PROMETEO

Bioprotection des fruits d'agrumes contre les pourritures à *Penicillium* moyennant des levures et bactéries épiphytes

Pr Najla SADFI, FST-UTM

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Coopération transfrontalière ITALIETUNISIE

Programme cofinancé par l'UNION EUROPÉENNE

Partenaire 2, Université de Tunis El Manar
Faculté des Sciences de Tunis (FST)

LMPB FST Mycologie Pathologies Biomarqueurs

Diagnosis, detection and identification of diseases and plant pathogens

Research topics of the laboratory of « Mycologie, Pathologies et Biomarqueurs » LR16ES05

Production of Bio-pesticides based on bacteria and Valorization at a large scale with socioeconomic partners

Objectifs du GT4: translating the results of the network's activity into concrete proposals and practical solutions.

Establishment of a technological platform and exchange of innovative solutions

Activities and roles of UTM under PROMETEO Project, GT4

Validation of a novel biocides and redaction of new protocols

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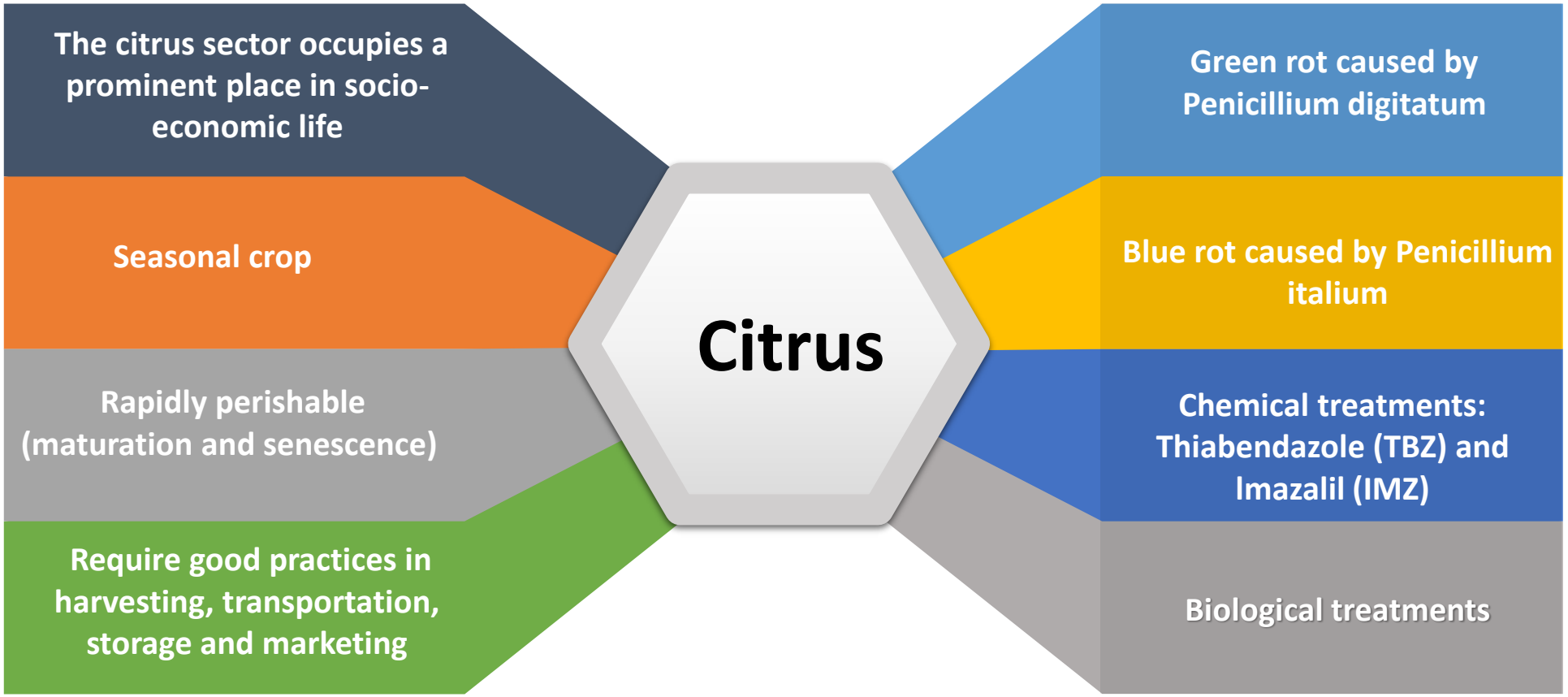

The citrus sector occupies a prominent place in socio-economic life
Seasonal crop
Rapidly perishable (maturation and senescence)
Require good practices in harvesting, transportation, storage and marketing
Citrus
Green rot caused by Penicillium digitatum
Blue rot caused by Penicillium italium
Chemical treatments: Thiabendazole (TBZ) and Imazalil (IMZ)
Biological treatments

Refrigerated storage of citrus fruits

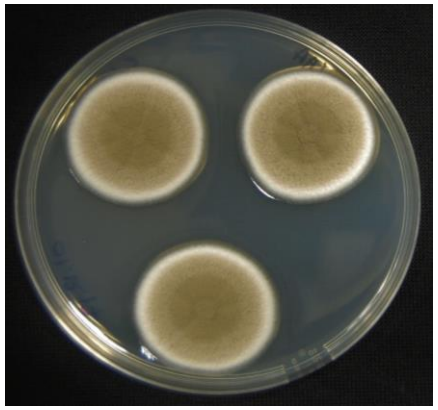
- Preserve post-harvest life and extend citrus market time
- Slow down the biological activity of the fruit, and reduce water loss
- Prevent the spread and invasion of pathogenic microorganisms

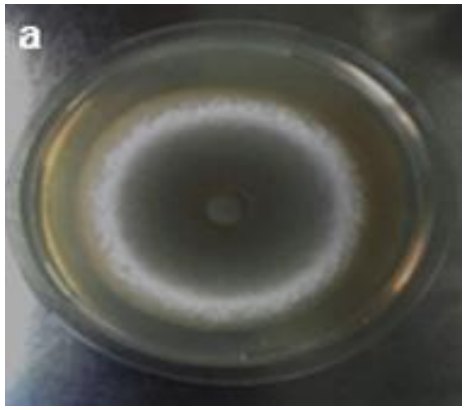
Phytosanitary problems of citrus fruits in post-harvest

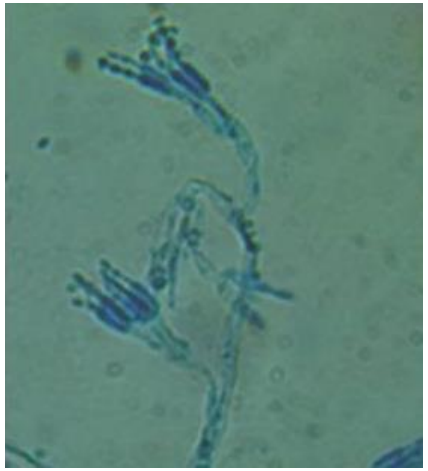
Blue mold rot caused by *Penicillium italicum*

Phytosanitary problems of citrus fruits in post-harvest

Green mold rot caused by *Penicillium italicum*



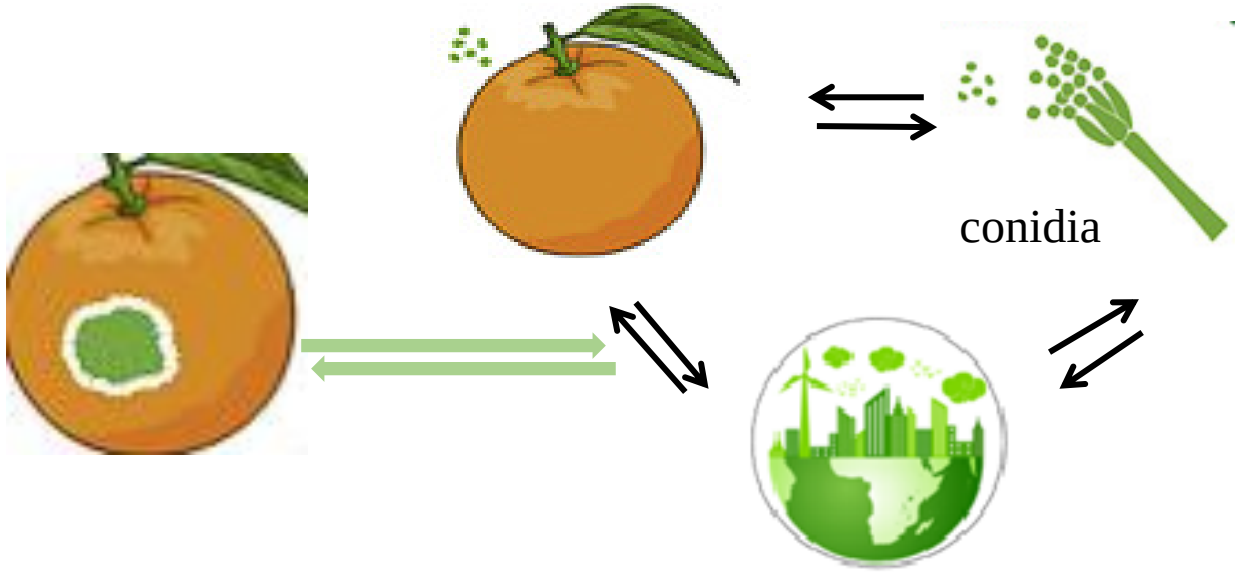





Fields


Harvest

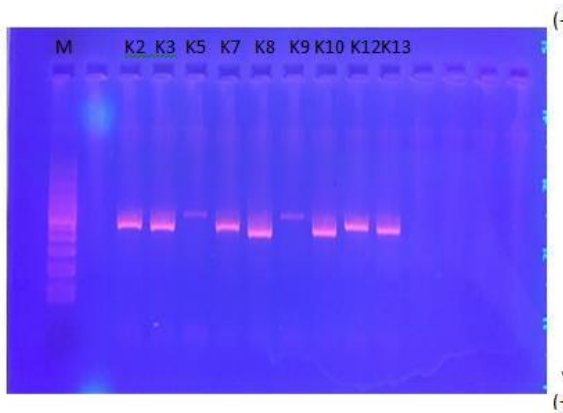

Packaging

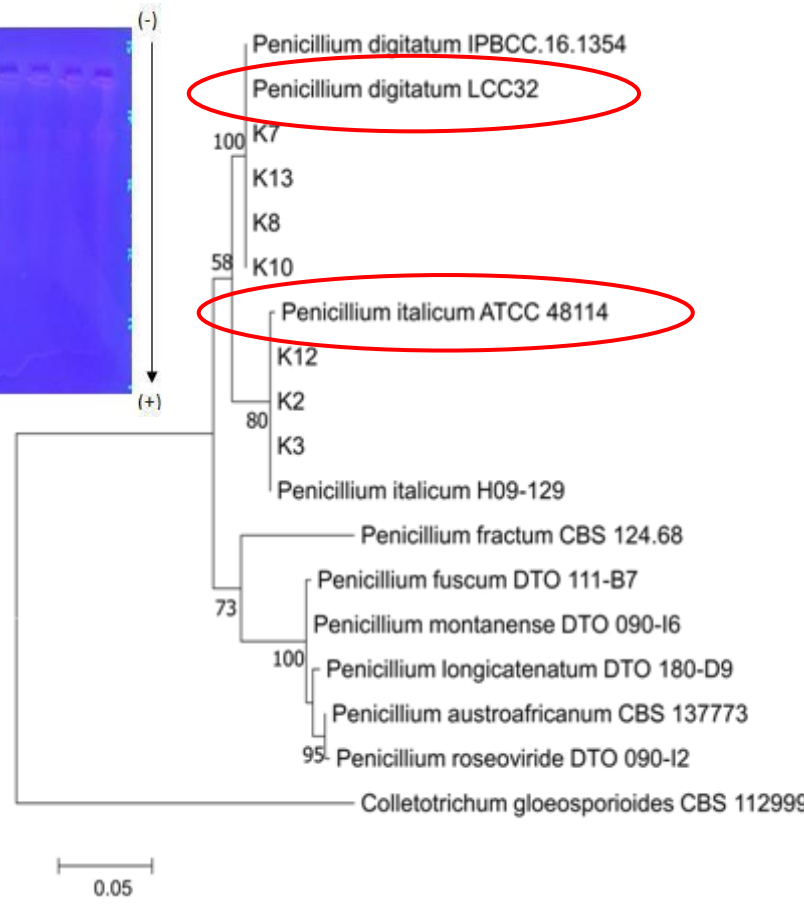


- Infections of fruits take place through wounds where the presence of nutrients stimulate conidium germination.
- The infection appears as a soft area surrounding the wound; soon, a white mycelium appears on the lesion and starts producing conidia.

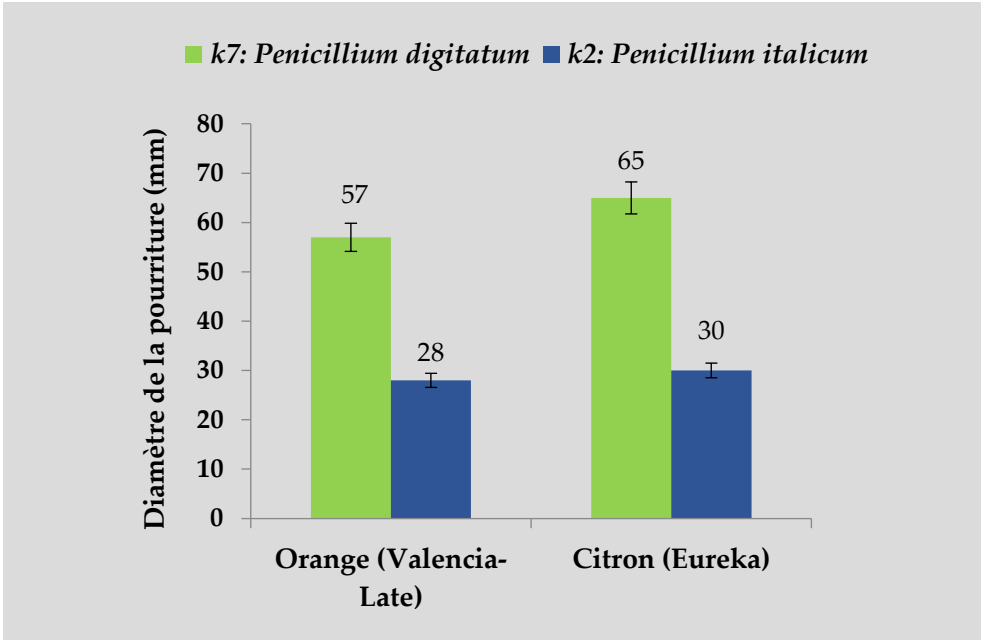
Characterization of the pathogen by amplification and sequencing of the ITS region

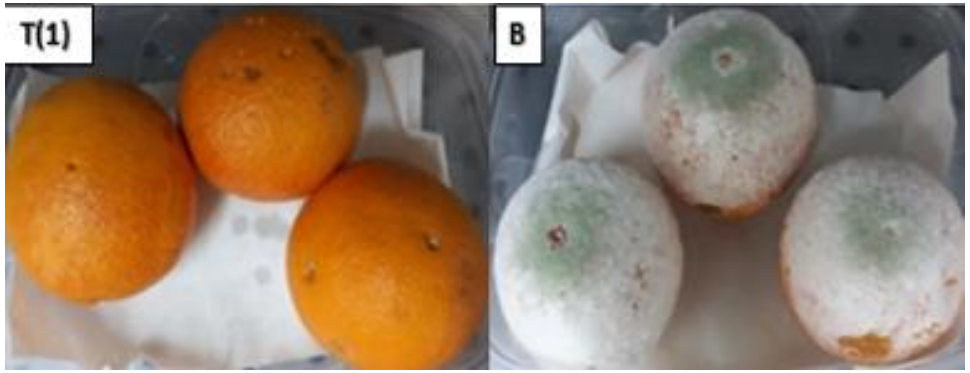
Code	Identity		Accession number
K2	*Penicillium italicum*	99%	MG956789.1
K3	*Penicillium italicum*	99%	HQ850905.1
K7	*Penicillium digitatum*	99%	AY373910.1
K8	*Penicillium digitatum*	99%	AY373910.1
K9	*Penicillium digitatum*	98%	MF527231.1
K10	*Penicillium digitatum*	98%	AY3739 10.1
K12	*Penicillium italicum*	99%	HQ850905.1
K13	*Penicillium digitatum*	99%	KY859376.1



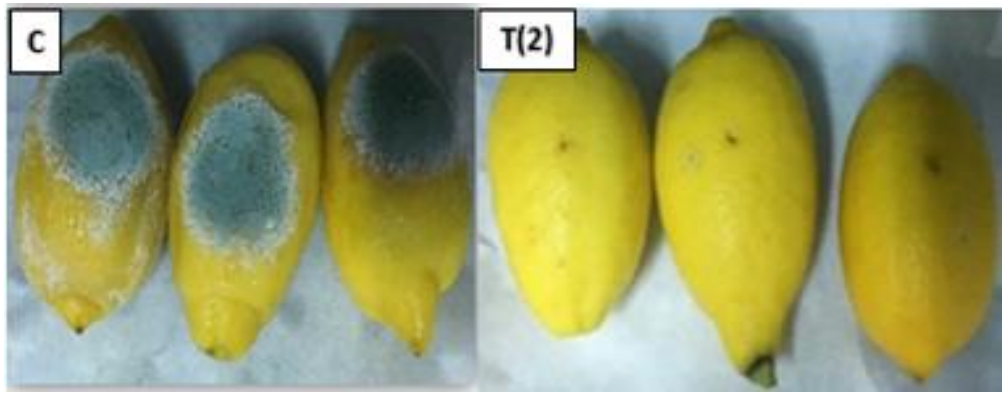


Pathogenicity tests

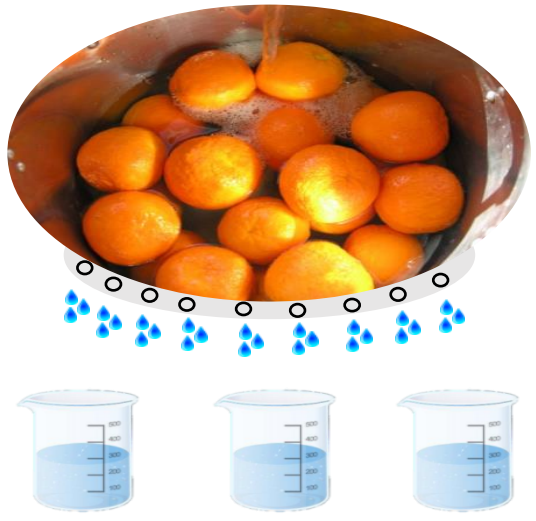




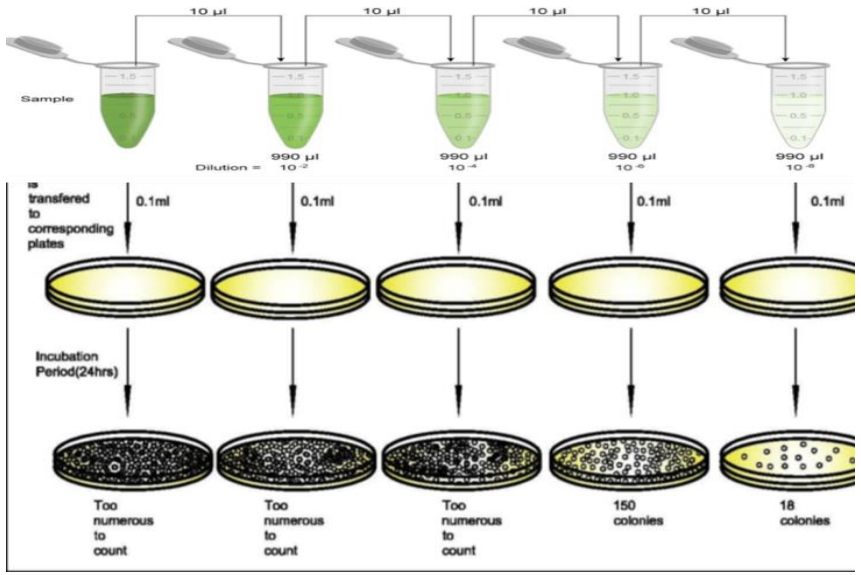
Tests confirming the pathogenicity of isolates P. *digitatum* K7 and *P. italicum* K2



Isolation of epiphytic bacteria and yeasts



Isolation was carried out from healthy citrus fruits



Dilutions were made from the washing waters and the last two dilutions were subcultured on TSA agar medium.

A total of 180 bacterial isolates and 30 yeasts has been established from the surface of citrus fruits

Antagonism in vitro Bacteria-*Penicillium*

Table 1. *In vitro* antagonistic activity of selected bacteria and yeast isolates on mycelial growth of *Penicillium digitatum* and *P. italicum*, determined by the dual culture test and expressed as % inhibition of mycelium growth.

Isolates	Mycelial growth inhibition of *Penicilllium digitatum* (%)	Mycelial growth inhibition of *Penicillium italicum* (%)
***Bacillus amyloliquefaciens* S15**	76.00 ± 2.00 a	45.00 ± 2.00 B
***B. subtilis* S57**	60.00 ± 0.00 b	73.33 ± 2.30 A
***B. pumilus* S67**	60.00 ± 0.00 b	32.00 ± 0.00 C
***Candida oleophila* L12**	32.00 ± 0.00 c	20.00 ± 0.00 C
***Debaryomyces hansenii* L16**	28.00 ± 4.60 c	20.00 ± 0.00 C

Values (± SD) followed by the same letters are not significantly different according to Duncan's multiple range test (P< 0.05).

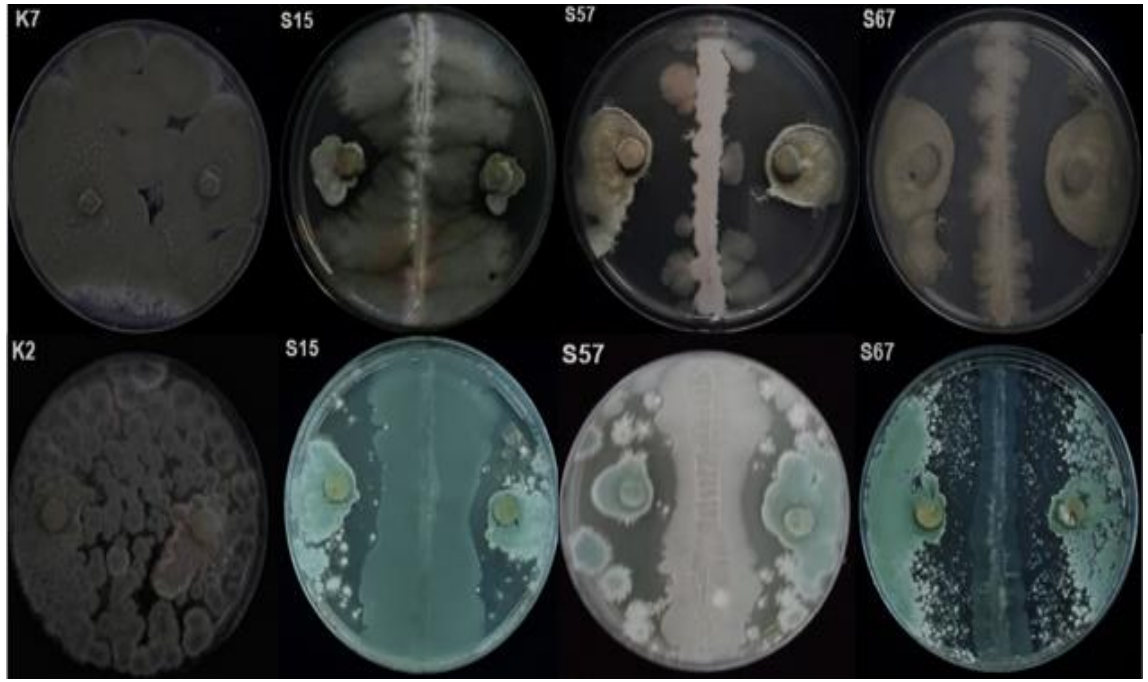


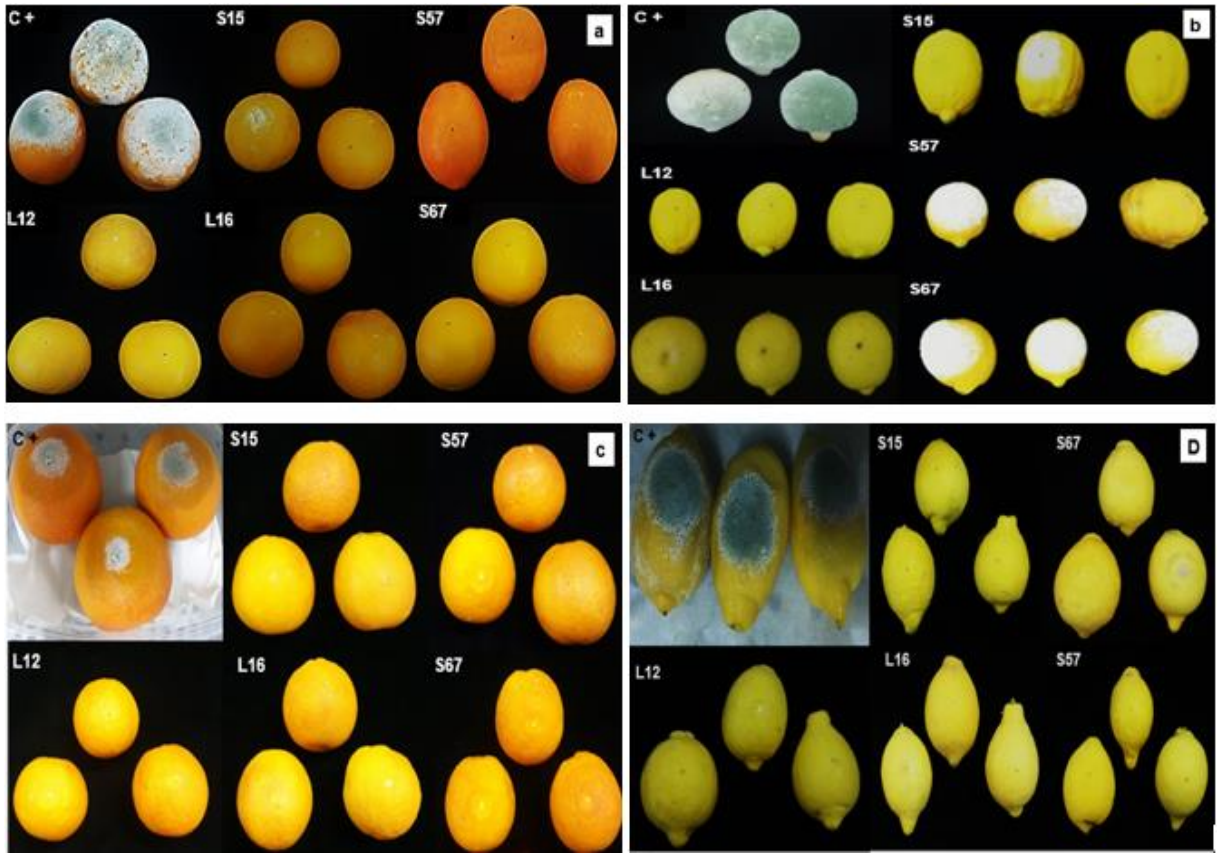
Fig 1. Dual culture test to evaluate the antagonistic activity of epiphytic bacteria (isolates S15, S57 and S67) against *Penicillium digitatum* and *P.italicum*

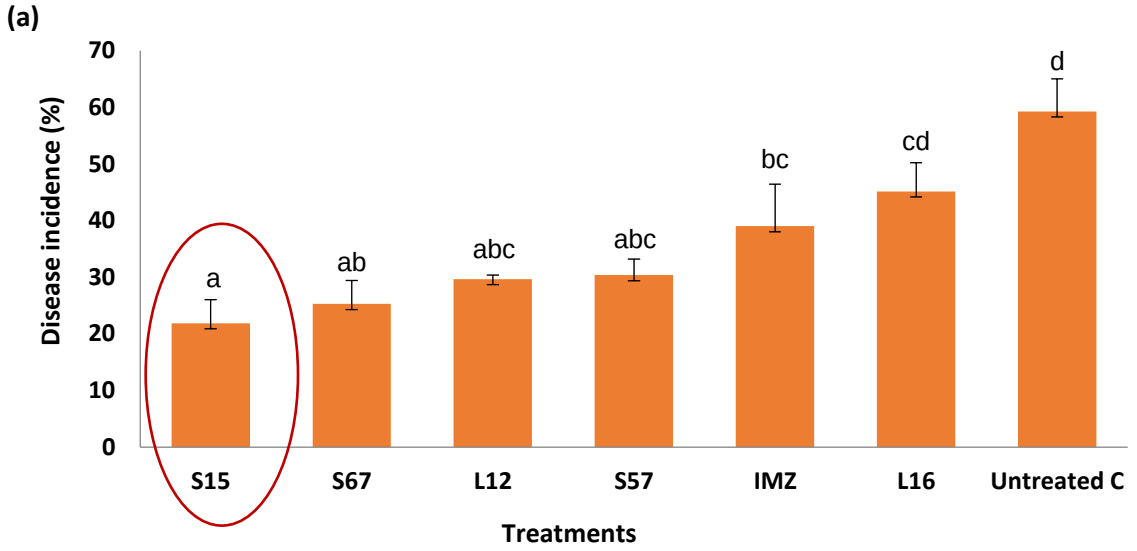
In vivo screening of antagonistic isolates at +25°C

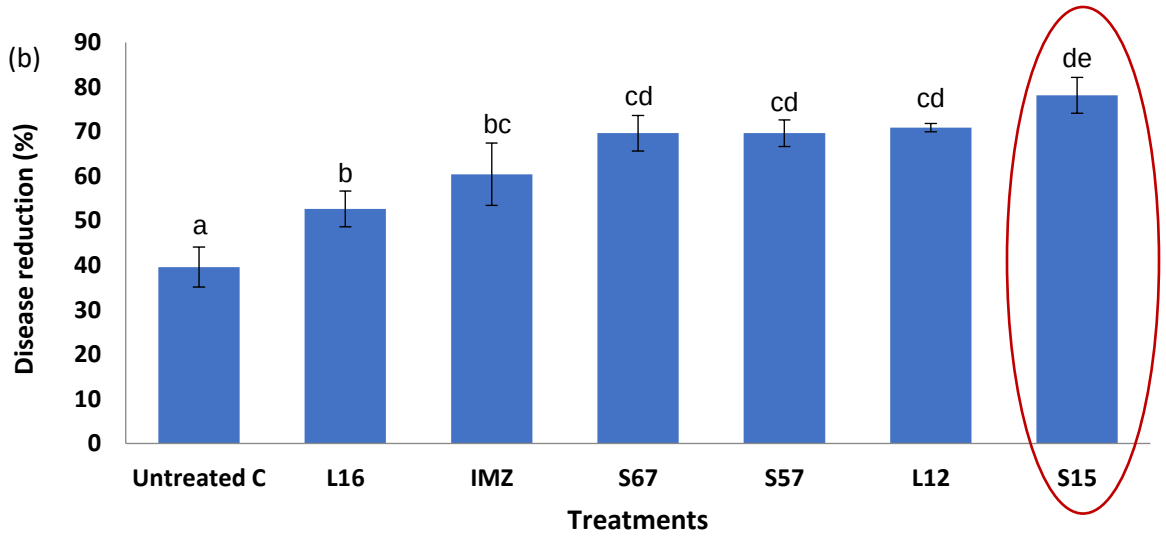
5 strains selected for *in vivo* assays on citrus fruits

- ***Bacillus amyloliquefaciens***
- ***Bacillus subtilis***
- ***Bacillus pumilus***
- ***Candida oleophila***
- ***Debaryomyces hansenii***

In vivo screening of antagonistic isolates at +25°C







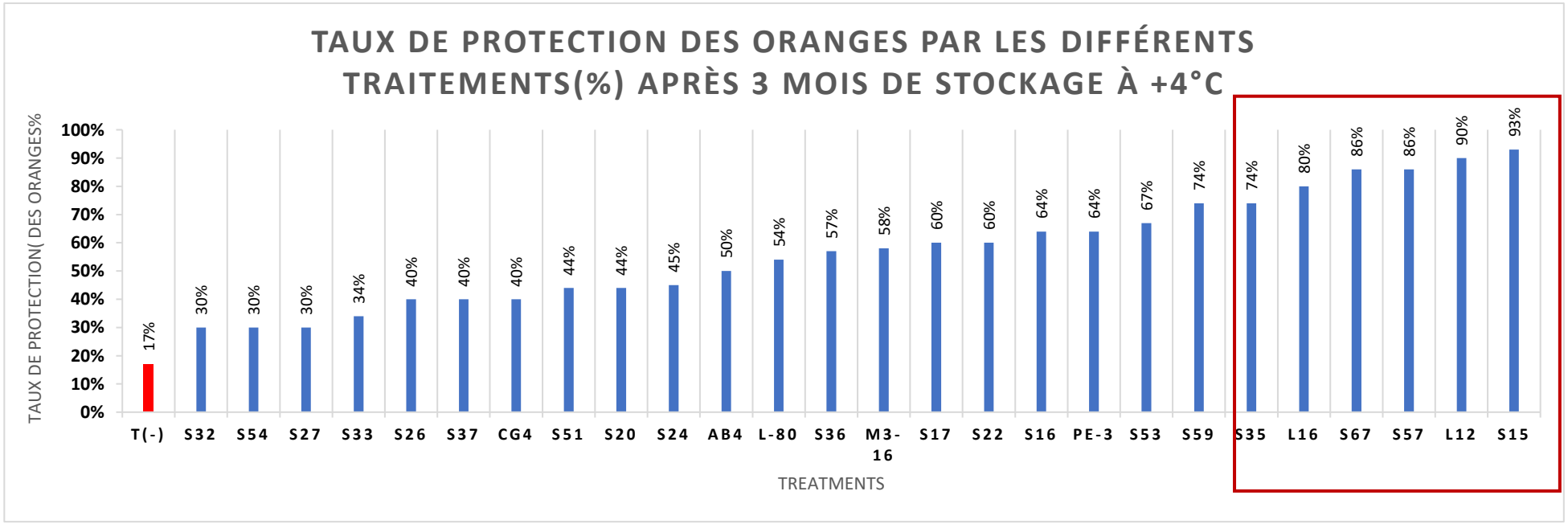
Effect of candidate BCAs epiphytic yeasts and bacteria (10^8 cells/ml) on natural decay incidence (a) and disease reduction (b)

In vivo screening of antagonistic isolates at +4°C

Bioassay on orange fruits: Immersion of fruits with a suspension of bio-product

Storage of fruits treated with biological agents at +4°C

Bio-protection of oranges at +4°C



Bacillus amyloliquefaciens **S15**

Bacillus subtilis **S57**

Bacillus pumilus **S67**

Candida oleophila **L12**

Debaryomyces hansenii **L16**

Mode of action of active bacteria

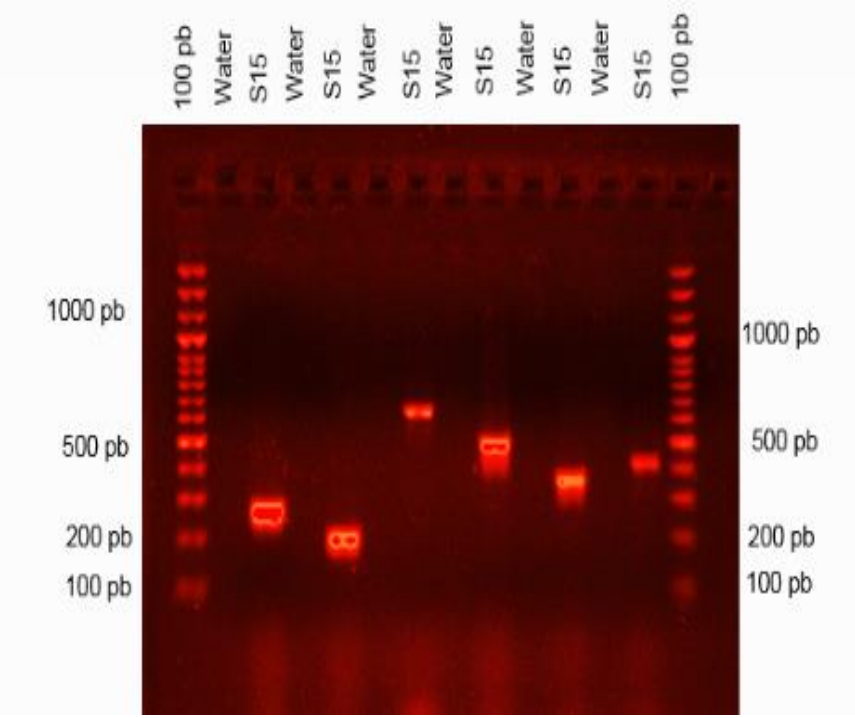


Figure 2. PCR amplification products of antibiotic biosynthetic genes of *Bacillus amyloliquefaciens* (S15), using primers for amplification of genes encoding (A) fengycin (FENDF/R) 269 bp; (B) surfactin (SRFAF/R) 201 bp; (C) iturin A (ITUD1F/R) 647 bp; (D) bacilysin (BCAF/R) 498 bp; bacillomycin (BMYBF/R) 370 bp; and (F) mycosubtilin (Am1F/Tm1R) 419 pb.

Table 2 Lytic enzyme activity of selected yeasts and *Bacillus* isolates as determined by spot-inoculation in Petri dishes.

Strains	Mannanase	Amylase	Protease	Cellulase	Chitinase	Pectinase
S15	+	+	+	+	+	+
S57	+	+	+	+	-	+
S67	+	-	+	-	-	-
L12	+	-	-	+	-	-
L16	+	-	-	+	-	+

Table 3. Ability to form biofilms of five candidate BCAs

Isolates	Optical Density (OD)
***Bacillus amyloliquefaciens* S15**	0.19 ± 0.35 c
***B. subtilis* S57**	0.26 ± 0.39 c
***B. pumilus* S67**	0.29 ± 0.00 c
***Candida oleophila* L12**	0.90 ± 0.28 a
***Debaryomyces hansenii* L16**	0.75 ± 0.24 b

Optimal culture conditions for cell growth of biocontrol agents

Effect of pH

pH varié (2, 4, 6, 7, 8, 10, 12)

PBD/ 30°C /48 h /130 rpm/min

Variation of temperature

Température variée (4°C, 10°C, 15°C, 20°C, 25°C, 30°C, 35°C, 40°C, 50°C)

PDB / pH 7/ 130 rpm/min / 48h

Inoculum (%)

Tailles d'inoculum testés (0.5%, 1%, 1.5%, 2%, 2.5%, 3%)

PDB / pH 7/ 30°C/ 48h / 130rpm/min

Selection of culture medium

Milieux de culture testés (LB, NB, NYDB, TSB, PDB, OJ 0.1%, OJ 1%, OJ 5%, OJ 15%, OJ 100%, FB, AB, AFB)

30°C, 48h / pH 7/ 130 rpm /min

Optimal culture conditions for cell growth of biocontrol agents

Candida oleophila

L12
pH8
20°C
OJ 100%
3%

S15
pH 7
30°C
LB
2%

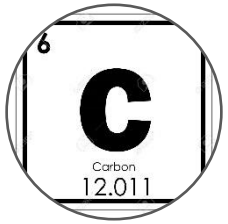
Bacillus amyloliquefaciens

S67
pH 4
40°C
TSB
2%

Bacillus pumilus

S57
pH 8
30°C et 40°C
TSB
1%

Bacillus subtilis

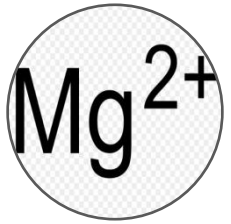


Carbon source

Les sources de carbone testées sont: le glucose, l'acide citrique, le saccharose, le fructose, le galactose, le xylose, le mannitol et le maltose

Nitrogen Source

Les sources d'azote testées sont : extrait de boeuf, peptone, extrait de boeuf peptone, extrait de levure, nitrate d'ammonium (NH4NO3), chlorure d'ammonium (NH4Cl), sulfate d'ammonium ((NH4)2 SO4), acide L-aspartique, L-proline, L-valine, L-arginine et L-tryptophane



Source of metal ions

Les ions métalliques testées sont : KH_2PO_4 (K^+), $FeSO_4$ (Fe^{2+}), $ZnSO_4$ (Zn^{2+}), $CuSO_4$(Cu^{2+}), $MgSO_4$(Mg^{2+}), NaCl (Na^+) et $CaCl_2$ (Ca^{2+}).

Other additives

vitamine C (acide ascorbique), la vitamine B2 (riboflavine), la vitamine B12(cyanocobalamine), la vitamine E (tocophérol), tween 20 et tween 80, l'huile de paraffine, l'huile minérale, l'huile de zeste de citron, l'huile de lentisque, l'huile de myrte et l'huile de romarin

1. **Effect of additive on cell growth of antagonists**
2. **Effect on mycelial growth of fungi**
3. **Effect on the antifungal activity of antagonists**

Development of a bioformulation of antagonists

ABCs	Temps (h)	Température	Proportion d'inoculation	pH	Milieux de culture	Source de carbone	Source d'ions métalliques	Source d'azote	Autres additives
L12	28	20°C	3%	8	OJ 100%	-	-	Arginine 0.5% (w/v)	Huile minérale 500µl/l
S15	48	30°C	2%	7	LB	-	-	-	-
S57	48	30°C, 40°C	1%	8	TSB	-	-	-	-
S67	48	40°C	2%	4	TSB	-	-	-	-

L12: Candida oleophila

S15: Bacillus amyloliquefaciens

S57: Bacillus subtilis

S67: Bacillus pumilus

Conclusion

- The yeast and bacterial isolates characterized in this study expand the list of promising BCAs that have the potential to be exploited commercially to control blue and green mold of citrus fruits.

- They could be formulated as either a single BCA or a consortium of diverse BCAs and applied alone or in mixture with natural substances as an alternative to conventional synthetic fungicides in ecofriendly management strategies of postharvest fruit decay.

Journal of *Fungi*

MDPI

Article

Epiphytic Yeasts and Bacteria as Candidate Biocontrol Agents of Green and Blue Molds of Citrus Fruits

Rania Hammami [1], Maroua Oueslati [1], Marwa Smiri [1], Souhaila Nefzi [1], Mustapha Ruissi [2], Francesca Comitini [3], Gianfranco Romanazzi [4], Santa Olga Cacciola [5] and Najla Sadfi Zouaoui [1,*]

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PROMETEO

Deux rives qui s'unissent
pour la défense des arboricultures
méditerranéennes

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Merci pour la gentille attention!