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## Effect of Four Fungicides on Development and Control of *Phytophthora* on Apple Tree *in vitro* and *in vivo*\*

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**Abstract:** Four fungicides were evaluated for their effectiveness against *Phytophthora cactorum* affecting apple trees and their ability to be absorbed and translocated by roots of apple tree. Ridomil gold MZ68 (metalaxyl+mancozeb) and curvax M (cymoxanil+mancozeb) suppressed the *in vitro* development of this fungus at high doses (100 and 1000  $\mu\text{g m}^{-1}$ ). Mikal flash (fosetyl-Al+folpet) and melody duo (iprovalicarb+propineb) reduced mycelium growth of *P. cactorum* when used at all tested doses. *In vivo*, melody duo was the less effective. The *in vivo* treatment of plant was more significant when the plant is not severely infested.

**Key words:** Apple trees, *Phytophthora cactorum*, fungicides, *in vitro*, *in vivo*

### Introduction

*Phytophthora* is one of the most damaging soilborne diseases-causing organism affecting the crown and roots of the fruit trees. The best way to protect apple orchards against *Phytophthora* crown rot is the resistant rootstocks. Disease incidence is especially high in orchards subjected to flood-irrigation and also where susceptible rootstocks have been used (Wilcox and Mircetich, 1985; Harris and Tobutt, 1986; Wilcox and Ellis, 1989; Jacobs and Johnson, 1996).

Rootstocks that are resistant to the pathogen have been developed and cultivated without, however, being completely resistant (Chitzanidis and Stylianides, 1987) and therefore the use of fungicides for the control of *Phytophthora* crown and root rot is essential. However, no apple rootstocks have been found immune to this disease (Chitzanidis and Stylianides, 1987). Therefore, chemical strategies must be applied to protect apple trees against *Phytophthora* sp.

Control of *Phytophthora* diseases is based mainly on the use of chemicals. Protectant fungicides kill germinating spores but have no effect on mycelium after it has penetrated the plant. The introduction of new systemic compounds has facilitated the control of *Phytophthora* sp. Fosetyl-Al and metalaxyl possess excellent systemic activity against several diseases caused by *Phytophthora* sp. Both can inhibit mycelial growth and sporulation of the fungus (Matheron and Matejka, 1991; Ferrin and Wadsworth, 1992; Jeffers, 1992). Additionally, reports suggest that dimethomorph and cymoxanil have high efficacy against diseases caused by several *Phytophthora* sp. (Erwin and Ribeiro, 1996). A variety of techniques has been developed to evaluate the effectiveness of fungicides against *Phytophthora* sp. The use of poisoned agar is the most common (Thomidis and Michailides, 2002). Timmer (1977) used bark disks from the trunk to evaluate the fungicide activity on treated trees. Matheron and Matejka (1988) reported that bark strip assay is a reliable and rapid technique for testing efficacy of fungicides for control *Phytophthora gummosis* of citrus.

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Most of these laboratory based methods evaluate only the protective functions of fungicides and not systemic activity. Evaluating systemic activity in the field is expensive especially for orchards crops, but could deal to an idea on the effect of fungicides in environmental conditions.

The purpose of this study was to determine the activity of four fungicides against diseases of apple trees in Tunisia caused by *Phytophthora cactorum*.

## **Materials and Methods**

### *Sampling and Isolation of Phytophthora sp.*

Samples were collected from root and crown of infected trees localized in Foussana and Oued Eddarb areas of Kasserine government in Tunisia in 2005. To isolate the pathogen(s) from the plant tissues, small pieces from the margin of the discolored area, including a healthy part of the tissue, were placed on malt medium amended with antibiotic. Isolates were maintained on Malt at 22°C in the culture collection of the Phytopathological laboratory of ESHE-CM (Tunisia). Fresh cultures were prepared by transferring an agar disc bearing actively growing mycelium of *Phytophthora* to plates containing fresh malt.

### *Identification of Phytophthora sp.*

*Phytophthora* sp. is an Oomycete, belonging to the order of Pythiales, family of Pythiaceae, characterized by a siphoned spawn. *Phytophthora cactorum* is characterized by its compact colonies without clean margin, that push a radial way and that are downy and present short aerial hyphes. The sexual reproduction of *Phytophthora* depends on species, either in the cloths of diseased plants (*P. cactorum*, *P. cambivora*) either only in the cultures (*P. infestans*). For *Phytophthora cactorum*, the antheridia applies against the oogona and send to its inside a tube copulate with a reproduction homothallic. Morphologically, the antheridia are spherical to globular. The oogona is uncolored and presents a smooth partition. The oospores germinate producing a sporangia or mycelium. The sporanges presents a characteristic papilla. They are terminal and rarely inset. They are deciduous and carry a short pedicel.

### *Laboratory Experiments*

#### *Fungicides Tested*

Four fungicides with different active matter were used in these tests:

Ridomil gold MZ68 (wp): 4% Metalaxyl and 64% Mancozeb

Melody Duo: 5.5% Iprovalicarb and 61.3% Propineb.

Curvax M: 4% Cymoxanil and 46.5% Mancozeb

Mikal flash: 50% Fosetyl-Al and 25% Folpet.

For each fungicide, four doses were applied D1 (2 µg mL<sup>-1</sup>), D2 (10 µg mL<sup>-1</sup>), D3 (100 µg mL<sup>-1</sup>) and D4 (1000 µg mL<sup>-1</sup>).

#### *In vitro Evaluation of Fungicides Against Phytophthora Cactorum Isolates*

The measure of the hyphal growth was noted daily until the mycelium covers the Petri dish for the controls.

#### *Fields Experiments*

The objective of these assays is to control the effect of fungicide, to specify the modes and the moments of treatment applications; following the physiological stage during which there will be absorption of the active matter and the sanitary state of the plant for which the protection is optimal. The same fungicides used *in vitro* were applied *in vivo* treatment. Two fields 1 and 2 at Foussana area where apple trees were highly frequent were chosen for these tests conducted in 2005.

#### *Treatment Methods*

Field 1: The orchard has been put in place in 1985 and contained 36 lines with 150 m of length (13 columns of 200 m of length). It is very heterogeneous with many varieties of apple (Golden Delicious, Richared, Red Delicious).

The treatments were applied on the first three columns with 3 replicates by fungicide and dose. They are applied by irrigation at the basis of every foot tree at the rate of 10 L of mush/m<sub>2</sub>/foot tree. We compared the following treatments:

Ridomil MZ68 Gold: 50 g tree<sup>-1</sup>

Melody Duo: 25 g tree<sup>-1</sup>

Curvax M: 25 g tree<sup>-1</sup>

Mikal flash: 50 g tree<sup>-1</sup>

Field 2: This test has been managed on 12 young plants newly planted in naturally infested soil in the objective to evaluate the effect of fungicides in preventive control. The same fungicides have been used.

#### *Evolution of Disease in the Time*

For this goal, we surveyed the field and we noted the external aspect of the tree according to the following key:

4 = Healthy plant having an optimal vigour indication

3 = Plant in beginning of attack and that shows the decay of a part of branches or the necrosis and the canker to the level of the collar

2 = Diseased tree that shows the fading of a complete branche or more

1 = Tree completely decayed

0 = A: plant pulled after its decay (R in case of the replantation)

We did three surveys, the 1st in August 21, 2004, at the end of the summery period and the beginning of the autumn, the second in April 7 and the latest in May 26, 2005.

#### *Evolution of Disease in the Space*

This survey consists in analyzing the results of the surveys as follows:

- The number of healthy trees.
- The number of trees in the beginning of attack
- The number of diseased trees.
- The number of decayed trees.

The survey of all the categories during a date and the comparison to the same plantations during another date could show the spatial evolution of the disease.

#### *Data Analyses*

The experiments were established using a randomized design. All *in vitro* experiments were conducted twice. To combine experiments, the Bartlett's test of homogeneity of variance was used. Data were analyzed by one-way Analyses of Variance (ANOVA) by SPSS Software program (SPSS Inc. Headquarters, Chicago, Illinois). Means values and standard errors were also calculated to compare the average length of cankers obtained with each isolates of *Phytophthora* sp.

**Results**

*In vitro* Evaluation of Fungicides Against *Phytophthora Cactorum* Isolates

The diameter of hyphal growth depends on the treatment and the length of incubation's duration (Fig. 1-4). After 24 h, ridomil gold MZ68 (metalaxyl+mancozeb) revealed to be the less effective at the dose of 2  $\mu\text{g mL}^{-1}$ , compared to the control. At the dose of 10  $\mu\text{g mL}^{-1}$ , ridomil gold MZ68 showed the diameter of mycelia growth (10.6 mm). With the doses of 100 and 1000  $\mu\text{g mL}^{-1}$ , ridomil gold MZ68 (metalaxyl+mancozeb) and curvax M (cymoxanil+mancozeb) inhibited completely the hyphal growth development of *Phytophthora cactorum* after 24 h of incubation. Whereas, mikal flash (fosetyl-Al+folpet) and melody duo (iprovalicarb+propineb) demonstrated a diameter of colonies of 6.7 and 7.5 mm, respectively (Fig. 1).

After 48 and 72 h of incubation, mycelium growth of *Phytophthora cactorum* was clear even when treated with mikal flash and melody duo at the doses of 1000  $\mu\text{g mL}^{-1}$ . In fact, after 24 h the total inhibition was instatement and then the fungus grows slowly with a diameter of 13.4 and 10.5 mm, respectively (Fig. 2 and 3).

The effectiveness of fungicide revealed to be not affected by the incubation's duration at low doses of 2 and 10  $\mu\text{g mL}^{-1}$ , as showed by the same effect on growth mycelia of *Phytophthora cactorum* after 24, 48, 72 and 96 h (Fig. 1-4).

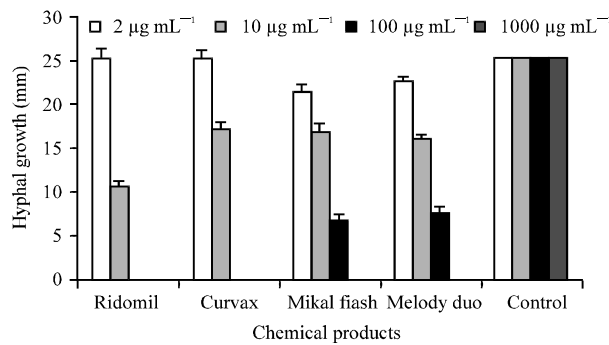


Fig. 1: Effect of four chemical products incorporated in malt medium on the hyphal growth of *Phytophthora cactorum in vitro* after 24 h of incubation at 22°C compared to the control

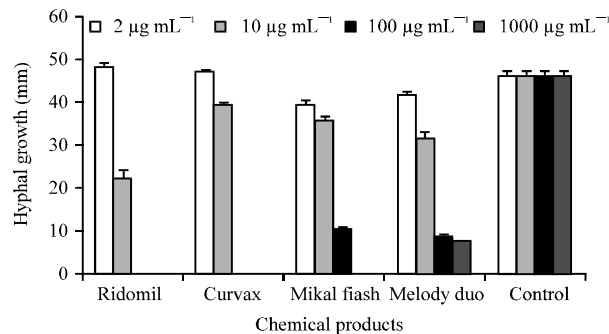


Fig. 2: Effect of four chemical products incorporated in malt medium on the hyphal growth of *Phytophthora cactorum in vitro* after 48 h of incubation at 22°C compared to the control

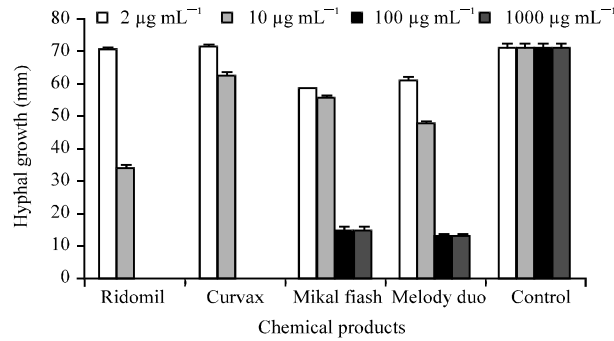


Fig. 3: Effect of four chemical products incorporated in malt medium on the hyphal growth of *Phytophthora cactorum* in vitro after 72 h of incubation at 22°C compared to the control

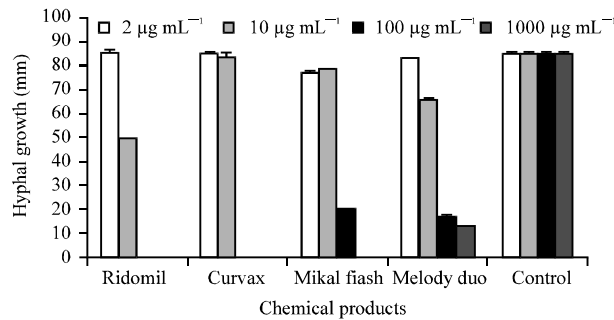


Fig. 4: Effect of four chemical products incorporated in malt medium on the hyphal growth of *Phytophthora cactorum* in vitro after 96 h of incubation at 22°C compared to the control

### Fields Experiments

#### Evaluation of Efficiency of Some Fungicides in vivo

Results showed no significant difference between the blocks, but a highly significant difference between the fungicides and demonstrated the influence of the initial sanitary state.

The comparison of fungicides showed that melody duo is the less effective among all the tested fungicides ridomil gold MZ68, curvax M, mikal flash is significant efficient.

The comparison of the combinations fungicides×initial phytosanitary state of plant permits to conclude that the treatment of a plant in advanced stage of attack is not effective.

#### Evolution of Disease in Space and Time

Results of the surveys of the evolution of disease in space and time are presented in Fig. 5 and 6. It revealed that after 9 months, field 2 evolve into an epidemic state, while field 1 evolves in a progressive way, but less intense than field 2. This difference could be due to the initial level of infestation determined as the percentage of plants completely infested, those at the beginning of attack, or the trees pulled and the infested soil could be a source of contamination. The level of infestation is 72% for field 2 and 43% for field 1 in 21/08/2005.

After 7 months, these levels increase toward rates of 94% for field 2 and 43% for field 1. It is necessary to signal that during this period (autumnal and wintry), the healthy plant evolution toward one some other categories are faster than the temporal other category evolution).

During the period between the two latest surveys (March 20 and May 24), these rates increase slightly to reach 46 and 96% for field 1 and 2, respectively. Furthermore, the number of decayed plant

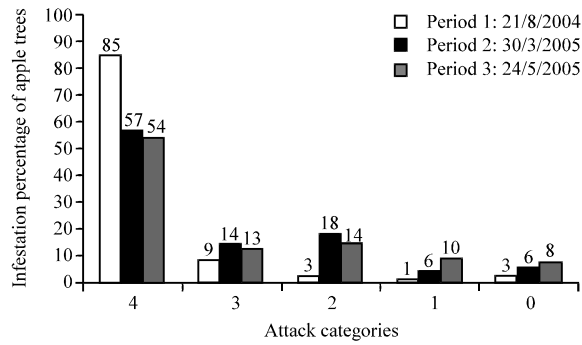


Fig. 5: Evolution in space of phytosanitary state of field 1 at different dates of surveys

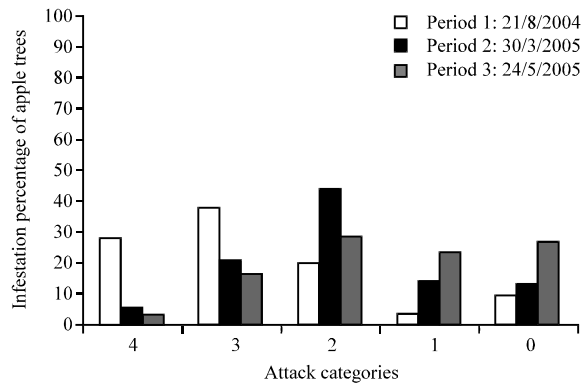


Fig. 6: Evolution in space of phytosanitary state of field 2 at different dates of surveys

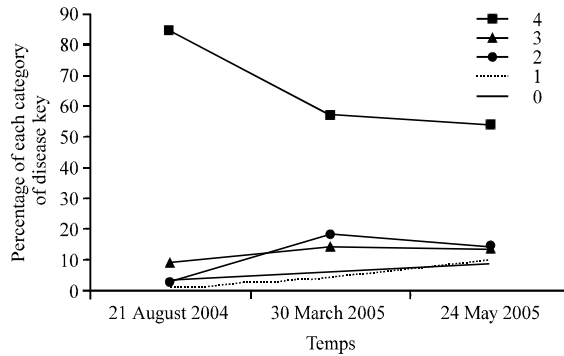


Fig. 7: Percentage of diseased trees according to each category of disease key evaluation in field 1 and period of survey

increases for the two fields. For example, this number is three times more during this period (2 months) compared the first period (7 months). We conclude that during vegetative growth stage of plant development, the disease revealed to be more epidemic (Fig. 7).

According to this evolution of disease in space and time, it revealed the presence of two phases in the epidemic process of this problem, a spatial evolution phase during the autumnal period and an internal evolution phase in period of vegetative growth.

## Discussion

Present results showed that *Phytophthora* sp. affecting apple trees could be controlled by chemical products *in vitro* as *in vivo* even at lower doses. These findings reported that ridomil gold MZ68 (metalaxyl+mancozeb) applied at dose of  $10 \mu\text{g mL}^{-1}$ , could be effective in reducing *Phytophthora* hyphal growth. Higher doses of ridomil gold MZ68 and curvax M (cymoxanil+mancozeb) ( $100$  and  $1000 \mu\text{g mL}^{-1}$ ) were able to suppress mycelium development of *Phytophthora cactorum* and could be used in preventing infection with this fungus. These results agree with those of Thomidis (2001) who demonstrated that growth of *Phytophthora cactorum* and *P. citrophthora* was inhibited *in vitro* by metalaxyl at rates of 10 ppm. In this research, Mikal flash (fosetyl-Al+folpet) and melody duo (iprovalicarb+propineb) were more effective when applied at dose of  $100 \mu\text{g mL}^{-1}$  in curative treatment but could be used in preventive control with higher rate also ( $1000 \mu\text{g mL}^{-1}$ ) and confirmed those of Thomidis and Michailidis (2002).

*In vivo* treatment, mikal flash (fosetyl-Al+folpet), ridomil gold MZ68 (metalaxyl+mancozeb) and curvax M (cymoxanil+mancozeb) were effective. Others reports confirmed our results and mentioned that metalaxyl and fosetyl-Al have the ability to be absorbed and translocated in plant tissues (Matheron and Matejka, 1991; Thomidis and Tsipouridis, 2001). Furthermore, El-Hamalawi *et al.* (1995) reported that fosetyl-Al is unique among fungicides in that it is translocated in both xylem and phloem. Thus as a practical result we could suggest that the fungi with active matter metalaxyl and fosetyl-Al would be used in treatment of trees to control diseases caused by *Phytophthora* sp.

Disease evolution in space and indicate that the colonization of the fungus in plant tissues could lead to the aggressiveness of symptoms during all year with a critical phase during the vegetative growth and a light regression in autumn period. Browne and Mircetich (1996) showed that the extension of epidemic period depends on the desirability of the varieties and the rootstocks and also on technical measures and chemical treatment that create that offer a continuous protection of apple orchards.

These results should be re conducted for others years in different climatic conditions to verify the interaction between trees-fungi-environmental conditions.

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