The PRIMA programme i
supported and funded unde
Horizon 2020, the Framewor
European Union's Programm
for Research and Innovation

Screening Of Beneficial Microorganisms To Control Root Rot Of Olive Trees

Presented by: IKRAM LEGRIFI

September 16th, 2024

INTRODUCTION

EXPERIMENTAL SECTION

- *Part 1: Screening of antagonistic bacteria for the biocontrol of olive root rot*
- *Part 2: Use of Trichoderma spp., for the management of olive root rot*

CONCLUSION & PERSPECTIVES

Introduction

IMPORTANCE OF THE OLEICULTURE SECTOR

23.6 M ha

World olive-growing surface (**2022**)

1.2 M ha

Olive-growing area in Morocco

1.9 M Tons

Average production of olives

50 M

working days per year

Contribution agricultural economy & employment

Introduction

PHYTOSANITARY PROBLEMS OF OLIVE TREES

Verticillium wilt

Verticillium dahliae

Cycloconium leaf spot

Spilocaea oleaginea

Anthracnose

Colletotrichum spp.,

Tuberculosis

Pseudomonas savastanoi

Introduction

ROOT ROT DISEASE OF OLIVE TREES

Oomycetes, ***Pythium*** genera, have been described as agents that induce:

- **Root rot**
- **Yellowing and leaves defoliation**
- **Branch wilt and dieback**

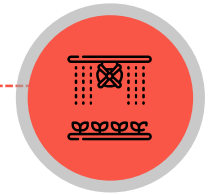
Disease conditions : heavy soils, excessive soil moisture, and poor drainage

Affect **olive nurseries** & **commercial groves**

Introduction

CONTROL STRATEGIES

1



Cultural Practices

Prophylaxis/sanitation practices are preventive measures that eliminate stress factors that predispose trees to attack by pathogens.

2

Chemical Control

Use of active fungicidal molecules to control plant pathogens.

3

Biological Control

Use of biological agents to control plant pathogens and establish an eco-friendly ecosystem.

Introduction

OBJECTIVES

Screening of beneficial microorganisms for the management of root rot in olive trees caused by *Pythium schmitthenneri*

Part 1: Screening of antagonistic bacteria for the biocontrol of olive root rot

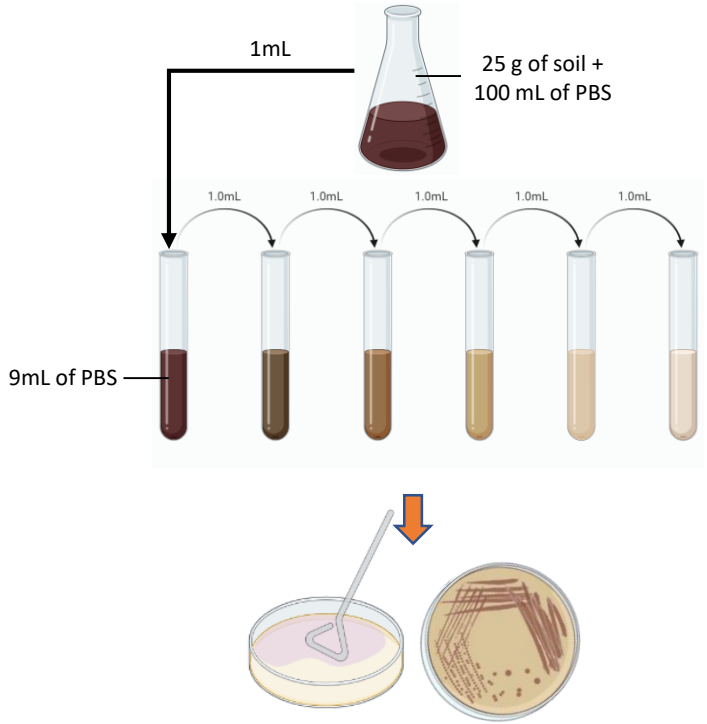
Methodology

Isolation

Samples

Olive Rhizosphere:
- **Fes**
- **Taza**
- **Taourirt**

Olive Rhizosphere



Incubation & purification

Conservation

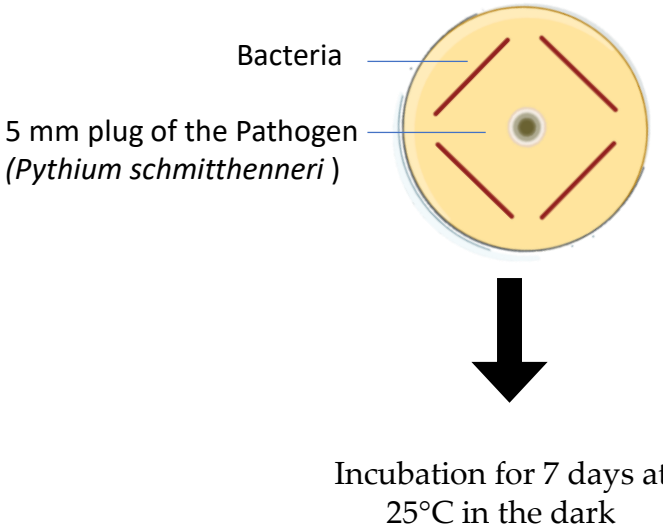
Cryoconservation (-80°C)

Methodology

Valorisation

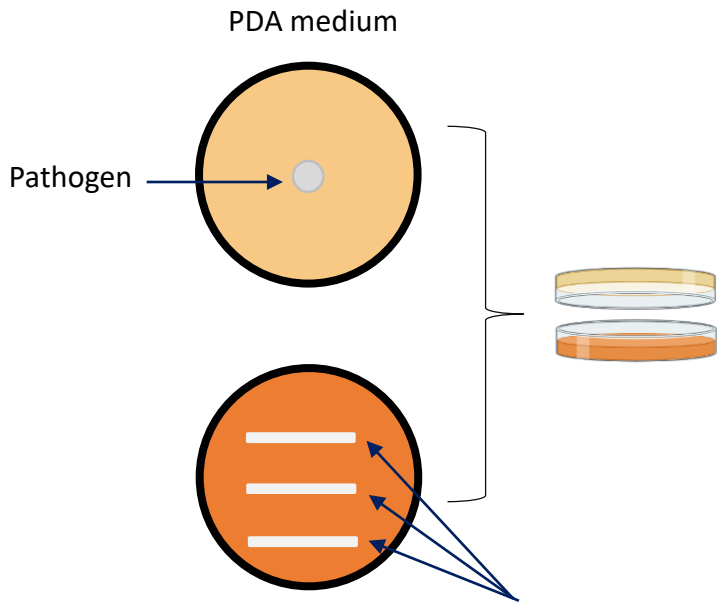
Search for bacterial antagonism

Screening & Dual culture

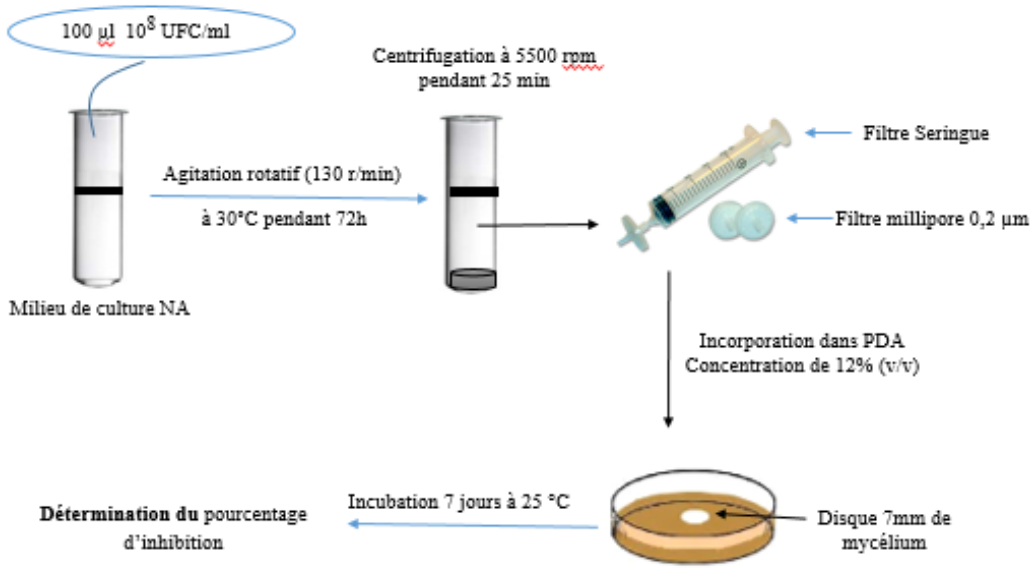


Indirect confrontations

VOCs



Filtrat



Methodology

Valorisation

Bacterial DNA extraction

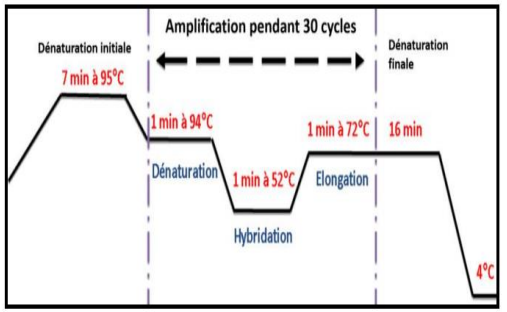
Bioneer MagListo TM 5M DNA extraction kit

Molecular identification

- **DNA amplification**

16s rDNA gene

Primer	Sequence
FD1	5′-AGAGTTTGATCCTGGCTCAG-3′
RP2	5′-ACGGCTACCTTGTTACGACTT-3′

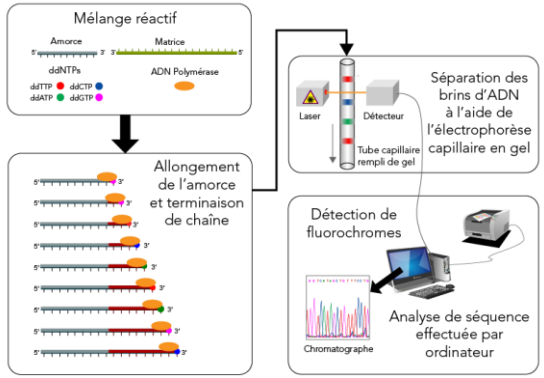


- **Amplicon revelation**

Evelution of DNA amplification by agarose gel electrophoresis

- **Sequencing**

Sanger technology



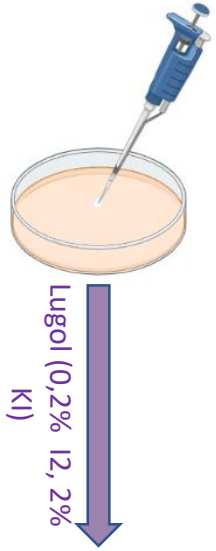
Methodology

Biochemical characterization

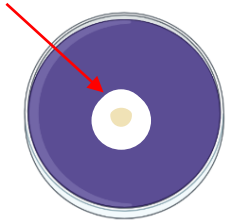
Production of lytic enzymes

Amylase

Starch-based medium

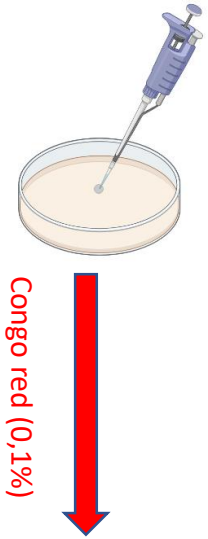


clear halos surrounding the colonies

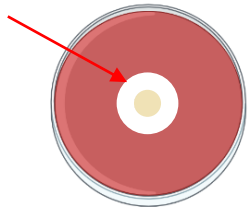


Cellulase

CMC-based medium

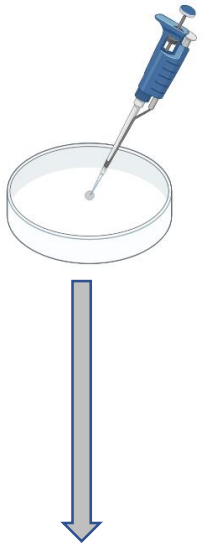


clear halos surrounding the colonies

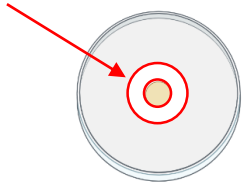


Protease

skim milk-based medium

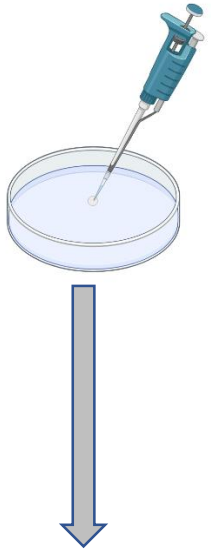


clear halos surrounding the colonies

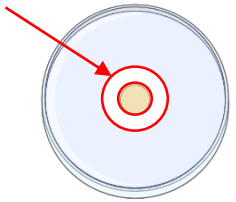


Chitinase

Chitine-based medium



clear halos surrounding the colonies



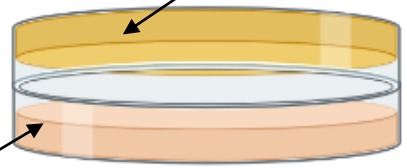
Methodology

Biochemical characterization

- **Volatile molecules production**

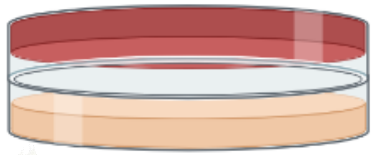
HCN production

Whatman filter paper impregnated with picrate solution



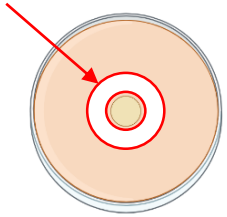
LPGA with 4,4 g/l of glycine

Change of paper color from yellow to orange, or red to reddish-brown



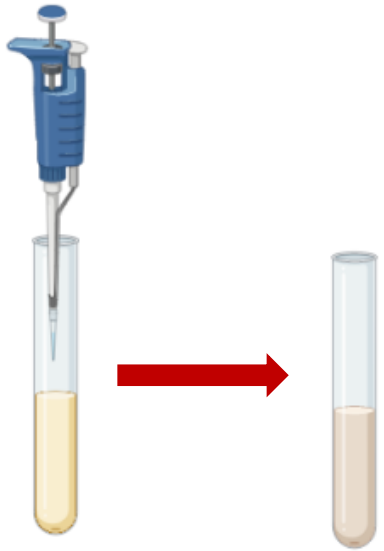
Phosphate solubilization

clear halos surrounding the colonies

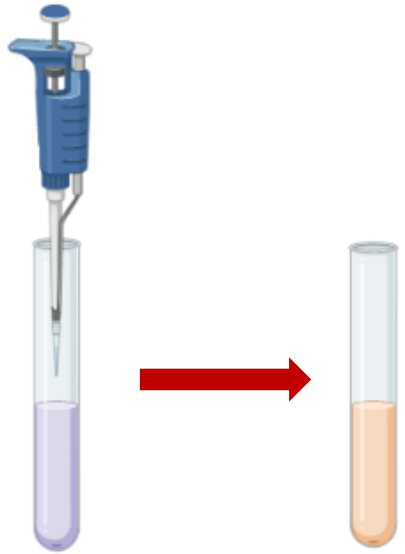


- **Promotion of plant growth**

IAA production



Siderophore secretion



Methodology

Application

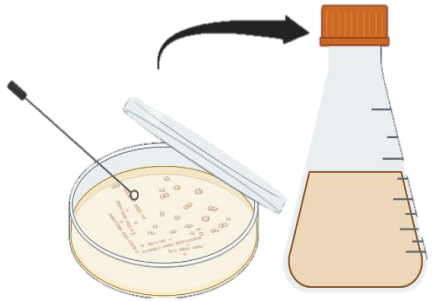
In vivo assay

1 Soil sterilization

- 121 °C
- 90 min (2 times)

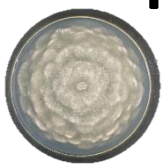
2 Treatment preparation

200 mL Bacterial suspension



3 Inoculum preparation

50 g of wheat seeds



Pathogen 50 mL of V8 jus

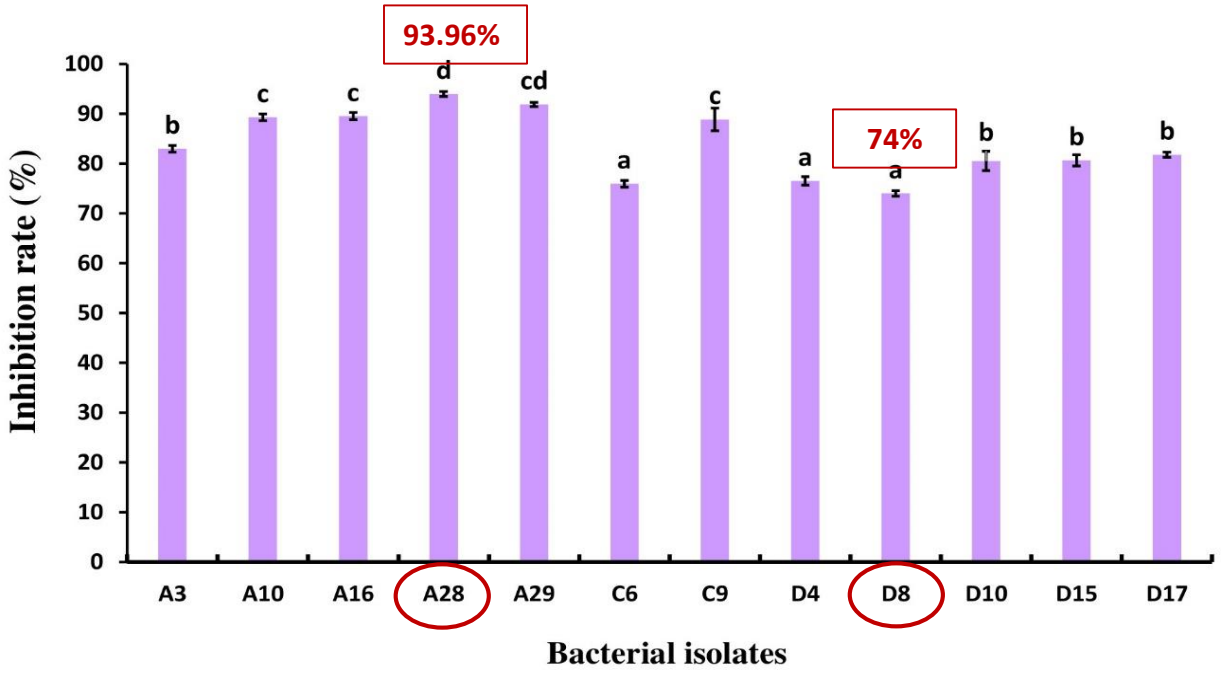
Incubation at 25°C/3 weeks

4 In vivo assay

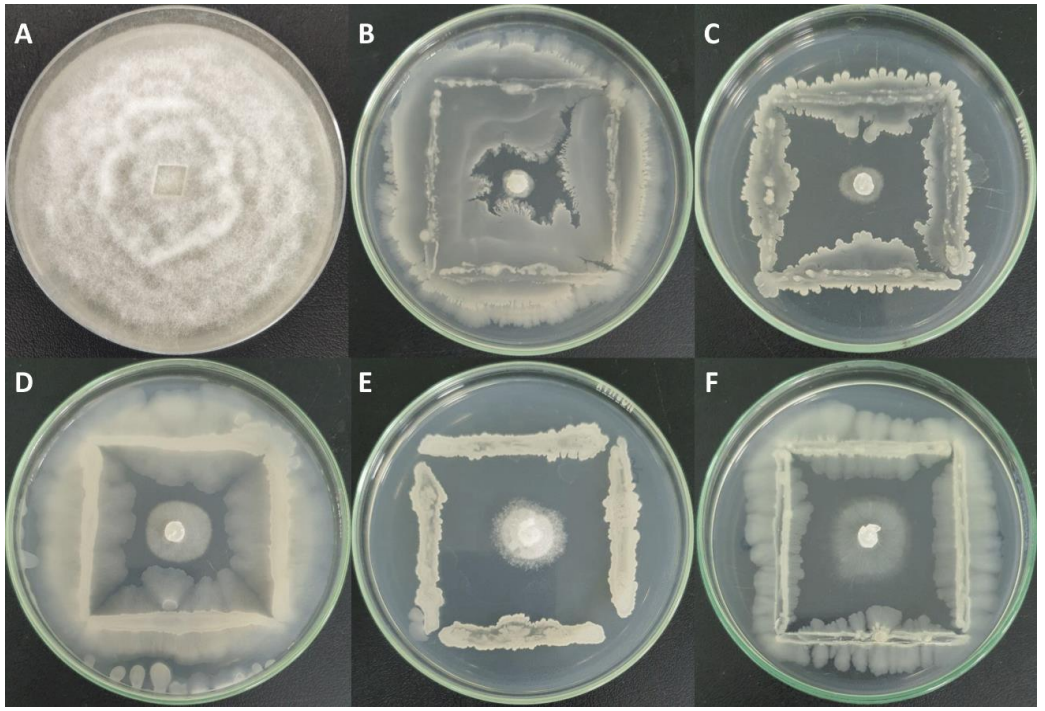
3 months Under greenhouse condition

Results

Direct antagonistic activity



Inhibition rate (%) of ***Pythium schmitthenneri*** mycelial growth by twelve selected bacterial isolates from the olive rhizosphere.



Double culture trial showing the antagonistic potential of selected bacteria against *P. schmitthenneri* on PDA medium following six days of incubation at 25°C. (**A**): untreated control; (**B**): A28; (**C**): C9; (**D**): A3; (**E**): D17; (**F**): C6.

Results

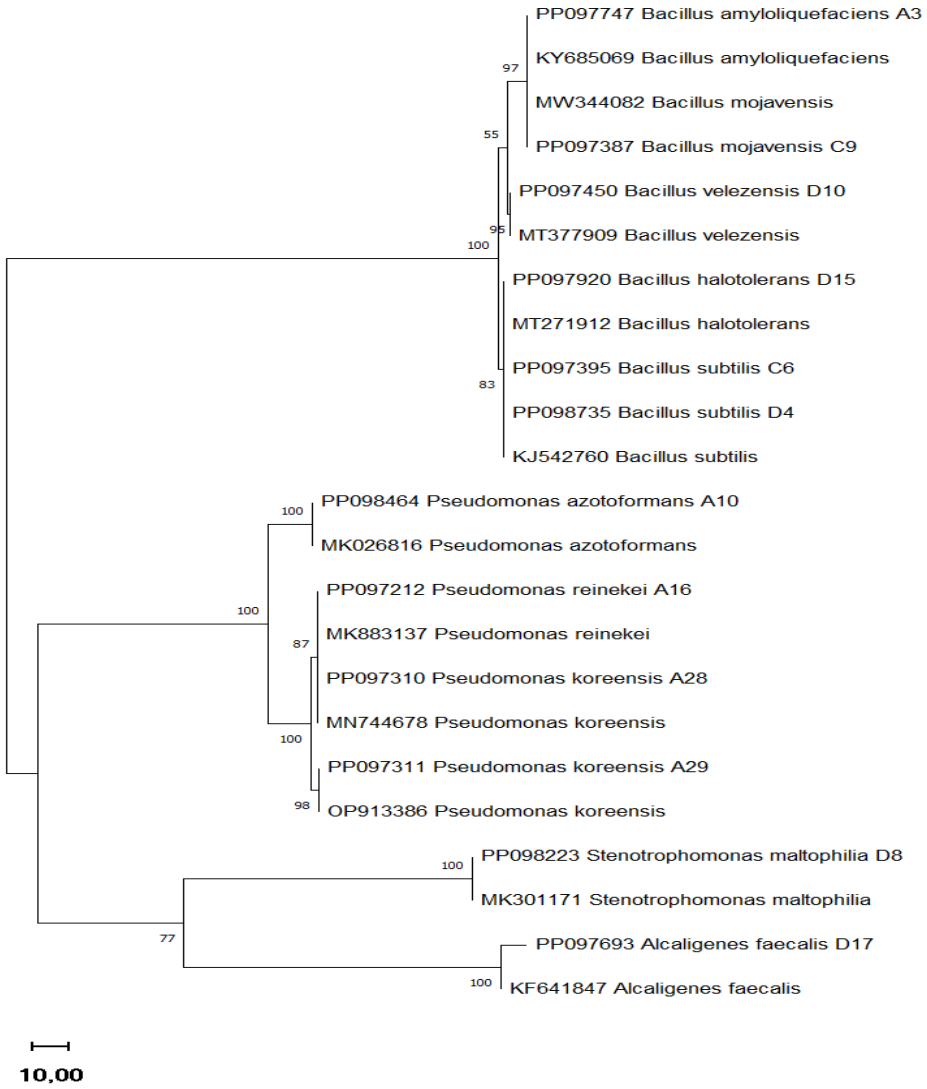
Indirect confrontations

Effects of bacterial volatile organic compounds (VOCs) and cell-free culture filtrates on the growth of *P. schmitthenneri* mycelia following 6 days incubation at 25°C.

Isolate code	Origine	Species	Accession number	VOCs	Cell-free filtrate
A3	Taza	*B. amyloliquefaciens*	PP097747	31.30 ± 0.43 ^{a}	75.48 ± .55 ^{d,e}
A10	Taza	*P. azotoformans*	PP098464	41.06 ± 1.04 ^{b}	75.78 ± .68 ^{d,e}
A16	Taza	*P. reinekei*	PP097212	68.28 ± 1.31 ^{f}	59.38 ± .72 ^{a}
A28	Taza	***P. koreensis***	PP097310	**89.65 ± 1.06** ^{i}	**85.55 ± .51** ^{g}
A29	Taza	***P. koreensis***	PP097311	**80.95 ± 1.38** ^{j}	**87.78 ± .88** ^{h}
C6	Taourirt	***B. subtilis***	PP097395	**75.20 ± 0.90** ^{g}	**74.04 ± .41** ^{d}
C9	Taourirt	***B. mojavensis***	PP097387	55.77 ± 1.64 ^{e}	**76.26 ± .64** ^{e}
D4	Fez	*B. subtilis*	PP098735	45.61 ± 1.18 ^{c}	70.77 ± .63 ^{c}
D8	Fez	*S. maltophilia*	PP098223	44.31 ± 0.90 ^{b,c}	63.62 ± .51 ^{b}
D10	Fez	***B. velezensis***	PP097450	55.50 ± 1.67 ^{e}	**82.29 ± .66** ^{f}
D15	Fez	*B. halotolerans*	PP097920	42.72 ± 1.32 ^{b,c}	58.79 ± .90 ^{a}
D17	Fez	*A. faecalis*	PP097693	49.25 ± .7 ^{d}	70.17 ± .59 ^{c}

Results

Molecular identification



- ***Bacillus subtilis* C6 & D4**
- ***Bacillus amyloliquefaciens* A3**
- ***Bacillus mojavensis* C9**
- ***Bacillus velezensis* D10**
- ***Bacillus halotolerans* D15**

- ***Pseudomonas koreensis* A28 & A29**
- ***Pseudomonas reinekei* A16**
- ***Pseudomonas azotoformans* A10**

- ***Alcaligenes faecalis* D17**

- ***Stenotrophomonas maltophilia* D8**

Results

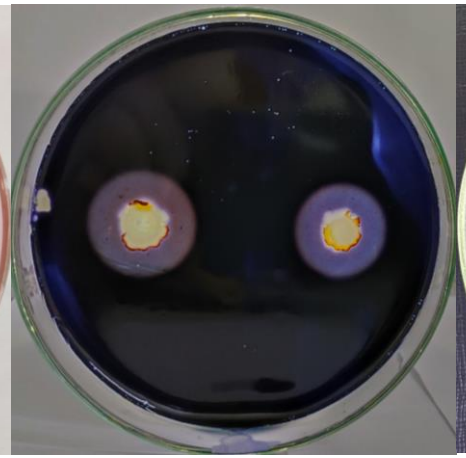
Biochemical characterization

Production of lytic enzymes

Cellulase

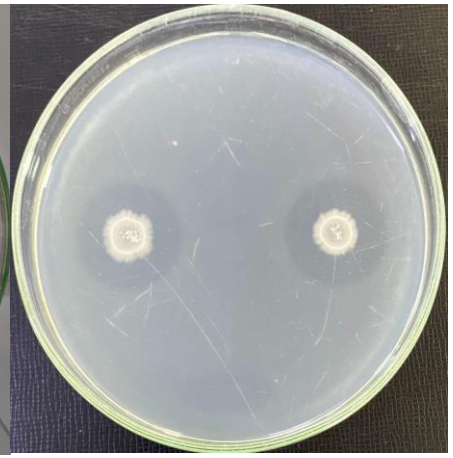
8 isolates

Amylase



8 isolates

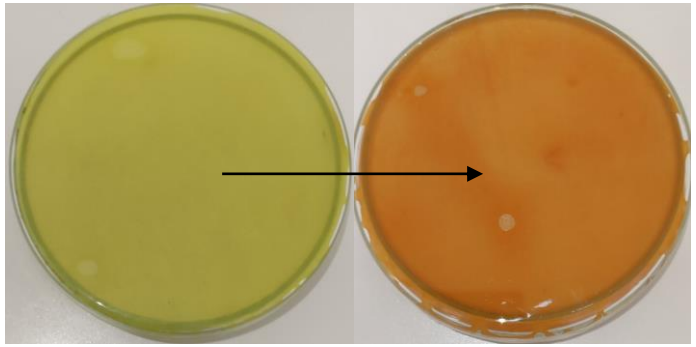
Protease



12 isolates

Volatile molecules production

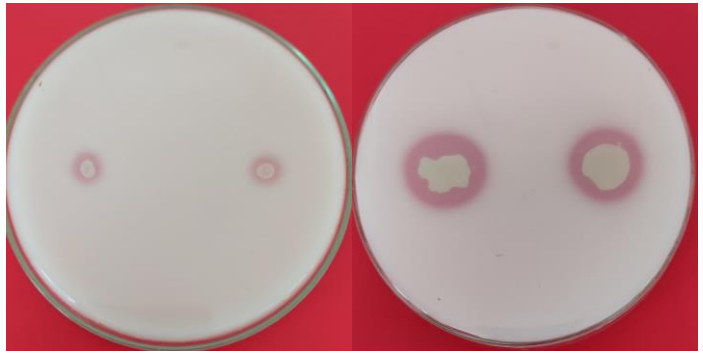
HCN Production



8 isolates

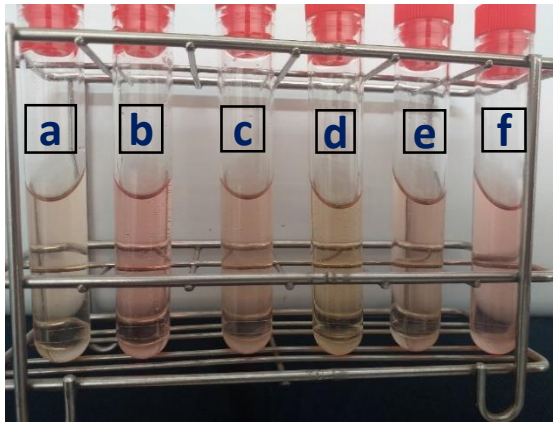
Promotion of plant growth

Phosphate solubilization



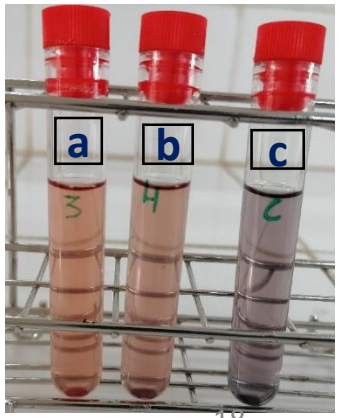
5 isolates

IAA Production



10 isolates

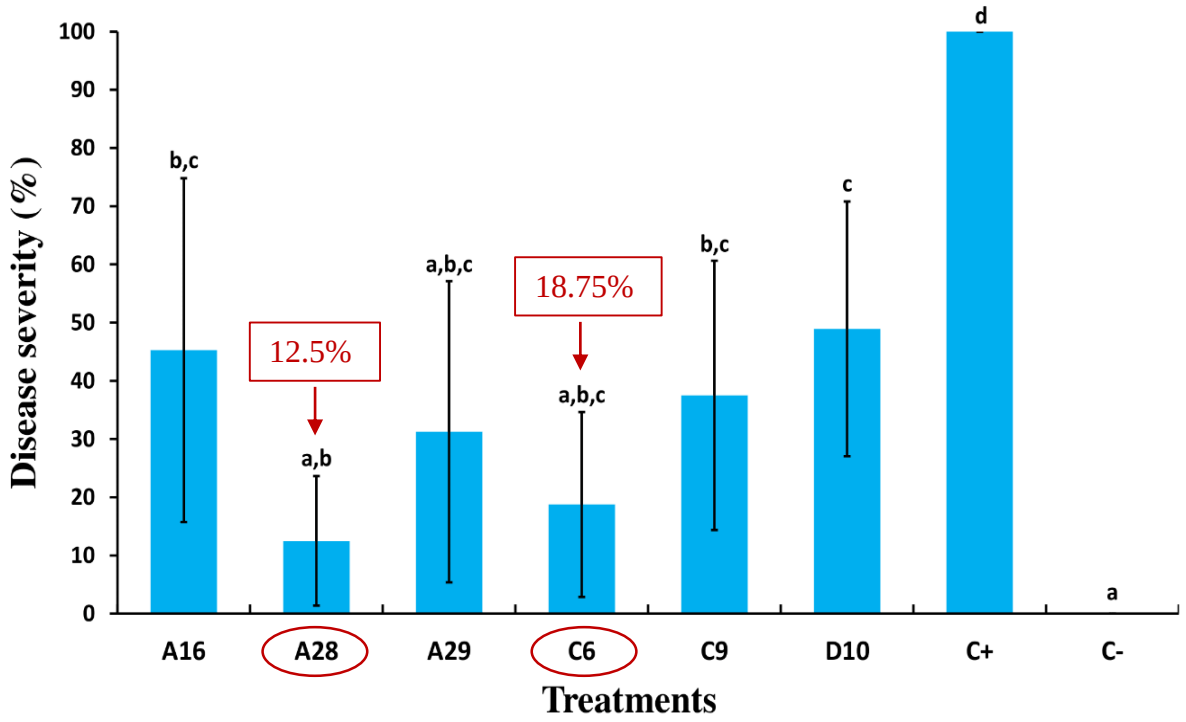
Siderophore secretion

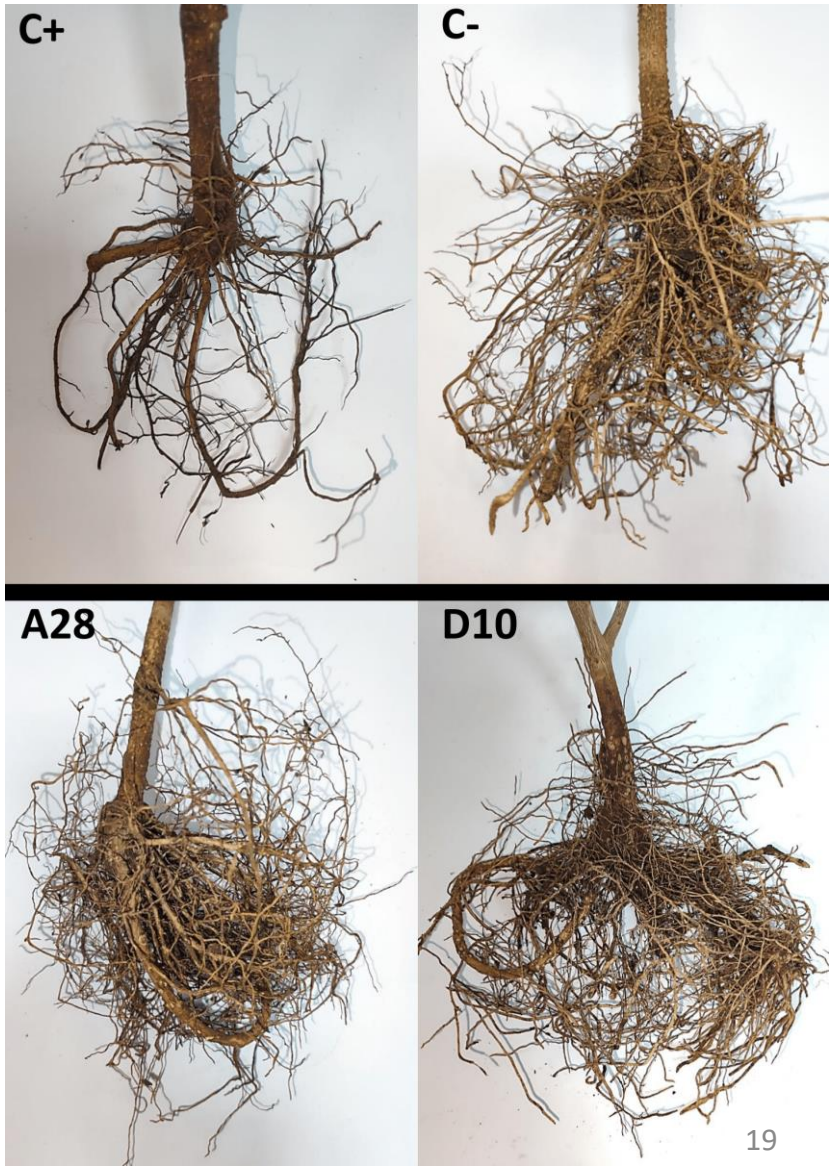


9 isolates

Results

Application





- *Pseudomonas Koreensis* A28 and *Bacillus subtilis* C6 exhibited higher effectiveness in disease suppression in comparison to the positive control

Part 2: Use of Trichoderma spp., for the management of olive root rot

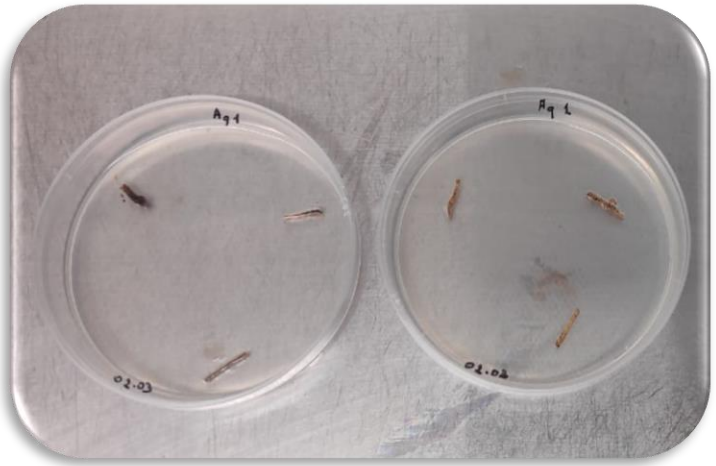
Methodology

Isolation

Surface sterilization of roots with sodium hypochlorite solution (2%) for 2 min.

Rinsing three times SDW & Drying on filter paper in a laminar flow hood

Culture in petri dishes containing PDA medium and incubated



Incubation
25ºC/5 days

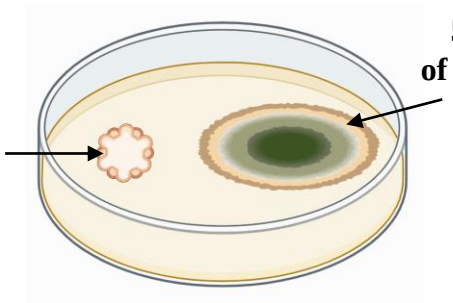
Purification

Methodology

Valorisation

Search for effective *Trichoderma* isolates

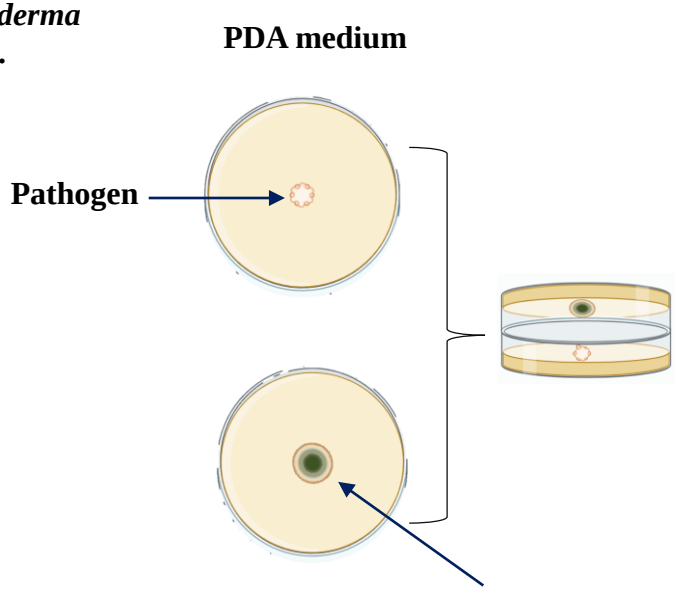
Screening & Dual culture



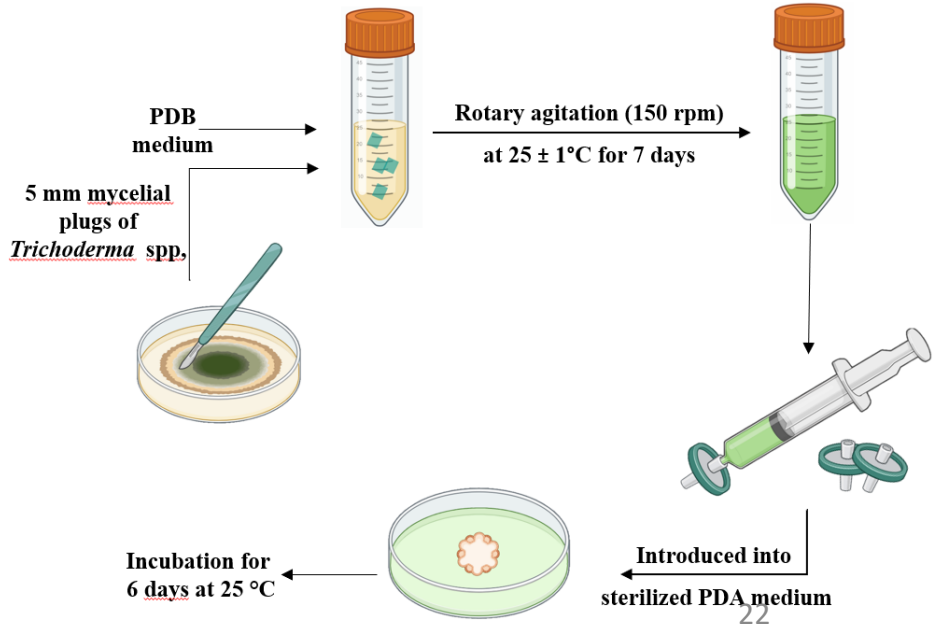
Incubation for 6 days at 25°C in the dark

Indirect confrontations

VOCs



Filtrat

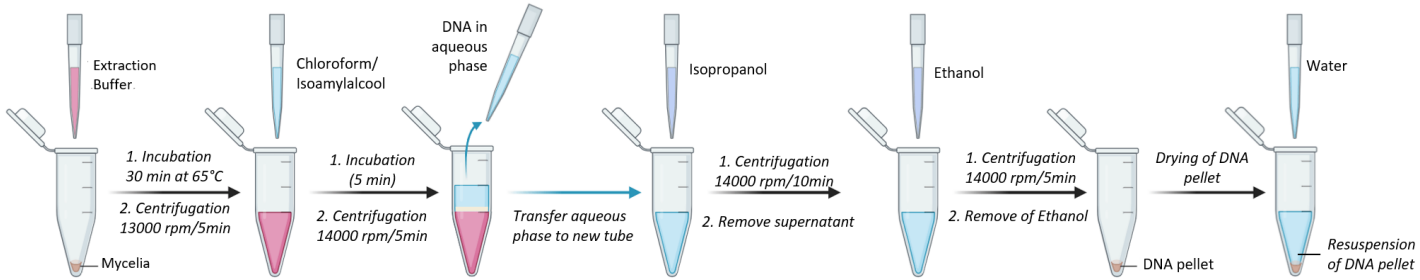


Methodology

Molecular identification

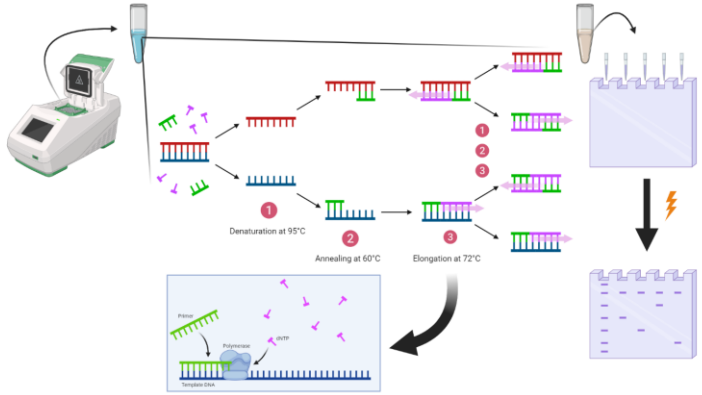
1 DNA EXTRACTION

Doyle & Doyle (2007)

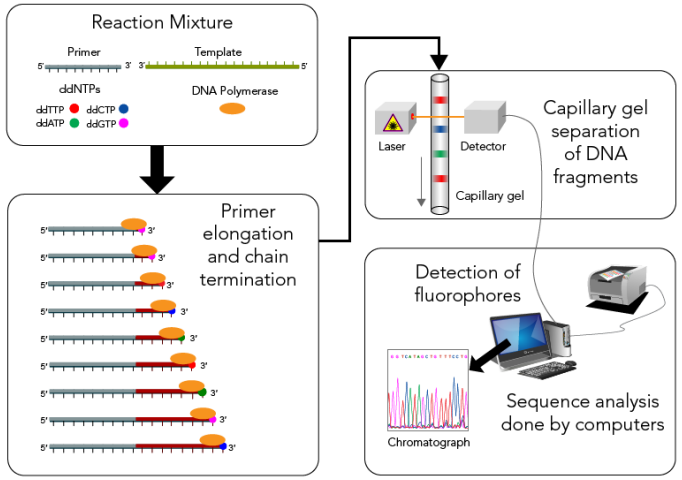


2 Polymerase Chain Reaction (PCR)

- Amplification of the internal transcribed spacer region **ITS**
- Primers **(ITS1/ITS4)**



3 Sequencing



Methodology

Biochemical characterization

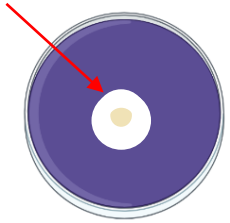
Qualitative screening of extracellular enzymes

Amylase

Starch-based medium

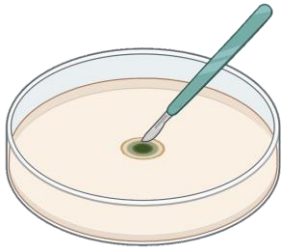
Lugol (0,2% I2, 2% KI)

clear halos surrounding the colonies



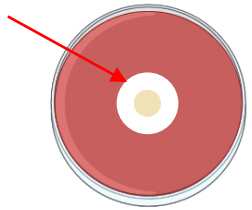
Cellulase

CMC-based medium



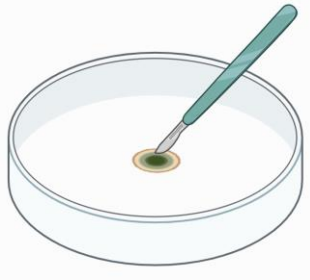
Congo red (0,1%)

clear halos surrounding the colonies

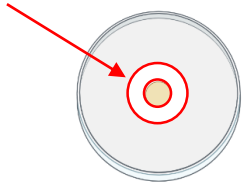


Protease

skim milk-based medium

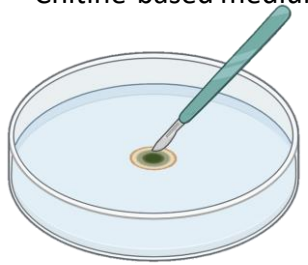


clear halos surrounding the colonies

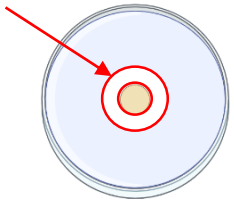


Chitinase

Chitine-based medium



clear halos surrounding the colonies



Methodology

Application

In vivo assay

Treatments applied in the greenhouse bioassay

Treatments	Composition	Repetition	Period
T-MK1	SS + pathogen + T-MK1		
T-BM6	SS + pathogen + T-BM6		
T-BM13	SS + pathogen + T-BM13		
T-KH4	SS + pathogen + T-KH4		
T-BK9	SS + pathogen + T-BK9	8	**3 months**
T-CS6	SS + pathogen + T-CS6		
T-E8	SS + pathogen + T-E8		
T-E11	SS + pathogen + T-E11		
C+	SS + pathogen		
C-	SS only		

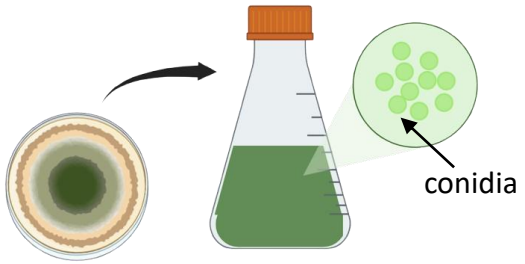
***SS: sterile soil**

1 Soil sterilization

- 121 °C
- 90 min (2 times)

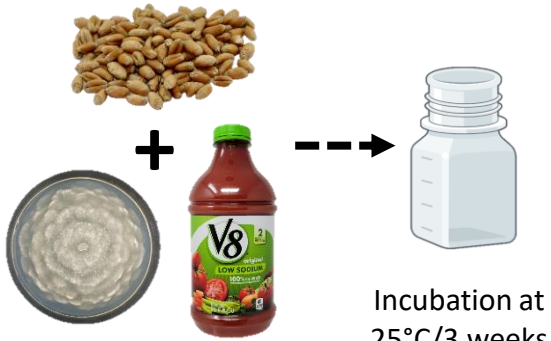
2 Treatment preparation

200 mL *Trichoderma's* conidial suspension



3 Inoculum preparation

50 g of wheat seeds



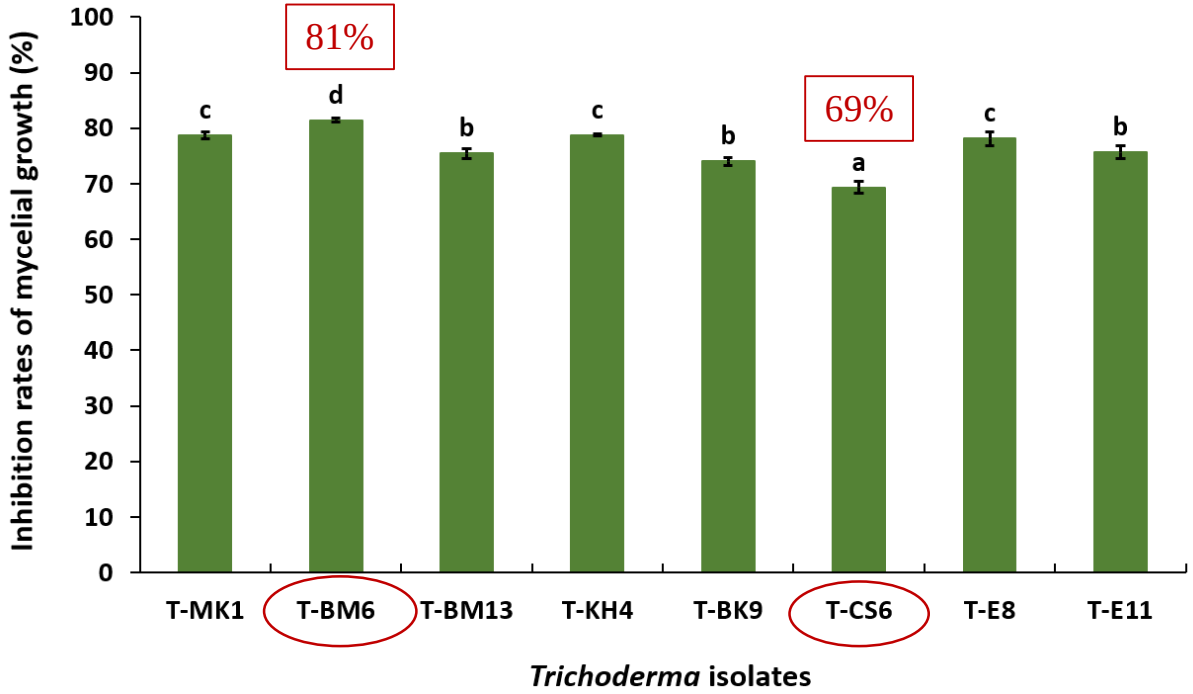
Pathogen 50 mL of V8 jus

Incubation at 25°C/3 weeks

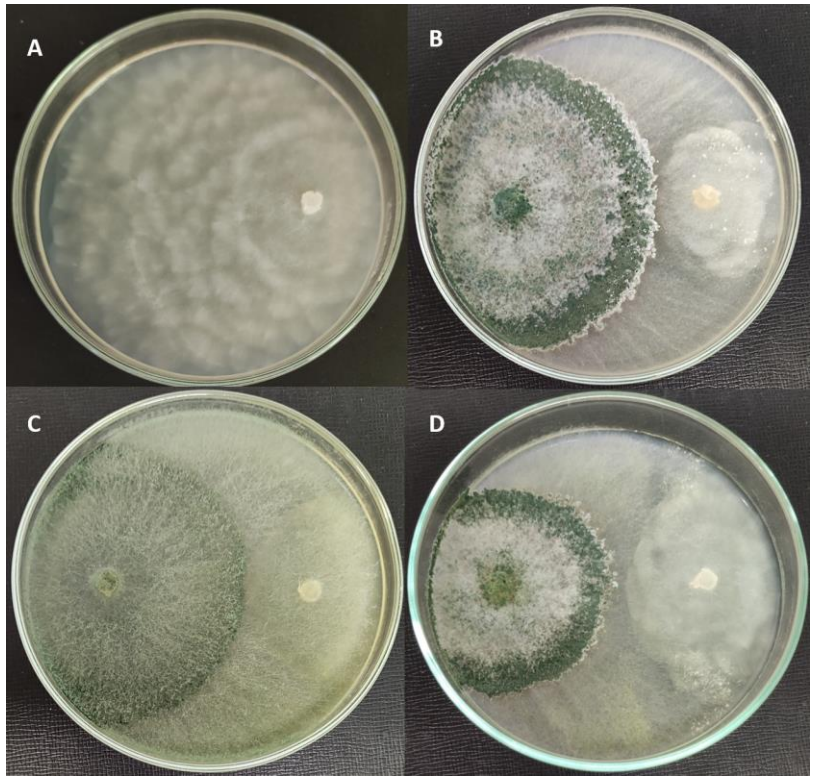
4 Greenhouse assay

Results

Direct antagonistic activity



In vitro inhibition rates (%) of mycelial growth of *P. schmittenneri*, obtained with eight selected *Trichoderma* isolates from olive roots.



(**A**) control; (**B**) T-BM6; (**C**) T-BM13; (**D**) T-CS6

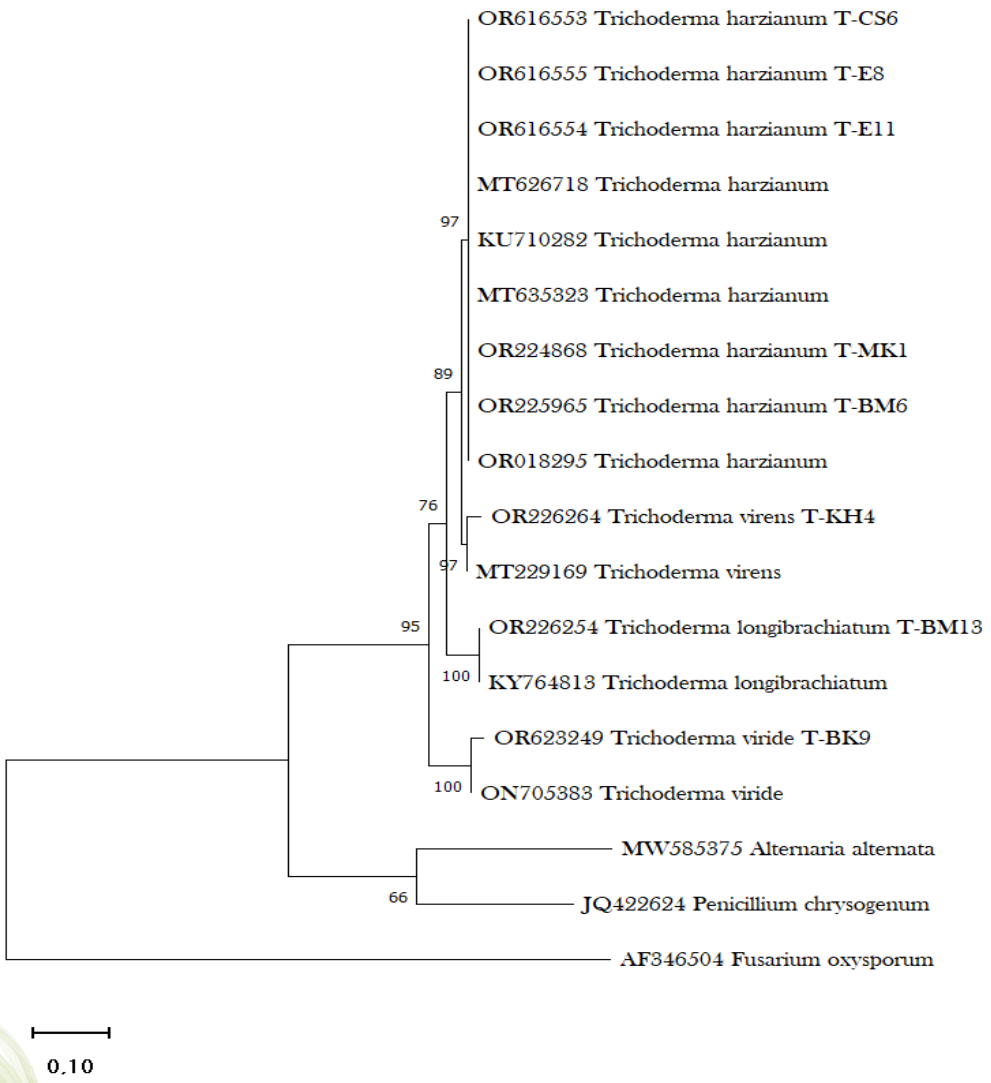
Indirect confrontations

Effect of Volatile organic compounds (VOCs) and *Trichoderma* spp. filtrates (at 10% v/v) on the *in vitro* mycelial growth of *P. schmithenneri*

Isolate code	Origin	Species	Accession number	VOCs	Filtrate
T-MK1	Meknes	*T. harzianum*	OR224868	65.65 ± 0.43 ^{d}	54.77 ± 0.40 ^{c}
T-BM6	Beni Mellal	*T. harzianum*	OR225965	74.92 ± 0.45 ^{f}	67.21 ± 1.57 ^{e}
T-BM13	Beni Mellal	*T. longibrachatium*	OR226254	60.74 ± 0.37 ^{c}	61.85 ± 0.45 ^{d}
T-KH4	Khenifra	*T. virens*	OR226264	69.57 ± 0.68 ^{e}	68.68 ± 1.26 ^{e}
T-BK9	Beni Mellal	*T. viride*	OR623249	57.46 ± 0.54 ^{b}	44.41 ± 0.66 ^{b}
T-CS6	Bouznika	*T. harzianum*	OR616553	49.71 ± 0.15 ^{a}	44.61 ± 1.03 ^{b}
T-E8	Errachidia	*T. harzianum*	OR616555	49.63 ± 1.24 ^{a}	41.44 ± 0.65 ^{a}
T-E11	Errachidia	*T. harzianum*	OR616554	56.67 ± 0.93 ^{b}	40.52 ± 0.27 ^{a}

Results

Molecular identification



- ***Trichoderma harzianum*** **(5 isolates)**
- ***Trichoderma longibrachatium*** **(1 isolate)**
- ***Trichoderma virens*** **(1 isolate)**
- ***Trichoderma viride*** **(1 isolate)**

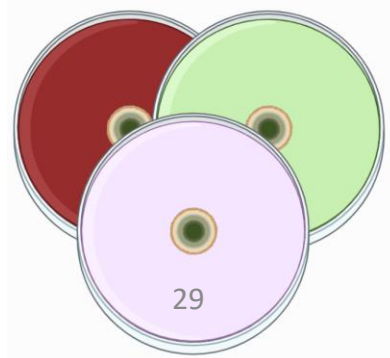
Results

Biochemical characterization

Qualitative primary screening of *Trichoderma* spp. for enzymatic activity

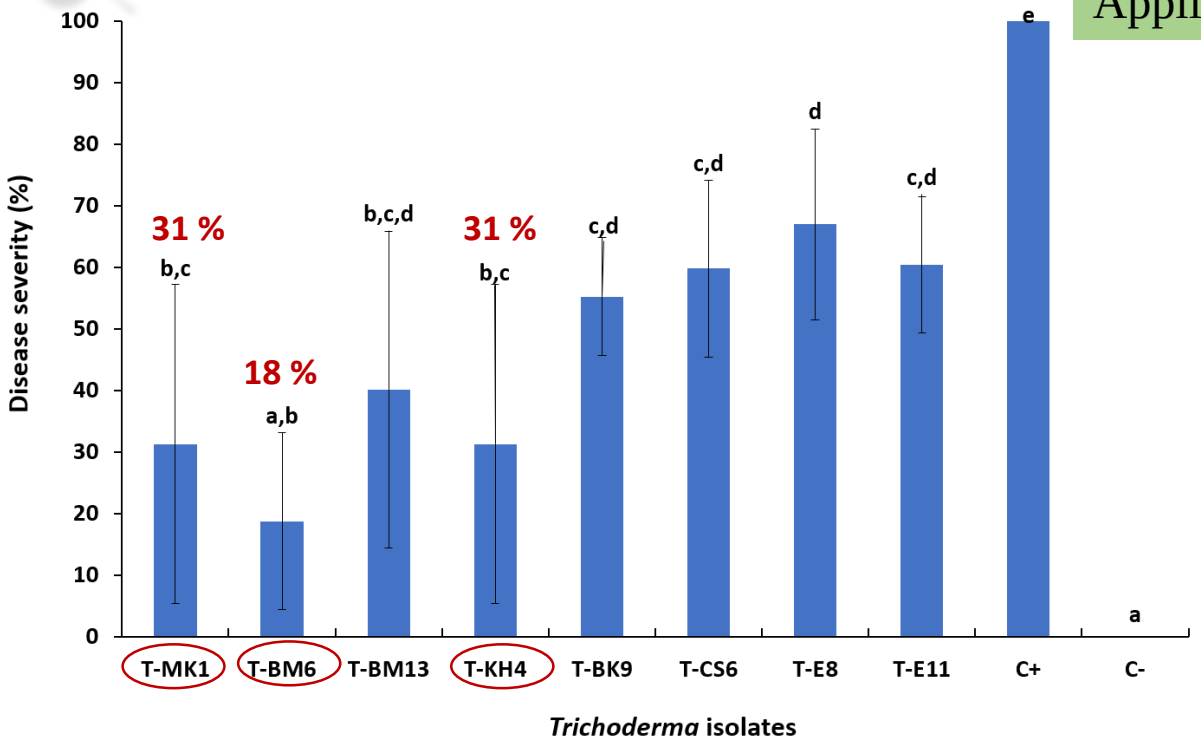
Isolate code	Amylase	Cellulase	Protease	Chitinase
T-MK1	+++	+	-	+
T-BM6	+++	+++	+	+
T-BM13	+	+	-	-
T-KH4	++	+	+	+++
T-BK9	+	++	+	+
T-CS6	++	+	+	+
T-E8	++	+	+	+
T-E11	++	+	+	+

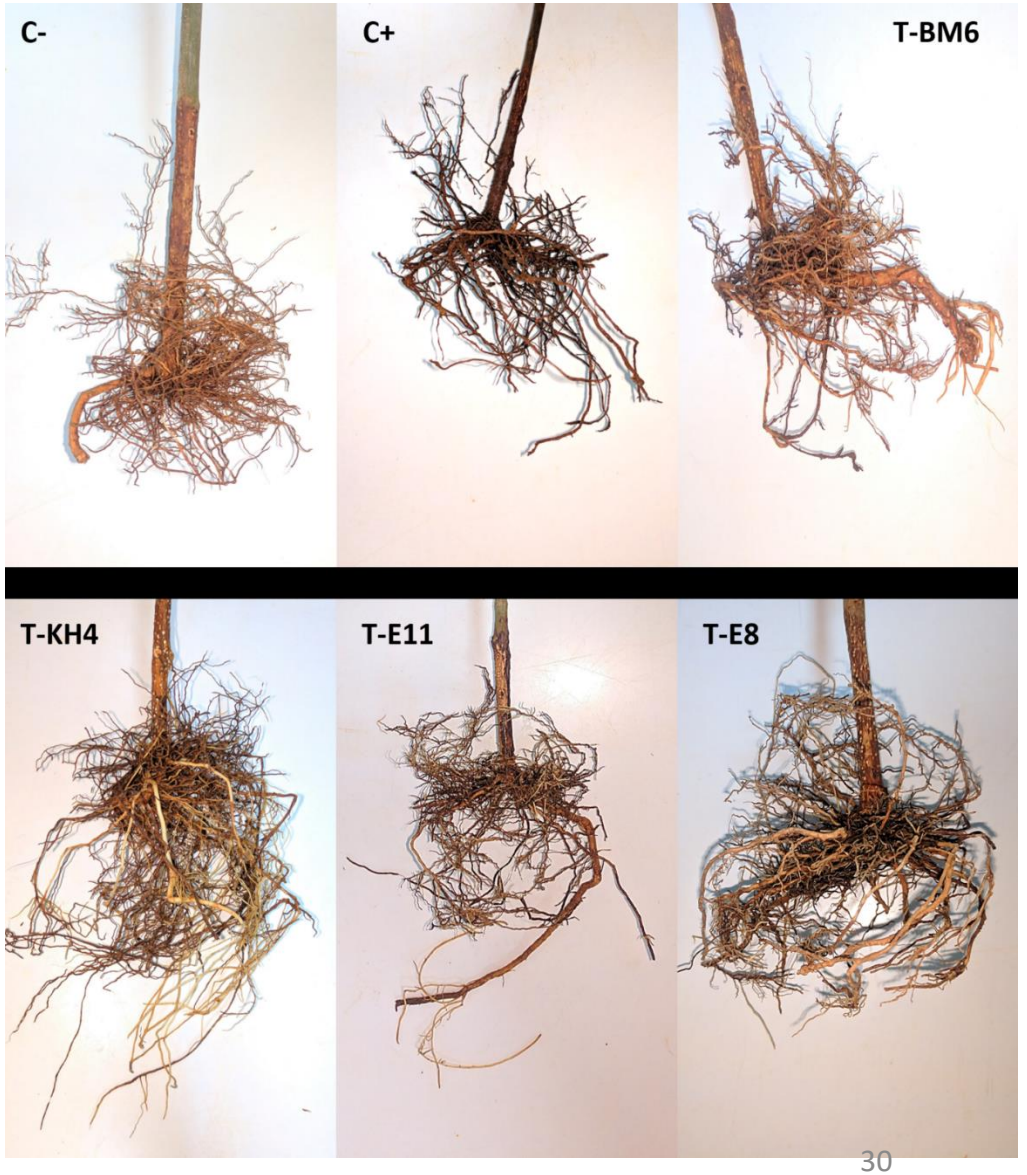
(-): no enzyme activity; (+): very low enzyme activity; (++): low enzyme activity; (+++): high enzyme activity.



Results

Application

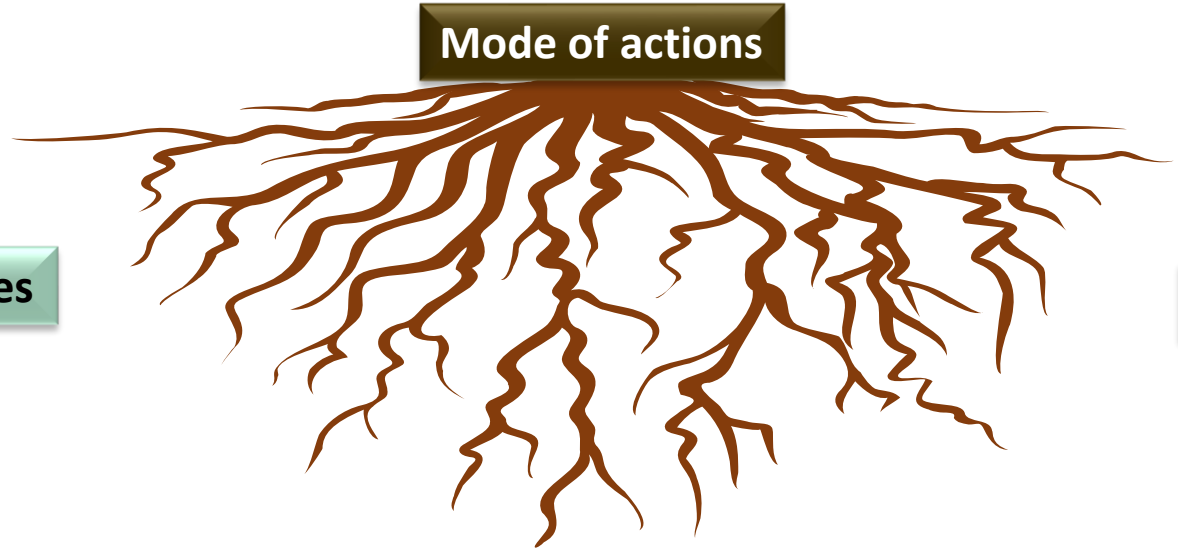




❖ *T . harzianum* T-BM6, *T . harzianum* T-MK1, & *T. virens* T-KH4 exhibited higher effectiveness in disease suppression in comparison to the positive control

Conclusion and Perspectives

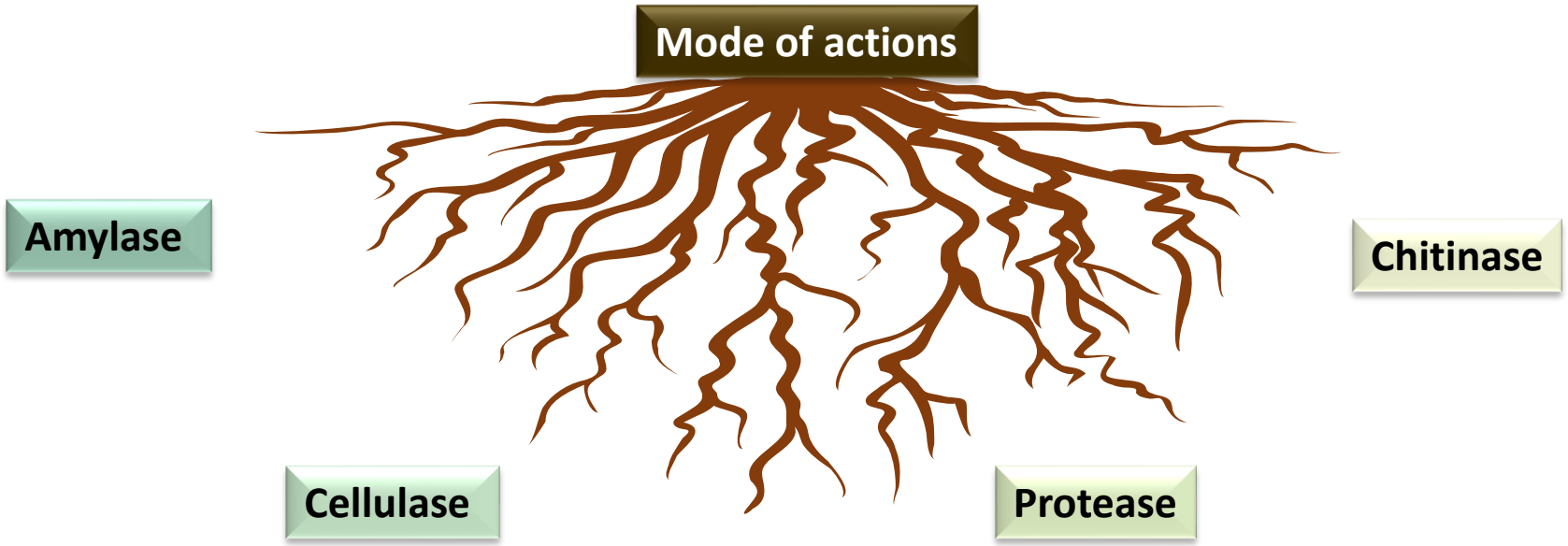
Screening and identification of 12 bacterial isolates with significant antifungal activity against the olive root rot pathogen



Pseudomonas Koreensis A28 and Bacillus subtilis C6 : Higher antifungal activity in vitro and in vivo

Conclusion and Perspectives

Screening and identification of 8 *Trichoderma* isolates with significant antifungal activity against *Pythium schmithhennei*



Trichoderma harzianum T-BM6 : Higher antifungal activity in vitro and in vivo

Thank you for your attention

LEGRIFI Ikram