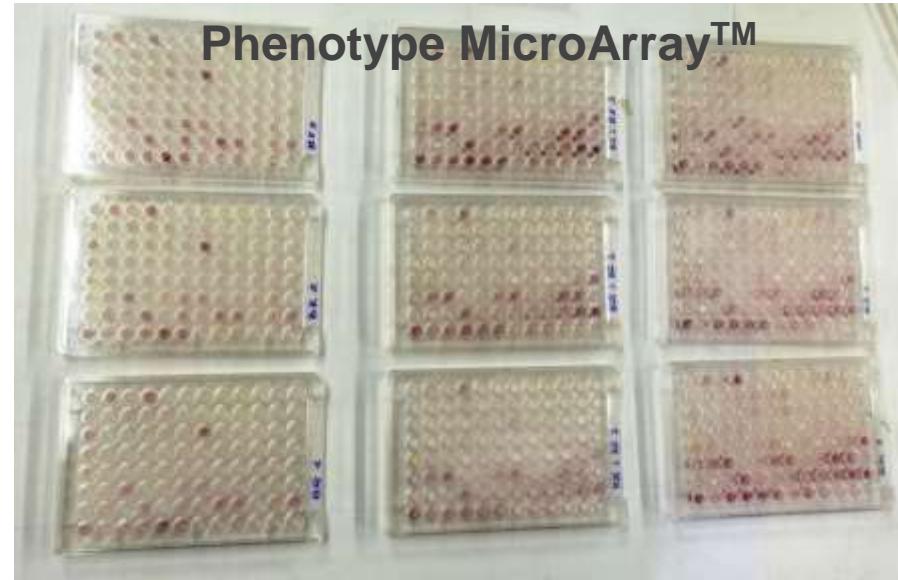
Biocontrol to reduce the incidence of *Melolontha melolontha* (European cockchafer), which is threatening the cultivation of strawberry plantations in several regions of Poland.



*Beauveria
bassiana*



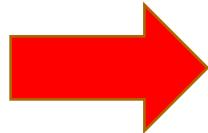
*Beauveria
brongniartii*



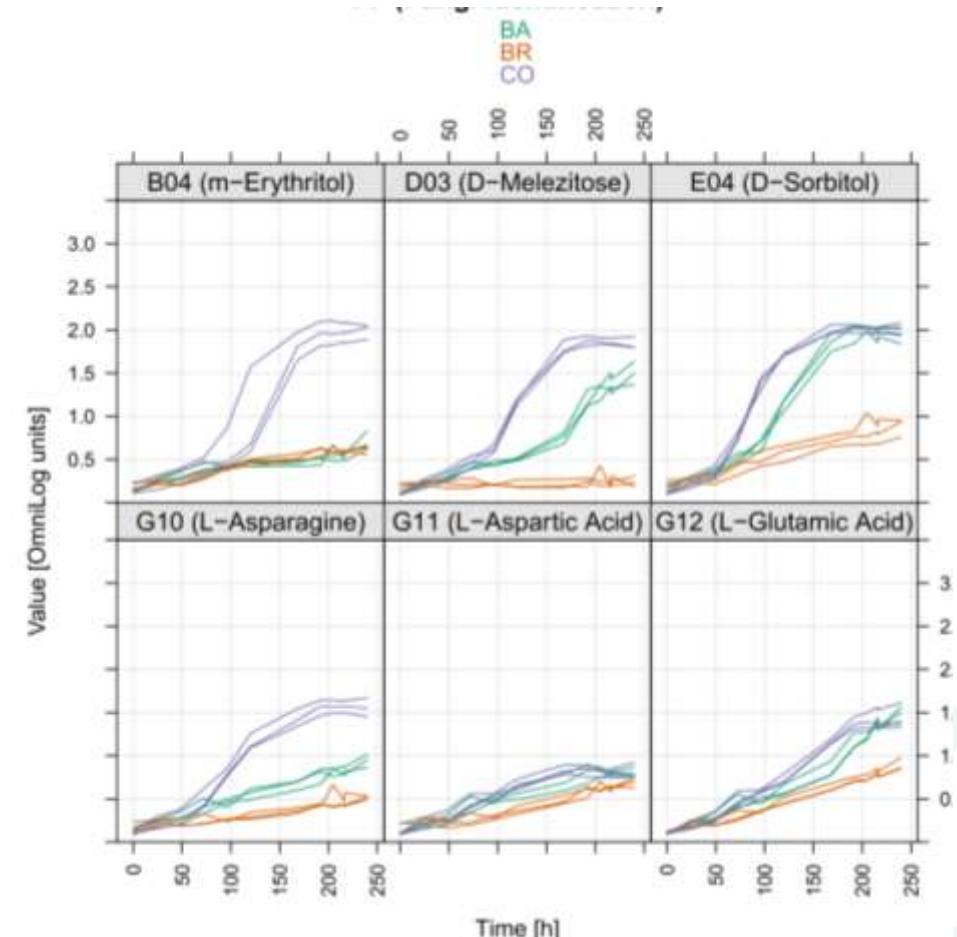
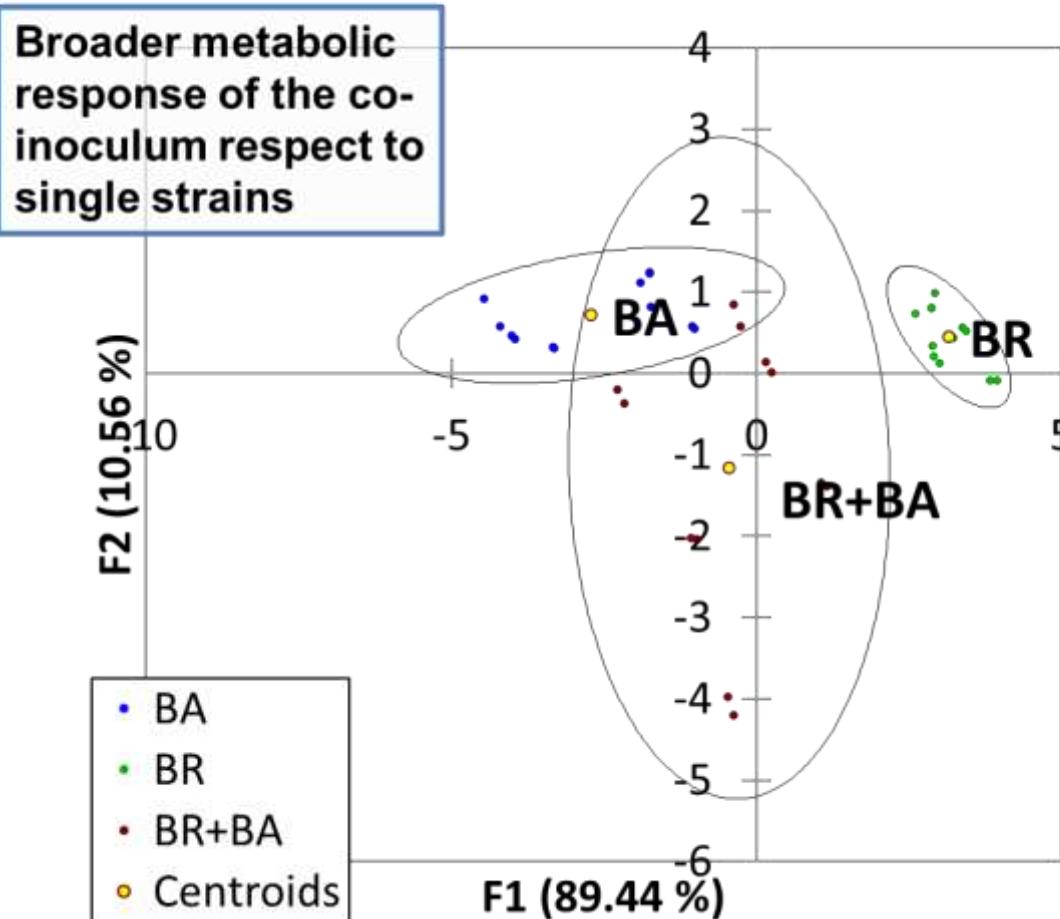
Phenotype MicroArray™

BA= *B. bassiana*
BR= *B. brongniartii*
BA+BR= the two fungi inoculated together

Canfora, L. et al. Co-inoculum of *Beauveria brongniartii* and *B. bassiana* shows *in vitro* different metabolic behaviour in comparison to single inoculums. *Sci Rep* 7, 13102 (2017). <https://doi.org/10.1038/s41598-017-12700-0>



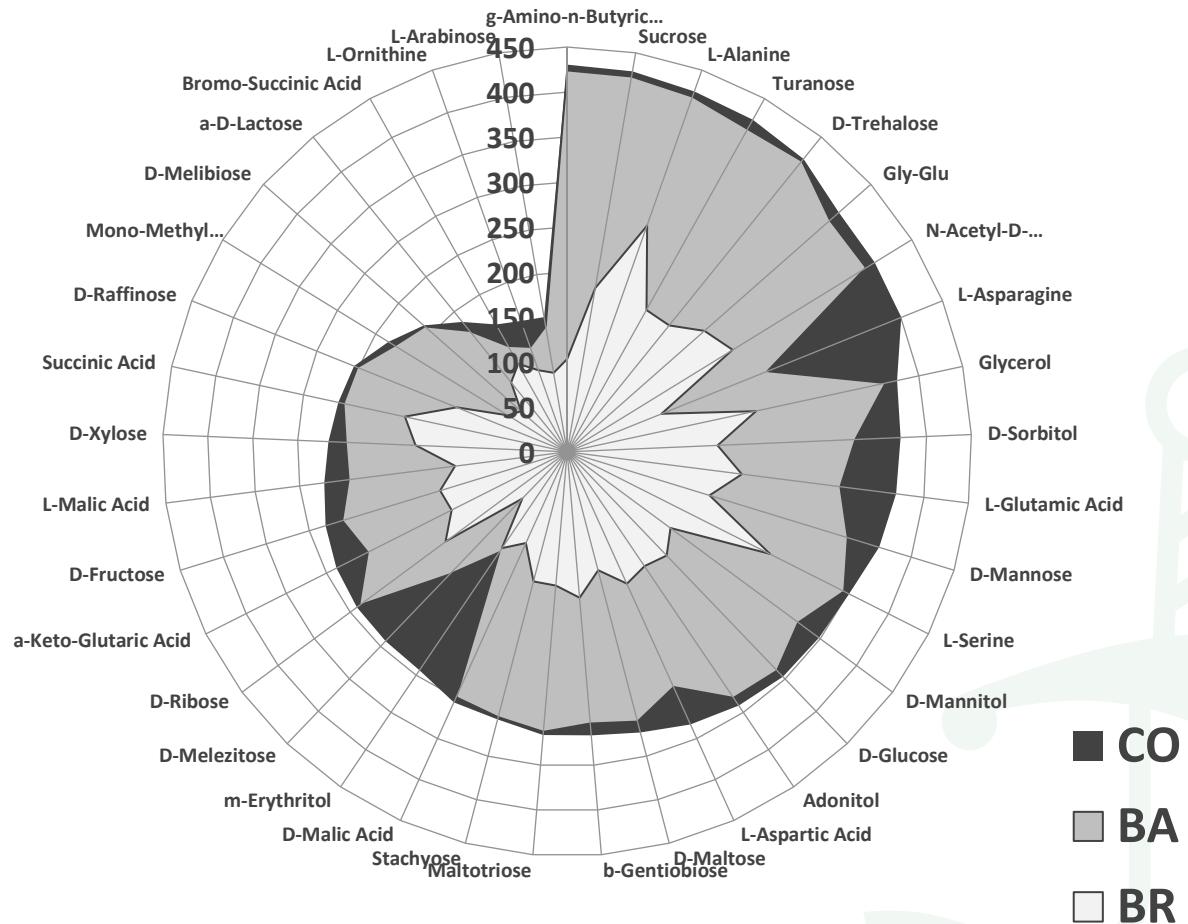
Metabolic activity and growth of the two fungi inoculated in PM microtiter plates Discriminant analysis , 48 hours incubation





Comparison of the metabolism of *Beauveria bassiana* (BA), *Beauveria brongniartii* (BR) and their the co-inoculum (CO) on simple carbon sources.

The comparison is based on the Area Under the Curve (AUC) values derived from the analyses of kinetic data of fungal growth on the substrates, in vitro, along 240h incubation.



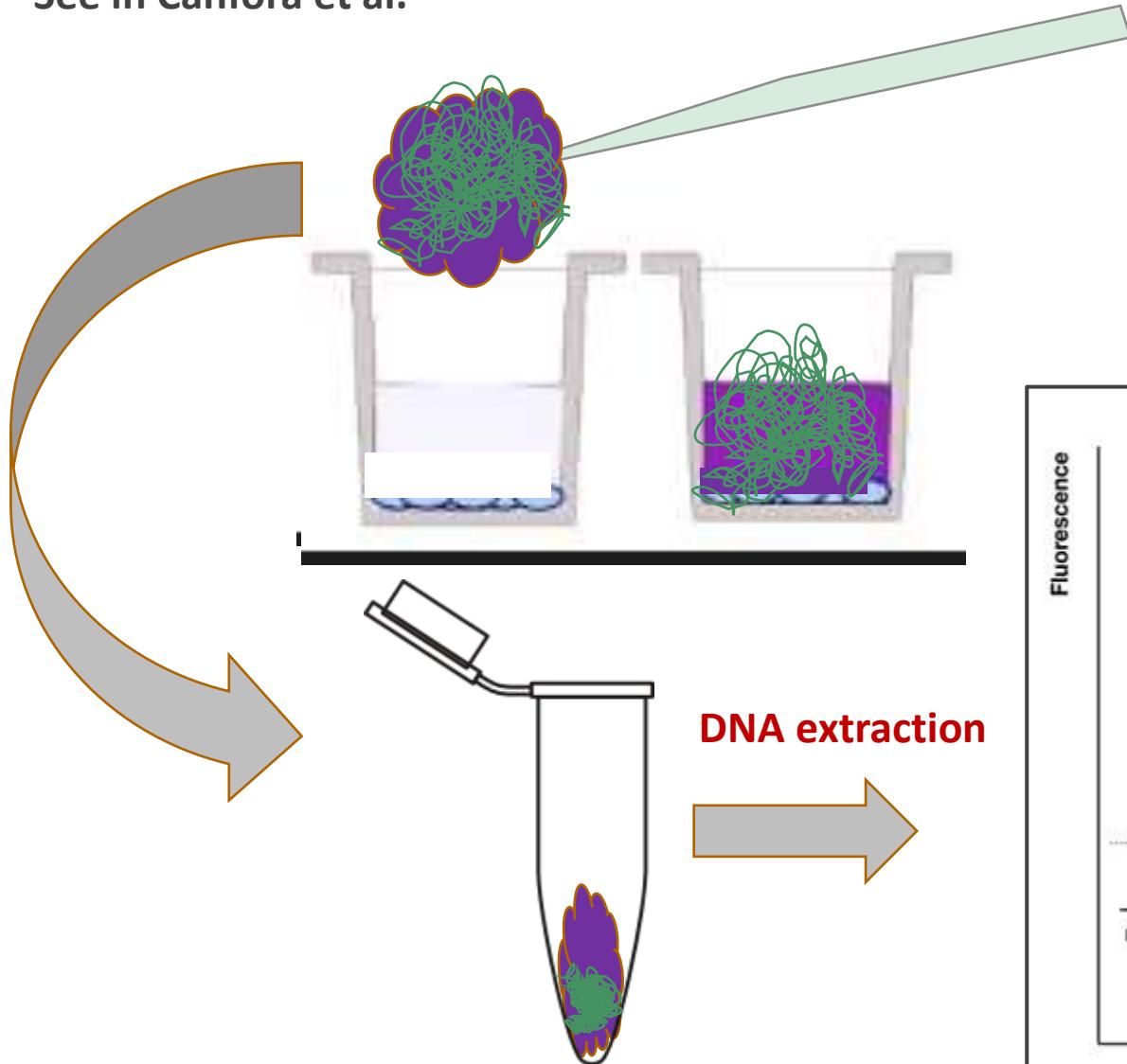
Together can metabolise more substrates and have higher fitness in a wider range of conditions and niches



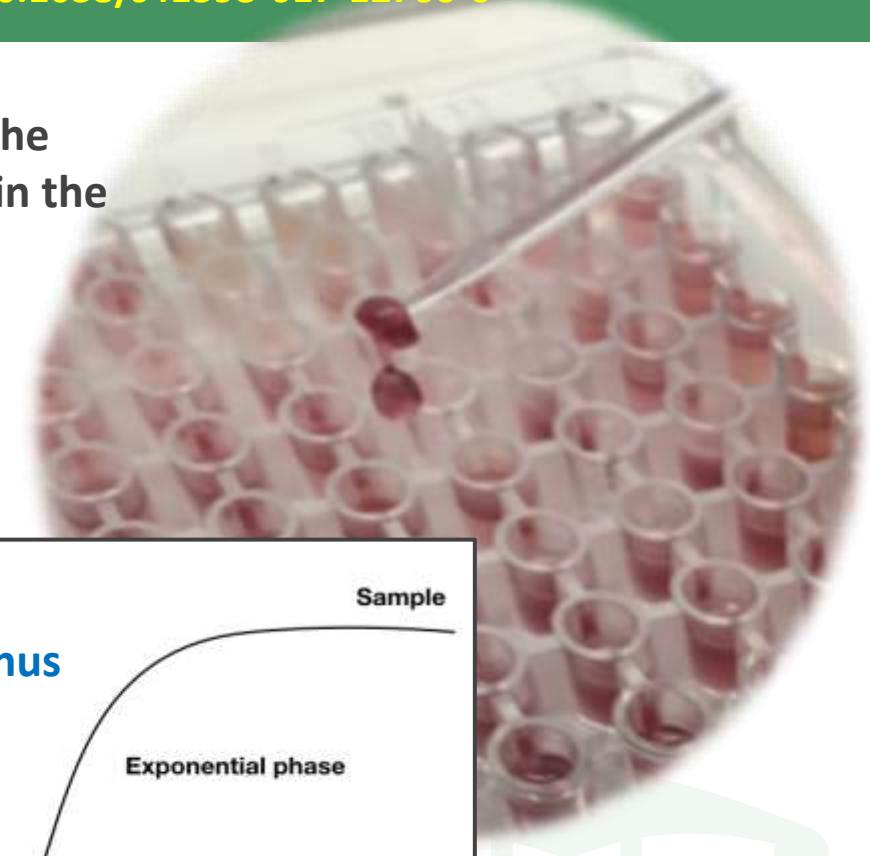
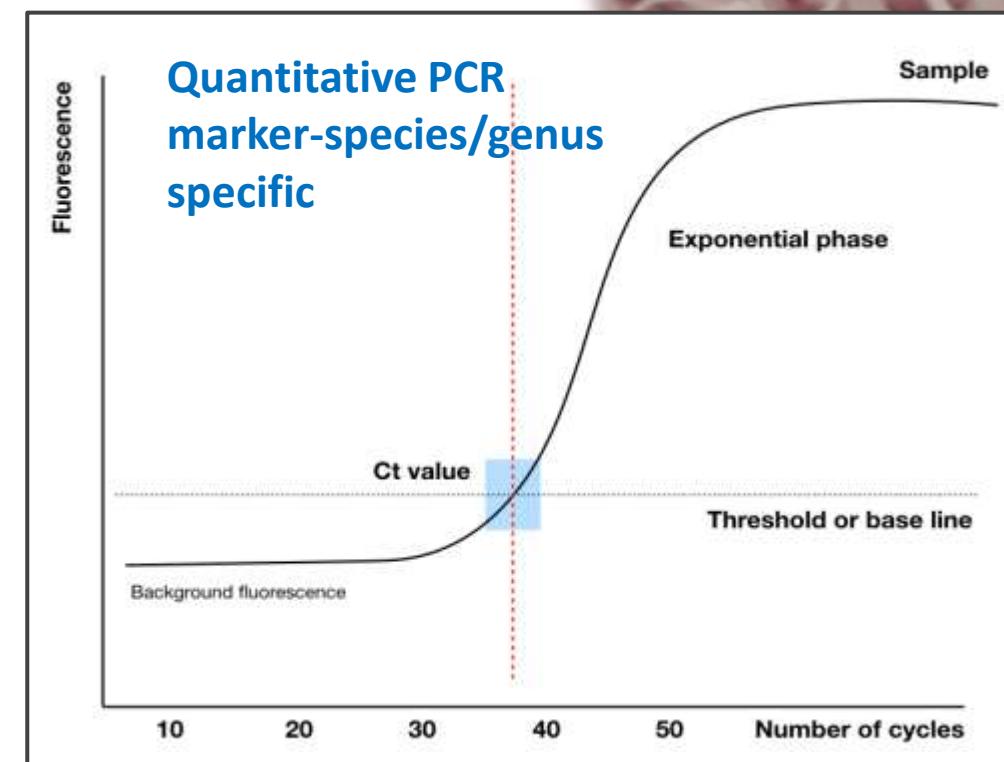
- In the co-inoculum it is necessary to quantify the two species to provide a correct model of niche partitioning and metabolic interaction.
- The proposed protocol combines the use of commercial Biolog^R microplates with the quantification of the biomass of the individual co-inoculated strains by means of molecular tracing techniques and **qPCR analysis**.



See in Canfora et al.



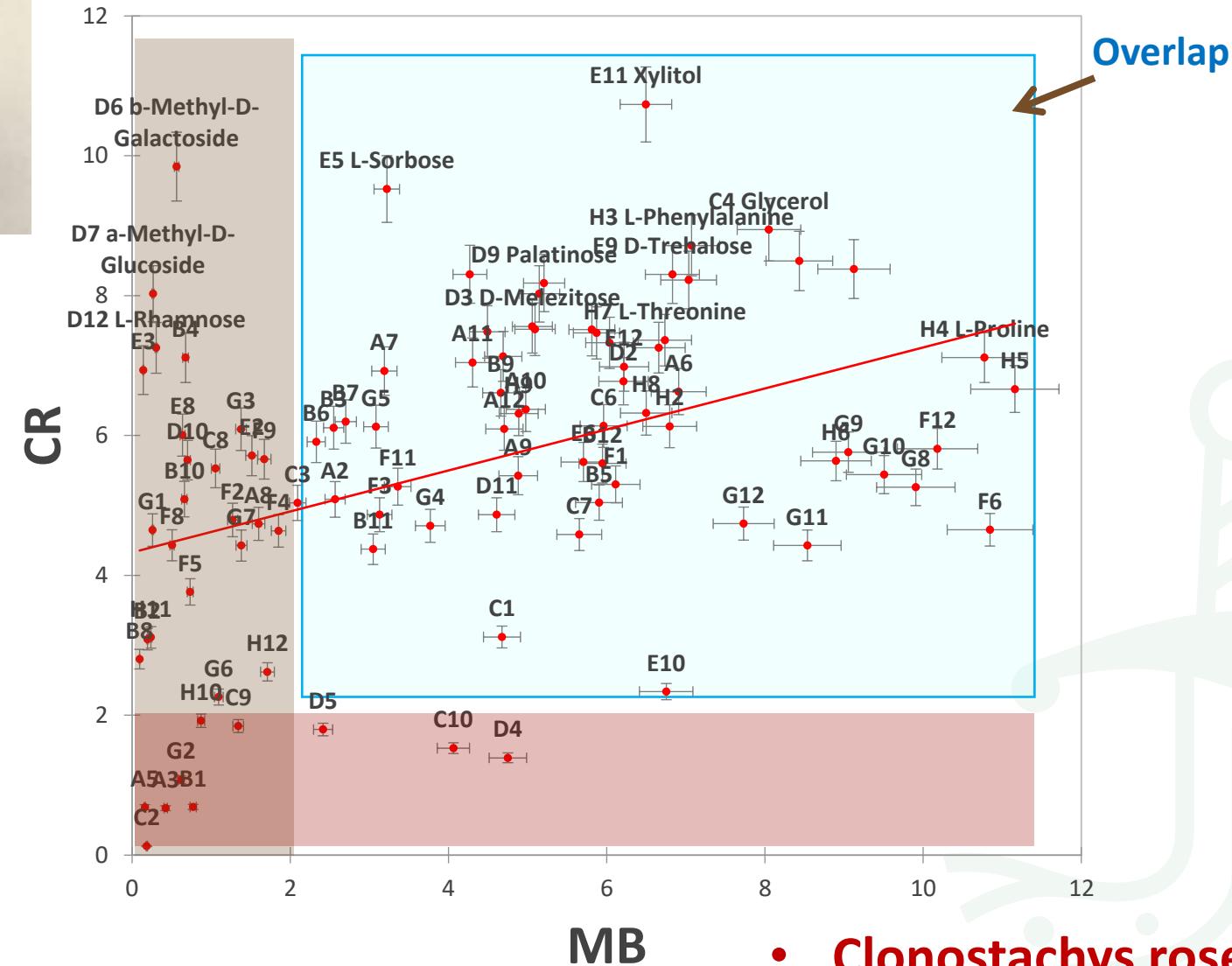
Recovery of ALL the mixed mycelium in the wells of interest





Niche overlap, competition and detection of nutrients (C, N) that trigger biomass and sporulation of co-inoculums or promote «co-habitation» of multiple inoculums in mixed formulations and in the field.

Application in Excalibur

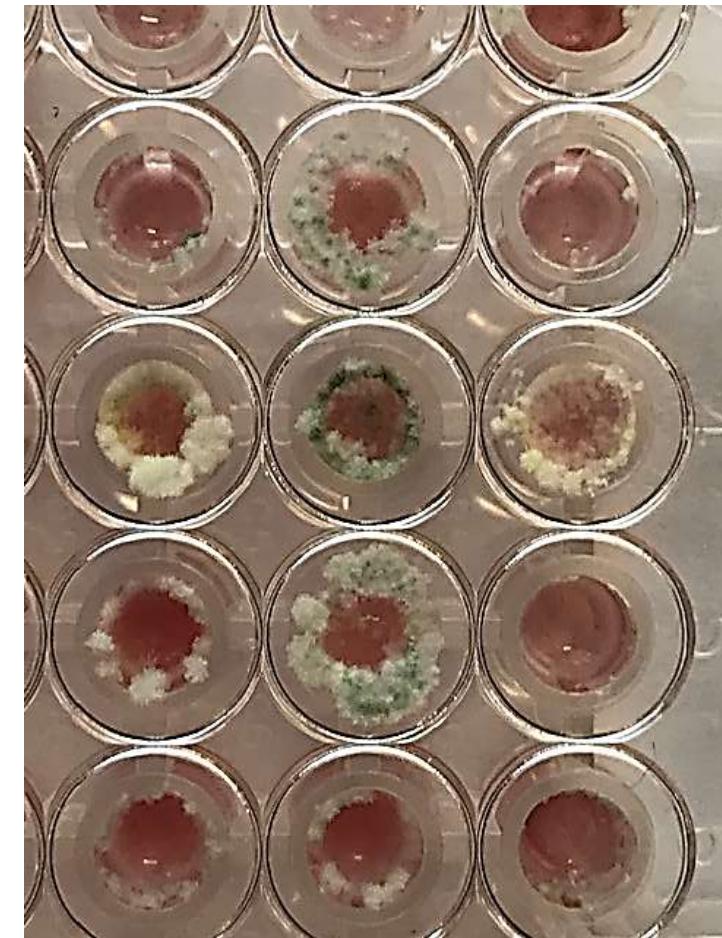
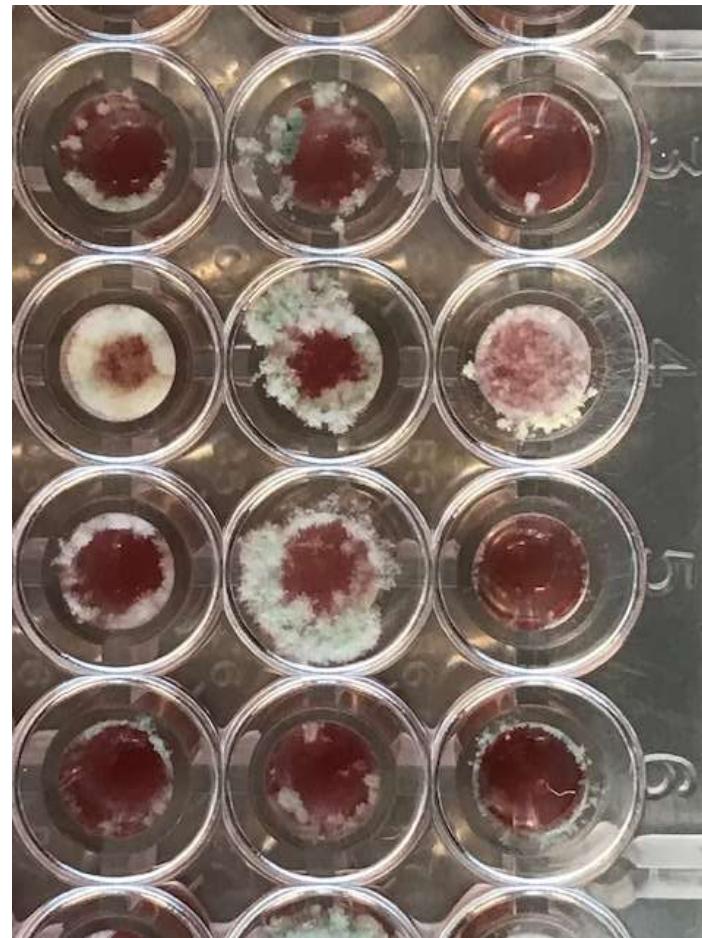


- *Clonostachys rosea*
- *Metarhizium brunneum*



- Future developments

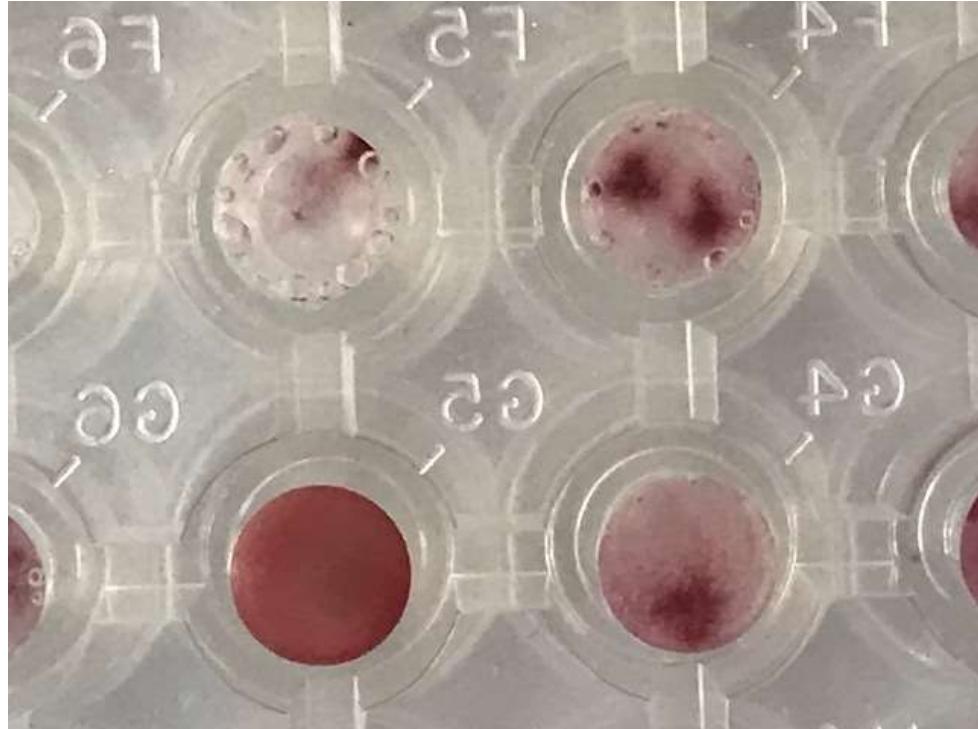
Sporulation/pigmentation pattern highly repeatable



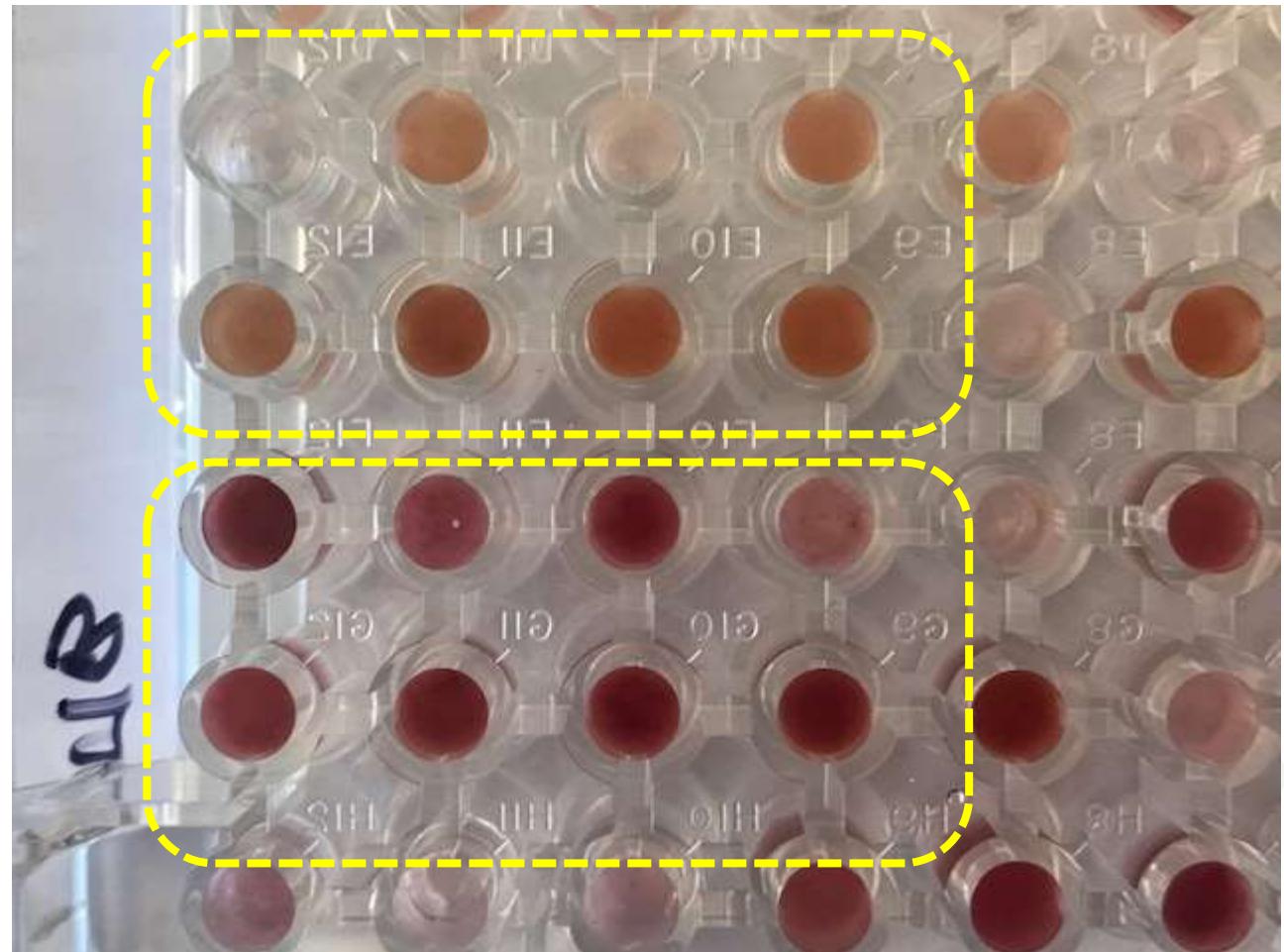
Sporulation density, intensity, speed, colour very different according to substrate.
Use Image analysis? Fractals? AI for pattern recognition and using also data on
sporulation/pigmentation?



- Future developments



Uniformity of colour different according to substrate: different growth pattern. Use Image analysis? Fractals? AI? For pattern recognition?



Not the same lambda (wavelength): use colour instead of OD?
But keep turbidity for biomass?





• Future developments

1. Tween 80	20. D-Ribose
2. Dextrin	21. D-Raffinose
3. Succinic acid mono-methyl ester	22. D-Melibiose
4. Glycerol	23. D-Melezitose
5. Amygdalin	24. D-Mannose
6. Succinic acid	25. D-Mannitol
7. Sebacic acid	26. D-Cellobiose
8. L-Malic acid	27. D-Arabinol
9. Fumaric acid	28. Adonitol
10. D-Malic acid	29. β -Methyl-D-glucoside
11. 2-Keto-D-gluconic acid	30. α -D-Lactose
12. Turanose	31. α -D-Glucose
13. Sucrose	32. L-Pyroglutamic acid
14. Stachyose	33. L-Proline
15. N-Acetyl-D-glucosamine	34. L-Phenylalanine
16. Gentibiose	35. L-Alanyl-glycine
17. D-Xylose	36. L-Alanine
18. D-Trehalose	37. Glycyl-L-glutamic acid
19. D-Sorbitol	38. γ -Amino butyric acid

A list of substrates present in Biolog® FF microplates and accounting for the most variance. Results obtained by running on a soil fungi dataset a Probabilistic Principal Component Analysis followed by robust clustering of substrates' principal components loadings with the Partition Around Medoids (PAM) algorithm.

See for ECOPLATES:

Hitzl W, Ranger A, Sharma S, Insam H. Separation power of the 95 substrates of the BIOLOG system determined in various soils. FEMS Microbiol Ecol 1997;22:167e74.



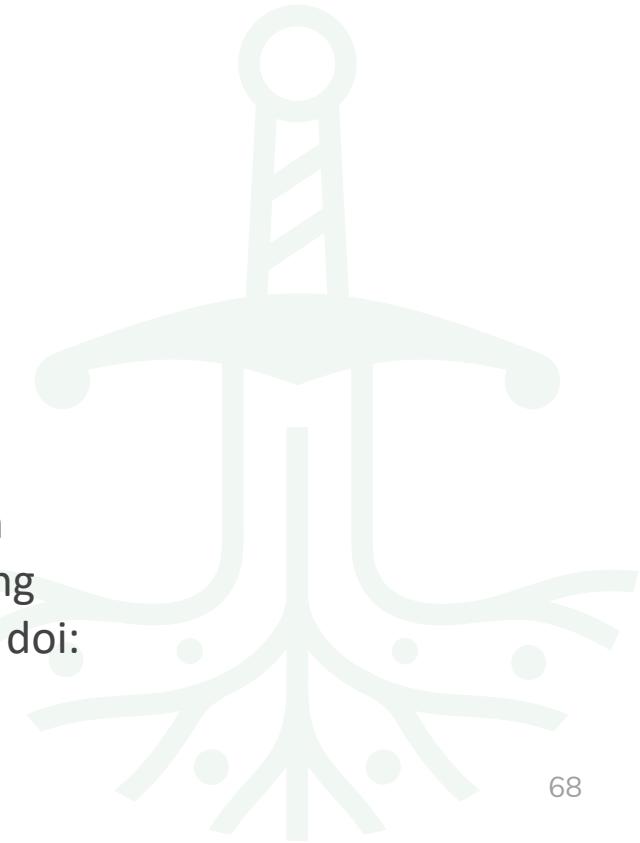
Some useful papers:

Atanasova L, Druzhinina IS. Review: Global nutrient profiling by Phenotype MicroArrays: a tool complementing genomic and proteomic studies in conidial fungi. *J Zhejiang Univ Sci B.* 2010 Mar;11(3):151-68. doi: 10.1631/jzus.B1000007.

Pinzari F, Ceci A, Abu-Samra N, Canfora L, Maggi O, Persiani A. Phenotype MicroArray™ system in the study of fungal functional diversity and catabolic versatility. *Res Microbiol.* 2016 Nov-Dec;167(9-10):710-722. doi: 10.1016/j.resmic.2016.05.008.

Blumenstein K, Macaya-Sanz D, Martín JA, Albrechtsen BR and Witzell J (2015) Phenotype MicroArrays as a complementary tool to next generation sequencing for characterization of tree endophytes. *Front. Microbiol.* 6:1033. doi: 10.3389/fmicb.2015.01033

Panek J, Frąc M, Bilińska-Wielgus N. Comparison of Chemical Sensitivity of Fresh and Long-Stored Heat Resistant *Neosartorya fischeri* Environmental Isolates Using BIOLOG Phenotype MicroArray System. *PLoS One.* 2016 Jan 27;11(1):e0147605. doi: 10.1371/journal.pone.0147605. PMID: 26815302; PMCID: PMC4729462.



Katherine Bernhardt
Diplo, 2022
Acrylic and spray paint on canvas
72 x 78 inches (182.9 x 198.1 cm)

Thank you!

flavia.pinzari@cnr.it



EXCALIBUR



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