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Laboratory and field efficacy of certain fungicides against gummosis of citrus disease in Tunisia

Najwa Benfradj, Naima Boughalleb-M'Hamdi*

Institut Supérieur Agronomique de Chott Mariem, Département des Sciences Biologiques et de la Protection des Plantes, 4042 Sousse, Tunisie.

Abstract

Gummosis, caused by *Oomycota*, is a devastating disease for citrus production in Tunisia. Due to the limited strategies available for this disease management, the laboratory and field applications of some commercially fungicides were conducted.Using the mycelia growrh ratios, from the tested fungicides, metalaxyl, cymoxanil, propamocarb, and mancozeb are the most efficient fungicides ,with 94.75%, 94.62%, 91.13%, and 90.75% in reducing the mycelial growth of *Oomycota* pathogens, respectively. While azoxystrobin and fosetyl-Al have generated a moderate growth reduction, with 81.25% and 74.25%, respectively. The lowest fungicides effect was obtained with trifloxystrobin, thiophanate-methyl and chlorothalonil, with 38.87%, 36.25%, and 15.25%. In field experiments, metalaxyl and fosetyl-Al were applied as paint trunk and as foliar spray, against infection by *Phytophthora nicotianae*. The results showed that the metalaxyl was more efficiency in reducing the length lesion caused by *P. nicotianae*, than the fosetyl-Al. Furthermore, paint treatments revealed to be more effective in reducing lesion expansion than foliar spray treatments both in curative and in residual activity. Moreover, citrus clementine variety is the most susceptible to *P. nicotianae* infection, while the most tolerant is tangerine variety.

Keywords: Gummosis, *in-vitro*, *in-planta*, systemic, contact fungicides.

* **Corresponding author:** Naima Boughalleb-M'Hamdi,
E-mail: n.boughalleb@laposte.net

Introduction

Citrus gummosis, caused by *Oomycota* is one of the main diseases affecting the citrus production in Tunisia. These soil borne pathogens are responsible of the mortality of newly planted trees, a slow decline and yield loss of mature trees (Graham & Menge, 1999). Many *Phytophthora* species have been reported to cause citrus gummosis disease (Ricci et al., 1990). However, the most widespread and important species are *Phytophthora nicotianae* and *Phytophthora citrophthora* (Graham & Timmer, 1992). In Tunisia, *P. nicotianae* is the major specie that has been found inciting this disease in the citrus-growing areas (unpublished data). Generally, the management of *Phytophthora* diseases includes the cultural practices such as the utilization of adequate soil drainage, the proper irrigation schedules, planting on ridges (Graham & Menge, 1999) and the use of resistant rootstocks (Erwin and Ribeiro, 1996). In spite of that, the application of fungicides remains an indispensable component for an integrated pest management (Thomidis & Elena, 2001). The losses attributable to *Phytophthora* gummosis have been reduced through the use of the systemic fungicides (Sandler et al., 1989). The traditional systemic fungicides used to control several diseases caused by *Oomyceta* and especially to control citrus gummosis are metalaxyl and fosetyl-Al (El-Hamalawi et al., 1995). Methods of these fungicides application include foliar spray, trunk injection with fosetyl-Al (Timmer & Castle, 1985; Darvas et al., 1984). Metalaxyl are characterized by his ability to inhibit rRNA synthesis by *Oomyceta*, there by inhibiting growth of hyphae (Davidse, 1995), while fosetyl-Al has little direct activity against mycelial growth of Oomycetes *in vitro* and may act indirectly by activating host defense mechanisms against pathogens (Khan, 1986; Guest, 1984; Bompeix et al., 1980). Chemical control success is affected by fungicide persistence over time, behavior on different plant hosts and in sensitivity among different genotypes of the pathogen (Garbelotto et al., 2009). *Phytophthora* species are known to develop resistance to metalaxyl after its repeated use (Gisi et al., 1997). Thind et al. (2009) reported the resistance of *P. parasitica* isolates to metalaxyl. These isolates are collected from Ferozepur district in Punjab where metalaxyl was applied for the past over 15 years and was not giving adequate control to gummosis since 4-5 years. This variation in sensitivity to chemical compounds among *Phytophthora* isolates belonging to the same species has also been reported by some authors (McCarren et al., 2009; Wilkinson et al., 2001). Recently, others fungicides such as the cymoxanil (Erwin & Ribeiro, 1996), the azoxystrobin (WeiCai et al., 2010), the chlorothalonil (Vawdrey et al., 2015) and the propamocarb (PMRA Health Canada, 2013) have been developed as effective for the control of plant pathogens in the order Peronosporales. Also, a variety of techniques has been developed to evaluate the effectiveness of fungicides against *Phytophthora* spp. (Thomidis, 2003). Fungicide application methods include foliar spray, trunk injection, trunk painting and soil drenching (El-Hamalawi et al., 1995). However, despite the seriousness of citrus gummosis in Tunisia, the fosetyl-Al is the only fungicides registered to control this disease (AVFA, 2015). No research work has been conducted before to evaluate the efficacy of chemicals fungicides for management of gummosis of citrus in Tunisia. The aim of this study was to: (i) evaluate the lab efficiency of some fungicides in reducing the radial growth

of *Oomycota*; (ii) investigate the effectiveness of metalaxyl and fosetyl-Al applied as trunk paint and foliar sprays against *P. nicotianae* infection of citrus trees.

Materials and methods

Oomycota isolates: Cultures of 7 *Oomycota* species have been used; two isolates of *Phytophthora (Phytophthora nicotianae* and *P. cryptogea)*, 3 isolates of *Pythium* (*Pythium dissotocum*, *P. aphanidermatum* and *P. ultimum*) and 2 isolates of *Phytopythium* (*Phytopythium vexans* and *P. mercuriale*). The isolates have been demonstrated in previous studies to be pathogenic to citrus trees. These species were originally isolated from naturally infected citrus orchards by gummosis in Tunisia and deposited in the GenBank as mentioned in table 1. For long-term storage, a 5- mm-diameter agar plug from the edge of each isolate was placed into a 25-ml tube with ~15 ml of sterile soil solution. Cultures were, then, maintained in the collection of the laboratory of Phytopathology, Department of Biological Science and Plant Protection, I.S.A of Chott Mariem, Tunisia. Fresh cultures were prepared by transferring agar disks with mycelium of each isolate to PDA medium (potato-dextrose-agar). The plates were then transferred to an incubator at 25±1°C, for 5 days, until the mycelium covered the agar surface.

Fungicides used and Effect on hyphal growth: Seven systemic and two contacts fungicides has been used in this assay. Fungicide concentrations were used as manufacturer guides (Table 2). The fungicides efficiency against *Oomycota* species was evaluated using the food poisoned technique as described by Nene and Thapliyal (1979). Stock solutions, of each fungicide, were dissolved in ethanol at the appropriate concentrations. To reflect the activity of fungicides, the prepared stock solutions were stored at 4°C in the dark. Fungicides were added to PDA medium after autoclaving when the medium had cooled to 55°C. Mycelial plugs, of 5-mm-diameter, excised from the edge of the actively growing colony of each pathogen, were transferred to the center of 9 cm petri plate containing ~20 ml of PDA medium amended with the tested fungicide. Plates of PDA medium without fungicide were included for each isolate also has been used as control. The plates were closed with parafilm, placed in a plastic container and incubated in the dark at 25±1°C.Four replicates per treatment of each fungicide and each isolate were used, and the test was repeated twice. The diameter of hyphal growth was measured when colonies in the absence of fungicide (control) had reached the edge of the plate. Colony diameters of each specie tested was measured, averaged and the percent of growth inhibition over control was determined. Percent growth inhibition for each isolate/fungicide was obtained by dividing colony diameter in the treated plates by that in the control plates. The percent of growth inhibition (PGI) was calculated using the formula below (Pandey et al., 1982):

$$PGI\ (\%) = \frac{a-b}{a} \times 100$$

Where, a= the mean colony diameter of the control plates, and b= the mean colony diameter of the fungicide amended plates.

Field experiments: Two isolates of *P. nicotianae* (P.66 and P.111) recovered from crown of citrus trees infected by gummosis in Tunisia were used for this assay. *Phytophthora nicotianae* has been selected as model specie of gummosis because it's the principal causal agent for the gummosis of citrus in Tunisia and the most virulent (Unpublished data). Isolates, of *P. nicotianae*, has been obtained from different regions in Northern Tunisia. Isolate P.66 were recovered in 28/05/2012 from the region of Bnikhaled. The isolate P.111 were recovered in 19/09/2013 from the region of Gobba. Two isolates were stored in 25- ml flasks containing~15 ml of sterile soil extract. Plug of PDA medium with mycelia of each isolate were transferred to Petri plates amended with PDA medium and incubated for 5 days at 25±1°C in the darkness. Two fungicides metalaxyl (Ridomil, 25% wt/wt WP; Syngenta Agro) and fosetyl-Al 80% (Aliette Express, 80%, wt/wt WG, Sepcm) were used in the field experiment. Distilled water was the control to estimate the quantity of fungicides solution needed. Fungicides, rates, and application methods used in each experiment are indicated in Tables 4 and 5. In field experiments were performed at commercial field of citrus in the region of Benikhaled in the Cap-Bon area, Northern of Tunisia. Five citrus varieties (Maltaise, Thomson, Clementine, Valencia and Tangerine) grafted onto rootstock Sour orange, have been used during these experiments. The age of the trees was between 25 and 28-years-old and the field was drip irrigated. Experiments had been start on 15 September 2015 and two experiments were performed in citrus field. In the first experiment, four lignified branches, of~25-30 mm-diameter, inside the canopy of tree of each variety of citrus were inoculated 30 days before the application of fungicides to evaluate their curative activity. For inoculation, about 5-mm-diameter plug of the bark of the scion of each branch was removed from the trunk with a cork borer. A five mm-diameter of PDA plug culture of each isolate of *P. nicotianae* was inserted in the wound, exposed to the cambium. The bark was replaced on the agar disk and the inoculated area was covered by cotton moistened with sterile water, sealed with a strip of parafilm, and wrapped with scotch to prevent wound desiccation. Four trees, for each variety of citrus, were inoculated for each fungicide treatment–isolate– inoculation date combination. For the control, four non-treated trees inoculated with each isolate of *P. nicotianae* were used. Each isolate–fungicide treatment combination was replicated four times on five trees and plots arranged in a randomized block design. Lesion length was quantified in inoculated branches of each tree at 15, 30 and 45 days after fungicide application (Figure 1). The lesion length was measured only from the inoculation point to the lower margin of the lesion. In the second experiment, branches of each trees of citrus was inoculated with *P. nicotianae* at 15, 30, 45, and 60 days after application of fungicides to evaluate their residual activity (Figure 2). Lesion length was quantified 30 days after each inoculation date. In all experiments, re-isolation from the infected branches has been made to confirm that lesions were caused by of *P. nicotianae* isolates. A hydraulic sprayer (Viola SP 600, Agrional) at 3,000 kPa with a hand-held

spray gun has been used for foliar sprays. Spray volumes varied according to the size and to the canopy density of the tree, but all trees were sprayed to runoff (~8-10 liters/tree). Inoculations were made in the center of the treated area. A paintbrush amended with solution of each fungicide, covering a section of 30 cm length of four branches, was applied for paint treatments. To determine the lesion length, the bark was stripped from the trunk and the lesions on the surface of the exposed wood were measured.

Statistical analysis: All data were subjected to variance analysis (ANOVA). Significant ANOVA tests were followed by multiple comparisons of means using Student Newman-Keuls test ($P<0.05$). Statistical analyses were performed with the software SPSS.18.0 (IBM Corporation, Somers, NY, USA).

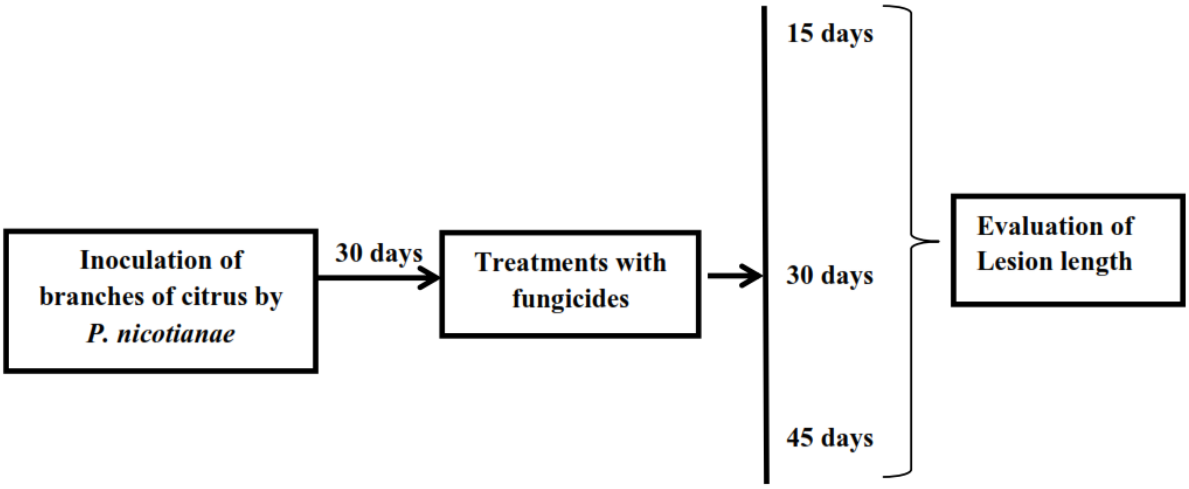

Figure 1: Methodology used for the test of the curative activity of fungicides against *P. nicotianae*, *in-planta* experiments.

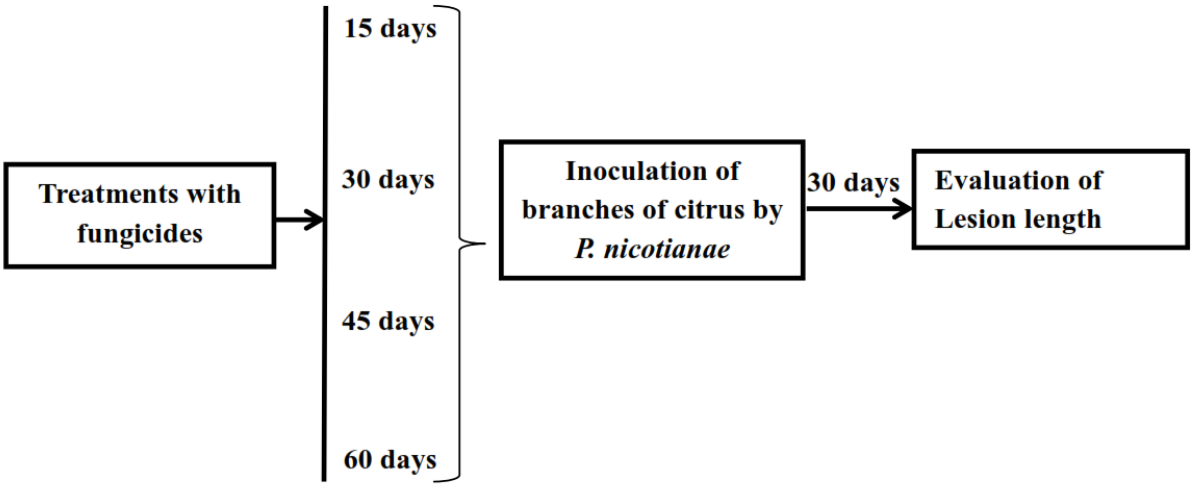

Figure 2: Methodology used for the test of the residual activity of fungicides against *P. nicotianae*, *in-planta* experiments.

Results

Laboratory experiments: Effectiveness of screened fungicides against *Oomycota* pathogens was investigated. All tested fungicides treatments were effective in reducing mycelium growth of pathogens. One-way ANOVA on data indicate a significant statistical difference between species of *Oomycota*, the fungicides tested and also between the interaction "pathogen- fungicide treatments". The highest percent of growth inhibition was observed with the metalaxyl, while the lowest percent of growth inhibition was noted with the chlorothalonil. For the metalaxyl, the interval of growth inhibition was ranged from 87.25% against *P. aphanidermatum* to 94.75% against *P. nicotianae*. The metalaxyl were followed by the mancozeb, with an average of growth inhibition ranged from 90% against *P. cryptogea* to 91.62% against *P. nicotianae*. In case of the cymoxanil the average of mycelium growth inhibition was ranged from 94.62% against *P. nicotianae* to 85% against *P. dissotocum*. For the propamocarb, the growth inhibition varied from 91.13% against *P. nicotianae* to 80.37% against *P. vexans*. However, for the azoxystrobin the interval of growth inhibition was ranged between 81.25% against *P. nicotianae* to 69.25% against *P. mercuriale*. In case of fosetyl –Al 80%, the growth inhibition ranged from 74.25% against *P. nicotianae* to 58.62% against *P. vexans*. The trifloxystrobin exhibited a hyphal inhibition ranged from 61.25% against *P. mercuriale* and against *P. aphanidermatum* and 38.87% against *P. nicotianae*. In case of thiophanate-methyl the growth inhibition varied from 51% against *P. dissotocum* and 36.25% against *P. nicotianae*). The chlorothalonil inhibited the hyphal growth at a level ranged from 34.25% against *P. nicotianae* and 15.25% against *P. mercuriale*. The highest percent of growth inhibition were noted by *Phytophthora* species with intervals of growth inhibition of 34.25%-94.75% and 32%-94.62%, respectively against *P. nicotianae* and *P. cryptogea*. Species of *Pythium*, showed an intermediate percent of growth inhibition, with intervals of 24.25%-91.12%, 22.18%-91.5% and 19.62%-90.25%, respectively, against *P. ultimum P. dissotocum* and *P. aphanidermatum*. However, the lowest percent of growth inhibition was noted with the two *Phytopythium* species, *P. vexans* (29.87%-94.5%) and *P. mercuriale* (15.25%-90.75%) (Table3).

Field experiments: Both in curative and in the residual experiments, branches of each citrus variety developed a canker in response to *P. nicotianae* isolates inoculation. One-way ANOVA indicate that fosetyl-Al 80% and metalaxyl had significantly reduced the lesions compared with the control. However, no difference was noted between the two used *P. nicotianae* isolates.

Curative experiments: In the curative activity experiments, a high significant difference has been observed in the treatments used, the varieties and the interaction between these factors, in each date of evaluation of lesion expansion (Table 4). In the first date of evaluation (15 days after fungicides applications/45 days after inoculation), the most susceptible variety was Maltaise variety

to *P. nicotianae* infection, with 0.05 cm of lesion length. This variety was followed by the Clementine variety (9.64 cm), Thomson (8.62 cm), Valencia (7.1 cm) and Tangerine (5.64 cm). For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk paints (3.1 cm). The lowest reduction of lesion expansion has been observed with fosetyl-Al 80%, foliar spray applied (8.47 cm). However, the fosetyl-Al 80%, trunk paints was in intermediate position. In the second date of evaluation (30 days after fungicides applications/60 days after inoculation), the most susceptible varieties were Clementine and Maltaise to *P. nicotianae* infection, with, 10.71 cm and 11.42 cm of lesion length, respectively. These two varieties were followed by Valencia (8.29 cm), Thomson (9.30 cm) and Tangerine (6.27 cm).

Table 1: Isolates of *Oomycota* employed *in-vitro* study, host, geographic origin, and year of isolation and GenBank accession number of sequences.

Species	Isolates	Date of collection	Origin	Organ of isolation	GenBank accession number of sequences
P. nicotianae	P.63	31/05/2012	Takilsa	Soil	KU248808
P. cryptogea	P.101	04/09/2013	Hawaria	Soil	KU248814
P. vexans	P.83	01/06/2012	Bouargoub	Crown	KU248801
P. dissotocum	P.79	12/04/2012	Bnikhaled	Soil	KU248782
P. mercuriale	P.121	29/09/2013	Takilsa	Crown	KU248804
P. aphanidermatum	P.95	23/02/2013	Menzel bouzalfa	Crown	KU248783
P. ultimum	P.67	31/05/2012	Takilsa	Soil	KU248785

Table 2: Characteristics of fungicides used to test the inhibition of hyphal growth of *Oomycota* isolates.

Active ingredients	Concentration	Commercial names	Manufacturers	Doses of uses (per hl)
Fosetyl Al 80%	80%	Aliette Express	Sepcm	250 g
Metalaxyl	2.5%	Ridomil	Syngenta Agro	300 g
Cymoxanil	4.8%	Aviso DF	Stima	150 g
Propamocarb	722 g/l	Proplant	Protagri	200 cc
Azoxystrobin	250 g/l	Amistar	Agriprotec	100 cc
Trifloxystrobin	50%	Zato 50	Atlas Agricole	15 g
Thiophanate-methyl	70%	Methyl-T	Bioprotection	250 g
Chlorothalonil	720 g/l	Bravo SC	Agriprotec	250 cc
Mancozeb	80%	Triziman80	Agriprotec	250 g

Table 3: Mycelial growth of *Oomycota* pathogen, after 6 days of incubation at 25±1°C, in PDA medium amended with chemical fungicides.

Fungicides	*Phytophthora* species		*Phytopythium* species		*Pythium species*		
	P. nicotianae	*P. cryptogea*	*P. vexans*	*P. mercuriale*	*P. ultimum*	*P. dissotocum*	*P. aphanidermatum*
Metalaxyl	94.75±0.7^{ay}	94.62±0.51^{a}	94.5±0.53^{a}	85.75±0.46^{e}	90±0.53^{c}	91.5±0.53^{b}	87.25±0.88^{d}
Mancozeb	91.62±1.4^{a}	90±0^{b}	91.12±0.35^{ab}	90.75±1.03^{ab}	90.5±0.75^{ab}	90.75±1.16^{ab}	90.25±0.46^{ab}
Cymoxanil	94.62±0.91^{a}	90.75±0.7^{b}	90.62±0.74^{b}	85±0.53^{d}	91.12±0.83^{b}	85±0.53^{d}	86±0.53^{c}
Propamocarb	91.13±0.97^{a}	80.75±1.03^{b}	80.37±0.74^{b}	80.62±1.06^{b}	81.62±1.18^{b}	80.87±1.12^{b}	81±1.06^{b}
Azoxystrobin	81.25±0.7^{a}	70.87±0.83^{b}	69±0.53^{c}	69.25±0.7^{c}	64.37±0.51^{d}	80.75±0.46^{a}	68.75±1.16^{c}
Fosétyl Al 80%	74.25±0.88^{a}	70.625±0.74^{b}	58.62±1.99^{d}	61.75±1.66^{c}	61.5±1.06^{c}	62.25±1.03^{c}	71.5±1.19^{d}
Trifloxystrobin	38.87±0.64^{c}	41.5±0.92^{b}	39.62±0.51^{c}	61.25±0.88^{a}	61±0.75^{a}	60.25±0.46^{a}	61.25±1.28^{a}
Thiophanate-methyl	36.25±0.88^{e}	36.37±0.74^{e}	38±0.92^{d}	49.75±0.46^{b}	45.87±0.64^{c}	51±0.75^{a}	50.25±0.46^{b}
Chlorothalonil	34.25±0.7^{a}	32±0.75^{b}	29.87±0.35^{c}	15.25±0.70^{g}	24.25±0.7^{d}	22.18±0.65^{e}	19.62±0.74^{f}

Y: Numbers within columns followed by the same letter are not significantly different (Student–Newman–Keuls testat P < 0.05).

For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk paints (3.05 cm). The fosetyl-Al trunk paints was in intermediate position. However, the lowest inhibition of lesion expansion has been observed with fosetyl-Al, foliar spray applied (9.05 cm). In the third date of evaluation (60 days after fungicides applications/75 days after inoculation), the most susceptible variety, to *P. nicotianae* infection, was Clementine variety with, 11.91 cm of lesion length. This variety was followed, respectively, by the varieties Maltaise (12.55 cm), Tangerine (8.30 cm) and Thomson (10.47 cm) and Valencia (9.3 cm). For the treatments used, the highest inhibition of lesion expansion has been observed with metalaxyl trunk paints (2.21 cm). The lowest inhibition of lesion expansion has been observed with fosetyl-Al, foliar spray applied (10.58 cm). However, the fosetyl-Al trunk paint was in intermediate position. In conclusion, the curative fungicides applications demonstrated that the length of lesion, caused by *P. nicotianae*, was retarded significantly by the metalaxyl trunk paints.

Residual activity experiments: In the residual activity experiments a significant difference has been also registered between treatments used, citrus varieties, and their interaction in each date of evaluation of lesion expansion (Table 5). In the first date of evaluation (15 days after fungicides applications/45 days after inoculation), the most susceptible variety, to *P. nicotianae* infection, was Maltaise variety with 11.34 cm of lesion length. The variety Maltaise was followed, respectively, by the varieties Clementine (10.45 cm), Thomson (9.35 cm), Valencia (8.2 cm) and Tangerine (5.7 cm). For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk paints (4.1 cm). The fosetyl-Al, branch paint applied was in intermediate position.

However, the highest inhibition of lesion expansion has been observed with fosetyl-Al 80%, foliar spray applied (4. 56 cm).

Table 4: Curative activity of fungicide treatments applied 30 days after the inoculation of branches with *P. nicotianae in-planta* experiments conducted in 2015.

Varieties	Treatments[y]	Dose (g a.i./liter)	Length of lesion (cm)[x]		
			Days after fungicide application[Z]		
			15 days	30 days	45 days
Clementine	Metalaxyl - branch paint	60	5.92±0.38[d]	6.55±0.17[d]	8.5±0.4[d]
	Fosetyl-Al - branch paint	80	6.53±0.22[c]	7.02±0.28[c]	10.97±0.41[c]
	Fosetyl-Al - foliar spray	2	8.47±0.25[b]	9.05±0.25[b]	10.58±0.24[b]
	Nontreated control		9.64±0.41[a]	10.71±0.2[a]	11.91±0.14[a]
Tangerine	Metalaxyl - branch paint	60	3.1±0.14[d]	3.05±0.33[d]	5.06±0.2[d]
	Fosetyl-Al - branch paint	80	3.5±0.42[c]	3.14±0.12[c]	5.85±0.19[c]
	Fosetyl-Al - foliar spray	2	4.35±0.19[b]	4.54±0.21[b]	6.11±0.19[b]
	Nontreated control		5.64±0.55[a]	6.27±0.28[a]	8.30±0.24[a]
Valencia	Metalaxyl - branch paint	60	4.58±0.25[d]	5.85±0.34[d]	2.21±0.17[d]
	Fosetyl-Al - branch paint	80	5.33±0.26[c]	7.18±0.29[c]	2.65±0.25[c]
	Fosetyl-Al - foliar spray	2	6.76±0.37[b]	7.88±0.3[b]	4.64±0.57[b]
	Nontreated control		7.1±0.45[a]	8.29±0.56[a]	9.3±0.42[a]
Thomson	Metalaxyl - branch paint	60	5.47±0.56[d]	4.84±0.58[d]	4.47±0.41[d]
	Fosetyl-Al - branch paint	80	6.4±0.31[c]	5.83±0.71[c]	4.53±0.32[c]
	Fosetyl-Al - foliar spray	2	7.52±0.55[b]	6.16±0.18[b]	6.29±0.34[b]
	Nontreated control		8.62±0.43[a]	9.30±0.35[a]	10.47±0.41[a]
Maltaise	Metalaxyl - branch paint	60	5.03±0.06[d]	6.22±0.33[d]	6.55±0.31[d]
	Fosetyl-Al - branch paint	80	7.3±0.29[c]	7.05±0.55[c]	6.17±0.36[c]
	Fosetyl-Al - foliar spray	2	9.36±0.47[b]	8.40±0.63[b]	7.60±0.43[b]
	Nontreated control		10.05±0.17[a]	11.42±0.35[a]	12.55±0.42[a]

X: Lesion length 30 days after inoculation with *P. nicotianae* (Mean of eight trees). Y: Paint treatments were applied to the branches. Foliar sprays were applied to runoff (~10 liter/tree). Z: Numbers within columns followed by the same letter are not significantly different (Student–Newman–Keuls test at $P < 0.05$).

In the second date of evaluation (30 days after fungicides applications/60 days after inoculation), the most susceptible varieties, to *P. nicotianae* infection, were Maltaise and Clementine varieties with, respectively, 11.7 cm and 11.9 cm of lesion length. These two varieties were followed, respectively, by the varieties Valencia (10.6 cm), Thomson (10.38 cm) and Tangerine (6.8 cm). For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk paints (4.05 cm). The fosetyl-Al, branch trunk paints was in intermediate position. However, the lowest inhibition of lesion expansion has been observed with fosetyl-Al foliar spray applied (10.05 cm). In the third date of evaluation (45 days after fungicides applications/75 days after inoculation), the most susceptible variety, to *P. nicotianae* infection, was

Clementine variety with 12.5 cm of lesion length; followed by the varieties, Maltaise (12.38 cm), Thomson (10.38 cm), Valencia (10.6 cm) and Tangerine (6.8 cm). For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk paints (3.2 cm).

Table 5: Curative activity of fungicide treatments applied 30 days after the inoculation of branches with *P. nicotianae in-planta* experiments conducted in 2015.

Varieties	Treatments[y]	Length of lesion (cm)[x]				
		Days from fungicide application to inoculation[Z]				
		Dose (g a.i./liter)	15 days	30 days	45 days	60 days
Clementine	Metalaxyl - branch paint	60 g	6.92±0.54[d]	7.55±0.68[d]	9.66±0.47[d]	10.64±0.76[d]
	Fosetyl-Al - branch paint	80	7.535±0.41[c]	8.02±0.2[c]	12.47±0.29[c]	11.71±0.4[c]
	Fosetyl-Al - foliar spray	2	9.475±0.48[b]	10.05±0.68[b]	11.58±0.43[b]	12.9±0.38[b]
	Nontreated control		10.45±0.52[a]	11.9±0.45[a]	12.5±0.57[a]	13.5±0.57[a]
Tangerine	Metalaxyl - branch paint	60	4.1±0.14[d]	4.05±0.33[d]	6.06±0.55[d]	6.64±0.47[d]
	Fosetyl-Al - branch paint	80	4.56±0.56[c]	4.14±0.29[c]	6.85±0.34[c]	7.27±0.22[c]
	Fosetyl-Al - foliar spray	2	5.35±0.45[b]	5.54±0.34[b]	7.11±0.16[b]	9.30±0.35[b]
	Nontreated control		5.7±0.47[a]	6.8±0.43[a]	8.3±0.46[a]	9.9±0.32[a]
Valencia	Metalaxyl - branch paint	60	5.58±0.68[d]	6.85±0.41[d]	3.21±0.41[d]	6.1±0.14[d]
	Fosetyl-Al - branch paint	80	6.33±0.45[c]	8.18±0.74[c]	3.65±0.6[c]	6.29±0.38[c]
	Fosetyl-Al - foliar spray	2	7.76±0.61[b]	8.88±0.35[b]	5.64±0.64[b]	8.3±0.08[b]
	Nontreated control		8.2±0.28[a]	10.6±0.48a	11.3±0.47[a]	12.7±0.24[a]
Thomson	Metalaxyl - branch paint	60	6.47±0.55[d]	5.84±0.65[d]	5.47±0.49[d]	4.62±0.48[d]
	Fosetyl-Al - branch paint	80	7.4±0.48[c]	6.83±0.62[c]	5.53±0.42[c]	5.30±0.21[c]
	Fosetyl-Al - foliar spray	2	8.52±0.6[b]	9.16±0.58[b]	7.29±0.34[b]	8.47±0.41[b]
	Nontreated control		9.35±0.43[a]	10.38±0.62[a]	11.84±0.35[a]	12.45±0.52[a]
Maltaise	Metalaxyl - branch paint	60	6.03±0.22[d]	7.22±0.33[d]	7.05±0.25[d]	7.05±0.45[d]
	Fosetyl-Al - branch paint	80	8.3±0.24[c]	8.05±0.06[c]	7.17±0.2[c]	7.42±0.38[c]
	Fosetyl-Al - foliar spray	2	10.36±0.47[b]	9.40±0.16[b]	8.60±0.48[b]	9.55±0.42[b]
	Nontreated control		11.34±0.43[a]	11.7±0.53[a]	12.38±0.73[a]	12.87±0.12[a]

X: Lesion length 30 days after inoculation with *P. nicotianae* (Mean of eight trees). Y: Paint treatments were applied to the branches. Foliar sprays were applied to runoff (~10 liter/tree). Z: Numbers within columns followed by the same letter are not significantly different (Student–Newman–Keuls test at $P < 0.05$).

The fosetyl-Al, branch trunk paints was in intermediate position. However, the lowest inhibition of lesion expansion has been observed with fosetyl-Al, foliar spray applied (11.58 cm). In the fourth date of evaluation (60 days after fungicides applications/90 days after inoculation), the most susceptible variety, to *P. nicotianae* infection, was Clementine variety with 13.5 cm of lesion length; followed by the varieties, Maltaise (12.87 cm), Tangerine (9.9 cm), Valencia (12.7 cm) and Thomson (12.45 cm). For the treatments used, the highest reduction of lesion expansion has been observed with metalaxyl trunk

paints (4.62 cm). The fosetyl-Al, branch trunk paints was in intermediate position. However, the lowest inhibition of lesion expansion has been observed with fosetyl-Al, foliar spray applied (12.91 cm). For the residual activity experiments, the present investigation demonstrate also that the metalaxyl applied as trunk paint, are the most efficiency in reducing the development of lesion caused by *P. nicotianae*.

Discussion

The results of this investigation showed that none of the fungicides tested were able to inhibit completely the growth of the tested *Oomycota* species. The tests, conducted in the laboratory showed that the effective fungicides were the metalaxyl, the cymoxanil, the propamocarb and the mancozeb, while a high resistance has been observed with the trifloxystrobin, the thiophanate-methyl and the chlorothalonil. However, the azoxystrobin and the fosetyl-Al 80% generated a moderate growth reduction. Also, the most sensitive pathogens were the specie *P. nicotianae* and the most resistance was the specie of *P. vexans*. The artificial inoculation methods used the in field experiment revealed that the application of metalaxyl by paint treatment is the most efficient in reducing the lesion caused by *P. nicotianae*, both in curative and in residual activity experiments. The Clementine variety was the most susceptible to *P. nicotianae* infection, while the variety tangerine was the most tolerant. Our results *in-vitro* experiments were in the same sense with other funding. Silva et al. (2016) found that fosetyl-Al was not effective in reducing mycelial growth *in-vitro*, while the metalaxyl is highly effective in reducing and even inhibiting sexual and asexual reproduction and growth of the pathogen. Thomidis and Michailidis (2002) showed that metalaxyl and fosetyl- Al were more effective than cymoxanil and dimethomorph in reducing mycelial growth of *P. cactorum* and *P. citrophthora*. For field experiments, our results were in agreement with those of previous works showing that both metalaxyl and fosetyl-Al provide effective to control *Phytophthora* gummosis of citrus (Feichtenberger, 1990; Cohen & Coffey, 1986; Menge, 1986). In addition, the results of these *in-planta* investigations agree with previous work showing that metalaxyl applied as trunk paint is highly effective for controlling *Phytophthora* infection on citrus tree trunks (Matheron & Matejka, 1988). Alvarez et al. (2008) noted that the residual activity of fungicides in field experiments was generally low, especially in foliar spray treatments, and none of the treatments evaluated inhibited completely lesion expansion. Also both metalaxyl and fosetyl-Al prevented growth of *P. cactorum* and *P. citrophthora* on peach trees when applied as stem trunk paint (Thomidis & Elena, 2001). Timmer (1977) demonstrated that trunk paint was a good preventive measure against *Phytophthora*. Fosetyl-Al and metalaxyl were more effective in reducing size of stem lesions incited by *P. citrophthora* in preventive applications than in curative applications (Feichtenberger, 1990). Menge (1986) recommended that preventive treatments of these fungicides in citrus nurseries but not in groves. The

differences in age of trees, the species of citrus, the time of application (during or between a growth flush) as well as different methodologies for measurement of the efficacy of chemicals could account for these discrepancies (Matheron & Matejka, 1988). The difference found between the effectiveness of fosetyl-Al and metalaxyl could result from a dose effect or from the application technique, instead of being by an intrinsic difference in the efficacy between both products. Alvarez et al. (2008) noted that by the artificial infection of the branch by the pathogen, the penetration phase of the pathogen's life cycle is greatly facilitated. By this technique it is possible to evaluate the ability of fungicides to inhibit lesion expansion, but not their preinfection protective activity. As lab experiment, the metalaxyl reveled to be the most efficient against the causals agents. However, the fosetyl-Al has been observed in intermediary position and it was not effective in reducing mycelial growth *in-vitro*. This dual behavior *in-vitro*/*in-planta* had already been reported in previous studies (Garbelotto et al., 2009). This behavior could be related to the dual action of the fungicide in the plant where it acts directly against the pathogen and indirectly by stimulating the defense mechanisms of the plant. The metalaxyl and fosetyl-Al are known to inhibit sporulation and suppress the development of *Phytophthora* (El-Hamalawi et al., 1995). It is possible that the fungicidal activity of this fungicide was influenced when the material was applied to plant tissues (Thomidis & Michailidis, 2002). This resistance to fosetyl-Al is also known in some *Oomyceta* fungi (Brown et al., 2004). Several studies showed that continued uses of fosetyl- Al may cause the increase in resistance in the pathogen populations (Wang et al., 1996). The inoculation technique adopted *in-planta* study is recommended by the EPPO guidelines for the evaluation of the efficacy of fungicides against *Phytophthora* gummosis (Anonymous, 1988). Adequate control of gummosis diseases in citrus should combine the use of systemic fungicides with other control measures, such as the use of more resistant or tolerant scion-rootstock combinations, and other biological and cultural methods. Future studies should continue to compare alternate fungicide chemistries, doses, and the seasonal timing of application for their effect on gummosis expansion.

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