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Phytophthora species in Tunisian citrus farms

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From Waste to eco-friendly Nanotools to control *Phytophthora* diseases of *Citrus* spp. (WaNaPhy2.0)

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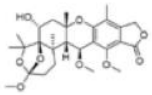
WaNaPhy 2.0

From Waste to eco-friendly Nanotools to control *Phytophthora* diseases of Citrus spp. (WaNaPhy2.0)

Citrus waste

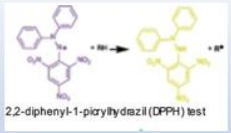
Essential oils

Encapsulated essential oil

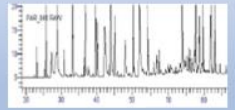


Fungal metabolites

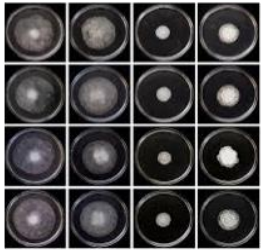
Bio-AlgNPs



Synergistic Fungicidal Activity

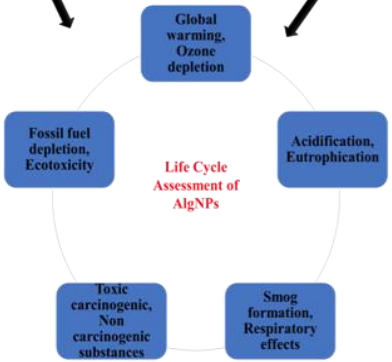


Characterization



In-vitro antifungal activity against *Phytophthora* spp.

In-vivo antifungal activity Against *Phytophthora* citrus diseases



Environmentally, safe, eco-friendly and effective biofungicide

The exploitation of the agricultural waste as resources for the synthesis of new nano-formulation(s) of bio-fungicides, based on nanoparticles charged with essential oils and fungal metabolites, against citrus brown rot and gummosis caused by *Phytophthora* spp.

SPECIFIQUES OBJECTIVES

Objective 1

Survey on the current management strategies for controlling *Phytophthoras* associated with Citrus diseases and waste;

Objective 2

Evaluation of the potential of agriculture waste in the synthesis of essential oils (EO) as source of biological control agents;

Objective 3

***In-vitro* evaluation of the antifungal activity of EOs and fungal metabolites against the causative agents of fruit brown rot and gummosis of Citrus**

Objective 4

Preparation and characterization of nanoparticles dispersion and *in-vitro* tests;

Objective 5

***In-vivo* effect of bio-AlgNPs against fruit brown rot and gummosis of Citrus.**

Objective 6

Life Cycle Assessment of bio-AlgNPs.

METHODOLOGY

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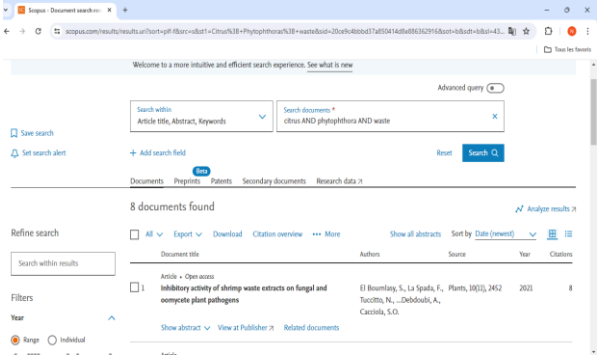
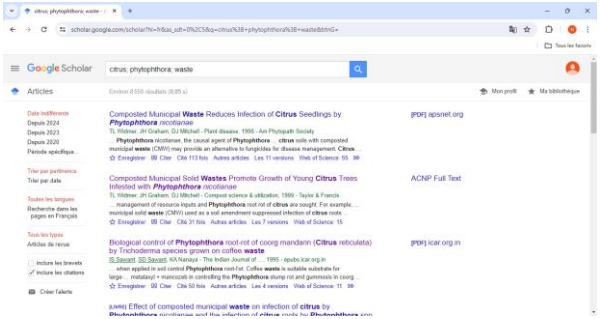
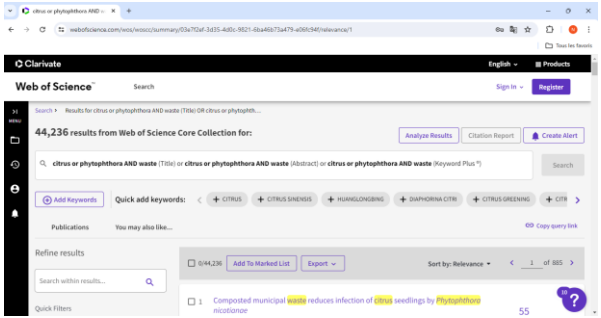






A scientific database (e.g., Scopus, Web of science, Google scholar…etc..), concerning citrus diseases associated with *Phytophthoras* and waste management, was used to create a template of investigation conducted.

Keywords: Citrus, *Phytophthora*, Diseases, Waste

Template of the investigation

Investigation N°	
Date of the investigation	

1. Personal information of the farmer	
Date of birth	

Gender ☐ Male ☐ Female

Education ☐ Primary ☐ Secondary ☐ Bachelors ☐ Masters ☐ Doctoral ☐

2. Orchard information	
Area (ha)	
Planting density	
Variety (ies)	
Percentage of plantation of each variety	
Age of plantation(year)	
Production /hear	

Legal status of the orchard

☐ Individual farm ☐ Company ☐ Group of natural persons

☐ Other legal (research institute, educational establishment, etc.)

3. Phytosanitary status			
Num.	Diseases	incidence	Severity
1	Gummosis		
2	Brown rot		
3	Dieback		
4	Wilting		
5	Damping-off		
6	Root rot		
7			
8			
9			
10			

4. Treatments	
Time of treatment (month)	
Type of treatment (...spray, irrigation etc.…)	
Product	
Name of the product	
The main component of the product	
Amount and period	

5. Disease incidence in the last 5 years								
Description of symptoms	Name	2022	2021	2020	2019	2018	Ask for analysis by expert	
							Yes	No

6. Knowledge of the environmental impacts of chemicals and cost

Knowledge of the health risks of chemicals ☐ Yes ☐ No

Use protection

☐ No ☐ Yes

☐ During treatment ☐ After treatment

Cost					
Period	**2022-2021**	**2021-2020**	**2020-2019**	**2019-2018**	**2018-2017**

7. Agricultural waste

Type of waste	
Waste amount	

Waste management strategies		
Methods	**No**	**Yes**
Burn		
Throw (If yes where ?)		
Compost it		
Sale (if yes cost of sale)		
Others (specify)		

8. How do the farmer prefer to be informed?

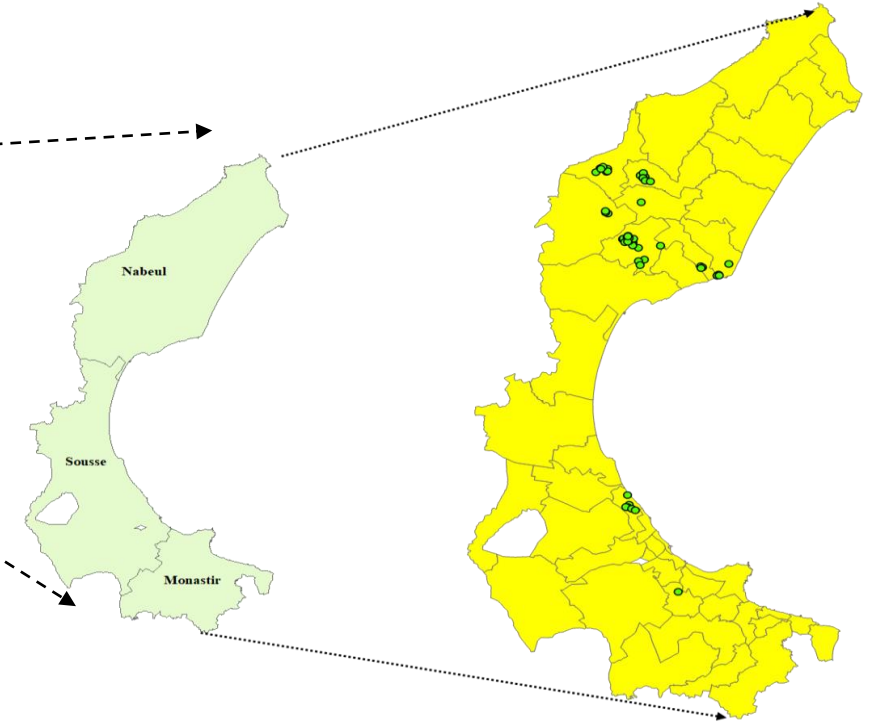
☐ **Email** ☐ **Workshop** ☐ **Persons**

☐ **Cooperative** ☐ **Newspapers** ☐ **Brochures**

☐ **Seminars** ☐ **Media** ☐ **Frameworks**

☐ **Others (specify)**

9. Suggestions of the farmer



- **A total 50 farmers were investigated.**
- **Period: Spring 2023.**
- **Localization: three major citrus-growing areas in Tunisia (Nabeul, Sousse and Monastir).**

SAMPLING

Infected trees were firstly examined visually for *Phytophthora* citrus diseases. From each infected tree, samples were taken from the trunk, the soil (with roots) and the fruits.

ISOLATION

From the tissue

- Samples were washed under running tap water to remove any adhering soil particles and excess water was removed by placed them between sterile absorbent filter paper.
- Surface sterilization by ethanol (70%).
- Cut from the edge of the lesions to pieces (about 2–5 mm wide) and when dried

From the soil

- Apples cv ‘Golden delicious’ were used as baits and three holes per fruit and 2 fruits per sample were used. Holes were then filled with sampled soil and incubated at room temperature. After three days, small pieces of tissue were aseptically removed from the inoculated apples fruits at the junction of the healthy and necrotic tissue

Plated onto 90 mm Petri dish, containing PARP-BH selective medium (CMA amended with Pimaricin, Ampicillin, Rifampicin, Benomyl, Pentachloronitrobenzene and Hymexazol). The plates were then incubated at 25 ± 1 °C in the dark and examined within 2–3 days. Pure cultures were obtained by sub-culturing hyphal tips onto potato dextrose agar (PDA).

Morphological identification

Colony morphology

- Recorded after 5 days of growth at 25°C in the dark on PDA (potato-dextrose-agar) and on Carrot agar (CA) medium.

***Phytophthora* structures**

For the sporangia production 5 mycelial plugs of each isolate, from a 5-day-old culture grown on CA medium, were transferred from the advancing margin of the colony into sterile soil extract and incubated at 20–25°C in daylight until sporangia were observed.

Chlamydospores and hyphal swellings were recorded directly on CA.

Measurement

The length and the breadth of 25 sporangia, as well as shape and diameters of 25 chlamydospores and hyphal swellings, were recorded.

Molecular identification

- Isolates were grown in CA and their genomic DNA extracted using Qiagen's DNeasy Plant Mini-kit (Qiagen GmbH). Amplification and sequencing of the Internal Transcribe Sequences (ITS1 and ITS4).
- PCR products were cleaned using a Qiagen Qiaquick PCR purification kit (Qiagen GmbH) and sequenced (Macrogen Inc.).
- Sequences of the isolates were compared with sequences available in the GenBank database.

Pathogenicity tests

Pathogenicity tests of 2 representatives isolates of *Phytophthora* were conducted using citrus fruits (cv. *limone bianchetto*).

- Four holes of 3mm-diam were made/ fruits
- 3 fruits/ isolates
- Incubation, 3 days at 25°C±1 in the darkness
- Evaluation of the existence of symptoms
- Re-isolation on PARP-BH medium

RESULTS

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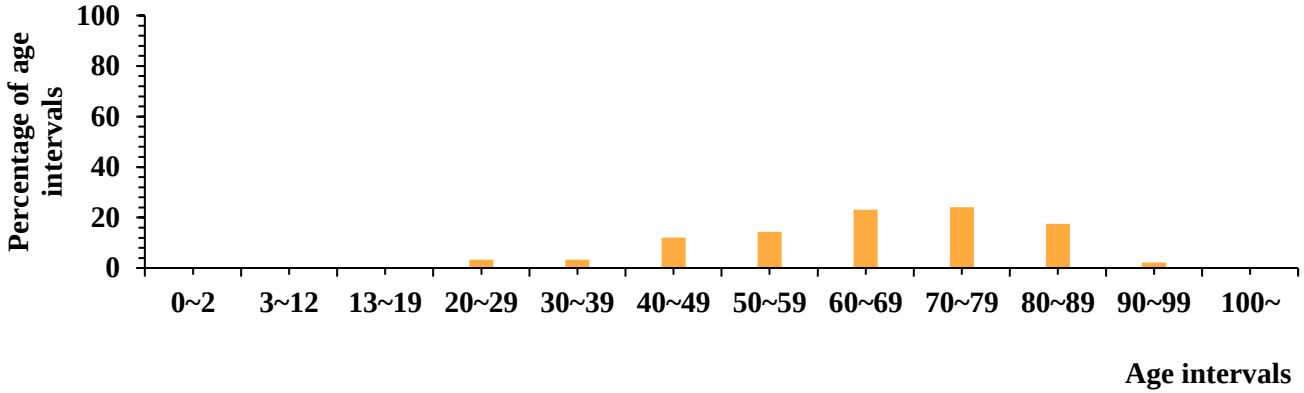
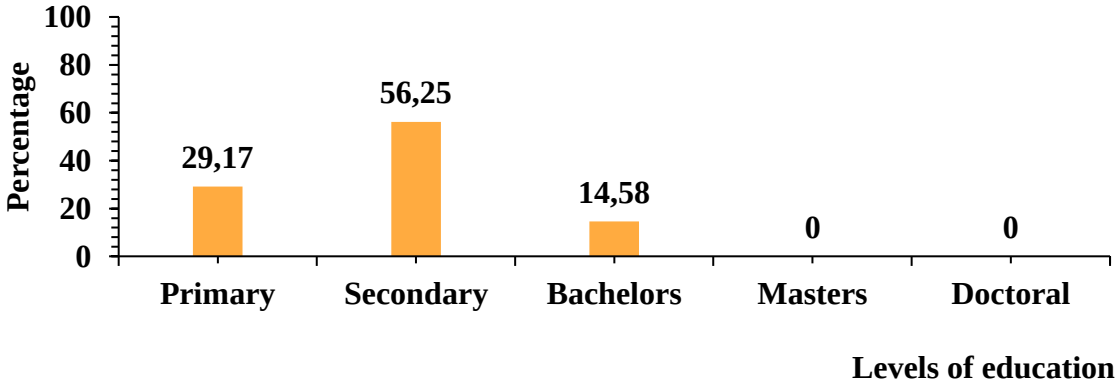
Personal information of the farmers

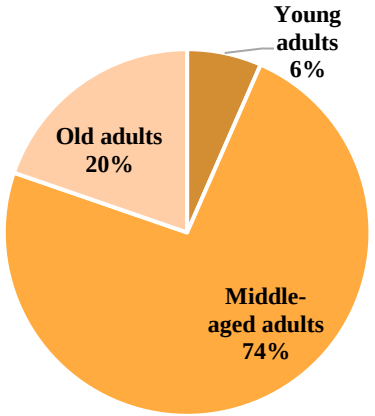


Gender

- **Male (95.83%)**
- **Female (4.16%)**

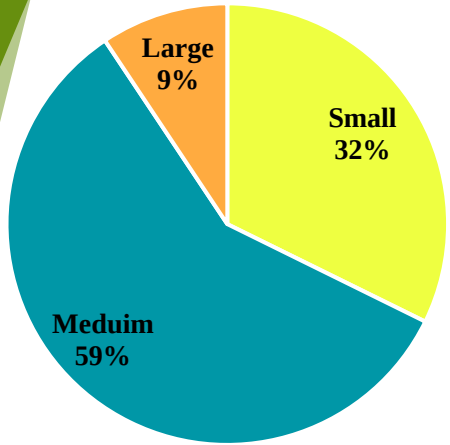
Classification of the age of the citrus farmers according to the age intervals





Classification of the farmers according to the level of education

Citrus orchards information



- A 59% of the citrus orchards was composed by a medium area (<1-10ha). A 32% of the citrus orchards was smalls (≤ 1ha), while just 9% of the orchards have a large area (>10ha).

- Most of the investigated citrus orchards was an individual farms (91.66%), and just 8.33% of the orchards was a company.

- Planting densities: 300ha (12.35%), 350ha (19.11%), 360ha (11.11%), 400ha (39.11%), 417ha (9.55%) and 500ha (8.77%).

Percentage of plantation, age and production of citrus varieties and rootstocks per hectare

	Species	Percentage of plantation	Age of plantation (year)	Production per hectare (Kg)
Varieties	*C. sinensis* L. *Osbeck* (Thomson Navel)	28.71	35.52	28241.66
	Maltaise demi-sanguine	03.00	16.11	17222.22
	C. reticulata (Mandarine)	01.81	25.00	11000.00
	C. Limon	25.10	26.75	13246.79
	C. sinensis (Maltaise)	15.68	28.54	19909.09
	C. clementina (Marisol)	03.61	35.00	15000.00
	C. tangarine	03.42	32.50	17000.00
	C. sinensis (Moro)	01.00	32.50	16000.00
	C. clementina (Hernandina)	07.03	14.16	18700.00
	C. reticulata (Nova)	02.61	27.50	14500.00
	C. sinensis (Navel)	00.81	12.50	12000.00
	C. sinensis (Valencia)	02.42	19.16	17666.66
	C. clementina (Nour)	00.81	08.33	15000.00
Rootstocks	*C. aurantium* (Bigaradier)	03.00	27.5	_
	C. volkameriana	01.00	07.50	_

Phytophthora diseases incidence and severity on citrus orchards

Diseases	Incidence	Severity	Analyze by expert
Gummosis	23.18	35.80	52.60
Dieback	14.50	22.90	62.50
Wilting	10.80	08.00	63.63
Damping-off	00.04	04.00	52.60

Phytophthora diseases incidence in the last five years (2018-2022)

	2018	2019	2020	2021	2022
Gummosis	3.42	4.18	5.41	6.73	7.07
Dieback	4.86	5.13	5.38	5.94	6.59
Wilting	17.40	22.31	27.04	31.90	32.91
Damping-off	0	0	0	0	1

Treatments

Modality of treatments

- Curatively by fungicides (80.90%)

- Foliar spray and using the recommended dose as described on the fungicides.

Application of the treatments:

- **2 to 4 times per year (44.61%)**
- **2 to 3 times per year (36.28%)**

Active ingredient	Percentage	Commercial name
Copper	64.34	Kocide 2000, Cuivox, Siscop, Hidrocobre, Maxil, CobraKey, Atlas Agricole, Cuprene
Fosetyl-Al	19.14	Aliette express, Filal, Alu-Vite WP, Magma Triple, Lotus pro, Katanga Man
Metalaxyl	12.63	Nufarm, Alial, Maxil
Mancozeb	3.88	Ridomil Gold, Dithane

Knowledge of the environmental impacts of chemicals and cost

A total of 88.46% of the farmers have an idea about the environmental impacts of the use of chemicals.

52% of them use protection against the treatments. Some of them prefer to protect them self during the treatment by have a mask (30.49%), while the majority of farmers prefer to protection them self after treatment by a doing a shower (69.50%).

Percentage of the cost of the chemical according to the production

	2017-2018	2018-2019	2019-2020	2020-2021	2021-2022
Percentage	**19.41**	**21.26**	**25.18**	**26.75**	**28.00**

Waste of citrus orchards

Types of waste

Branches, Leaves and fruits

Management of agricultural waste

- Burn (92.22%)
- Throw (6.66%)
- Compost (1.11%).

The farmers throw the agricultural wastes in the same orchard, under walls or windbreaks.

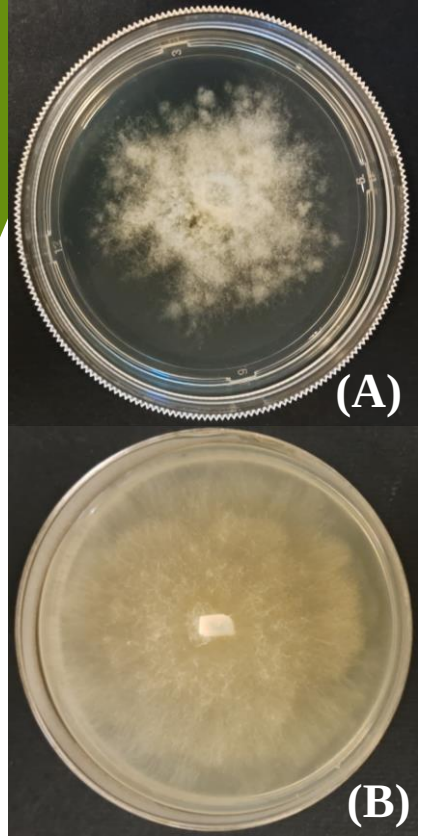
How do the farmers prefer to be informed?

Methods	Percentage
Email	01.48
Seminars	10.05
Workshop	12.15
Cooperative	04.34
Newspapers	10.24
Media	18.39
Persons	17.44
Frameworks	12.46
Brochures	12.88
Others (specify)	00.53

Suggestions of the farmers

- **Financial support from the government;**
- **Dissemination of information related to citrus production in general and the results of investigation (companies or technical center).**

Identification



Morphological aspect of the colony on PDA medium (A) and CA medium (B)

Molecular identification: *Phytophthora nicotianae*

Pathogenicity tests

After 3 days of inoculation with *P. nicotianae*

Control

https://www.facebook.com/profile.php?id=100094062848168

https://www.instagram.com/wanaphy2.0?igsh=YzRtN3pud3FpNmt2