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Fungitoxicity of some fungicides against to pathogens responsible of olive trees decline in the Chebika's area in Tunisia

Ben Salem I., N. Boughalleb*, M. Souli, S. Selmi, and M. E. Romdhani

[1] Institut Supérieur Agronomique de Chott Mariem, Laboratoire de Phytopathologie, 4042 Sousse- Tunisie
*Corresponding author: n.boughalleb@laposte.net

The incidence of the disease seems very important on young trees and tends to be moderate with the aging of the tree. In fact, olive trees have a shallow root system and are still vulnerable to pathogens especially the irrigated varieties. Chemical and biological control against *Fusarium solani, Fusarium oxysporum, Rhizoctonia solani* and *Verticillium dahliae* have revealed that the application *in vitro* of Prodazim and of Methyl-thiophanate have showed a very good efficacy up to 100%. Ridomil and Tachigaren have indicated a regular efficiency, while the two bio-fungicides Fungstop and the compost juice have demonstrated a low efficiency. The two bio-control agents *Trichoderma harzianum* and *Gliocladium virens* have showed a relatively high effectiveness *in vitro*. *In vivo*, obtained results have revealed that the nature of the product, the doses applied and the condition of the olive trees are highly correlated factors. The treatment doesn't appear to have a positive effect on the beginning of stage 1 and on plots presented a good structured soil. Going beyond this stage, whatever the product and the doses used, the attack is irreversible.

Keys words: Decline, olive trees, chemical and biological control, *in vitro*, *in vivo*

Olive (*Olea europaea* L.) is one of the most traditional and important tree crops in the Mediterranean region. The plant originated in an area extending from the southern Caucasus to coastal Syria. However, some problems could limit the production such as the decline of the tree due to a fungal complex and causing a various types of symptoms like yellowing, defoliation, dwarfism, deterioration and drying of the seedlings. Boulila (1994) has observed this phenomenon in different cultivation area in Tunisia: from the south-west (Gafsa, Chebiba, Ben Aoun) to the center (Ennadour, Kairouan, sbikha). Four fungal species were identified from young decayed olive-trees: *Macrophomina phaseolina, Nectria haematococca, Armillaria mellea,* and *Corticium rolfsii*. Shabi *et al.*(1994) have shown the presence of *Verticillium dahliae, Cheatomium sp* and *Phoma sp* by the investigations were carried out in the field and in the laboratory. *Verticillium* wilt caused by *Verticillium dahliae*, is the most important disease in Iran. Starting from the roots of the decayed trees, *Fusarium solani*, *Rhizoctonia solani* and *Macrophomina phaseolina* was isolated (Sistani *et al.*, 2009). In Algeria, the most known varieties such "Sigoise" in the North-West of this country and "Chemlali" in Kabilye are susceptible to the *verticillium* wilt (Al Aribi *et al.*, 2008). Several approaches have been used to control the decay in established olive orchards including chemical control (Tawil and Abdin, 1994), soil solarization (Tjamos *et al*., 1991), solar chamber (Al- Ahmad and Duksi, 1994), cultural practices (Al-Ahmad, 1993), integrated control (Abu-Qamar and Al-Momany, 2002) and biological control

(Tjamos, 1993). Among the evergreen fruit tree species used in a study performed in the Jordan Valley, olive was able to survive the post-plant soil solarization treatment (Abu-Gharbieh *et al*., 1998). Grafting onto resistant varieties could be a solution against this disease (Porras *et al*., 2003). The most common Tunisian varieties, tested in Morocco, appeared very sensitive to this disease (Jardak *et al*., 2004). Al Aribi *et al.* (2008) have shown the effectiveness of *Trichoderma spp* against *Drechslera sp*., *Cladosporium* sp., *Actinopelte* sp., *Fusarium* sp. and *Nigrospora* sp. It is good ideas to follow the control of fungal pathogens by other fungi such as *Trichoderma* and *Gliocladium* species which is an exciting and rapidly developing research area (Butt *et al*., 2001). Recently the management of *V. dahliae* as a soil borne pathogen has been occurred by application of *T. viride* (Saremi, 2003). It has been also reported that microsclerotia of *V. dahliae* can be killed by *Gliocladium roseum* in soil (Butt *et al*., 2001). There is considerable interest in the exploitation of naturally occurring organisms such as bacteria and fungi for the control of plant diseases. The control of this soil-born fungus remains limited. Indeed, the objective of this research is to evaluate the efficiency in *vitro* and *in situ* of some chemical and biological products on the development of the fungic agents responsible for the deterioration of the olive-tree in the area of Chebika.

Materials and methods

Chemical control test in vitro

Fungal strains

The purpose of this trial is to test, *in vitro*, the effectiveness of some fungicides on the mycelia growth of four fungal species identified (*Fusarium solani*, *F. oxysporum*, *Rhizoctonia solani* and *Verticillium dahlia*) from infected olive plants collected from different areas in Kairouan.

Mycelial growth assay

Commercial formulations of four fungicides and two bio-fungicides, representing different chemical families, were used in this study (Table 1). Appropriate volumes of each fungicide and bio-fungicides were added to PDA at approximately 50°C in amounts to achieve final concentrations at the rate of the half, the same and the double of the registered dose (Table 1). Mycelia plug (6mm in diameter), obtained from the margins of actively growing cultures, and were transferred to fungicide amended plates. Control PDA plates were prepared similarly but adding sterile distilled water (SDW) instead the fungicide or the organic compound solution. There were two replicates of fungicide concentration. The dishes were incubated of 25°C in the dark and the diameter of each colony was measured twice perpendicularly. Measurements were made at the same time and averaged.

Aggressiveness of bio-control agents in vitro

The antagonism assay *in vitro* was performed by two bio-control fungi *Trichoderma spp*. and *Gliocladium spp*. tested. Each antagonist was incorporated into the culture medium PDA in the form of a spore suspension of 10^8 spores / ml, as, three doses tested (1000ppm, 2000ppm and 4000ppm). For the control plates we added to the culture medium an equivalent volume of spore suspension of each antagonist in the form of sterile distilled water. Each 6mm mycelia plug of fungal species tested namely: *Fusarium solani*, *F. oxysporum*, *Rhizoctonia solani* and *Verticillium dahlia* were transferred to amended plates. We have two replicates per treatment. The dishes were incubated of 25°C in the dark and the diameter of each colony was measured twice perpendicularly. Measurements were made at the same time and averaged.

Analyses statistiques

The trial assay is a completely random factorial experiment (with repetition of experience) with two factors (treatments and isolates). Data were subjected to an

analysis of variance (GLM, ANOVA), the means comparison and homogeneous groups were defined according to Duncan's test at 5%by SPSS 12.0 program.

Chemical control test *in vivo*

Disease management trials were conducted in fields naturally infested which is composed of 5 elementary plots presented in Table2. Each plot has showed the same trait such as, the homogeneity of the plant material (same age and variety), the same agronomic conditions (density, cultivation techniques), and the same soil characteristic (texture and salinity). The four most effective fungicides and bio- fungicides in the *in vitro* test, Prodazim, methyl-thiophanate and the two bio-fungicides: Fung stop and the juice of compost also were evaluated under field conditions. We have chosen testing three doses of each product that is the half, the same, and the double of the registered concentration. The doses tested are different, we have for Prodazim (25g, 50g, 100g/100 l), methyl-thiophanate (50, 100, 200g/100 l), and for the two bio-fungicides: Fung stop (50.100, 200ml/100 l) and Juice of compost (200, 100 and 250ml/100 l).

Fig 1: Treatment by watering a sampling olive

Table 1 : Characteristics and dose used of fungicides and biological compounds tested

Trade Name	Active Ingredient	Chemical group	Mode of action	Formulation[a]	Dose tested (ppm)
Prodazim	Carbendazim 50%	Benzimidazolyl -2 Methyl carbamate	Systemic	50g/l WP	250 500 1000
Ridomil gold 480 SL		Unknown			1000 2000 4000
Methyl-Thiophanate	Thiophanate – Methyl 70 %	Benzimidazole	Contact and Systemic	100g/l WP	500 1000 2000
Tachigaren	Hymexazol 30%	Oxazole	Systemic	200 cc /hl SL	1000 2000 4000
Organic compound					
Fung stop	Citrique acide 16% Peppermint oïl 0.8%		Blocks protein synthesis and causes the destruction of fungal cells	200 cc/hl SC	1000 2000 4000
Compost juice	1 / 3 juice sheep manure, 1 / 3 juice and cow manure 1 / 3 juice by products of poultry				1000 2000 4000

[a] *WP, wettable powder ; SC, suspension concentrate ; SL, soluble concentrate*

Table 2 : Characteristics of elementary plots

Elementary plots	Soil Texture	Soil Salinity (total salts kg / g soil)	Cultivars	Total Number of Trees
S1	Clayey silty sand	3,12	Chemlali Sfax	5146
S2	Sandy loam	2,44	Chemlali Sfax	4000
S3	Sandy loam	2,52	Chemlali Sfax	4200
S4	Sandy loam	2,68	Koroneiki	2366
S5	Sandy	0,26	Koroneiki	3525

The fungicides were applied through the submersion of the trees with an aqueous solution of each test product about 10 liters per tree (Fig 1), the treatment was carried out arbitrarily at each elementary plot and it was made once in May 2008. After three weeks of treatment we've estimated the condition of treated trees compared with the untreated control trees.

Results

Chemical control test in vitro

Mycelial growth assay

The different fungal species showed different response to the chemical trial *in vitro* and the effect of the treatment was significant ($p<0.05$).

Based on the mean comparison results, Prodazim and Methyl- thyiophanate had significantly high efficiency compared to the other products (Table 3). Moreover, these two fungicides have caused a complete inhibition of mycelial growth. According to statistical analysis (ANOVA), the doses tested have shown a significantly effect on radial growth of all fungi.

Effect of Ridomil and Tachigaren were tested at 3 different doses (1000, 2000 and 4000ppm) on radial growth of the pathogens. Data in table3 show that radial growth of all fungi tested were reduced gradually as the fungicide concentration was increased and all doses significantly reduced the mycelia development of the tested fungus, as compared to the check (0 concentrations). The highest two tested concentrations (2000, 4000 ppm) showed almost significant complete inhibition to growth of *F. solani* and *V. dahlia*, therefore, this two fungus seems to be the most sensitive. On the other hand the lowest concentration showed inhibition of more than 50% of the radial growth at 1000 ppm of *F. oxysporum* (15.62mm) and *R. solani* (16.13mm), also obtained data indicated that this fungus are the most resistant to the tested treatment.

Data presented in table 3 revealed that in presence of Ridomil, the mycelia development of all tested fungi showed inhibition of more than 50% of the radial growth at 4000ppm for all fungal species. *Verticillium dahliae* seems to be the most sensitive with the lowest values: 9.13 mm (4000ppm) to 33.37 mm (1000ppm), however, *F. solani* has recorded the highest values with 51.37 mm (4000ppm) to 67.75 mm (1000ppm) and it is the resistant one in comparison with the other fungus.

Fung Stop is moderately effective on inhibition of the mycelial growth on all fungi, the different values were moderately low recorded at 2000ppm from (58.75 mm) *F. oxysporum* to (71.5mm) *F. solani* and at 4000ppm is from 37,87mm (*F. oxysporum*) to 62.25mm (*R. solani*). Both *F. solani* and *R. solani* are the most resistant fungus in comparison with the other ($p <0.05$) (Table 4).

Unlike other products are tested and proved effective, the compost juice seems to have no inhibitory effect on the mycelia development of all fungi. Indeed, 4000ppm is the only dose that reduced the mycelial growth from 11% (*R. solani*) to 40% (*F. oxysporum*), compared to the other two doses forming the last group (Table 4).

Statistical analysis (GLM) revealed that the nature of product has affected the mycelial growth of all fungi independently of the isolates and the doses ($p < 0.05$). Results demonstrated the existence of clear effect of concentration of different product applied on the radial growth. Also, data presented in table 3 and table 4 revealed the interaction of the concentration and the majority of fungi. We have an only exception on the application of Fungstop which demonstrated no interaction (Table 4).

Table 3 : The radial growth (mm) of each fungus species following the three doses of the 4 fungicides tested

Products	Doses tested	*Fusarium solani*	*Fusarium oxysporum*	*Rhizoctonia solani*	*Verticilium dahliae*
Ridomil	Control	90a [a]	90 a	90 a	90a
	1000	67,75b	58,25 b	76 b	33,375 b
	2000	61,625 bc	50,625 bc	43,625c	20,25 c
	4000	51,375c	44,25 c	36,375 c	9,125 d
	Means	67,69	60,78	61,5	38,19
			p values [b]		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,000*		
Tachigaren	Control	90a	90a	90a	90a
	1000	0b	15,62b	16,125b	0,25 b
	2000	0b	11,87 b	13,375 b	0±0 c
	4000	0b	5,375 c	6c	0±0 c
	Means	22,5	30,72	31,375	22,56
			p values		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,000*		
Prodazim	Control	90	90	90	90
	250	0	0	0	0
	500	0	0	0	0
	1000	0	0	0	0
	Means	22,50	22,50	22,50	22,50
			p values		
	Effect of Isolate		-		
	Effect of Dose		-		
	Effect of Interaction		-		
Methyl-thiophanate	Control	90	90	90	90
	500	0	0	0	0
	1000	0	0	0	0
	2000	0	0	0	0
	Means	22,50	22,50	22,50	22,50
			p values		
	Effect of Isolate		-		
	Effect of Dose		-		
	Effect of Interaction		-		

[a] *Values represent the average of 8 measurements (four Petri dishes) by treatment. The averages presented vertically followed by the same letter are not statistically different ($p < 0.05$) by Duncan test.*

[b] *p Values (0,05) by ANOVA*

Table 4 : The radial growth (mm) of each fungus species following the three doses of the two organic compounds tested

Organique compound	Doses tested	*Fusarium solani*	*Fusarium oxysporum*	*Rhizoctonia solani*	*Verticilium dahliae*
Fungstop	Control	90 a [a]	90 a	90 a	90 a
	1000	71,5 b	58,75 b	71,125 b	59,375 b
	2000	68 bc	52,87 bc	67,75 bc	55,62 b
	4000	60,87 c	37,78 c	62,25 c	44 b
	Means	72,59	59,85	72,78	62,25
			p values [b]		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,22		
Compost juice	Control	90 a	90 a	90 a	90 a
	1000	71,875 b	68 b	80 b	75,75 b
	2000	57,625 c	65,75 b	80 b	70,37 c
	4000	56,375 c	54 c	80 b	61,25 d
	Means	68,97	69,44	82,5	74,34
			p values [b]		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,000*		

[a] *Values represent the average of 8 measurements (four Petri dishes) by treatment. The averages presented vertically followed by the same letter are not statistically different (p <0.05) by Duncan test.*
[b] *p Values (0,05) by ANOVA*

Table 5 : The radial growth (mm) of each fungus species following the three doses of the two biological agents tested

Antagonists agents	Doses tested	*Fusarium solani*	*Fusarium oxysporum*	*Rhizoctonia solani*	*Verticilium dahliae*
Trichoderma harzianum	Conrol	90 a [a]	90 a	90 a	90 a
	1000	12,75 b	17,5 b	34 b	16,125 b
	2000	10,875 b	13,625 bc	29,625 bc	15,875b
	4000	10,5 b	6,75 c	26,875 c	12,125 c
	Means	31,03	31,97	45,13	33,53
			p values [b]		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,000*		
Gliocladium virens	Control	90 a	90 a	90 a	90 a
	1000	18,625 b	22,125 b	34,125 b	29,75 b
	2000	14 c	14,75 c	31,25 c	23 bc
	4000	12 c	10,625 c	21,375 d	19,5 c
	Means	33,66	34,38	46,44	40,56
			p values		
	Effect of Isolate		0,000*		
	Effect of Dose		0,000*		
	Effect of Interaction		0,000*		

[a] *Values represent the average of 8 measurements (four Petri dishes) by treatment. The averages presented vertically followed by the same letter are not statistically different (p <0.05) by Duncan test.*
[b] *p Values (0,05) by ANOVA*

Aggressiveness of antagonists in vitro

Radial growth of the fungi tested was determined after three days of growing on PDA. Data presented in table 5 indicated that all bio-agents tested were significantly decreased the radial growth of all fungi in comparison with the control ($p<0.05$).

The mycelial growth of *Fusarium solani* in the presence of the bio-agents *Trichoderma harzianum* (31.03mm) and *Gliocladium virens* (33.65mm) was significantly reduced in comparison with the other fungi ($p<0.05$).

Trichoderma harzianum overlapped and the best inhibited the growth of *V. dahlia, F. oxysporum* and *F. solani* (inhibition of radial growth more than 50%) at 2000ppm while *Gliocladium virens* retarded the mycelial growth of the fungus at a distance by producing inhibitory zone against and reduced the growth (less than 50%). Generally, when the bioagents inoculated after inoculation by all fungi, at 4000ppm, the two bio-agents were the most effective (reduction of growth 84.4% and 84.85% respectively).

According to the GLM statistical analysis, there is no significant difference between the behaviors of fungi on PDA amended by *T. harzianum*. However, *R. solani* showed a considerable resistance against this bio-agent with 45.13mm (Table 5). Also obtained data indicated that the doses at 1000 and 2000 ppm have no difference between them for all fungi tested (Table 5).

The last group is constituted by the third dose (4000ppm) recording the smallest diameter of 6,75mm (*F. oxysporum*) to 26.875mm (*R. solani*) for *Trichoderma harzianum* and 10.625mm (*F. oxysporum*) to 21.375mm (*R. solani*) for *Gliocladium virens* (Table 5).

Gliocladium virens at the three doses tested showed a significant response for all fungi, data presented in table 5 reveal that *F. oxysporum* and *F. solani* proved no significant deference and exhibited the lowest radial growth with (34.375 and 33.65mm, respectively). Though, *Rhizoctonia solani* revealed the highest value with 64.44mm.

Obtained Data indicated that from 1000ppm, *Gliocladium virens* and *Trichoderma harzianum* can reduce the colonization of all fungi tested with an exception of *Rhizoctonia solani* (Table5). For *Verticillium dahliae*, after 6 days of incubation on PDA contaminated by *Trichoderma harzianum* at 1000ppm, the reduction rate of the colony is high with 82.08% (16.13 mm) compared to the control. Same for *Gliocladium virens* with a reduction rate about 66.94% (29.75 mm). *Verticillium dahliae* revealed sensitive against the two antagonists applied to three doses 1000, 2000 and 4000ppm. The two antagonists *Trichoderma harzianum* and *Gliocladium virens* are able to inhibit the mycelial development of the 4 fungus species with slowing their growth.

Chemical control test in vivo

Knowledge of the spatiotemporal pattern and spread of plant diseases (i.e., the arrangement of diseased plants relative to each other on a time basis) in susceptible crops may provide valuable information about the nature and role of inoculums sources for development of epidemics, and thus contribute to designing adequate strategies for plant disease management.

The spatiotemporal spread of plant diseases was very fast because the space occupied by olive trees attacked have increased during a very short period of time and the tree have completely decayed. We observed three stages of attack on trees: i) stage 1, which represents the beginning of leaf curl; ii) stage 2 shows a yellowing of the plant with a rosette appearance and iii) stade3 which is the beginning of defoliation.

For both varieties Koroneiki and Chemlali Sfax, the number of trees treated with the four products tested have significantly exceeded natural recovery of untreated control trees which could be attributed to the nearly elimination of the microslerotia of *V. dahliae* in the soil. Doses, stage of attack and culture conditions have proved an

effect on treatment. At the elementary plot S6 (sandy soil), the Prodazim was effective and there was an improvement in the condition of trees in stage 1 at the dose of 50 g/100 l. However, the application in stage 2 has no effect on the variety Koroneiki treated.

The same product at the dose of 50 g/100l of applied in elementary plot with a sandy loam soil has no effect on the disease. Trees suffered from defoliation from the tips of young shoots treated with Prodazim at 100 g/100 l have completely defoliated after three weeks, this could be explained by the fact that such treatment should be established at the beginning of attack.

Methyl-thiophanate, used as a preventative treatment at the plot S6, has been effective against the decline of trees in stage 1 showed an increase in tree condition even at a relatively low dose (50g/100l).

Fung stop showed a suppressive effect on the disease for both varieties, whatever the soil type at a dose 200ml/100 l, at stage 1 of the disease (the beginning of attack). For the other stages this product has no effectiveness.

The variety Koroneiki, at early defoliation (stage 3), and grown in plot S5 was treated with 200ml/100l, have been stable and not be completely defoliated. It is the same for a tree of the variety Chemlali Sfax, grown in plot S2 at the stage 2.

The compost juice also showed an inhibitory effect of the disease at a dose of 100ml/100l water compared to the other doses. So we can conclude that the stage 1 is a reversible stage of the disease as compared to the other stages.

Discussion

This study provides new information's: the use of Prodazim, and Methyl-Thiophanate has been officially cited the screen on the olive tree. The ability of reducing the decay of olive trees at the dose to 25 g / 100 l in the case of Prodazim. The application of the fungicide Methyl-Thiophanate at the dose of 50 g / l provide a good efficiency on the development of the symptoms.

Among the fungus species tested, it was found that *Rhizoctonia solani* and *Fusarium solani* were the most resistant to various treatments followed by *Fusarium oxysporum* showing a moderate resistance, while *Verticillium dahliae* proved to be the most sensitive to all products tested. Moreover, the chemical trials in fields were successful in recovering the infected olive tree, but the perennial and the seasoning nature of olive trees should be more evaluated at a longer period (one year or more), by studying other parameters such as the yield, the volume of the canopy and the leaf area.

The results of chemical trial *in vitro* revealed that the two fungicides Prodazim and Methyl-Thiophanate have showed a significant effectiveness against the mycelial growth of all fungi tested. Tachigaren and Ridomil have exhibited a moderately effectiveness. While, the two bio-fungicide : Fungstop and the compost juice have divulged a low efficiency.

For the biological trial *in vitro* the bio-agents tested *Trichoderma harzianum* and *Gliocladium virens* have shown efficient against *V. dahliae*, this result was approved by many other researches like Tjmos (1993), Butt *et al.* (2001), Saremi (2003) and Al Aribi *et al.* (2008).

In hot, dry climates, the disease is usually not a major problem, because the cool wet weather is needed for the development of the epidemic. The main method used to control this epidemic throughout the survival of olive cultivation in the regions of the world is the use of chemical fungicides (Granti, 1993).

The most common fungicides used against *Verticillium* wilt of olive trees are based in copper, and this include Bordeaux mixture, copper hydroxide, copper oxide. Though some of them like Chlorothalonil were applied as a preventive fungicide to control the disease but it has a long persistence (Shabi *et al.*, 1994).In the Mediterranean area, fungicides are generally applied before the main infection periods, which often coincide with the main growing seasons (spring and / or autumn) (Shabi *et al.* ,

1994). Obanor *et al.* (2008) also reported that copper sulfate and a mixture of kresoxim-methyl and copper hydroxide was very effective, reducing the disease incidence, respectively 85-96% and 63-93%. It seems that the effectiveness of fungicides is increasingly linked to environmental conditions. It would be linked to the time of application of fungicides. That's why sometimes the results are not consistent (Sistani *et al.*, 2009). Application of antagonist like *Talaromyces flavus* (Klöcker) was effective enough to contain the disease (Tjamos, 1991; Tjamos, 1993). The use of resistant varieties of olive tree is the most economical and environmentally sustainable as a control. Research has shown that the cultivars Frantoio, Coratina, Frangivento, and oblong Kalamon have interesting properties of resistance (López-Escudero *et al.*, 2004; Tjamos, 1993). Cultivars as Ascolana, Cellini, Leccino, Manzanillo, Chemlali, Konservolia, Mission and Picual are sensitive (Citrulli *et al.*, 2008; López-Escudero *et al.*, 2004; Tjamos, 1993).
Studies on biological control against the microsclerotia of *Verticillium dahliae* have focused on the use of the antagonist *Talaromyces flavus* having an effect on microsclerotia in vitro, due to the production of glucose oxidase converts glucose to hydrogen peroxide. The bacterial antagonists, such as *Pseudomonas spp*. have been also used against *verticillium* wilt (Fahima *et al.*, 1992).

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