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Italiadomani PIANO NAZIONALE DI RIPRESA E RESILIENZA
SUS-MIRRI.IT

Isolation, cultivation and preservation of microorganisms

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ENEA-SSPT-BIOAG-SOQUAS

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Training course SUS-MIRRI.it
ENEA CR Casaccia

It has been estimated that the fraction of already cultured bacterial species ranges between 0.1% and 0.001% (Overmann et al., 2017)

- **Culturing microorganisms**

The growth of microorganisms is controlled by a wide range of environmental variables, including pH, temperature, salinity, oxygen, light, osmotic pressure, nutrient availability….

- **Culture media**

Culture media are used to support the artificial growth of microrganisms in laboratory by providing the nutritional needs.

Minimal: precise amounts of highly purified chemicals

Complex: high amount of partially unknown chemical compounds

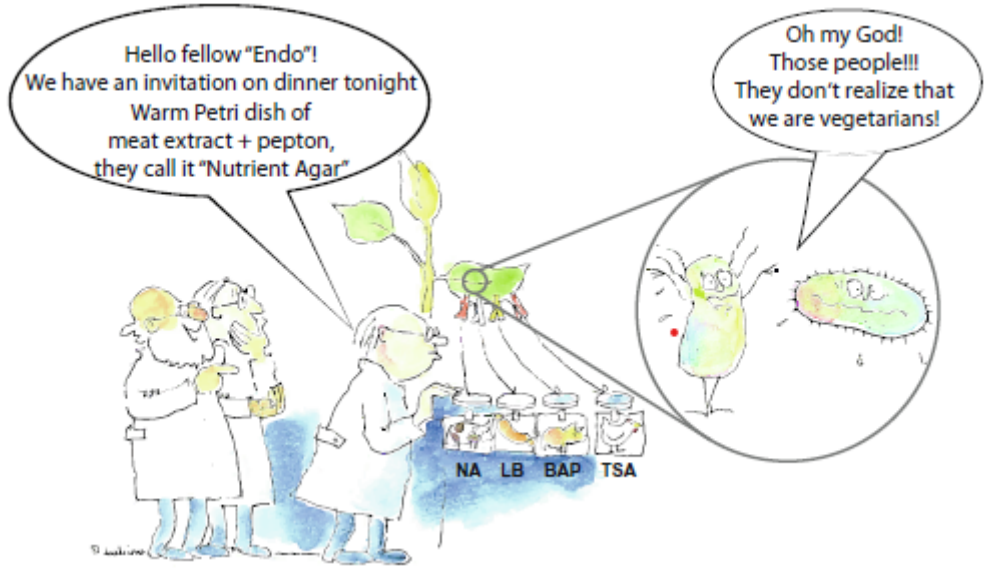
Selective: contains compounds that selectively promote the growth of a specific microorganism

Differential: contains indicator. It is used to distinguish one microorganism from another growing on the same medium

Culture media commonly used to isolate soil microorganisms

1/10 Reasoner's 2A agar (R2A)
1/100 Reasoner's 2A agar (R2A)
1/10 Tryptic soy agar (0.1×TSA)
1/100 Tryptic soy agar (0,01 TSA)
1/10 Nutrient agar (0.1×NA)
1/100 Nutrient agar (0,01 NA)

Heterotrophic and oligotrophic bacteria



From: Saharan *et al*. J Adv Res (2019) | | https://doi.org/10.1016/j.jare.2019.04.002

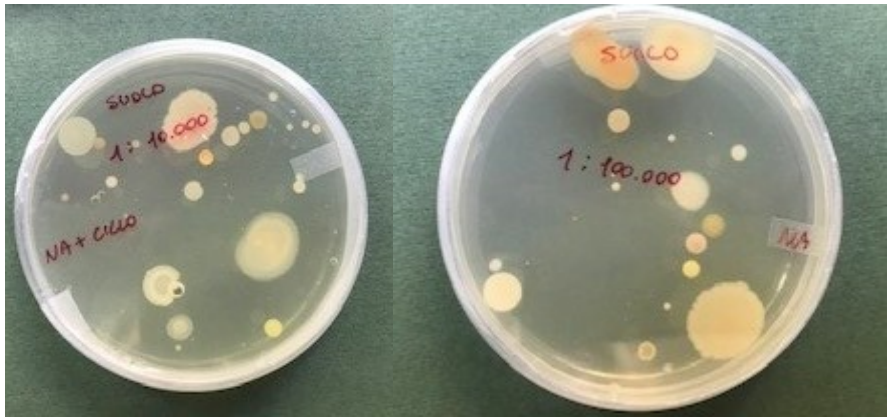
Isolation of soil bacteria

1 grams of of soil = 10^{10} batteri -10^{3} - 10^{4} different species

Rhizosphere soil

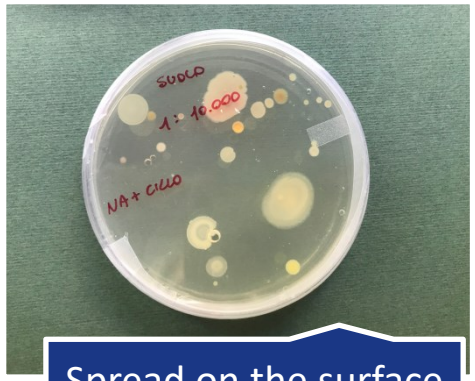
Bulk soil

- *Sampling site*
- *Sampling protocol*

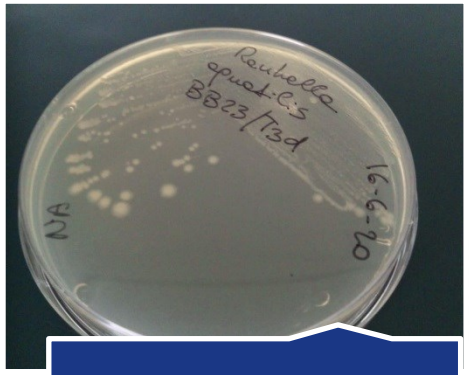


Soil and rhizosphere samples recovered from sampling site must be processed as soon as possible to have a more reliable picture of the diversity of the microbial community

Isolating aerobic bacteria



Spread on the surface of agar plates



Streak on agar plates

Bacterial culture in Erlenmeyer flask

Colonies count

Isolation of pure colonies

Microbial growth (OD 600 nm)

Isolating microaerophilic bacteria: semisolid media

Isolation of nitrogen-fixing bacteria on semisolid medium

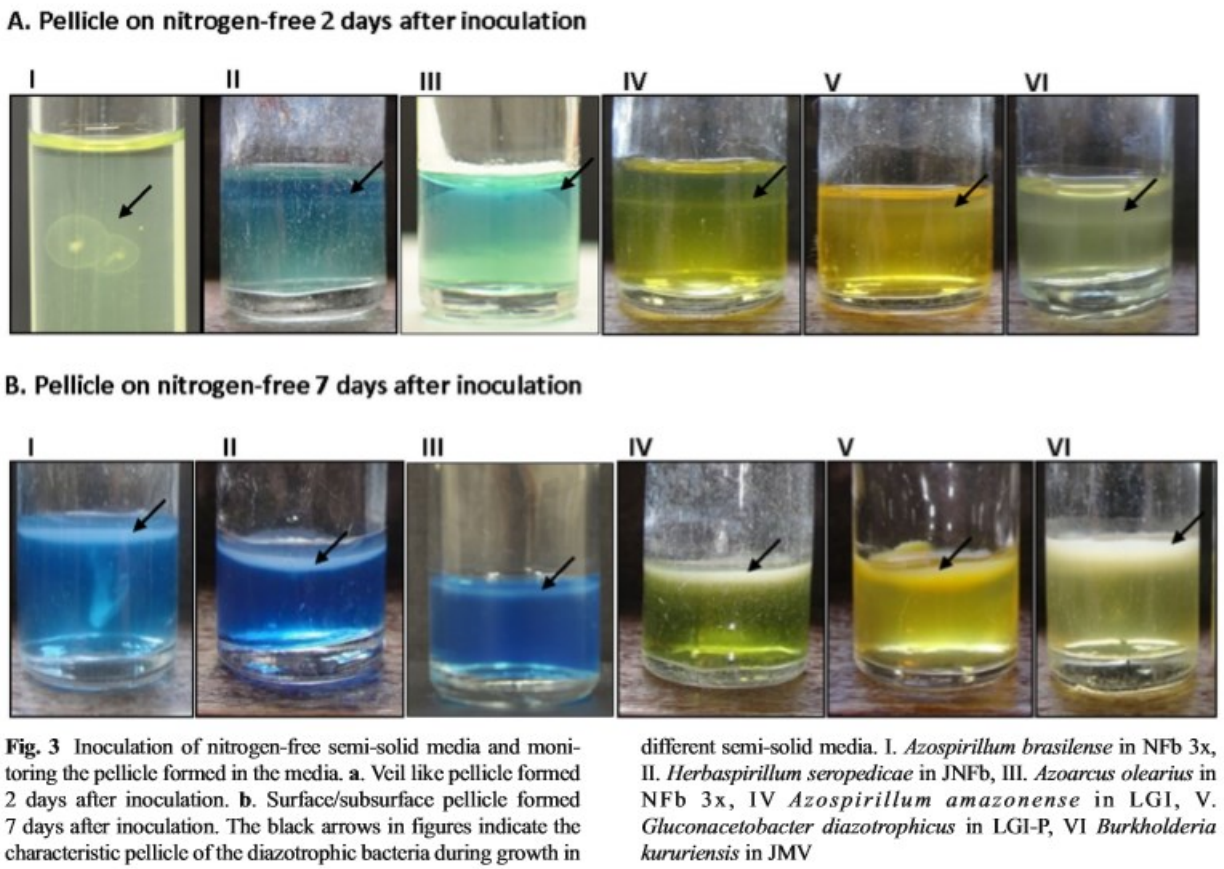


Fig. 3 Inoculation of nitrogen-free semi-solid media and monitoring the pellicle formed in the media. **a**. Veil like pellicle formed 2 days after inoculation. **b**. Surface/subsurface pellicle formed 7 days after inoculation. The black arrows in figures indicate the characteristic pellicle of the diazotrophic bacteria during growth in different semi-solid media. I. *Azospirillum brasilense* in NFb 3x, II. *Herbaspirillum seropedicae* in JNFb, III. *Azoarcus olearius* in NFb 3x, IV *Azospirillum amazonense* in LGI, V. *Gluconacetobacter diazotrophicus* in LGI-P, VI *Burkholderia kururiensis* in JMV

From: Baldani *et al*., (2014) Plant Soil | https://doi.org/ 10.1007/s11104-014-2186-6

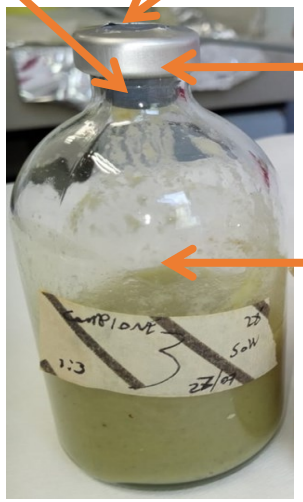
Isolating anaerobic bacteria

Anaerobic glove-box

Anaerobic jar

Butyl rubber stopper

Insert syringe needle to flux gas and a needle to mantain the gas equilibrium



Aluminium screw cap with an opening

Headspace sparged with N_2 or gas **mixture**, 80% **N_2** and 20% **CO_2** to remove oxygen

Traditional and innovative culturing techniques

Soil sample

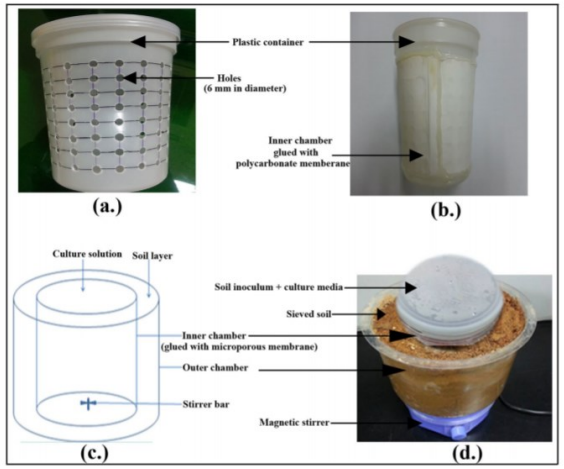


Figure 2. Design of the diffusion bioreactor for the cultivation of previously uncultured bacteria. (**a**) Plastic container perforated throughout with holes; (**b**) polycarbonate membrane glued inner chamber; (**c**) schematic diagram of the diffusion bioreactor; (**d**) overall experimental setup using the newly developed diffusion bioreactor.

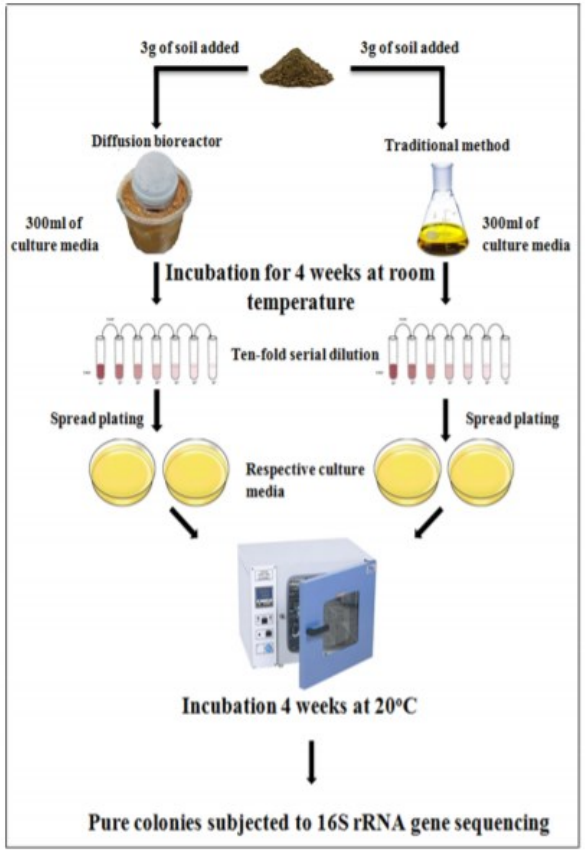
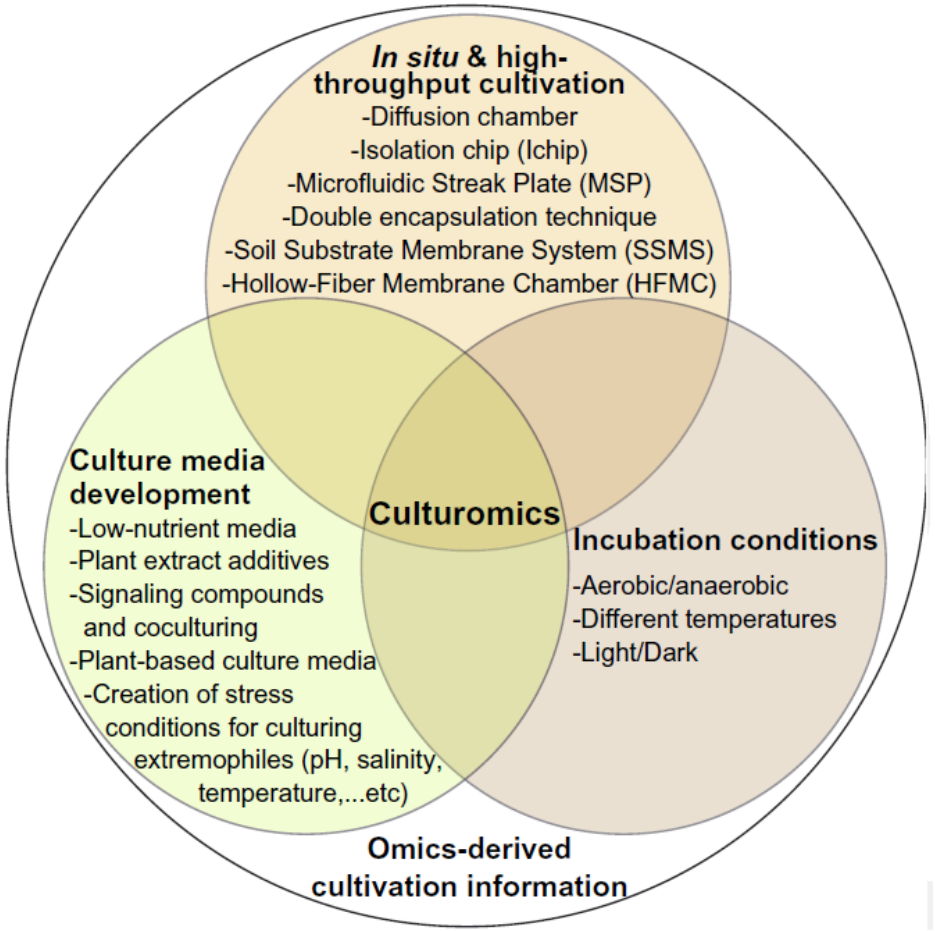


Figure 1. Scheme of the optimized protocol for the cultivation of previously uncultured microorganisms from forest soil.

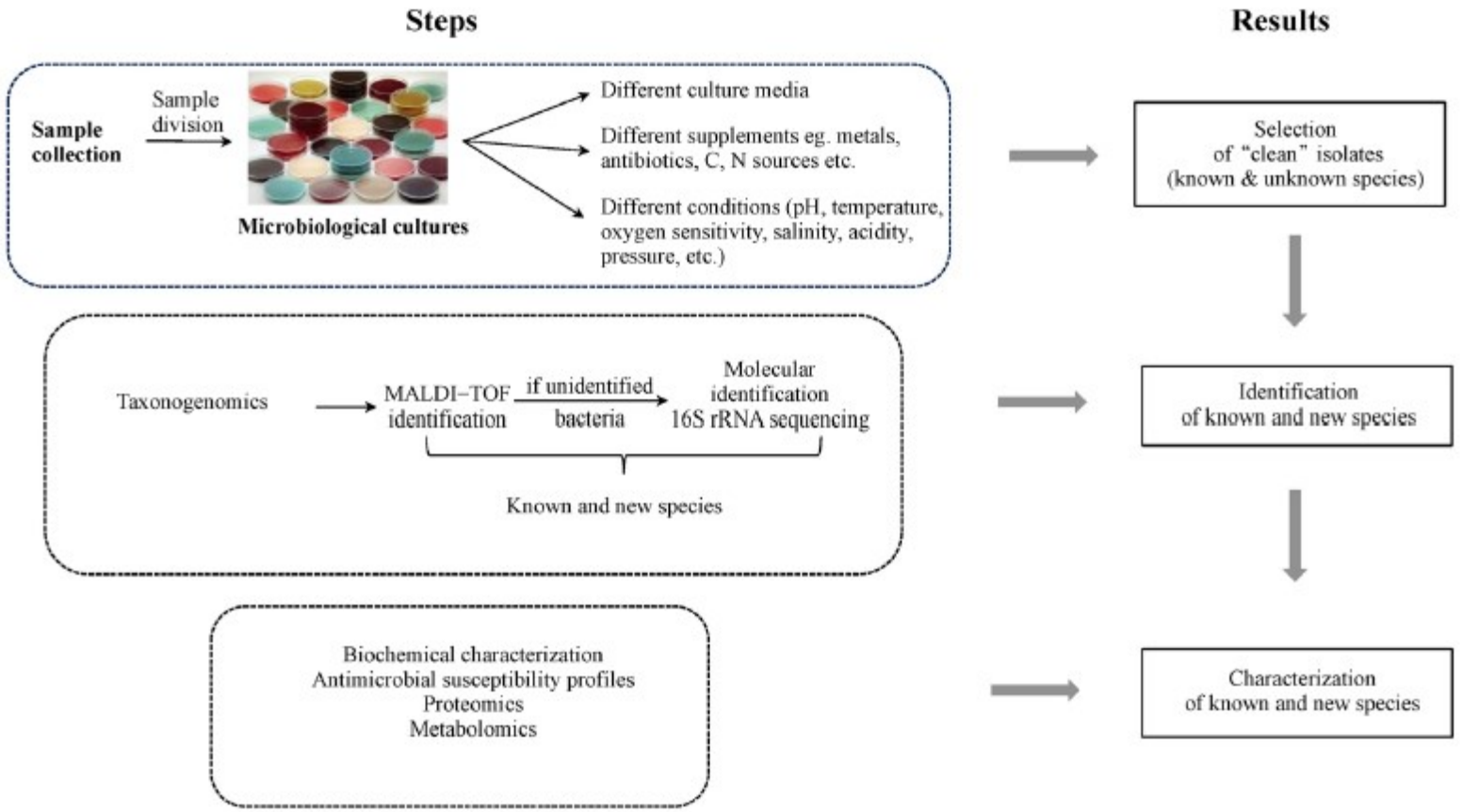
From : Chaudary *et a*l., (2019) Sci.Rep. | https://doi.org/10.1038/s41598-019-43182-x

Strategies to improve culturability of environmental microbiomes



From: Sahran et al., (2019) | https://doi.org/10.1016/j.jare.2019.04.002

Scheme of the culturomics approach



From: Nowrotek *et al*. (2019) | https://doi.org/ https://doi.org/10.1007/s11783-019-1121-8

ARTICLE OPEN

Check for updates

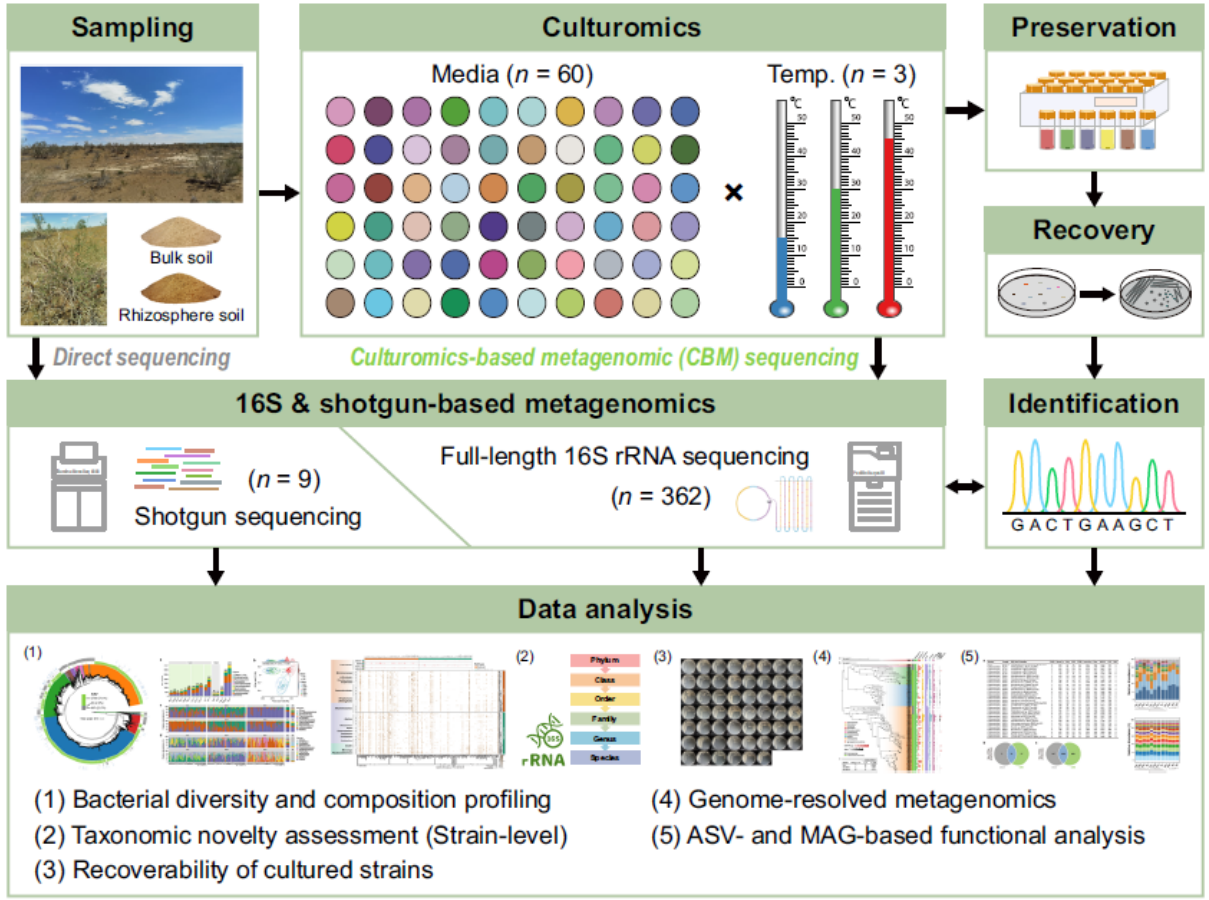
Capturing the microbial dark matter in desert soils using culturomics-based metagenomics and high-resolution analysis

Shuai Li[1,2], Wen-Hui Lian[1], Jia-Rui Han[1], Mukhtiar Ali[1], Zhi-Liang Lin[1], Yong-Hong Liu[3], Li Li[3], Dong-Ya Zhang[4], Xian-Zhi Jiang[4], Wen-Jun Li[1,3]✉ and Lei Dong[1]✉

Deserts occupy one-third of the Earth's terrestrial surface and represent a potentially significant reservoir of microbial biodiversity, yet the majority of desert microorganisms remain uncharacterized and are seen as "microbial dark matter". Here, we introduce a multi-omics strategy, culturomics-based metagenomics (CBM) that integrates large-scale cultivation, full-length 16S rRNA gene amplicon, and shotgun metagenomic sequencing. The results showed that CBM captured a significant amount of taxonomic and functional diversity missed in direct sequencing by increasing the recovery of amplicon sequence variants (ASVs) and high/medium-quality metagenome-assembled genomes (MAGs). Importantly, CBM allowed the post hoc recovery of microbes of interest (e.g., novel or specific taxa), even those with extremely low abundance in the culture. Furthermore, strain-level analyses based on CBM and direct sequencing revealed that the desert soils harbored a considerable number of novel bacterial candidates (1941, 51.4%), of which 1095 (from CBM) were culturable. However, CBM would not exactly reflect the relative abundance of true microbial composition and functional pathways in the in situ environment, and its use coupled with direct metagenomic sequencing could provide greater insight into desert microbiomes. Overall, this study exemplifies the CBM strategy with high-resolution is an ideal way to deeply explore the untapped novel bacterial resources in desert soils, and substantially expands our knowledge on the microbial dark matter hidden in the vast expanse of deserts.

npj Biofilms and Microbiomes (2023)9:67 ; https://doi.org/10.1038/s41522-023-00439-8

Culturomic-based metagenomic approach



Li *et al*., Biofilms and Microbiomes (2023) | https://doi.org/10.1038/s41522-023-00439-8

Preservation of bacteria

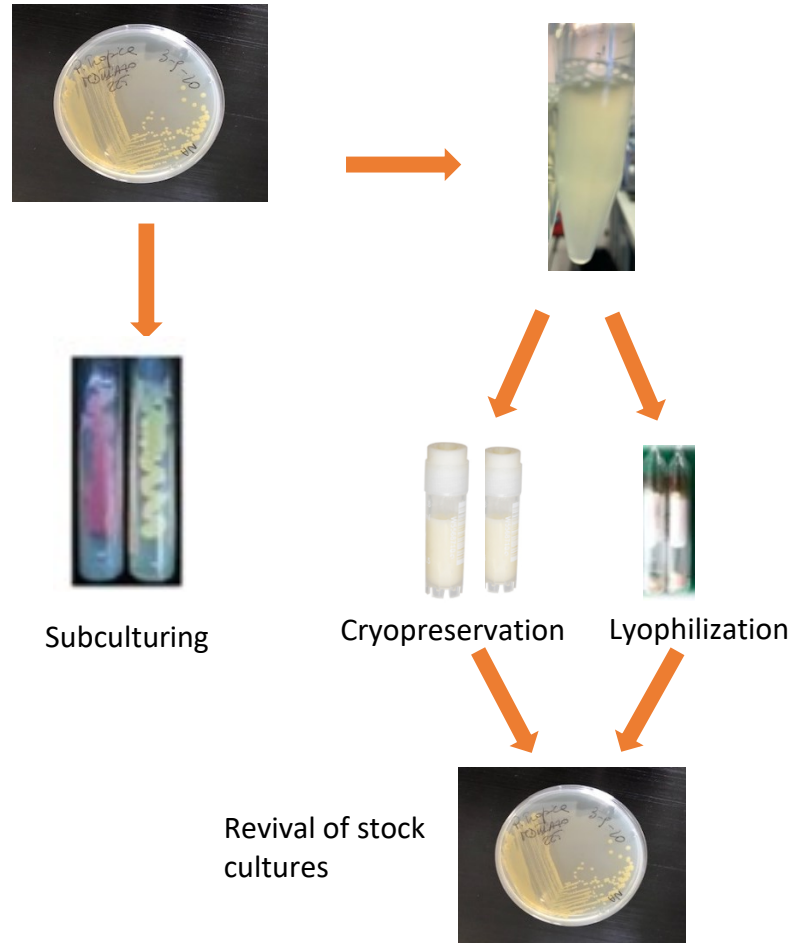
Storage method	Temperature	Storage time	Notes
Short term	Depend from the bacterium (room temperature, +4°C)	Individual property of the strain	Contamination, genetic drift
Medium term	-20°C, -30°C	Up to 1-2 years	Incomplete freezing (damage of cells)
Long term Cryopreservation	-80°C, -150°C	Up to 5-10 years	Expensive
Long term Freeze dry	+4°C, +8°C	Up to several decades	Standard method adopted by culture collections

- ***It is good practice to preserve strains that you work with for long term storage as repeated subculturing can leave these cultures vulnerable to contamination and genetic drift***

- ***A cryoprotectant must be used for medium and long term storage***

Main steps of bacterial preservation

- *Check for the the purity*
- *<u>Do not use a single cell to preserve a bacterial culture</u>*
- *Growth the bacterium at the optimal conditions (T, culture medium, atmosphere)*
- *Cryopreservation or freeze-dry*
- *Revival of the preserved bacterial culture at regular intervals*

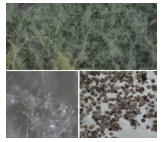


Fungi identification

- ✓ The most common traditional method used for fungi identification is the classification based on phenotypic characters For the identification of the fungal species **macromorphological** and **micromorphological** criteria are evaluated such as color of the mycelia, diameter of mycelia and conidia, production of conidiophores, production of sclerotia, etc,

- ✓ Analysis of macromorphological characters is performed with Stereomicroscope (magnification of 10-100 times)

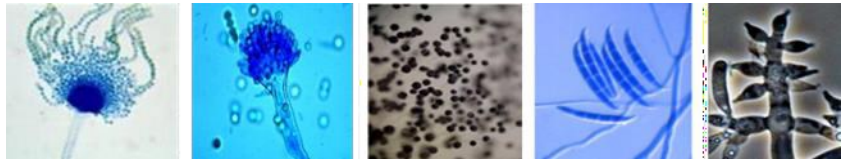
Mycelia of different fungal species



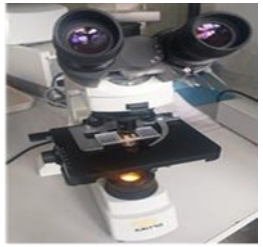
Fruiting bodies and conidial orientation from stereomicroscope

- ✓ **Micromorphological analysis** of sexual and asexual reproductive structures can be performed with Optical Microscope at increasing magnifications (up to 100X) .

Conidiophores



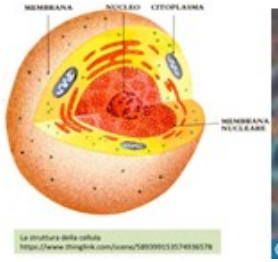
A. Flavus *A. Parasiticus* *A. Carbonariuus* *F. graminearum* *Trichoderma harzianum*

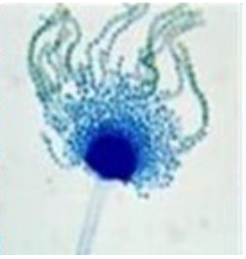


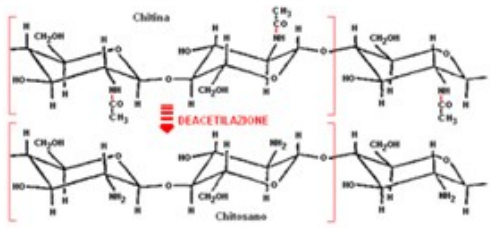
Optical Microscope

FUNGI

- ✓ Eukaryotic, macroscopic or microscopic organisms
- ✓ Single-cell such (yeasts), multicellular microscopic organisms (filamentous molds)
- ✓ Saprotrophs/ Symbionts/ Parasites
- ✓ Asexual or sexual reproduction by spores
- ✓ Cell wall (mainly containing chitin as well as other components including polysaccharides, glucans, proteins and melanins





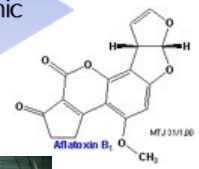
Pictures source:google

Ubiquitous in terrestrial and aquatic habitat, universal spread (Maharachchikumbura et al. 2016)

FUNGI RELEVANCE FOR AGRIFOOD SYSTEM

FOOD SPOILAGE (e.g. altherative yeasts/molds

FOOD BOORNE OUTBREAKS (e.g. toxigenic molds)



SUSTAINABLE AGRICULTURE (e.g Biostimulants PGPMs Plant Growth-Promoting Microbs)

AGRO- INDUSTRIAL PROCESSES (e.g. pro-technological yeasts and molds)

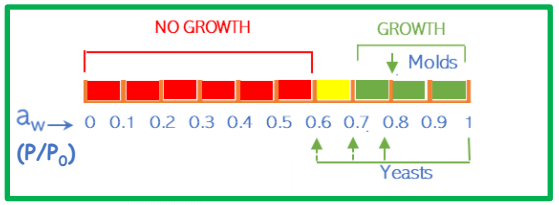
High adaptability to different environmental and host-related factors

Main parameters affecting fungal growth

✓ **Activity/metabolic water (a_w)**

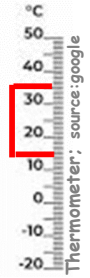
Fungal colonization can take place at a_w > 0.80
Xerophilic molds and osmophilic yeasts are viable ≤0.70

Water activity scale; source:google



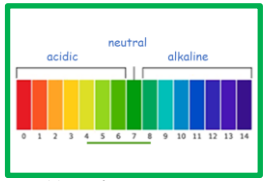
✓ **Temperature**

Growth in the range 15 - 35 °C. Ther mophilic, psychrophilic
or psychrotolerant fungal species/strains can grow outside this range



✓ **pH**

Fungi grow at pH among 4 and 8
Some species are able to colonize their host even in unfavorable acid or alcaline conditions



pH Scale; source:google

✓ **Oxygen**

Generally obligate aerobes; several species can also grow in microaerophilic or anaerobic conditions

FUNGI ISOLATION, WHY?

- ✓ Fungi are in soil, water and food as (saprophytes/pathogenic/symbiontes) components of mixed fungal-yeast-bacterial) microbial communities

- ✓ The species of interest must therefore be isolated from the microbial community to which it belongs and grown in ***genetically pure culture, useful for:***

  - Morphological identification, physiological and chemical profiling of fungal strains
  - Production of fungal biomass for DNA extraction and for molecular characterization/identification
  - Microbial Culture Collection
  - Agro-industrial applications

CULTURE MEDIA FOR FUNGI

- ✓ Sintetic media ensuring easily nutrients and suitable moisture, **favoring both the growth and sporulation of fungi. Potato Dextrose Agar (PDA)** (Potato Dextrose Broth-PDB is its liquid form) **Enrichment medium** is the most used.

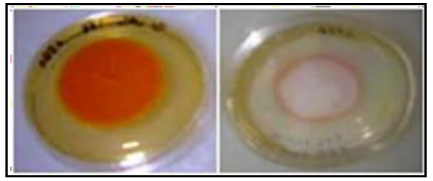
PDA/PDB plates/flask

- ✓ **Czapek Dox Agar (CDA) or Czapek Dox Broth (CDB)**, having sodium nitrate as the only source of nitrogen, and sucrose as the only carbon source, is instead **a selective medium,** widely used for saprophytic fungi (particularly soil fungi) able to use inorganic forms of nitrogen

- ✓ Other commercial selective/ differential media (e.g. Aspergillus flavus-parasiticus agar selective medium)…

.

Aspergillus flavus **on PDA growth medium after 7 days of incubation at 27ºC.**



Aspergillus flavus **on AFPA selective/colorimetric growth medium after 7 days of incubation at 27ºC.**

ISOLATION METHODS

Direct plating

✓ **To detect, enumerate and isolate fungi from particle samples (g.e. seeds, cereal…..).**

1. Put particles often after surface disinfection (e.g. 0.4% chlorine solution) essential for example to isolate endophytic fungi, directly on PDA plates containing antibiotic (Streptomycin 300 ppm and Neomycin 150 ppm) to avoid bacterial growth and then incubate in thermostatic chamber at 25 -30 °C in darkness for .7 days.

2. .Results usually expressed as percentage of particles with fungal growth

Direct plating on PDA growth medium of maize kernels Incubation at 25°C for 7 days.

2. Isolate fungi of interest by subculturing into new media picking up a small agar plug of each mycelium (approx. 5mm diameter), putting it on fresh PDA plates and then incubating at 25 -30 °C in darkness. This operation must be replicated for several generations until to obtain the pure strains of fungal isolate .

ISOLATION METHODS

Dilution plating

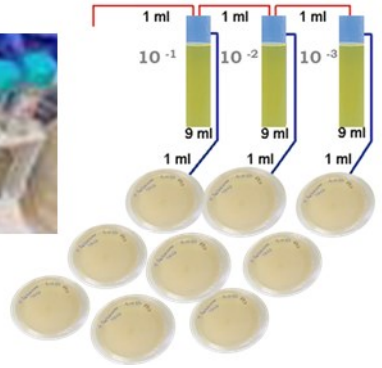
✓ For **liquid, complex matrices or powdered samples**

1. Dilute/resuspend in sterile aqueous solution (e.g. physiological saline solution 0.9% NaCl or 0,01% Triton x- 100) and then blend/homogenize.

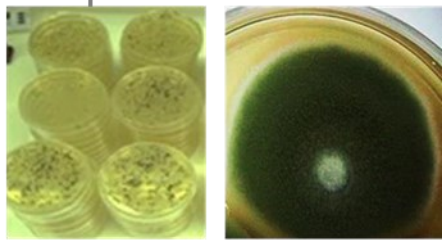
2. Prepare Serial dilutions, plate on PDA plates containing antibiotic (Streptomycin 300 ppm and Neomycin 150 ppm) to avoid bacterial growth and then incubate in thermostatic chamber at 25 °C in darkness for 7 days.

3. Pick up colonies of interest and reinoculate on PDA plates until pure colonies are obtained

Tomato Sauce dilution with sterile 0.9% NaCl physiological solution



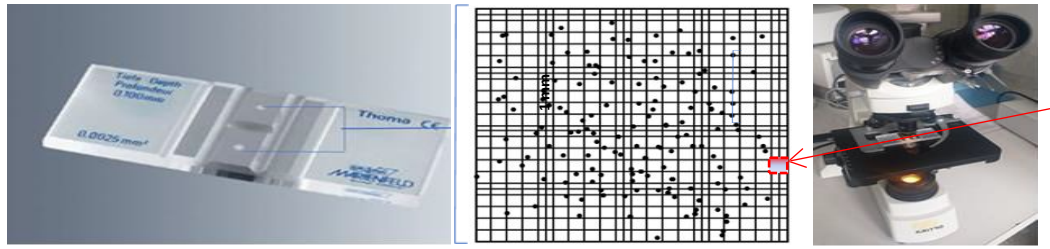
Preparing Serial dilutions, plating on PDA plates and incubation in thermostatic chamber at 25 °C in darkness for 7 days



Isolation and growing of pure fungal strain on PDA

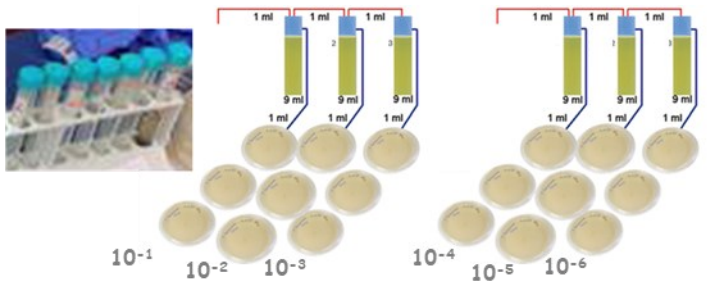
EVALUATION AND QUANTIFICATION OF FUNGAL GROW

- ✓ **Direct count (CFU/ml)** by **Thoma cell counting chamber** and **optical microscope;**



N (CFU/mL): [(N colonies counted/n counting units)/$2.5*10^{-7}$ mL]

- ✓ **Colonies count on PDA agar plates (viable fungi) (N CFU/mL)**



Preparing Serial dilutions, plating on PDA plates and incubation in thermostatic chamber at 25 -30 °C for 7 days

PDA PLATE fungal colonies counted

EVALUATION AND QUANTIFICATION OF FUNGAL GROW

FILAMENTOUS FUNGI- MOLDS

- ✓ Critical Issues
  1. **Fungal Hyphal filaments**: more difficult quantification by traditional microbiological counting techniques
  2. **Spores production**: high increasing of viable counts, low correlation with fungal biomass.

- ✓ Due to the fungi morphological characteristics, the spectrophotometric method based on the evaluation of the optical density is not commonly applicable for the quantification of fungal growth
- ✓ For *Thricoderma* inocula was developed a spectrophotometric method evaluating the influence of spore morphology.

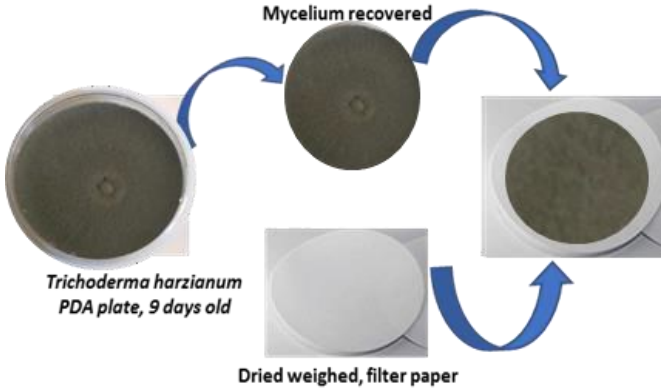
  Optical density (OD) measurement at 550 nm using a UV/Visible specrophotometer (Conidia/mL);

  (Schütz G., Faltrich D., Atanasova L., 2020; https://doi.org/10.2144/btn-2019-0152

EVALUATION AND QUANTIFICATION OF FUNGAL GROW

- ✓ **Mycelium dry weight (mg)**

  (cut from Agar Petri plate or recovered from liquid culture medium)

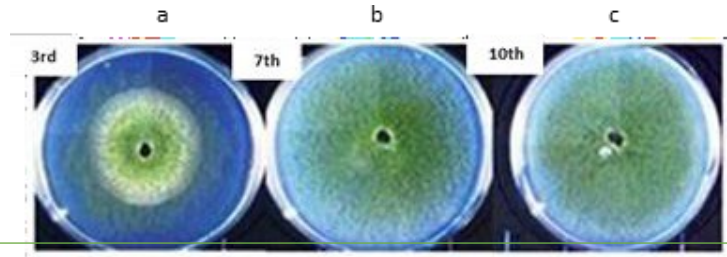


- ✓ Oven-drying at 80°C for 18 h;
- ✓ Dry weight mycelium calculated by difference †

- ✓ **Colonies diameter (mm) measurement**

  Evaluation and quantification of fungal growth

  (linear correlation between colony diameter and growth rate)



PDA Petri plates ***Aspergillus flavus 3357***, **3(a), 7(b)** and **10(c)** days after inoculation. Incubation a 25°C. Plates Diameter 9 mm.

Chemical assays

- ✓ <u>HPLC determination of ERGOSTEROL</u>.

  Prevalent/ sterol in most fungi , not significant extent in plants, animals or bacteria. Marker for fungal biomass.

FUNGI IDENTIFICATION

How do we proceed?

1. Preliminary, morphology-based classification, by using phenotypic approach

2. Identification and taxonomic classification by chemical, ecological or physiological and molecular analyses

A morphology-based classification allows fungi to be classified at genus and species level. It is crucial for a large number of existing species for which only morphological and ecological data and not molecular ones are available. It therefore allows to compare such data among different taxonomic or ecological groups of fungi even in the absence of molecular data.

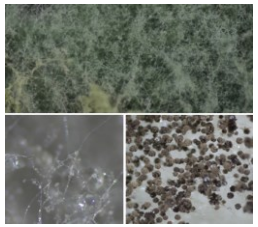
Molecolar analysis allows the identification and taxonomic classification of fungi. Phylogenetic, biological and genetic analysis allows to understand the diversity and inter- and intra-relationships of fungal groups

Fungi identification

- ✓ The most common traditional method used for fungi identification is the classification based on phenotypic characters. **Macromorphological** and **Micromorphological** criteria are evaluated such as color of the mycelia, diameter of mycelia and conidia, production of conidiophores, production of sclerotia, etc,

- ✓ Analysis of macromorphological characters is performed with **Stereomicroscope** (magnification of 10-100 times)

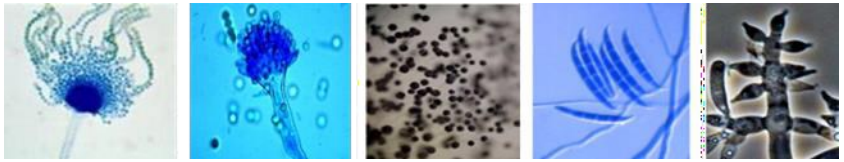
Mycelia of different fungal species



Fruiting bodies and conidial orientation from stereomicroscope analysis

- ✓ **Micromorphological analysis** of sexual and asexual reproductive structures can be performed with Optical Microscope at increasing magnifications (up to 100X) .

Conidiophores



A. Flavus *A. Parasiticus* *A. Carbonariuus* *F. graminearum* *Trichoderma harzianum*

EXPERIMENTAL ACTIVITY: Evaluation of In Vitro Compatibility *T. harzianum* TH01* –Bacteria

(Tabacchioni et al.,2021; https://doi.org/10.3390/microorganisms9020426)

***Trichoderma harzianum* TH01** Antagonistic fungi, biocontrol agent against pathogens and "growth promoter"

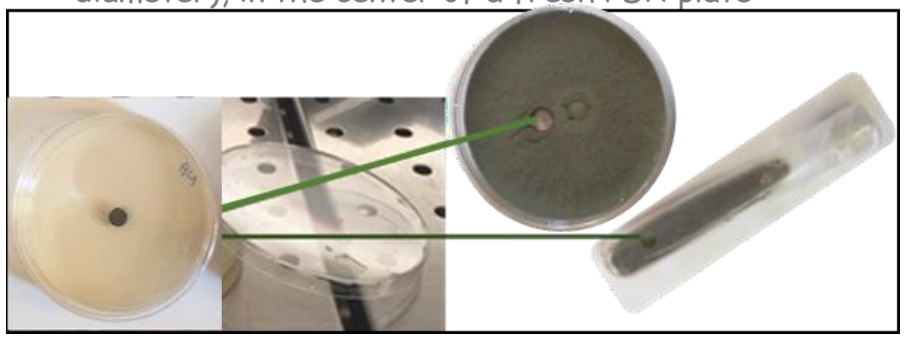
1.FUNGUS REFRESHING /REACTIVATION

9 days PDA-grown slant/plate or 30% glycerol stocks

- Pick up a loopful of the fungus from the cryotube conidial suspension in 30% glycerol and place in the centre of a fresh PDA plate

or

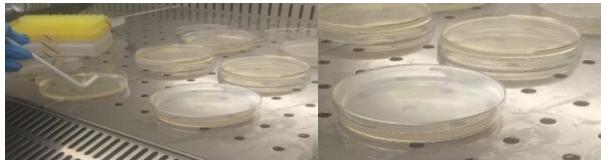
- Transfer a small agar plug of the mycelium (5mm diameter), in the center of a fresh PDA plate



Plating of fungal mycelium on PDA growth medium. Incubationin in thermostatic chamber at 28°C for 9 days.

2.INOCULUM PREPARATION

- Add sterile distilled water (10 ml) to a 9 days PDA-grown slant or plate of the fungus and scrape the surface slightly with a sterile plastic microbiological loop to recover spore and conidial suspension. Filtrate the resulting suspension through two layers of sterile gauze to remove residual hyphae;
- Determinate the concentration of conidial suspension (n. CFU/ mL) by using the Thoma Chamber and an optical microscope at 10-40X magnification;
- Count the CFU/mL value by the plate dilution method, spreading 100 µL of 10^{-4}, 10^{-5} and 10^{-6} dilution of fungal suspension PDA plates (in triplicate for each dilution) and incubating in thermostatic chamber at 28 °C for 4-7 days;



- Measure the OD at 550 nm to correlate the absorbance value to the concentration. (Schütz G., Faltrich D., Atanasova L., 2020; https://doi.org/10.2144/btn-2019-0152

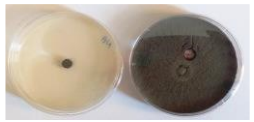
EXPERIMENTAL ACTIVITY: Evaluation of In Vitro Compatibility *T. harzianum* TH01* –Bacteria

(Tabacchioni et al.,2021; https://doi.org/10.3390/microorganisms9020426)

3.INOCULATION AND GROWING

SOLID PHASE INOCULATION AND GROWING

- Transferring a small agar plug of the mycelium (5 mm diameter) in the center of a fresh Potato Dextrose Agar (PDA) plate and allowing it to grow at 28°C for 9 days in thermostatic chamber .



LIQUID PHASE INOCULATION AND GROWING

- Inoculation of 1ml of *T.harzianum* conidial suspension (4-5*10^8 conidia/ml) in 200 ml of potato dextrose broth (PDB) contained in a 500 ml flask; Incubation at 28°C and 120 r.p.m for 72h; Recover, filtration and washing the mycelium.

Mycelium from liquid culture suitable to starting with a large scale production ofT. harzianum biomass.

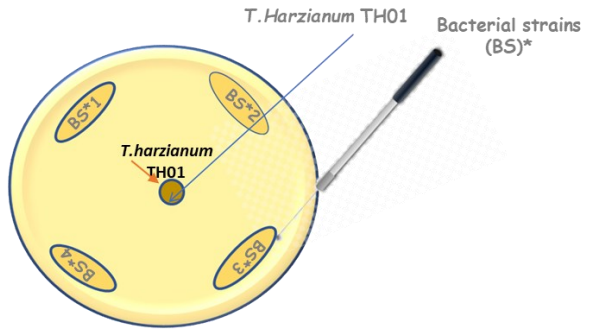
4.PRESERVATION

- Short term preservation: Fungal strains grown on to PDA plates at 4°C for daily or weekly use.
- Medium term preservation: Fungus PDA slants Overlaid with autoclaved mineral oil to prevent the fungus from drying out.
  Slants can be kept at 4°C for two months.
- Long term preservation: Spore and conidial suspension (aliquot) cryopreserved at -80°C in 30% glycerol, in sterile cryovials (1.5 mL capacity); lyophilization

Evaluation of In Vitro Compatibility T. harzianum TH01 – bacteria by Agar-Plate method

(Tabacchioni et al.,2021; https://doi.org/10.3390/microorganisms9020426)

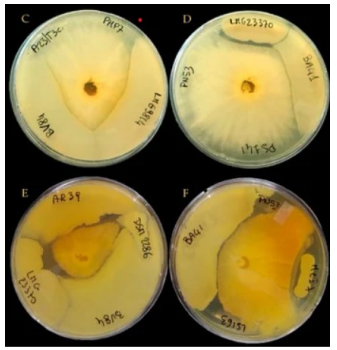
1. **Bacterial strains refreshing:** A loop of each microbial strain (bacteria and/or yeast) was picked up from glycerol stocks stored at −80 ° C and streaked onto nutrient agar (NA). The plates were incubated at 28 °C for 24–48 h to allow the microbial growth.
2. **Bacterial strains and fungus inoculum:** A loop of each bacterium or yeast to be tested (maximum four strains for each plate) was streaked near the edge of potato dextrose agar (PDA) plates at fixed positions. A mycelium agar plug (5-mm diameter) of the (9 days old) fungus was transferred to the centre of the previous inoculated PDA plates.
3. **Incubation**:The plates were incubated in darkness at 28 °C, and the zone of inhibition (if any) was recorded after 48–96 h of microbial growth.



For each microbial combination (bacteria-fungus),two independent experiments were performed with three replicates.

4. **Evaluation**:

Any overgrowth of the fungus on microbial (bacteria) streaks without a zone of inhibition were considered as bacteria-fungus compatibility.



Source: Tabacchioni et al.,2021

SOIL FUNGI ISOLATION PROTOCOL

https://journals.plos.org/plosone/article?id=10.1371/journal.pone.0105515

1. Resuspension of 1 gram of rhizospheric soil with 10 ml of Phosphate Buffer Saline (PBS) pH 7.0
2. Homogenization with Ultra-Turrax Thyristor Regle 50 (Janke & Kunkel IKA-Labortechnik), 30" at low speed
3. Transfer suspension (10 ml) into Erlenmeyer flasks (100 ml) sterilized in autoclave for 20 minutes at 121°C before use, containing 10 g of glass beads (2 mm) and stirring for 1 h at 180 rpm. and 28°C
4. Transfer of the soil suspension into a 15 ml falcon and make serial dilutions (in sterile 0.9% NaCl) up to 10^{-6}
5. Plating 100 µl aliquots of the diluted soil suspensions on agar enrichment media (potato dextrose agar - PDA) or (Czapek dox agar – CDA) supplemented with antibiotic (50 mg L-1 of chloramphenicol)
6. Incubation at 28±2°C for 5 days
7. Count of fungal colonies developed on PDA/CDA plates after incubation
8. Isolation of single colonies: collection of circular sections (about 5 mm) of fungal mycelium with a sterile tip and transfer onto PDA and CDA plates
9. Analysis of the morphological, conidial (fruiting bodies) and cultural characteristics of the isolates using a stereomicroscope and optical microscope
10. Short-term storage of isolates at 4°C on PDA plates11. Long-term storage at -80°C in 30% glycerol

Thank you for your attention!

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