Università di Modena e Reggio Emilia
Dipartimento di Scienze della Vita

with CCS Aosta S.r.l. Company

QUALITY CONTROL OF MICROBIALS

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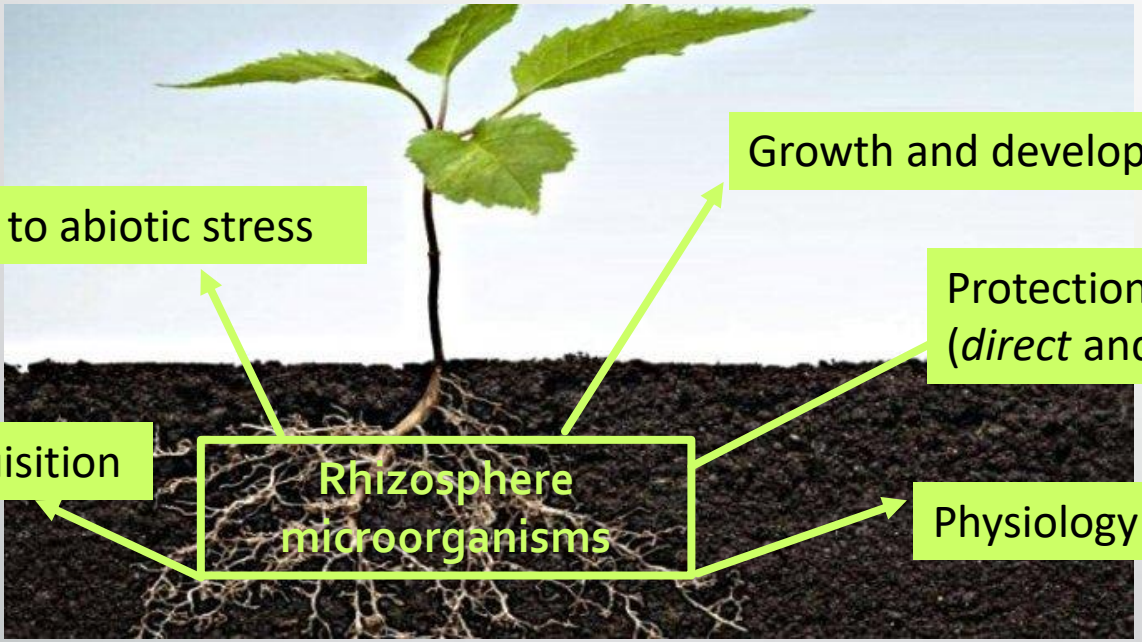
XXXII CYCLE
III year

INTRODUCTION

SYMBIOTIC AGRICULTURE

New cultivation process that involves the use of **beneficial microorganisms** such as fungi, bacteria and yeasts, for growth promotion, soil fertility and plant health.



- **Low** environmental impact (less chemical inputs)
- **Less** resources needed (*e.g*. water)
- **Resilience** (less care).

AIM OF THESIS

To set up **rules**, **procedures** and **protocols** to be used during the industrial production of microbials, in order to obtain an **efficient**, **effective** and **sustainable** microbial formulation.

Starting from a commercial biostimulant as **Micosat F UNO**, made up of a microbiological consortium, develop a "**quality protocol**" considering both the individual microorganisms presents and the consortium as a whole.

Only by performing quality control checks, microbials can be a valid alternative to chemical pesticides.

ELEMENTS THAT DETERMINE THE QUALITY OF A MICROORGANISM

- Origin and identity;
- Antagonistic activity;
- Plant growth promoting features;
- Biosafety;
- Consortium composition and viability;
- Quality control during production and shelf life;
  - *Efficacy of single microbes in the final commercial product(s);*

- (Dynamics of microbes in agricultural environments).

MATERIAL

Micosat F UNO is a microbial consortium produced by **the CCS Aosta** company.

Microbial formulation:

- **Rhizosphere bacteria**

Agrobacterium radiobacter AR39
Bacillus amyloliquefaciens BA41

- **Actinomycetes**

Streptomyces spp. SB14
***Steptomyces spp*. SA51**

- **Saprophytic fungi**

Pochonia chlamydosporia PC50
Trichoderma harzianum TH01

- **Yeasts**

Pichia pastoris PP59

- **Crude inoculum**

mycorrhizal and crushed roots containing:
Glomus (*Glomus spp*. GB67)
Funelliformis (*Funelliformis mosseae* GP11)
Septoglomus (*Septoglomus viscosum* GC41)

IDENTIFICATION AND CHARACTERIZATION of SA51

Colony morphology

Molecular characterization

Material and methods:

- SA51 isolated from the rhizosphere of an olive tree
- 3 successive sub-cultures on ISP-2 medium
- Pure cultures grown in Tryptic soy broth (TSB) for 3 days at 28°C.

- DNA extraction and purification: DNeasy Blood and Tissue Kit (Qiagen)
- NanoDrop One Microvolume UV - quantity
- Gel electrophoresis - quality

- Illumina HiSeq2000 sequencer for DNA sequencing was used: quality control, cutting and assembly phases were also performed using predefined parameters.

Sequence alignment was performed: ClustalW and "Map to a reference"

PCR amplification of the short subunit (SSU) 16S rRNA with primers:
strepB (5'-ACAAGCCCTGGAAACGGGGT-3')
strepE (5'-CACCAGGAATTCCGATCT-3')

Results:

Genome annotation with RAST:

- 6,040 coding sequences (CDS),
- 32 tRNAs, and
- 13 rRNAs in the SA51 genome.

SA51 preliminarily identified as ***Streptomyces avermitilis*** with identity of 97% with the e value of 0-3e-77 with the strains present in NCBI GenBank.

Furthermore, using the Kyoto Encyclopedia of Genomes and Genomes (KEGG) a construction of the metabolic profile of SA51 was carried out to identify the genes involved in promoting growth.

Statistics	Unassembled Reads	All Contigs	Contigs>=100 bp	Contigs <=1000 bp
Number of	2,959	792	792	74
Min Length (bp)	398	707	707	1,001
Median Length (bp)		2,179	2,325	2,179
Mean Length (bp)	1,088	2,832	2,832	2,969
Max Length (bp)	14,732	23,079	23,079	23,079
N50 Length (bp)		3,517	3,517	3,565
Number of Contigs >= N50		199	199	193
Length Sum (bp)	3,221,808	2,243,264	2,243,264	2,197,473

CULTIVATION

Growth is defined as the increase in the number of bacterial cells in a population; this increase can also be measured as an increase in microbial mass.

To study of a liquid medium suitable for the growth of the BF90 strain (*Bacillus firmus*)

Material and methods:

Different liquid media:

T1

nutrient medium (www.dsmz.de) for *Bacillus firmus:*
5 g of peptone
3 g of meat extract
1 liter of distilled water.
pH 7.0

T2

nutrient medium (www.dsmz.de) for *Bacillus firmus* + soil extract component

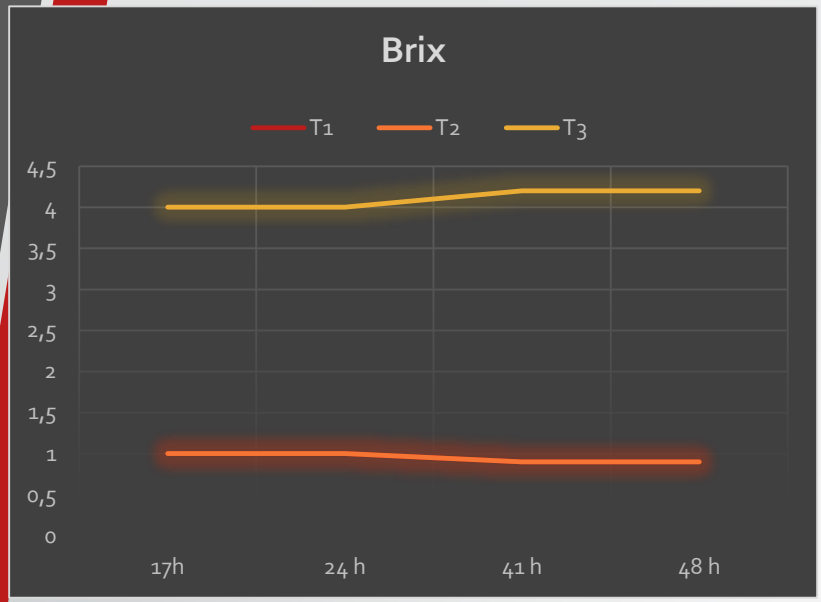
T3

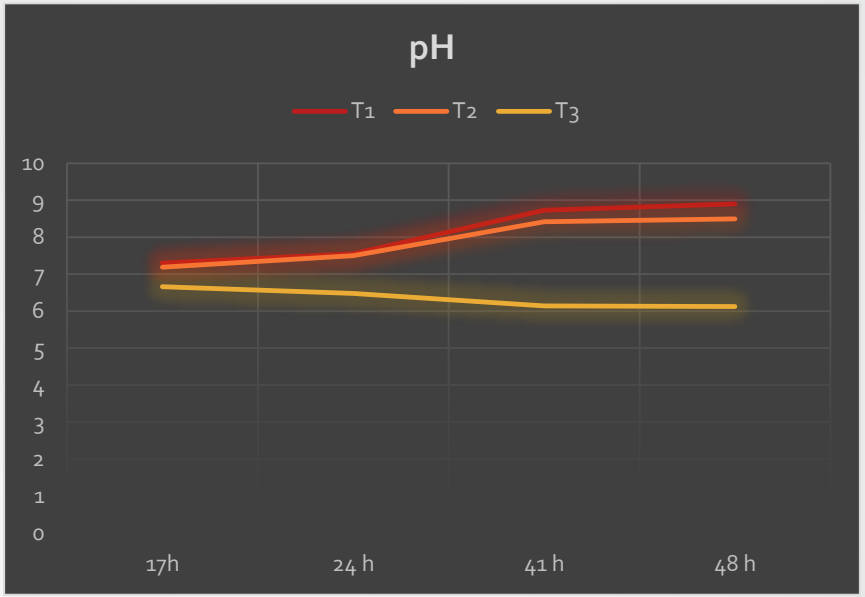
nutrient medium used by CCS Aosta for *Bacillus:*
20 g of glucose
10 g of malt extract
5 g of yeast extract
10 g of corn steep atomized (CSA)
1 g of casein peptone
1 liter of distilled water.
pH 7.0

Each flask containing a different growth medium was inoculated with BF90 and placed in a shaker at 30 ° C and 100 r.p.m.

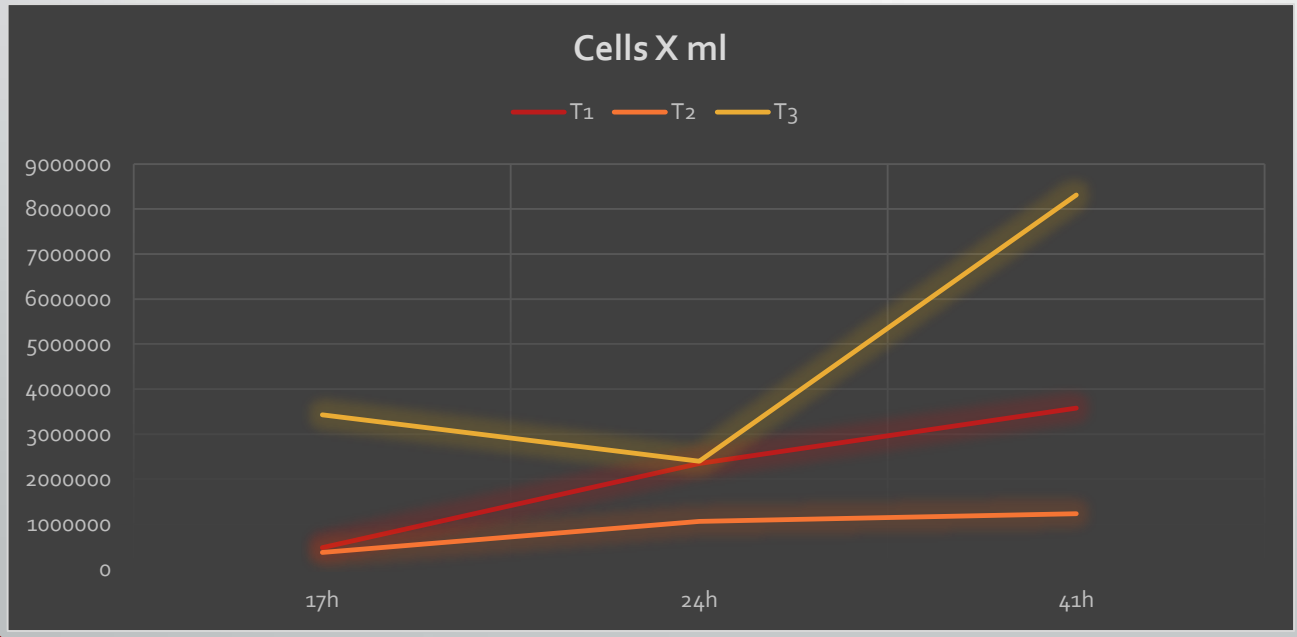
Checks at regular intervals of 17, 24, 41 and 48 hours:

- pH,
- Brix
- Optical Density





the Brix and pH levels in the three substrates



the turbidity levels (O.D. 600 nm, 0.1)

DEFINITION OF ANTAGONISTIC ACTIVITY

In vitro antimicrobial activity

Material and methods:

Interactions *in vitro* between selected microorganisms from the CCS Aosta collection

PATHOGENS

FUNGI: SP75 - *Sclerotium* sp.
FP76 - *Fusarium* sp.
RP77 - *Rhizoctonia solani*

BACTERIA: XP89 - *Xanthomonas pruni*
XJ88 - *Xanthomonas juglandis*
PS87 - *Pseudomonas syringae*

ANTAGONISTS

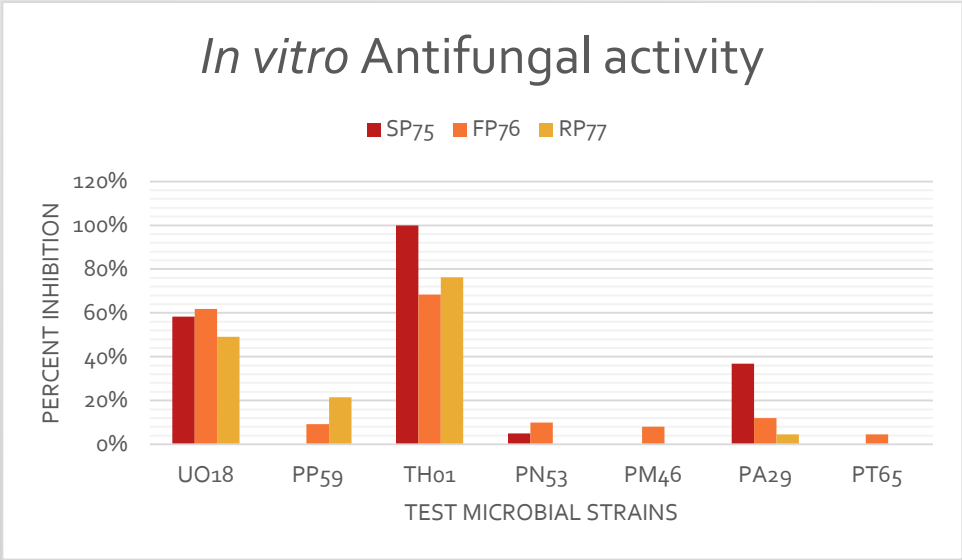
ACTINOMYCETES: SA51 - *Streptomyces avermitilis*

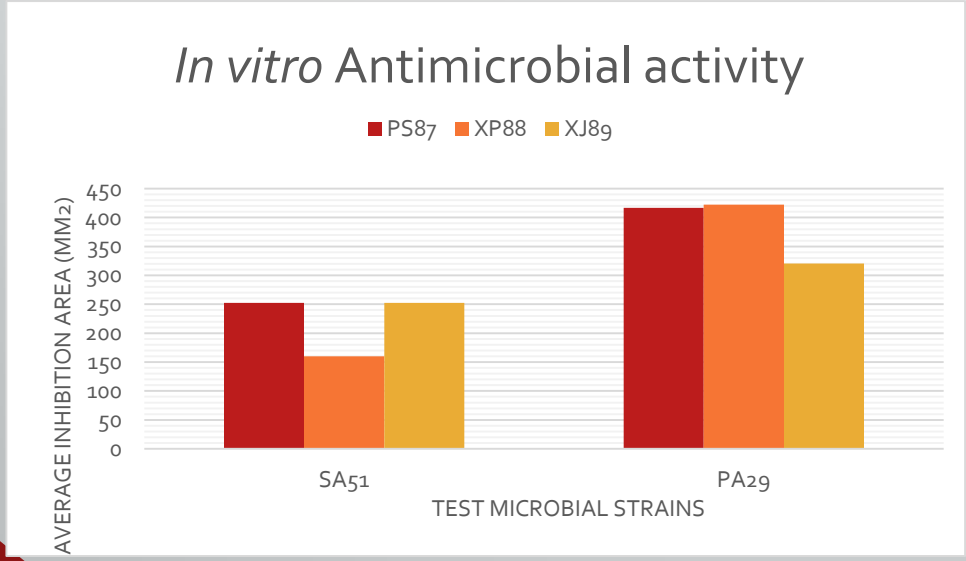
PSEUDOMONAS: PT65 - *Pseudomonas Spp.*
PA29 - *Pseudomonas fluorescens*
PM46 - *Pseudomonas Spp.*
PN53 - *Pseudomonas fluorescens*

FUNGI: UO18 – *Ulocladium oudemansii*
TH01 - *Trichoderma harzianum*

YEASTS: PP59 – *Pichia pastoris*

Results:





The study of the single microorganisms and the interaction between the antagonist and pathogenic strains is extremely useful for the development of a targeted and certified microbial product.

VOCs production

Many fungal species produce low concentrations of volatile organic compounds (VOCs), some of them have antimicrobial activity.

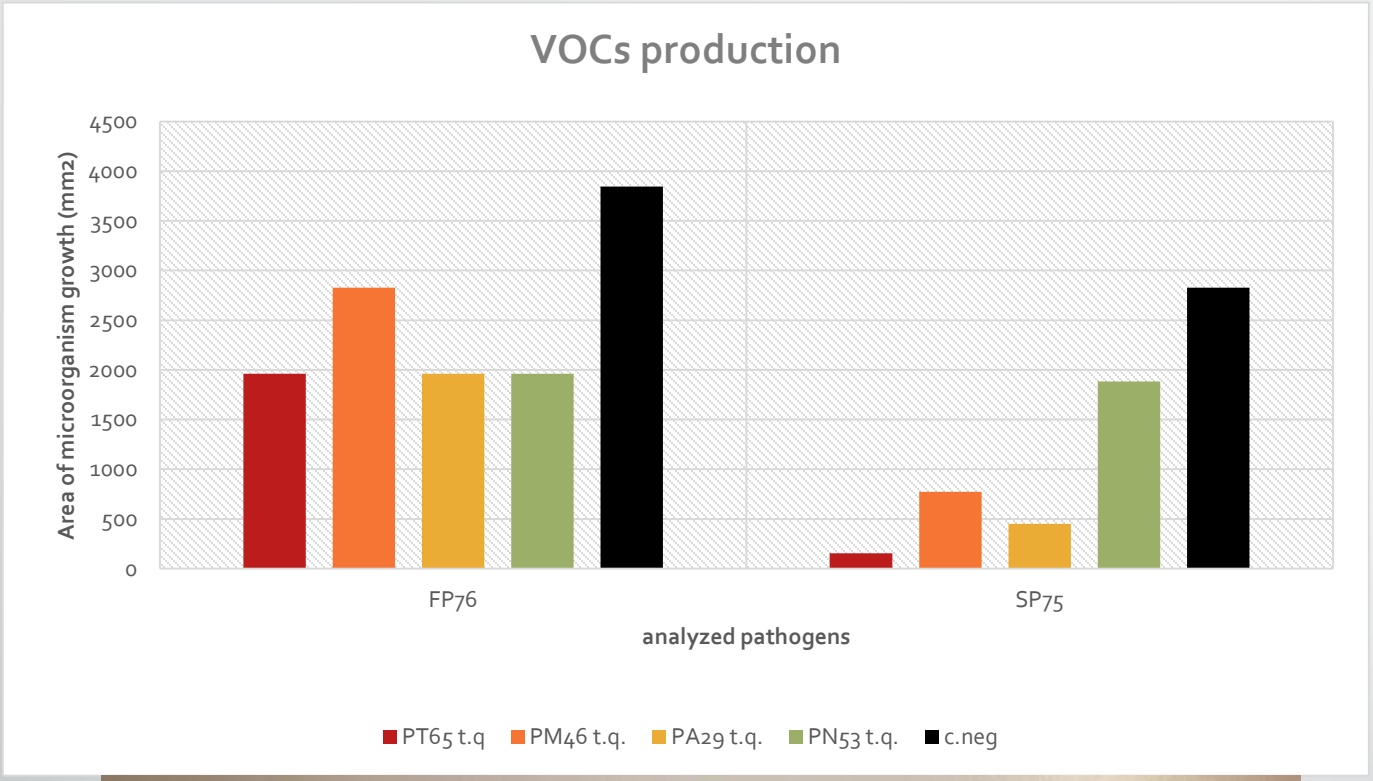
Material and methods:

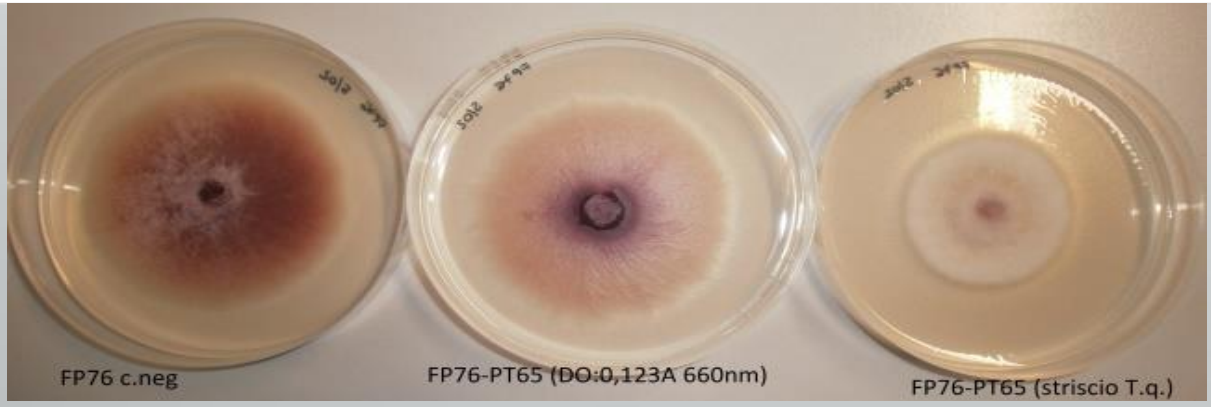
The inoculated plates were inserted open (without the lid) inside the plastic boxes:

- One box with a plate with the pathogen and a plate with the presumed antagonist organism together;
- One box with only the pathogenic microorganism;
- One box with only the antagonist organism

The sealed boxes were stored at a temperature of 27(±2)° C to allow the growth of microorganisms

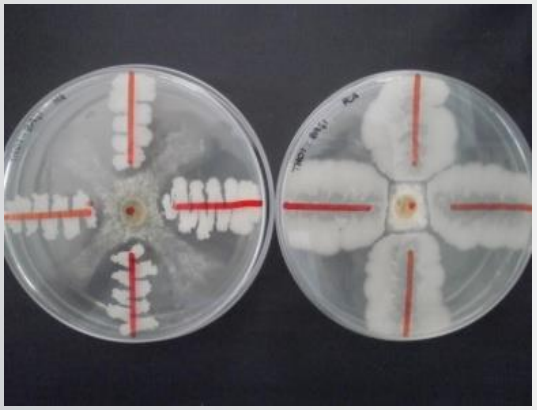
Results:


VOCs production
Area of microorganism growth (mm2)
4500
4000
3500
3000
2500
2000
1500
1000
500
0
FP76
SP75
analyzed pathogens
PT65 t.q
PM46 t.q.
PA29 t.q.
PN53 t.q.
c.neg

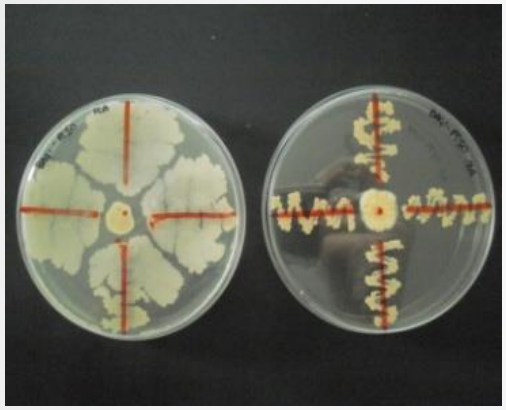

FP76 c.neg
FP76-PT65 (DO:0,123A 660nm)
FP76-PT65 (striscio T.q.)

CONSORTIUM COMPOSITION

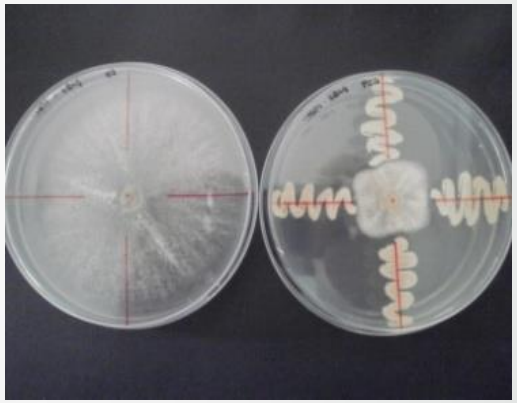
- Investigate the **interactions among beneficial microorganisms** towards each other.



BA41-TH01



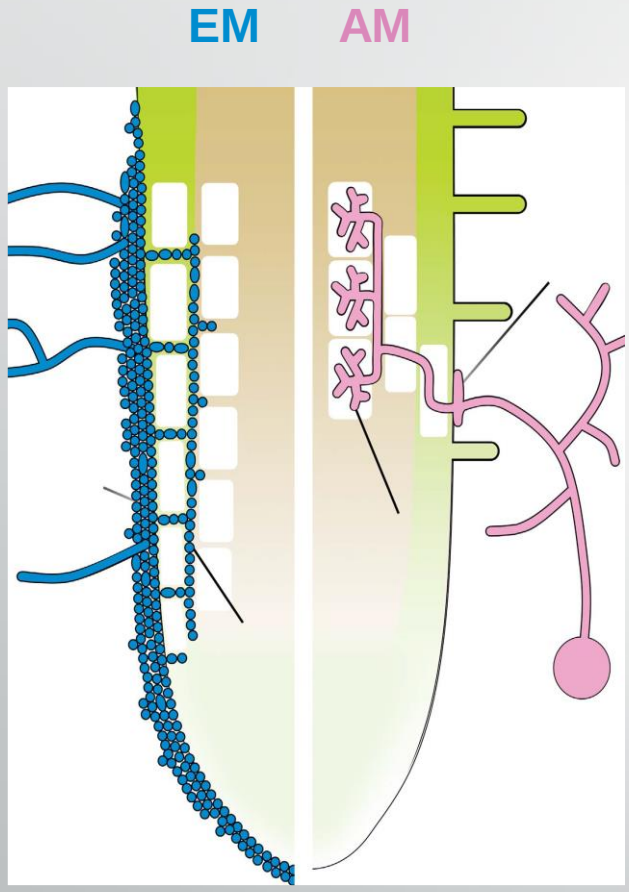
BA41-PC50



TH01 – SB14

Both BA41 and AR39 (bacteria) strains inhibit the growth of TH01 (*Trichoderma harzianum*) and partially inhibit the growth of PC50 (*Pochonia clamydospora*). Some microorganisms have different effects based on the growth medium used.

- Investigate the **mycorrhizal component** in the product.



Root colonization structures in ectomycorrhizal (blue, EM) and arbuscular mycorrhizal (pink, AM) interactions.

Materials and Methods:

Three different substrates were tested
GU53 – *Glomus coronatum*

- Roots were dyed overnight in blue lactc for microscopic observations.
- Samples observation by optical microscope for the calculation of the mycorrhizal index: class 0 (absence of infection),
  class 1 (traces of infection),
  class 2 (less than 10%),
  class 3 (from 11% to 59%),
  class 4 (from 51% to 90%),
  class 5 (over 90%)

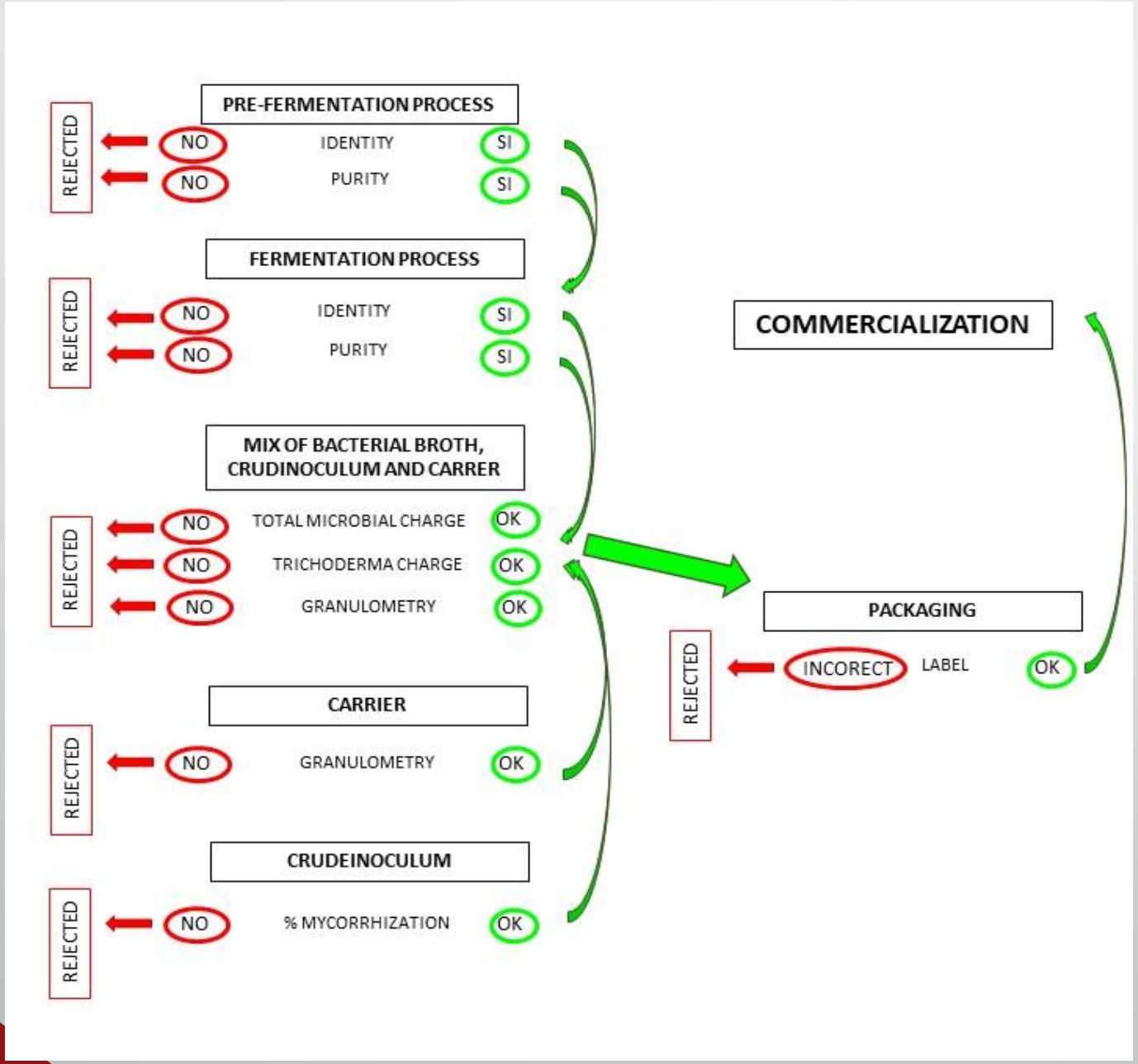
$$M\% = (95n5 + 70\,n4 + 30n3 + 5n2 + n1)/N$$

n1, n2, n3, n4, n5: the numbers of pieces belonging respectively to classes 1, 2, 3, 4, 5
N is the total number of pieces examined.

Results:

Substrate	Mycorrhization	Negative control	Δ mycorrhization
T1	61,83%	5,90%	55,93%
T2	55,73%	0,53%	55,20%
T3	39,57%	1,40%	38,17%

QUALITY CONTROL IN THE PRODUCTION INDUSTRIAL PROCESS



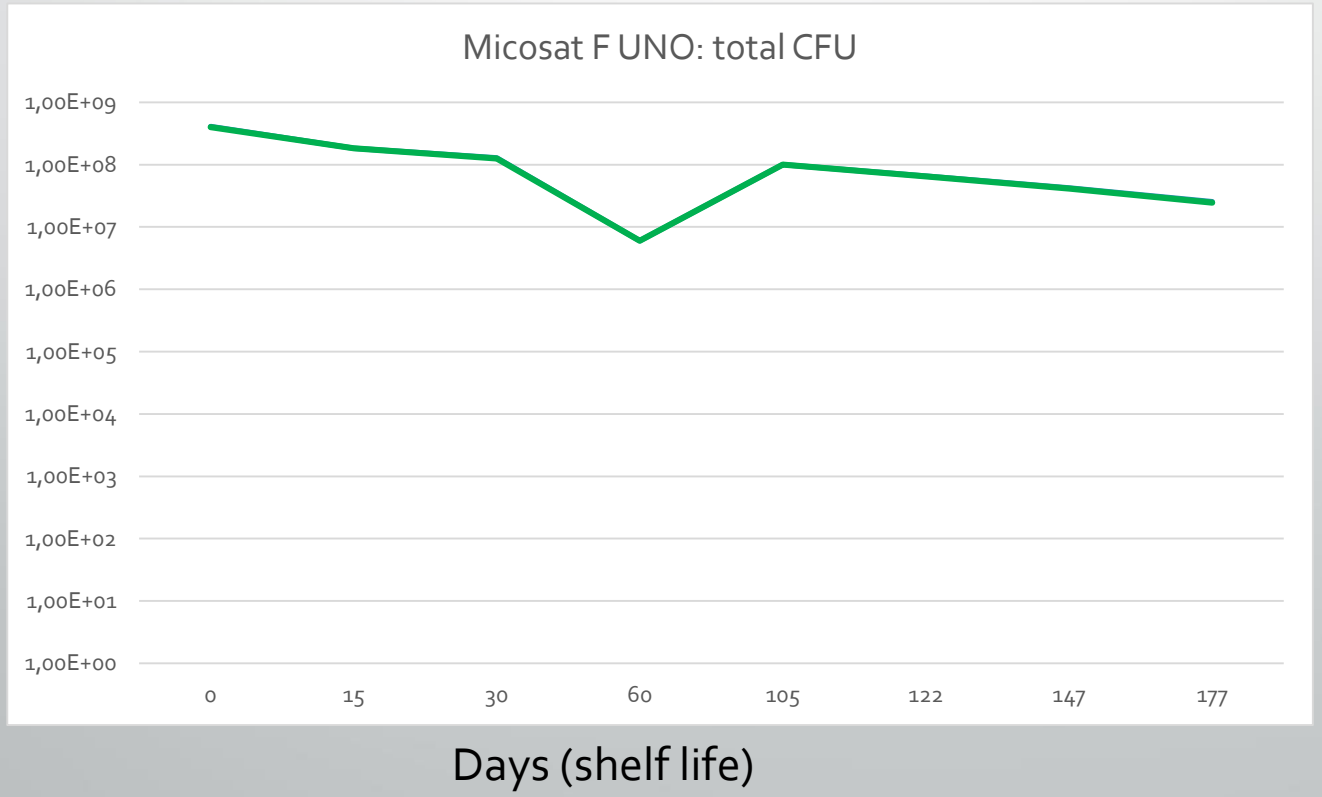
MICROBIAL VIABILITY OF THE CONSORTIUM AND SHELF LIFE

Materials and Methods: CFU count on serial dilutions

10 g sample of product dissolved in **90 ml** of water through a 10-minute stirring.

20 µl of the dilutions -4, -5, -6 and -7 are plated on selective media and then placed to grow in a small oven.

Results:



DYNAMICS OF MICROBES IN AGRICULTURAL SOILS.

GREENHOUSE TESTS:

The product is tested first on a **small scale**, under controlled conditions

FIELD TESTS:

- **Site selection and preparation:**

It is important to **select a site** where the conditions are as uniform as possible.

- Orchard
- Vineyard
- Cereal fields
- Pastures

- **Treatments:**

Dosages, dates and climatic conditions at the time of treatment.

- **Monitoring (phytopathometry, growth promotion), sampling (evaluation of quality):**

Sampling time and samples may vary according to the objectives.

CONCLUSIONS AND FUTURE PERSPECTIVES

Companies producing microbial consortia should **increasingly aim to ensure high quality** of their products

In-depth knowledge of microbe-microbe and plant-microbe interactions is needed to produce targeted and functional microbial consortia.

Process and product qualty control is, therefore, essential during the production of **high quality** microbials.

A critical point emerged from the analysis of Micosat F consortium: the **crude inoculum (mycorrhizae)** requires a different preparation process that ensures its efficacy.

CONCLUSIONS AND FUTURE PERSPECTIVES

The commercial product "Micosat F UNO" satisfied the requirements for a quality product.

Research is always necessary to develop and improve **innovative microbials**, focusing on a better understanding of functions and mechanisms of beneficial microbes *in planta*.

Additional research is required to assess the role of such beneficial microbes during the production of functional food, *e.g.*, increase the nutritional value of agricultural products through a focussed management of the microbiota.

Thank you