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# *In vitro* and *in vivo* effects of some chemical fungicides against *Pythium ultimum* and *Phytophthora citrophthora* associated with peach seedlings decline

Sabrine Mannai[1*]; Naima Boughalleb-M'Hamdi[1]

[1]Department of Biological Sciences and Plant Protection, High Institute of Agronomy of Chott Mariem, 4042 Sousse, L21AGR05, University of Sousse, Tunisia

[*]Corresponding author E-mail: sabrinemannai@hotmail.com

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## Abstract

Peach decline, responsible for seedlings root and collar rot in nurseries, is an important disease that causes reduction in plant production. Several oomycetes species were associated with this disease. The aim of this study was to control this serious peach decline disease using several assays such as; *in vitro* poisoned food technique and *in vivo* greenhouse assay. About six chemical fungicides were evaluated for their *in vitro* and *in vivo* inhibitory potentials against *Pythium ultimum* and *Phytophthora citrophthora* associated with this disease, respectively. The *in vitro* poisoned food technique demonstrated highly significant difference in the efficacy of the fungicides used at the five tested doses (10, 25, 50, 100 µg\ l and application rate). Carbendazim inhibited *Pythium ultimum* and *P. citrophthora* by 75.30 % and 100 % at 250 µg\ l. For Mancozeb, the inhibition % achieved by the registered dose was 100 % for *Pythium ultimum* and 50 % for *P. citrophthora* at 2000 µg\l. The registered dose (2000 µg\ l) of Fosetyl-Al inhibited *Pythium ultimum* and *P. citrophthora* by 51 % and 100 %, respectively. The highest rates of inhibition induced by Hymexazol were recorded at 60 µg\ l (90.55 % for *Pythium ultimum* and 94.49 % for *P. citrophthora*). In case of Chinosol, inhibition percentages of 90.30 % and 90.96 % for *P. citrophthora* and *Pythium ultimum*; respectively, were achieved at the tested concentration of 50 µg\ l, and the same inhibition values were also recorded for both tested concentrations of 100 and 2000 µg\ l. The highest inhibition rates for Metalaxyl-M against *Pythium ultimum* were observed at the dose of 100 µg\ l (79.70 %), whereas the used dose of 120 µg\ l recorded 86.59 %. The *in vivo* greenhouse assays demonstrated the efficacy of the Fosetyl-Al and Metalaxyl-M in reducing the peach seedling root browning induced by *Pythium ultimum* by 62.55 %. However, all the tested chemicals neither improved the growth and health status of the peach seedlings, nor reduced root browning of the seedlings inoculated with *P. citrophthora*.

**Keywords**: Chemical fungicides, Nurseries, Peach decline, *Pythium ultimum*, *P. citrophthora*

## 1. Introduction

The peach tree (*Prunus persica*) has an important place in the world; in terms of total fruit yield within the fruits industry. It represents the 3rd position in the world production of fruit trees after citrus and apple (Yuan *et al*., 2018). Development of the peach sector could be affected by various phytosanitary problems such as the trees decline. Occurrence of this complex syndrome could be linked to several biotic factors including the pathogenic fungi such as the Oomycetes (Bent *et al*., 2009).

Previous studies on peach and apple decline disease have revealed that the Pythiaceae spp. could be the pathogens responsible of this disease, as they reduced the seedling growth parameters (Utkhede and Smith, 2000; Mazzola *et al*., 2002; Boughalleb *et al*., 2006b; Bent *et al*., 2009; Souli *et al*., 2011a; Jabiri *et al*., 2021). The correlation analyses conducted in South Africa on concentration of the pathogen DNA and apple seedlings weight and height; recorded significant negative correlation between seedlings weight and *P. irregulare* DNA concentration (Tewoldemedhin *et al*., 2011). Similarly, previous investigations carried out in California by Bent *et al*., (2009) on stunting of peach seedlings caused by a replant soil; using sequence-selective quantitative PCR assays, demonstrated that *Pythium ultimum* was negatively associated with peach plant top weights. In Tunisia, several *Phytophthora* and *Pythium* spp. were associated to apple trees decline in orchards (Boughalleb *et al*., 2006b; Souli et al., 2011a, b). In Tunisian nurseries, infected seedlings showed symptoms of browning of the apical part of the scion and/or at the collar, which finally resulted in complete decline (Mannai *et al*., 2018a). Surveys in the Tunisian nurseries and diagnosis of the peach seedlings decline; demonstrated that *Fusarium*, *Phytophthora*, *Pythium* and *Phytopythium* spp. were associated with the decline of peach, and proved that these species were pathogenic (Mannai *et al*., 2018a).

For management measures of the peach decline disease in the Tunisian nurseries, a previous study was conducted by Mannai *et al*., (2018b) to evaluate the *in vitro* and *in vivo* activity of six fungicides against *Fusarium oxysporum* and *F. solani*. Results of this study showed the efficacy of some chemical products such as Alliette express (fosetyl-Al); Ridomil Gold (mancozeb+ metalaxyl-M), Dithane-M45 (mancozeb), Prodazim (carbendazim) and Beltanol (chinosol). Besides, previous works carried out in different areas throughout the world have shown that several species of *Pythium* and *Phytophthora* including *Pythium ultimum*, *Pythium spiculum*, *P. cinnamomi*, *P. infestans*, *P. cactorum* and *P. citrophthora* were sensitive to metalaxyl-M and fosetyl-Al (Boughalleb *et al*., 2006a; Weiland *et al*., 2014; Khadka *et al*., 2020; González *et al*., 2020; Molin *et al*., 2021).

The goal of the present investigation was to evaluate the *in vitro* and *in vivo* effectiveness of the six fungicides in management of *Pythium ultimum* and *P. citrophthora* pathogens associated with the peach seedlings decline.

## 2. Materials and methods

### 2.1. Isolation of the fungal isolates

The pathogenic fungal isolates were isolated from samples of peach seedling roots collected from Tunisian nurseries in 2013. Peach roots samples collected from surveyed nurseries were washed under tap water to remove adhering soil; cut into small pieces of 3 to 5 mm in lengths using a sterile scalpel, surface disinfected with ethanol (70 %) for 30 s, rinsed in sterile dist. water and then dried using a sterile Whatman filter paper. The root segments were placed aseptically on the surface of petri plates (90 mm in diameter) containing PARP (pimaricin + ampicillin + rifampicin + pentachloronitrobenzene [PCNB]) agar selective medium composed of; 17 g of corn meal agar (CMA); amended with 10 µg\ l of pimaricin, 200 µg\ l

of ampicillin, 10 µg\ ml of rifampicin and 25 mg\ ml of pentachloronitrobenzene (PCNB), in reference to Jeffers and Martin, (1986). All petri plates were incubated at 25 °C in the darkness and examined within 2-3 d. After incubation, recovered oomycete-like isolates were purified using the hyphal tip method (Mannai, 2019).

### 2.2. Morphological and molecular identification of the recovered oomycete isolates

Two oomycete isolates were recovered, and then identified based on their morphological criteria; according to the descriptions of Plaats-Niterink, (1981); Möller *et al*., (1992); Mounde e*t al*., (2012). Both isolates were grown on PDA medium at 25°C in the darkness to assess their growth rates and colonies aspects. These isolates were placed on the surface of V8 agar medium (200 ml of V8 juice, 2 g CaCO3, 20 g agar, 800 ml dist. water), and then incubated at 25°C for 5 d in the darkness; in order to develop different growth structures (Erwin and Ribeiro, 1996). After incubation, a 5 mm-diameter mycelial plug of each isolate cut using a sterile cork borer was inoculated individually into 20 ml of sterile soil extract (100 g of soil, 900 ml of dist. water), and then incubated for 2-3 d at room temperature. The presence or absence of oogonia; antheridia, oospore, chlamydospores and sporangia produced by each isolate was observed, and an average diameter of 25 structures of each diagnostic character was measured using a compound Leica microscope (DM 2500, Germany), according to Mannai, (2019).

Molecular identification of both isolates was made through genomic DNA extraction of each isolate according to the method adopted by Möller *et al*., (1992). The ITS region was amplified with universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3'). The Polymerase chain reaction (PCR) analysis was performed in a 50 µl volume reaction mixture composed of; 2 µl of DNA (50 ng/ µl), 0.5 µl of Taq polymerase (5U/ µl), 3 µl of MgCl2 (1.25 mM), 5 µl of PCR buffer (10 x), 5 µl of dNTP (1.25 mM), 5 µl of each of 5 µM forward and reverse primers, and 24.5 µl of sterile dist. water. The PCR product was separated using electrophoresis in an agarose gel (1 %), purified and then sequenced. Obtained sequences were analyzed by BLAST (Basic Local Alignment Search Tool), compared to several sequences of Pythiaceae species from GenBank, and then deposited in the GenBank (Ristaino *et al*., 1998; Mannai, 2019).

### 2.3. *In vivo* pathogenicity assay in the greenhouse

The *in vivo* pathogenicity was assay carried out in the greenhouse for each isolate. Peach seedlings of 18-months-old were used in this essay. These plants were grown in the greenhouse in plastic pots (23 cm diameter × 23 cm deep) containing the growth substrate. The used substrate was composed of 50 % sterile soil, 25 % peat and 25 % sand, inoculated with sand-oat inoculum at the rate of 1% (v/v), and then incubated for 24 h at 25 °C prior to planting. The sand-oat medium composed of; 200 g of sterile sand, 20 g of oat, and 30 ml of dist. water, which was added to 500 ml Erlenmeyer's flask, and then autoclaved for 20 min. at 120 °C for 2 consecutive d. After sterilization, each flask was inoculated with 10 mycelial plugs (6 mm diameter) cut using a sterile cork borer from a 1-week-old of each oomycete culture grown on PDA. For the control treatment, the oomycete plugs were replaced by PDA plugs. The inoculated flasks were incubated at 25°C for 1 week, and were shaken every 2 d to ensure the uniform colonization (Tewoldemedhin *et al*., 2011; Mannai, 2019). The seedlings were grown in the plastic pots containing the seeded substrate. The experiment was conducted as a complete randomized block design, with three replicates per isolate. The seedlings were harvested 3 months after inoculation. At harvest, the seedlings roots were washed with dist. water, and then the disease severity was evaluated using 2 parameters mainly; (i) the root weight and (ii) the root browning index (Tewoldemedhin *et al*., 2011).

### 2.4. Chemical fungicides

Six chemical fungicides namely; Prodazim 50 wp (Carbendazim), Dithane M-45 (Mancozeb), Alliette

Express (Fosetyl-Al), Tachigazol 300 (Hymexazol), Beltanol-L50 % (Chinosol ) and Ridomil Gold MZ (Metalaxyl-M+Mancozeb)); were tested for their inhibitory potentials against *Pythium ultimum* and *P. citrophthora* associated with the peach seedlings decline in Tunisia (Table 1). These fungicides had different active ingredients and were tested previously against *Fusarium* spp. associated to the same disease in Tunisian nurseries (Mannai *et al*., 2018b).

**Table 1.** Chemical fungicides used to control growth of *Pythium ultimum* and *P. citrophthora* associated with peach decline seedlings in the Tunisian nurseries

| Trade Name | Chemical name | Active concentration | Registered dose |
|---|---|---|---|
| Prodazim 50 wp | Carbendazim | 50 % | 50 g/ hl (250 µg\ l) |
| Dithane M-45 | Mancozeb | 80 % | 250 g/ hl (2000 µg\ l) |
| Alliette Express | Fosetyl-Al | 80 % | 250 g/ hl (2000 µg\ l) |
| Tachigazol 300 | Hymexazol | 30 % | 20 ml/ hl (60 µg\ l) |
| Beltanol-L50% | Chinosol | 50 % | 4 l/ha |
| Ridomil Gold MZ | Metalaxyl-M<br>+Mancozeb | 4 %<br>64 % | 300 g/ hl (120 µg\ l Metalaxyl) |

Where; Hl: hectoliter

### 2.5. Evaluation of *in vitro* effects of the chemical fungicides on mycelial growth of the pathogens

Using the *in vitro* poisoned food technique according to Mannai *et al*., (2018b); for each chemical product about 5 doses of the active ingredient were tested mainly; 10, 25, 50 and 100 µg\ l. The fungicides concentrations were adjusted using sterile dist. water; where 10 ml of each fungicide concentration was added individually to 250 ml flasks of Potato dextrose agar (PDA), and then poured aseptically into the Petri plates. After solidification, a plug of 6 mm diameter was cut individually from 6-days-old cultures of *Pythium ultimum* and *P. citrophthora* using cork borer, and then placed in the center of the PDA plates (90 mm in diameter). The control plate was maintained by placing the fungal disc at the center of non-treated PDA plate. All plates were incubated in the darkness at 25°C for 4 d. Three plates were used for each fungicide concentration, and the assay was repeated twice. After incubation, the diameter of radial mycelial growth of the fungal pathogen was measured (mm) in each treated plate using a calibrated ruler; and compared to the control. The used statistical design was a completely randomized factorial model with two factors (The first factor is the fungicides, while the second one is their doses). The percentage (%) of growth inhibition of each pathogen was calculated using the following formula of Mannai *et al*., (2018b):

$$\text{Inhibition (\%)} = \{(C - T)/C\} \times 100$$

Where; T: mean diameter (mm) of the colonies in presence of the fungicide; C: average diameter (mm) of the control colonies

### 2.6. *In vivo* potential of the chemical fungicides on disease severity of the peach seedlings in the greenhouse

About 4 weeks-old peach seedlings variety 'Garnem' were used in this study. These seedlings were grown in pots (23 cm diameter × 23 cm deep) in the greenhouse. Effectiveness of the fungicides on causing inhibition of the growth of *Pythium ultimum* and *P. citrophthora* were evaluated *in vivo* using the method of Utkhede and Smith, (1991) with little modifications.

The fungal inoculum was prepared by inoculating 10 agar plugs of *Pythium ultimum* or *P. citrophthora* isolates individually into an Erlenmeyer flask (500 ml) containing sand-oat (200 g of sand, 20 g of oat and 30 ml of dist. water, which had been autoclaved twice at 120 °C for 20 min.). The flasks were then incubated for one week at 25 °C; shaken every 3 d to ensure homogenous colonization. After incubation, the sand-oat inoculum was added individually to the potting mix (peat and sand in 2:1 v/v) at the rate of 1 % (v/v), which was then placed in 23 cm diameter plastic pots. The un-inoculated control treatment contained a potting mix without the sand-oat inoculum; whereas the positive control plants were inoculated with the pathogen inoculum only (Tewoldemedhin *et al*., 2011).

After plantation in this prepared potting mix; each peach seedling was treated with the chemical fungicide using the registered dose for each product (50 ml/ plant), in reference to Utkhede and Smith, (1991). The pots were arranged in a completely randomized design and watered when needed. Three seedlings were used per each individual treatment and the assay was repeated twice. Disease severity including root browning; sanitary state and plant growth parameters (i.e. plant height and root fresh weight) were recorded 3 months post- inoculation.

The sanitary state of the plants vegetative parts was scored based on a 0-5 scale according to Santini *et al*., (2006); where: 0= no obvious symptoms of peach decline; 1= moderate discoloration of plant leaves (≤ 25 %); 2= moderate discoloration and falling leaves (≤ 50 %); 3= moderate discoloration of plant collar, stem and leaves (≤ 75 %); 4= extensive discoloration of plant collar and stem with falling leaves (> 75 %); and 5= dead plants.

Root rot was rated onto a 0-5 scale (0= no obvious symptoms; 1= moderate discoloration of the root tissue; 2= moderate discoloration of root tissue with some lesions; 3= extensive discoloration of the root tissue; 4= extensive discoloration of the tissue with girdling lesions; and 5= dead plants), in reference to Tewoldemedhin *et al*., (2011).

### 2.7. Statistical analysis

All data were subjected to a one-way analysis of variance (ANOVA) by using Statistical Package for the Social Sciences software (SPSS), version 20.0. The *in vitro* assay was analyzed according to a completely randomized factorial model with two factors (tested fungicides and their doses). The *in vivo* assays were analyzed in a completely randomized model. For all the *in vitro* and *in vivo* assays, means were separated using Student-Newman-Keul's (SNK) test ($p \leq 0.05$).

## 3. Results

### 3.1. Isolation, identification and pathogenicity of the fungal isolates

Isolation of the fungal pathogens from peach seedling roots led to the recovery of 28 fungal isolates. About 16 isolates were identified morphologically and molecularly as *Pythium ultimum* (GenBank Accession no. MF993110); whereas 12 isolates were identified as *P. citrophthora*. Pathogenicity of both isolates were confirmed in the greenhouse; recording disease severity of 100 % for *Pythium ultimum* and 65 % for *P. citrophthora*.

### 3.2. *In vitro* effects of the fungicides on radial mycelial growth of *Pythium ultimum* and *P. citrophthora*

The mean inhibition percent (%) of the mycelial growth of *Pythium ultiumum* and *P. citrophthora* that was measured 4 d post- incubation of the plates at 25 °C, varied according to the type and dose of each fungicide. A highly significant interaction was observed between both fixed factors (fungicides and their doses) at $p \leq 0.001$. The *in vitro* assay of the six fungicides gave highly significant difference ($p \leq 0.001$) for efficacies of the 5 tested doses against *Pythium ultimum* and *P. citrophthora*, as presented in Table (2). For Carbendazim applied at 100 µg\ l and the registered dose, the inhibition % of *Pythium ultimum* mycelial growth was 70.06 % and 75.30 %, respectively (Table 3 and Fig. 1). However, carbendazim was recorded to be effective at the lowest dose (10 µg\ l) against *P. citrophthora* with a growth inhibition of 72.42 %, which increased to 100 % for the registered dose of 250 µg\ l.

Results demonstrated that mancozeb was ineffective at the low doses (10 and 25 µg\ l), but effectiveness was positively correlated with the used doses. The registered dose of 2000 µg\ l was the most effective recording 100 % inhibition for *P. ultimum* and 50 % for *P. citrophthora* (Table 2 and Fig. 1). It was also observed that Fosetyl-Al at the 4 first doses of 10, 25, 50 and 100 µg\ l was unable to reduce the mycelial growth of *P. ultimum* and *P. citrophthora*. However, on application of the registered dose (2000 µg\ l), the recorded percentages of mycelial inhibition of *Pythium ultimum* and *P. citrophthora* were 51 % and 100 %, respectively (Table 2 and Fig. 1).

For Hymexazol, the highest rates of growth inhibition were noted (90.55 % for *Pythium ultimum* and 94.49 % for *P. citrophthora*); at the registered dose of 60 µg\ l (Fig. 1). Chinosol was observed to be effective against *P. citrophthora*, with an inhibition percent greater than 67 % for all the tested doses. However, this product was ineffective against *P. ultimum* at low tested doses of 10 and 25 µg\ l. In fact, the best inhibition percentages were achieved at the concentrations of 50 µg\ l, 100 µg\ l and at the registered dose of 2000 µg\ l; with inhibition values of 90.30 % and 90.96 % for *P. citrophthora* and *Pythium ultimum*, respectively, as obvious in Table (2) and Fig. (1). The highest inhibition rates for metalaxyl-M against *Pythium ultimum* were noted at the concentration of 100 µg\ l and the registered dose of 120 µg\ l, recording inhibition percentages of 79.70 and 86.59 %, respectively (Table 2 and Fig. 1). Nevertheless, the 5 tested doses of this product were ineffective against *P. citrophthora*; with an inhibition percent lower than 33.46 % (Table 2 and Fig. 1).

### 3.3. *In vivo* impacts of the chemical fungicides on growth of *Pythium ultimum* and *P. citrophthora* in the greenhouse

The variance of analysis (ANOVA) revealed significant effects ($p \leq 5\%$) of Fosetyl-Al and Metalaxyl-M on root browning of the peach seedling induced by *Pythium ultimum*. Indeed, these two fungicides reduced significantly the root browning by 62.55 %, compared to the inoculated control. However, the different active ingredients tested did not significantly improve the health status of the plants. Nevertheless, Carbendazim and Fosetyl-Al fungicides reduced the plants health status index by 42.92 % and 57.08 %; respectively, compared to the inoculated control ($p \geq 5$ %) (Table 3). Regarding the seedling growth parameters; the six chemical products did not improve the height and root weight of the peach seedlings inoculated with *Pythium ultimum*. In fact, the seedling height didn't exceed 49.33 cm for the peach seedlings treated with Metalaxyl-M. However, the highest root weight of 4.40 g was recorded by treatment with Chinosol (Table 3 and Fig. 2). On the other hand, in the case of *P. citrophthora*; the Mancozeb and the Fosetyl-Al fungicides reduced the root browning index in the peach seedlings non-significantly ($p \geq 5$ %) by 10.22 and 28.4 9% respectively. Hymexazol was the only chemical product that reduced the health status index non-significantly ($p \geq 5$ %) by 6.18 %. Concerning the plants growth; Fosetyl-Al fungicide improved the root weight by 42.57 %. Meanwhile, the six tested chemical fungicides did not improve the heights of the plants significantly ($p \geq 5$ %) (Table 3).

**Table 2.** Percent of growth inhibition of *Pythium ultimum* and *P. citrophthora* upon treatment with the different doses of the active ingredients of each tested chemical fungicides; recorded 4 d after incubation at 25°C in the dark

| Fungal Pathogens | Active substance | Doses (µg\ l) | | | | Registered dose | *p*-value |
|---|---|---|---|---|---|---|---|
| | | 10 | 25 | 50 | 100 | | |
| ***Pythium ultimum*** | **Carbendazim** | 10.45± 1.93$^{BCa*}$ | 45.76± 2.92$^{Fb}$ | 67.80± 2.35$^{Dc}$ | 70.06± 2.16$^{Dc}$ | 75.30± 1.17$^{Bd}$ | ≤ 0.001 |
| | **Mancozeb** | 0.80± 0.00$^{Aa}$ | 5.65± 0.65$^{Bb}$ | 24.01± 2.67$^{Bc}$ | 43.79± 1.69$^{Cd}$ | 100.00± 0.00$^{De}$ | ≤ 0.001 |
| | **Fosetyl-Al** | 0.00± 0.00$^{Aa}$ | 0.00± 0.00$^{Aa}$ | 0.00± 0.00 | 0.00± 0.00$^{Aa}$ | 51.83± 11.37$^{Ab}$ | ≤ 0.001 |
| | **Hymexazol** | 8.19± 2.32$^{Ba}$ | 13.56± 3.28$^{Cb}$ | 22.03± 1.28$^{Bc}$ | 32.20± 0.77$^{Bd}$ | 90.55± 6.71$^{CDe}$ | ≤ 0.001 |
| | **Chinosol** | 13.84± 6.35$^{Ca}$ | 27.68± 2.06$^{Eb}$ | 90.96± 0.00$^{Ec}$ | 90.96± 0.00$^{Fc}$ | 90.96± 0.00$^{CDc}$ | ≤ 0.001 |
| | **Metalaxyl-M** | 8.66± 0.26$^{Ba}$ | 21.65± 0.66$^{Db}$ | 43.29± 1.32$^{Cc}$ | 79.70± 1.91$^{Ed}$ | 86.59± 2.63$^{Ce}$ | ≤ 0.001 |
| ***p*-value** | | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | |
| ***P. citrophthora*** | **Carbendazim** | 72.42± 0.61$^{Ea}$ | 81.82± 1.71$^{Db}$ | 85.45± 0.99$^{Ec}$ | 84.85± 0.70$^{Dc}$ | 100.00± 0.00$^{Cd}$ | ≤ 0.001 |
| | **Mancozeb** | 1.44± 0.00$^{Aa}$ | 5.15± 2.69$^{Aa}$ | 6.36± 5.54$^{Ba}$ | 29.70± 0.99$^{Bb}$ | 50.00± 2.69$^{Bc}$ | ≤ 0.001 |
| | **Fosetyl-Al** | 1.21± 1.48$^{Aa}$ | 5.45± 2.62$^{Ab}$ | 0.61± 0.76$^{Aa}$ | 2.12± 2.29$^{Aa}$ | 100.00± 0.00$^{Cc}$ | ≤ 0.001 |
| | **Hymexazol** | 30.00± 1.16$^{Ca}$ | 48.48± 0.70$^{Bb}$ | 59.70± 2.86$^{Dc}$ | 70.91± 2.62$^{Cd}$ | 94.49± 2.03$^{Ce}$ | ≤ 0.001 |
| | **Chinosol** | 67.88± 0.70$^{Da}$ | 74.24± 1.16$^{Cb}$ | 90.30± 0.00$^{Fc}$ | 90.30± 0.00$^{Ec}$ | 90.30± 0.00$^{Cc}$ | ≤ 0.001 |
| | **Metalaxyl-M** | 33.27± 0.64$^{Ba}$ | 8.47± 1.63$^{Aa}$ | 16.95± 3.56$^{Cb}$ | 31.47± 6.75$^{Bc}$ | 33.46± 6.85$^{Ac}$ | ≤ 0.001 |
| ***p*-value** | | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | ≤ 0.001 | |

(*) Means ± standard error in the row followed by the same lower case letter are not significantly different according to SNK test at $p \leq 0.05$;

(**) For each pathogen, means ± standard error in a column, followed by the same capital letter are not significantly different according to SNK test at $p \leq 0.05$.

The registered dose was 2000 µg\ l for Mancozeb, Chinosol and Fosetyl-al; 250 µg\ l for Carbendazim; 60 µg\ l for Hymexazol and 120 µg\ l for Metalaxyl-M

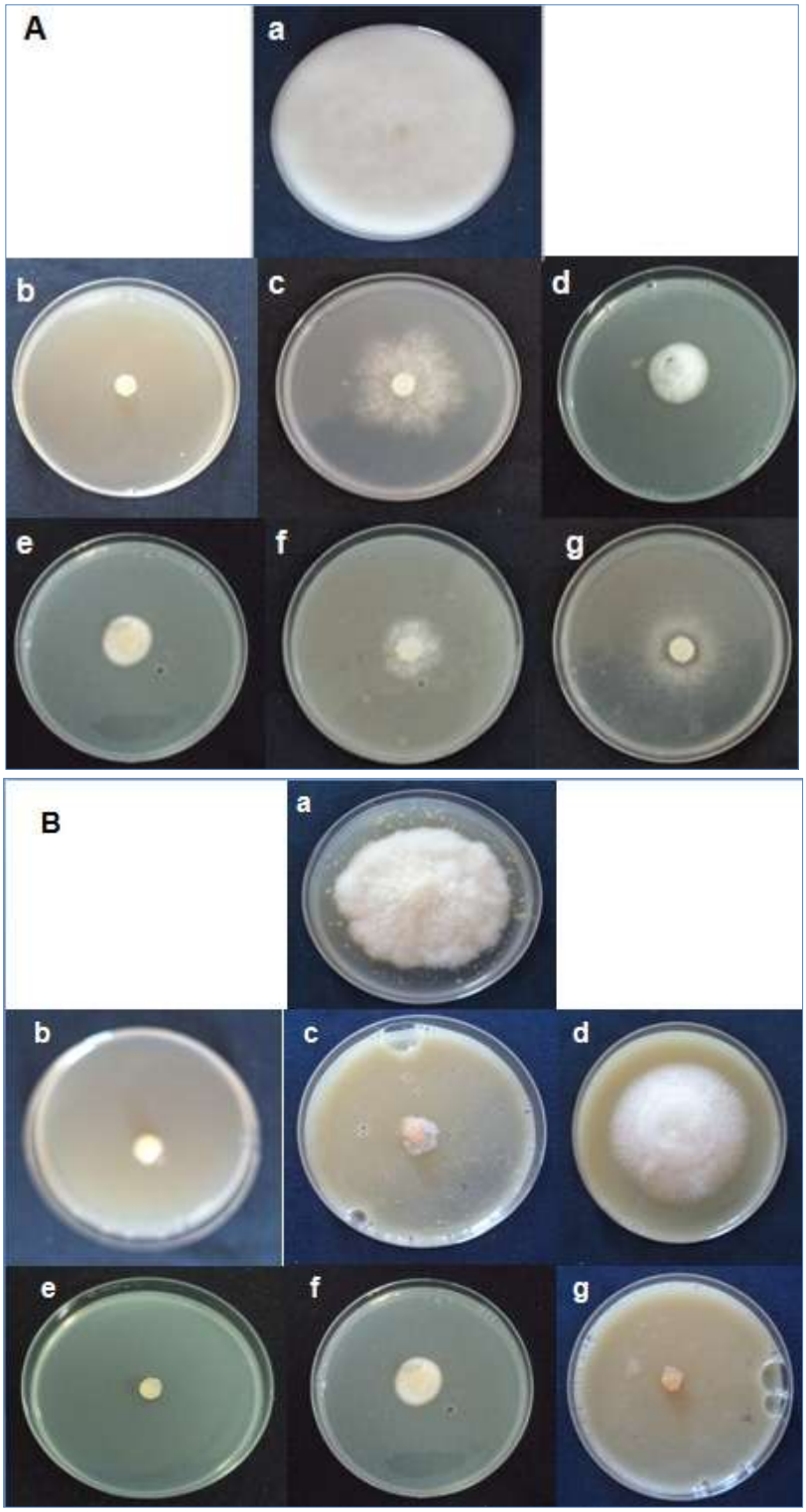


**Fig.1.** Effects of the registered dose of mancozeb (b), fosetyl-Al (c), hymexazol (d), chinozol (e), metalaxyl-M (f) and carbendazim (g) on mycelial growth of *Pythium ultimum* **(A)**, and *P. citrophthora* **(B)**, compared to the control colony (a); recorded after 4 d of incubation at 25 °C

**Table 3.** *In vivo* effects of the tested fungicides on severity of symptoms of the peach decline disease induced by *Pythium ultimum* and *P. citrophthora*; noted 3 months after inoculation and treatment with the fungicides in the greenhouse

| Fungal Pathogens | Treatment | Root browning (0-5) | Sanitary state (0-5) | Height (cm) | Root weight (g) |
|---|---|---|---|---|---|
| ***Pythium ultimum*** | **CNI** | 1.33± 0.58 $^{b*}$ | 1.67± 0.58 $^{bc}$ | 62.40± 2.26 $^{a}$ | 4.79± 0.37 $^{a}$ |
| | **CI** | 2.67± 0.58 $^{a}$ | 2.33± 0.58 $^{abc}$ | 55.00± 10.11 $^{ab}$ | 4.50± 0.55 $^{a}$ |
| | **Carbendazim** | 1.67± 0.58 $^{ab}$ | 1.33± 0.58 $^{bc}$ | 46.23± 6.88 $^{ab}$ | 4.06± 1.80 $^{a}$ |
| | **Mancozeb** | 1.67± 0.58 $^{ab}$ | 2.33± 0.58 $^{abc}$ | 38.10± 9.38 $^{b}$ | 3.17± 1.36 $^{a}$ |
| | **Fosetyl-AL** | 1.00± 0.00 $^{b}$ | 1.00± 0.00 $^{c}$ | 47.50± 5.77 $^{ab}$ | 3.58± 0.61 $^{a}$ |
| | **Metalaxyl-M** | 1.00± 0.00 $^{b}$ | 2.67± 1.15 $^{abc}$ | 49.33± 5.13 $^{ab}$ | 4.29± 0.75 $^{a}$ |
| | **Hymexazol** | 2.33± 0.58 $^{ab}$ | 3.00± 1.00 $^{ab}$ | 45.73± 4.11 $^{ab}$ | 3.60± 0.94 $^{a}$ |
| | **Chinosol** | 2.33± 0.58 $^{ab}$ | 3.67± 0.58 $^{a}$ | 47.67± 2.52 $^{ab}$ | 4.40± 0.80 $^{a}$ |
| ***p*-value** | | $p \leq 0.05$ | $p \leq 0.05$ | $p \leq 0.05$ | $p \geq 0.05$ |
| ***P. citrophthora*** | **CNI** | 1.33± 0.58 $^{b}$ | 1.67± 0.58 $^{a}$ | 62.40± 2.26 $^{a}$ | 4.79± 0.37 $^{a}$ |
| | **CI** | 1.86 ± 1.00$^{ab}$ | 1.78± 0.58 $^{a}$ | 51.80± 14.63 $^{ab}$ | 3.50± 1.04 $^{a}$ |
| | **Carbendazim** | 2.67 ± 0.58$^{ab}$ | 3.67± 0.58 $^{a}$ | 41.67± 10.02 $^{ab}$ | 3.02± 1.34 $^{a}$ |
| | **Mancozeb** | 1.67± 0.58 $^{b}$ | 2.00± 1.00 $^{a}$ | 37.00± 16.04 $^{ab}$ | 3.21± 2.11 $^{a}$ |
| | **Fosetyl-AL** | 1.33± 0.58 $^{b}$ | 2.00± 1.73 $^{a}$ | 45.27± 10.65 $^{ab}$ | 4.99± 1.24 $^{a}$ |
| | **Metalaxyl-M** | 3.67± 1.15 $^{a}$ | 3.33± 1.53 $^{a}$ | 31.53± 14.76 $^{b}$ | 1.78± 1.43 $^{a}$ |
| | **Hymexazol** | 2.33± 0.58 $^{ab}$ | 1.67± 0.58 $^{a}$ | 44.50± 3.50 $^{ab}$ | 2.93± 0.33 $^{a}$ |
| | **Chinosol** | 2.44± 0.58 $^{ab}$ | 2.33± 0.58 $^{a}$ | 40.43± 1.53 $^{ab}$ | 3.24± 0.22 $^{a}$ |
| ***p*-value** | | $p \leq 0.05$ | $p \geq 0.05$ | $p \geq 0.05$ | $p \geq 0.05$ |

(*) For each pathogen, the means ± standard deviation, in the same column followed by the same lower case letter are significantly comparable according to the SNK test at $p \leq 0.05$

Where; CNI: Un-inoculated control, CI: Inoculated positive control

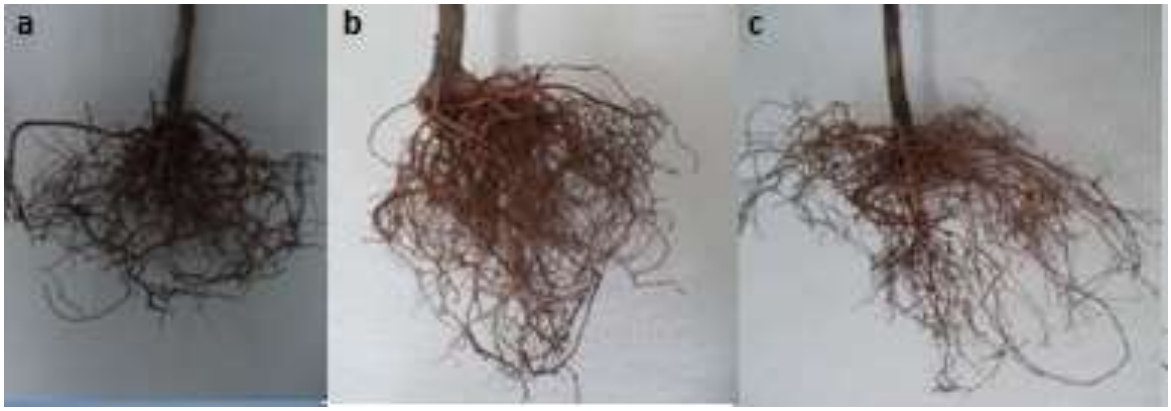


**Fig. 2.** *In vivo* impacts of Fosetyl-AL **(b)** and Metalaxyl-M **(c)** on root browning of peach root stock 'Garnem' after 3 months of inoculation with *Pythium ultimum* and treatment with both fungicides, compared to the non-treated control **(a)**

## 4. Discussion

The present investigation demonstrated that using chemical fungicides to control *Pythium ultimum* and *P. citrophthora*; the pathogenic agents associated with peach seedlings decline, revealed significant differences in efficacies between the active ingredients and the used fungicides doses.

The *in vitro* assay showed that Carbendazim was effective against *Pythium ultimum* at 50 µg\ l. It was also effective at the lowest tested dose of 10 µg\ l against *P. citrophthora*. Similarly, previous studies reported that this fungicide was very effective in causing *in vitro* inhibition of mycelial growth of *Fusarium* spp. and *Rhizoctonia solani* (Gaur and Chakrabarti, 2009; Iqbal *et al*., 2010; Kumar *et al*., 2017; Mannai *et al*., 2018b; Sameer, 2019).

The test of *in vivo* efficacy of Carbendazim showed that this product was ineffective; as it reduced only the health status index of the peach plants inoculated by *P. ultimum* by 42.92 %. This chemical is a broad-spectrum systemic benzimidazole fungicide, which was commonly used previously to control leaf and soil borne diseases caused by fungi (Yu *et al*., 2009). Carbendazim was recorded to be significantly effective in reducing the soil population of *F. oxysporum* f. sp. *dianthi*; the causal agent of wilt in Carnation, and reduced the wilt incidence under greenhouse conditions (Manasa *et al*., 2017), suppressed cashew wilt disease caused by *F. oxysporum*, and enhanced cashew recovery after 120 d of application (Mbasa *et al*., 2021). Furthermore, this fungicide also controlled *F. oxysporum* in chickpea (Golakiya *et al*., 2018); cotton (Rajput *et al*., 2006), Cumin (Khalequzzaman *et al*., 2016), *F. solani* infection in coriander (Bhaliya and Jadeja, 2014) and *F. graminearum* infection in wheat (Ivic *et al*., 2011). However, the oomycete pathogens such as *P. citrophthora* were insensitive to benzimidazole, because they have different binding sites known as tubulin compared to the true fungi (Johnson *et al*., 1997). Similarly, previous field experiments conducted to evaluate the efficacy of fungicides on management of late blight caused by *P. infestans* and on potato yields in the western plains of Nepal; showed the ineffectiveness of carbendazim to control this disease (Khadka *et al*., 2020).

All the tested doses of Mancozeb have no inhibitory effects except for the 2000 µg\ l, which has been shown to be effective *in vitro* against *Pythium ultimum* and *P. citrophthora*. Mancozeb was previously recorded amongst the best tested fungicides causing *in vitro* inhibition of the growth of *Fusarium* spp. (Ahmad *et al*., 2012; Mannai *et al*., 2018b).

The greenhouse assay demonstrated that Mancozeb reduced the root browning index induced by *P. citrophthora* in the peach seedlings by 10.22 %. This result is in agreement with a recent study conducted by Khadka *et al*., (2020) showing that Mancozeb effectively reduced the late blight (*P. infestans*) severity, and increased the potato yield as well. Nonetheless, there were no previous studies about its efficacy against the Pythiaceae species associated with peach seedlings decline.

The different tested doses of Fosetyl-Al presented low *in vitro* inhibitory efficacy except for the registered dose of 2000 µg\ l. In accordance, similar results were recorded during the previous study conducted by Utkhede and Smith, (1991), which showed that Fosetyl-Al was effective against *P. cactorum*, *P. cambivora* and *Pythium ultimum* at high concentrations. Furthermore, a recent study of Tawil *et al*., (2020) proved that Fosetyl-Al inhibited the *in vitro* mycelial growth of *Verticillium dahlia*; the causal agent of olive tree wilt disease by 100 % and 81.79 % at the tested concentrations of 5000, 2000 µg\ l, respectively. Moreover, previous work of Weiland *et al*., (2014) revealed that several species

of *Pythium* were sensitive to this chemical fungicide; including *Pythium ultimum*.

Results of *in vivo* testing's of Fosetyl-Al demonstrated that it caused significant inhibitory effects on the peach seedling root browning (62.55 %), and plants health status (57.08 %) induced by *Pythium ultimum*. Moreover, this fungicide improved the root weight of the peach seedlings inoculated with *P. citrophthora* by 42.57 %. Fosetyl-Al is a phosphonate fungicide that had been widely used in the management of diseases caused by Peronosporales; including some *Phytophthora* diseases of trees (Silva *et al*., 2016). In fact, Fosetyl-Al as a phosphonate fungicide; was experimentally proved to protect cork oaks against root rot caused by *P. cinnamomi* and *Pythium spiculum* in the pots and in the field experiments, and this fungicide also exhibited a therapeutic effect on the preexisting infections (González *et al*., 2017; González *et al*., 2020).

For Hymexazol, all the used doses were ineffective against *Pythium ultimum*; however, the registered dose of 60 µg\ l was effective against *Pythium ultimum* and *P. citrophthora*. Previous studies conducted by Ayed *et al*., (2006); Hibar *et al*., (2007) have shown the efficacy of Hymexazol in reducing the *in vitro* mycelial development of *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *tuberosis*; the causal agents of wilt in tomato and potato plants, respectively.

The *in vivo* assay revealed that Hymexazol fungicide reduced the health status index induced by *P. citrophthora* by 6.18 %. In accordance, previous studies conducted by Ayed *et al*., (2006); Hibar *et al*., (2007) have shown the efficacy of Hymexazol in reducing the *in vivo* wilt disease in tomato and potato plants induced by *F. oxysporum* f. sp. *radicis-lycopersici* and *F. oxysporum* f. sp. *tuberosis*, respectively. Moreover, Daami-Remadi, (2001) also reported the effectiveness of Hymexazol in inhibiting potato leak syndrome caused by *Pythium aphanidermatum*. However, several studies indicated the development of resistance to Hymexazol among several *Fusarium* spp. and *Pythium* spp.; including *Pythium ultimum* and *Pythium aphanidermatum*, because this chemical product is widely used in several countries (Ali-Shtayeh *et al*., 2003; Al-Sadi *et al*., 2015; Al-Balushi *et al*., 2018).

Chinosol was effective against *P. citrophthora* at all the tested doses. However, it was ineffective at low doses of 10 and 25 µg\ l against *Pythium ultimum*. The registered dose of Chinosol reduced the *in vitro* mycelial growth of *P. citrophthora* and *Pythium ultimum*. These current results confirm the earlier results reported by Vaartaja, (1964), who proved the capacity of this fungicide to manage the different oomycetes (*Pythium* spp.). Besides, a recent study conducted by Mannai *et al*., (2018b) revealed that Chinosol was effective at different doses against *F. oxysporum* and *F. solani*; recording growth inhibition of 88 % at 50 µg\ l, respectively.

The *in vivo* assay showed that Chinosol application neither reduced the disease severity, nor improved the peach plants growth. However, this fungicide was effective against *Pythium* spp. and several fungal spp. including; *R. solani*, *F. avenaceum*; on its application to the soil of different crops such as; turf, pines and some garden plants. In addition, Chinosol improved the peach seedling growth inoculated by *F. solani* (Mannai *et al*., 2018b).

Metalaxyl-M induced reduction in the mycelial growth of *Pythium ultimum*. However, the 5 tested doses of this chemical were ineffective against *P. citrophthora*. Previous works have shown that several species of *Pythium* were sensitive to this fungicide; including *Pythium ultimum* (Brantner and Windels, 1998; Mazzola *et al*., 2002).

The *in vivo* assay recorded the efficacy of Metalaxyl-M in reducing the root browning induced

by *Pythium ultimum*. Similarly, Thomidis and Tsipouridis, (2001) reported that application of Metalaxyl-M as a soil treatment provided effective control of the stem cankers in 2-year-old peach trees. They deduced also that Metalaxyl-M was effective against *P. cactorum* and *P. citrophthora* infecting the peach plant. In addition, Thomidis and Elena, (2001) reported that application of Metalaxyl-M through staining the trunk or treating the soil; was effective in controlling root rot in peach plants induced by *P. cactorum*. Furthermore, Mazzola *et al*., (2002) found that *Pythium* species associated with apple trees were sensitive to Metalaxyl-M. Several previous studies carried out on apple trees demonstrated that Metalaxyl-M was effective in causing *in vitro* and *in vivo* control of *P. cactorum* associated with the apple trees (Ellis *et al*., 1982; Boughalleb *et al*., 2006a).

A recent study carried out by Kongtragoul *et al*., (2021) in Thailand tested several isolates of *P. palmivora* for *in vitro* mycelium-growth sensitivity to Metalaxyl-M, and revealed that some isolates of this pathogen were resistant to Metalaxyl-M with 50 % effective concentration.

## Conclusion

The present study demonstrated the efficacies of the Fosetyl-Al and Metalaxyl-M fungicides on reducing disease severity of the peach seedlings decline induced by *Pythium ultimum*. However, all the tested chemical products were inefficient *in vivo* against *P. citrophthora*. Thus, in the future; it is necessary to test the potentials of these fungicides to control diseases incited by Pythiaceae *and Fusarium* spp., because the peach seedlings decline disease is associated with complex of pathogens.

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## Conflict of interests

The authors declare that there is no conflict of interests related to this article.

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## Ethical approval

Non-applicable.

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