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Comparative study of pathogenicity tests for *Verticillium dahliae* and *Phytophthora palmivora* causing wilt and decline of olive tree (*Olea europaea* L.)

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ABSTRACT

Haouzia and Dahbia Olive plants were inoculated with *Phytophthora palmivora* and *Verticillium dahliae* with two inoculation techniques. These two species were respectively isolated from Souk El Arbaa and Meknes. Both of *V. dahliae* and *P. palmivora* provoke defoliation and decline symptoms with a difference on the period of incubation. Using Technique 1 of inoculation (plants were sprayed with the inoculum suspension), the aerial fresh weight of the non inoculated Dahbia olive plants was 400 g and it was respectively 178 and 155 g for the olive plants inoculated with *P. palmivora* and *V. dahliae* and 87 and 73 leaves were count on the olive plant inoculated respectively with *P. palmivora* and *V. dahliae* relative to the controls (480 leaves). Using Technique 2 (roots of the plants were dipped in the prepared inoculum). The aerial and root fresh weight of Haouzia olive plants inoculated with *P. palmivora, V. dahliae* were respectively (382-124 g) and (462-305 g) compared to the control (654-904 g). *V. dahliae* had a negative effect on the height of the inoculated Dahbia and Haouzia olive plants; height of Haouzia olive plants were affected (85 cm) compared to control (111 cm). *P. palmivora* was highly isolated from the stems of Dahbia (95 %) and Haouzia (83 %) olive plants and it was weakly isolated from the leaves petiole of the Dahbia (33.33%) and Haouzia (22 %) olive. *V. dahliae* was only isolated from the roots of the inoculated Dahbia (18%) and Haouzia (22.22 %) olive plants. The importance of these two pathogens of the olive tree has been discussed in this work.

Keywords: *Verticillium dahlia, Phytophthora palmivora, Wilt, Olive tree.*

INTRODUCTION

Verticillium wilt of olive (*Olea europaea* L.) is economically important in the entire Mediterranean region; it is one of the most serious diseases affecting olive (*Olea europaea* L.) worldwide[1] and may cause severe losses and plant death[2, 3]. Verticillium wilt of olive was first described by Ruggiere[4] in Italy and after wards reported from all regions of olive cultivations. Recently had a great increasing particularly in newly established olive orchards[5].

Growth suppression, defoliation and wilting may occur on part of the branches because this is a vascular disease[6, 7, 8] and the olive is a highly sectored tree with direct vascular connection of specific roots and shoots[9]; in severe attacks, trees die[10].

In *Verticillium*-susceptible hosts several phases can be distinguished in the parasitic phase of *V. dahliae* life cycle: (i) infection of the root, (ii) colonization of the vascular system, and (iii) symptom development[11]. In addition, *V. dahliae* can survive in soils for prolonged period of time by means of the production of latent, resistant structures [microsclerotia (MS)] which upon exposure to appropriate stimuli may germinate[12]. Germinating hyphae can then penetrate into root tissues initiating the infection process. Damaged root hairs or dead epidermal cells are preferred microsites for *Verticillium* spp. penetration. In addition, in nature extensive root damage by human or animal activities can provide pathogen penetration sites[13,14]. Following penetration into the root vascular system, pathogen colonization of above-ground vascular tissues of trees can be very rapid, finally reaching leaf petioles[15, 16].

Some species of Phytophthora are able to tackle the olive and to induce almost the same disease cycle on this host species that presents almost the same life cycle: *P. megasperma*[17, 18], *Phytophthora nicotianae*[19], *Phytophthora inundata*[17]. *Phytophthora palmivora,* for example, attacks young olive trees in southern Spain and causes wilt or dieback and death[20], pathogen of olive tree in Italy that provoked leaf chlorosis, defoliation, wilting, twig dieback and eventual plant collapse associating the symptoms with the root rot[21], same symptoms on young olive trees in Sicily[22] and in Morocco[23].

In Morocco, Verticillium wilt of the olive tree induced by *Verticillium dahliae* was studied by several authors[24, 25]. The disease is spread across several olive regions[26, 27]. *Phytophthora palmivora* was reported in Morocco in 2013[23] and the disease has also been confirmed in regions where verticillium wilt was observed[28].

Both pathogens *Verticillium dahliae* and *Phytophthora palmivora,* causing almost the same symptoms on the olive tree. In this study Pathogenicity of these two pathogens was compared on two varieties of the olive tree (*Olea europaea* L.) in the nurseries conditions.

MATERIALS AND METHODS

Pathogens

Two isolates were used in this study. The isolate OMV_5 of *Verticillium dahliae* was isolated from roots of Olive trees growing in Meknes (north-central of Morocco) showing decline symptoms. The isolate OPSL of *Phytophthora palmivora* was isolated from stems segments of olive crops in Souk El Arbaa (northwest of Morocco) showing wilting symptoms.

Plant materials

Twenty four plants of Dahbia and Haouzia olive plants in the age of two years were used for this experiment. Six plants were used for each lot.

Inoculum preparation

A 4-day-old polyspore culture of *Verticillium* on PDA was aseptically washed with sterilized distilled water. The final concentration of spores was 10^5 spores mL^{-1} (Douira and Lahlou, 1989).

Zoospores of *P. palmivora* were produced by growing cultures on oatmeal agar at 28°C in the dark for 14-21 days. The mycelium was transferred to a sterile Petri dish, covered with sterile distilled water (SDW) and incubated overnight at 28°C, under lights. The mycelia plates were chilled for 5 min at -20°C to induce zoospores release. The concentration of the inoculum was adjusted at 10^5 zoospores/ml of SDW.

Substrate Preparation

Mamora's sand was sterilized three times at the interval of 24 hours at 200 °C for 2 hours, and then it was mixed with the peat. This mixture constituted the experiment substrate of the olive plants (Table 1).

Table1. Chemical characteristics of experiment substrate

physicochemical parameters	pH	Organic matter (%)	Total calcium (%)	Organic carbon %	Ammoniacal nitrogen (ppm)	Nitric nitrogen (ppm)	Mineral nitrogen (ppm)	Assimilable phosphorus (ppm)	Potassium (ppm)
Experiment substrate	6.7	3.11	0.3	1.8	126.36	255.44	381.8	83	176

Inoculation test

Using two inoculation techniques on 2 years old of Dahbia and Haouzia olive plants, pathogenicity test was studied:

Technique 1. Olive plants were planted in plastic pots filled the substrate and plants were sprayed with 250 mL of the inoculum suspension (control plants were watered 250 mL with sterile distilled water).

Technique 2. Plants were inoculated according to the method described by Olbricht *et al.*[29]. The roots of investigated plants were washed under running water to discard soil remnants, trimmed to 2/3 of their length, and subsequently dipped in the prepared inoculum during 6 hours. The olive trees plants were potted into universal soil substrate, watered with the remaining fungal suspension (about 20 ml per plant), and cultivated in a greenhouse. For control plants, sterile water was used instead of fungal suspension.

Measuring parameters

Roots and the aerial part fresh weight, height of aerial part were measured and dwarfing index was calculated. The number of leaves was counted (only leaves those showed no symptoms of chlorosis and necrosis were counted). The incubation period of each pathogen was taken into consideration.

Reduction of the epicotyl size of the inoculated plants compared to control was estimated by dwarfing index of (D.I.) calculated according to the formula[30]:

D.I. = Mt-X / Mt x100

X: The increase of the inoculated plants epicotyl.

Mt: The average growth of the control.

Pathogen reisolation

Thin sections from the roots, stems and petiole of olive plants inoculated with *P. palmivora* and *V. dahliae* and non inoculated plants were cuts and placed in alcohol at 95 ° for 2 min, rinsed several times with sterile distilled water, quickly dried on sterile filter paper and placed on agar (agar : 20 g ; l000 ml of distilled water)[30] and on PSA plates. Then, these agar plates were incubated in the darkness at 22°C. Isolation test were affected also on the stems showing Verticillium and Phytophthora wilt after they were conserved for 2 years in the laboratory conditions (15- 28 ^{o}C).

The isolation percentage (Pr %) was obtained by applying the following formula:

$$Pr = NsPx/ NT \times 100$$

N_SPx: Number of segments containing the fungal specie x.

NT: Total number of segments used in the isolation.

RESULTS

Using technique 1 of inoculation: Dwarfing symptom was appeared after 48 weeks of Dahbia olive plants inoculation with *V. dahliae*. Thus, inoculation with *P. palmivora* showed chlorosis and defoliation after 24 weeks. As the same, using technique 2, *P. palmivora* showed chlorosis and defoliation symptoms on the Dahbia olive plants 5 weeks after inoculation, in the other hand *V. dahliae* induced dwarfing and defoliation symptoms on the same olive plant variety after 12 weeks of inoculation (Table 2).

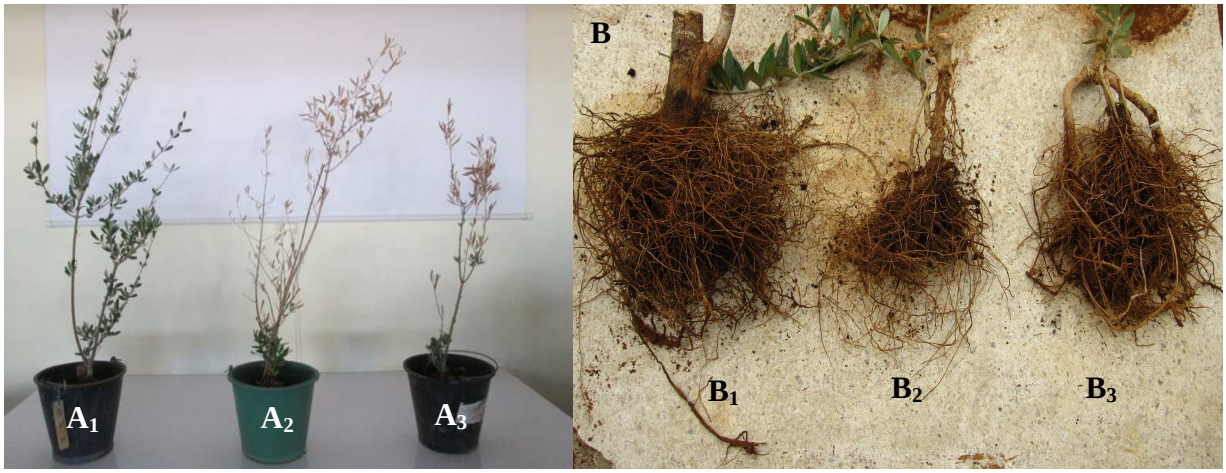
Chlorosis and defoliation symptoms appeared on the Haouzia olive plant inoculated with *P. palmivora* after 24 weeks of inoculation (technique 1). However, *V. dahliae* have taken 96 weeks to provoke the same symptoms on the same olive plant variety (Table 2). Therefore, using technique 2, *P. palmivora* showed defoliation symptoms 5 weeks after inoculation, in the other hand, *V. dahliae* showed same symptoms with dwarfing on the Haouzia olive plants 12 weeks after inoculation (Table 2).

Table 2. Incubation period and type of symptoms of the inoculated Dahbia and Haouzia olive plants with *Phytophthora palmivora* and *Verticillium dahliae* using two inoculation techniques

		Phytophthora palmivora		***Vertcillium dahliae***	
		Incubation period (week)	**Type of symptoms**	**Incubation period (week)**	**Type of symptoms**
Dahbia	**Technique 1**	24 a	chlorosis and defoliation	48 b	dwarfing
	Technique 2	5 b	chlorosis and defoliation	12 c	dwarfing and defoliation
Haouzia	**Technique 1**	24 a	chlorosis and defoliation	96 a	chlorosis and defoliation
	Technique 2	5 b	chlorosis and defoliation	12 c	dwarfing and defoliation

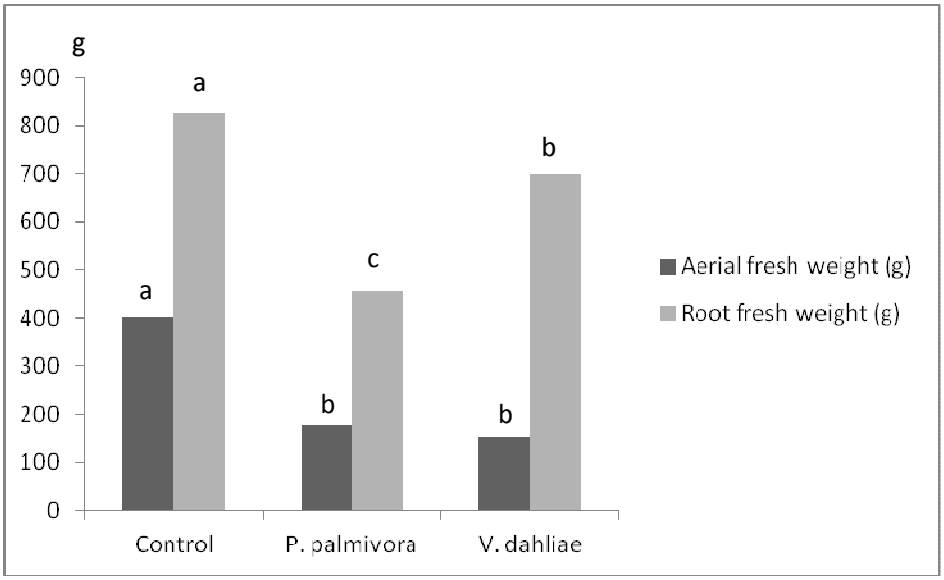
Using two inoculation techniques, chlorosis was the most common symptoms of disease appeared on the inoculated olive plants; Dahbia and Haouzia olive plants inoculated with both of *P. palmivora* and *V. dahliae* showed wilt and decline symptoms (Figure A_2 and A_3).

Fig. 1 Aerial part of the control olive plants (A_1) and inoculated with *P. palmivora* (A_2) and *V. dahliae* (A_3); root system of the control olive plants (B_1) and inoculated with *P. palmivora* (B_2) and *V. dahliae* (B_3).



Using technique 1 of inoculation, *Phytophthora palmivora* and *Verticillium dahliae* affect both of the aerial and root fresh weight of Dahbia olive plants. Thus, the aerial fresh weight of the non inoculated plants was 400 g and it was respectively 178 and 155 g for the olive plants inoculated with *P. palmivora* and *V. dahliae* (Figure 2). *P. palmivora* affect the root fresh weight (457g) of Dahbia olive plants more than *V. dahliae* (700g) relative to the control (825g) (Figure 2).

Fig. 2 Aerial and root fresh weight of the Dahbia olive plants inoculated with *Phytophthora palmivora* and *Verticillium dahliae* using technique 1 of inoculation



Both of *P. palmivora* and *V. dahliae* showed a defoliation symptom, they decrease the number of leaves: 87 and 73 leaves were count on the olive plant inoculated respectively with *P. palmivora* and *V. dahliae* relative to the controls (480 leaves) (Table 3). Furthermore, *V. dahliae* affect the height of Dahbia olive plants: So, it decreases the height of the Dahbia olive plants (80 cm) relative to the controls (113 cm) (Table 3) (Figure A_3).

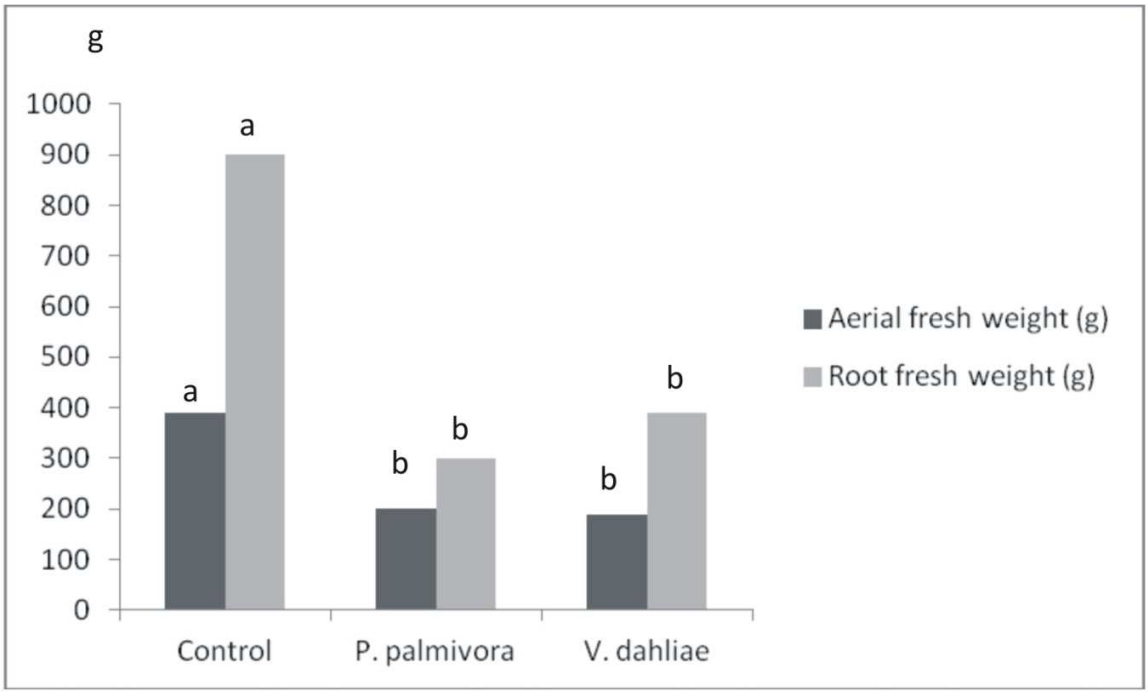
Table 3. Leaves number, height and dwarfing index of Dahbia olive plants inoculated with *P. palmivora* and *V. dahliae* using the technique 1 of inoculation

Dahbia technique 1	Leaves number	Height (cm)	Dwarfing index
Control	480[a]	113[a]	0[b]
P. palmivora	87[b]	100[a]	11.5[ab]
V. dahliae	73[b]	80[b]	29.2[a]

Numbers in the same column followed by the same letter (a, b) are significantly different at the 5% level of significance.

Using Technique 2 of inoculation, *P. palmivora* and *V. dahliae* decrease both of the aerial and root fresh weight, aerial fresh weight of the control was higher (391g) than those of Dahbia olive plants inoculated with *P. palmivora* (200g) and *V. dahliae* (189g) (Figure 3). There was no difference between the root fresh weight of Dahbia olive plant inoculated with *P. palmivora* (300g) and *V. dahliae* (391g) (Figure 3).

Fig. 3 Aerial and root fresh weight of the Dahbia olive plants inoculated with *Phytophthora palmivora* and *Verticillium dahliae* using technique 2 of inoculation.



Leaves number of Dahbia olive plants was affected after the inoculation respectively with *P. palmivora* (80) and *V. dahliae* (91) relative to the controls (403). Using the technique 2 of inoculation, *V. dahliae* showed the dwarfing symptoms on the Dahbia olive plants. Thus, it decreases the height of the olive plants (77cm) relative to the control (109cm) (Table 4).

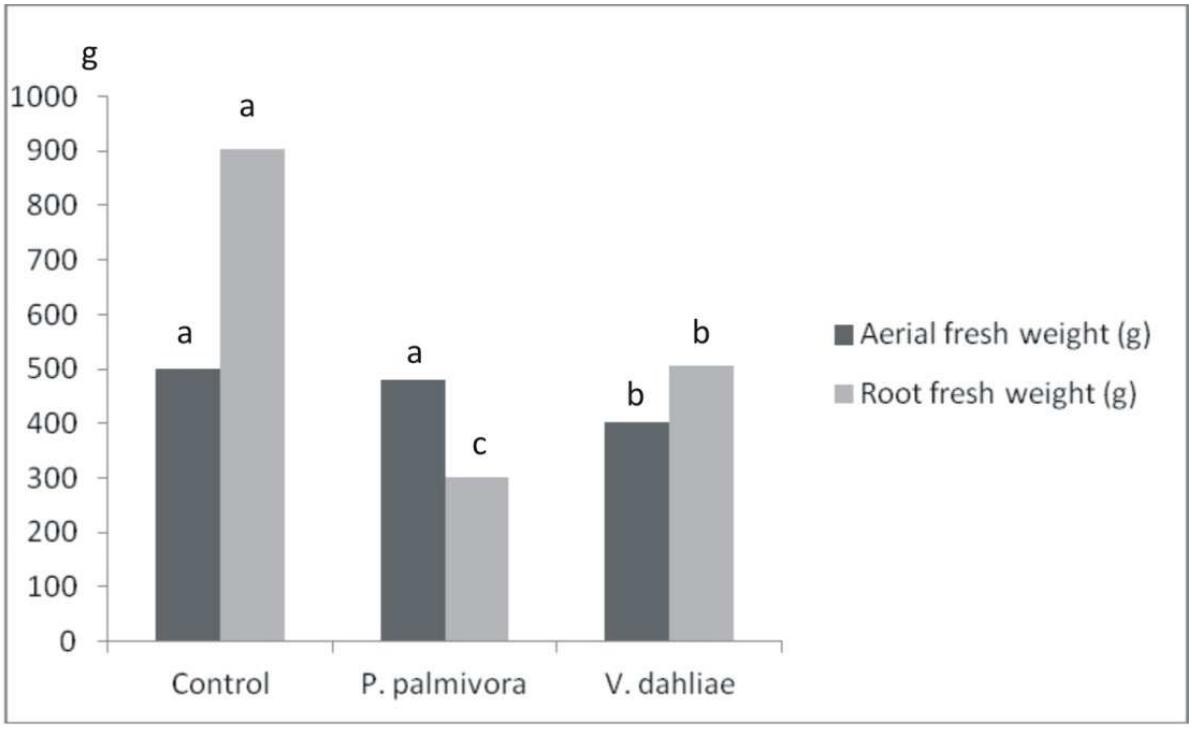
Table 4. Leaves number, height and dwarfing index of Dahbia olive plants inoculated with *P. palmivora* and *V. dahliae* using the technique 2 of inoculation

Dahbia technique 2	Leaves number	Height (cm)	Dwarfing index
Control	403[a]	109[a]	0[b]
P. palmivora	80[b]	101[a]	7.33[b]
V. dahliae	91[b]	77[b]	29.35[a]

Numbers in the same column followed by the same letter (a, b) are significantly different at the 5% level of significance.

Using the technique 1 of inoculation, *P. palmivora* affected the root fresh weight of the Haouzia olive plants (300 g) more than *V. dahliae* (505 g) which in turn had a negative effect on the root fresh weight relative to the control (904 g). The same inoculation technique showed that *V. dahliae* decrease the aerial fresh weight (401 g) relative to the control (480 g) and also showed that *P. palmivora* had no effect on the aerial fresh weight of the Haouzia olive plants (501 g) (Figure 4).

Fig. 4 Aerial and root fresh weight of the Haouzia olive plants inoculated with *Phytophthora palmivora* and *Verticillium dahliae* using technique 1 of inoculation



The results in Table 5, showed that *V. dahliae* had a negative effect on the leaves number of Haouzia olive plants (88 leaves), this number was lower than what was count on the Haouzia olive plants inoculated with *P. palmivora* (312 leaves). This last was also lower than that of the control (424 leaves) (Table 5).

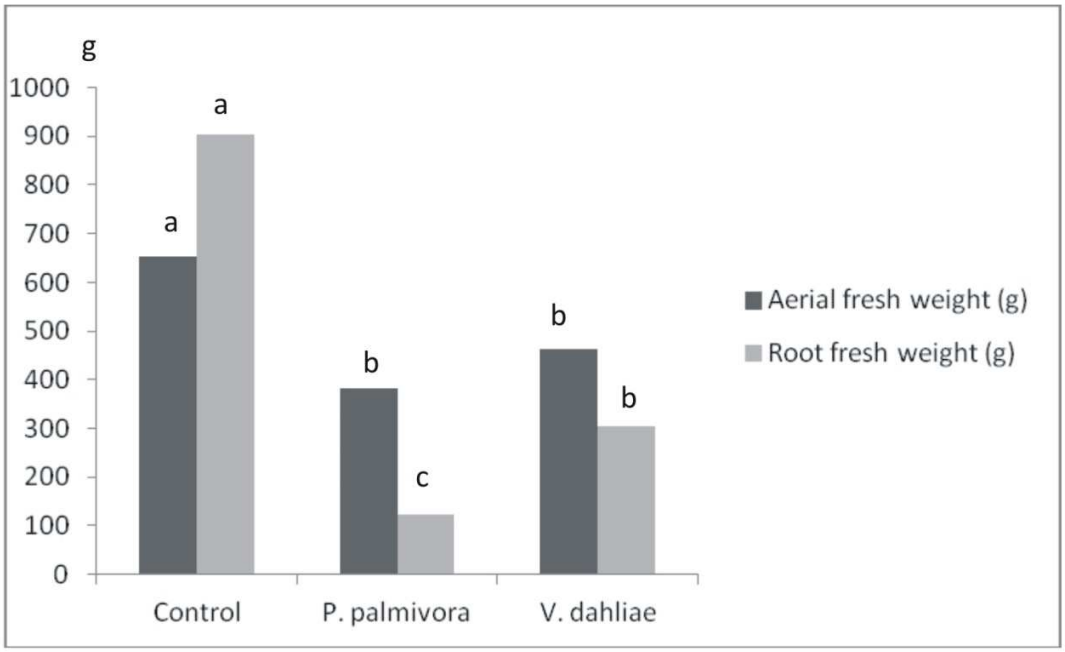
Table 5. Leaves number, height and dwarfing index of Haouzia olive plants inoculated with *P. palmivora* and *V. dahliae* using the technique 1 of inoculation

Haouzia technique 1	Leaves number	Height (cm)	Dwarfing index
Control	424^{a}	121^{a}	0^{c}
P. palmivora	312^{b}	101^{a}	16.52^{b}
V. dahliae	88^{c}	69^{b}	42.97^{a}

Numbers in the same column followed by the same letter (a, b,c) are significantly different at the 5% level of significance.

After inoculating Haouzia olive plants with *P. palmivora* and *V. dahliae* using technique 2 of inoculation, both of *P. palmivora* and *V. dahliae* affect the aerial and root fresh weight of the Haouzia olive plants. Thus, the aerial fresh weight of Haouzia olive plants inoculated with *P. palmivora* was 382 g, *V. dahliae* (462 g) compared to the control (654 g) (Figure 5). The root fresh weight was too affected after the inoculation with *P. palmivora* (124g) and with *V. dahliae* (305 g) relative to the control (904 g) (Figure5).

Fig. 5 Aerial and root fresh weight of the Haouzia olive plants inoculated with *Phytophthora palmivora* and *Verticillium dahliae* using technique 2 of inoculation



P. palmivora had a negative effect on the leaves number of the Haouzia olive plants. It decreased the leaves number (60 leaves) compared to *V. dahliae* (103) and to the control (400). Six week after inoculation with *V. dahliae* using technique 2 of inoculation, height of Haouzia olive plants were affected (85 cm) compared to *P. palmivora* (102.33 cm) and control (111 cm) (Table 6).

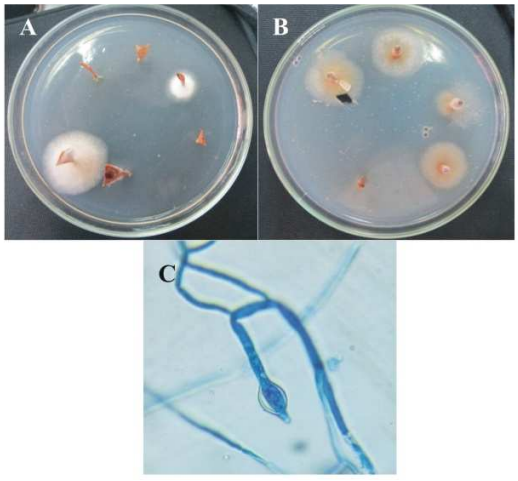
Table 6. Leaves number, height and dwarfing index of Haouzia olive plants inoculated with *P. palmivora* and *V. dahliae* using the technique 2 of inoculation

Haouzia technique 2	Leaves number	Height (cm)	Dwarfing index
Control	400^{a}	111^{a}	0^{c}
P. palmivora	60^{c}	102.33^{a}	7.81^{b}
V. dahliae	103^{b}	85^{b}	23.42^{a}

Numbers in the same column followed by the same letter (a, b,c) are significantly different at the 5% level of significance.

P. palmivora was highly isolated from the stems of Dahbia (95%) and Haouzia (83%) olive plants and it was weakly isolated from the leaves petiole of the Dahbia (33.33%) and Haouzia (22%) olive plants (Table 7) (Figure 5). *V. dahliae* was only isolated from the roots of the inoculated Dahbia (18%) and Haouzia (22.22%) olive plants (Table 7) (Figure 6).

Fig. 6 Isolation of *Phytophthora palmivora* from the petioles (A) and from stem segments (B); sporangia of *P. palmivora* (C)



Phytophthora palmivora was inoculated from different parts of Dahbia and Haouzia olive plants inoculated with this fungus (roots, stems and petiole). However, *Verticillium dahliae* was isolated from the roots of the olive plants inoculated with it (Table 7).

Table 7. Mean percentage of the isolation percentage after the appearance of decline symptoms on Dahbia and Haouzia olive plants inoculated with *P. palmivora* and *V. dahliae*

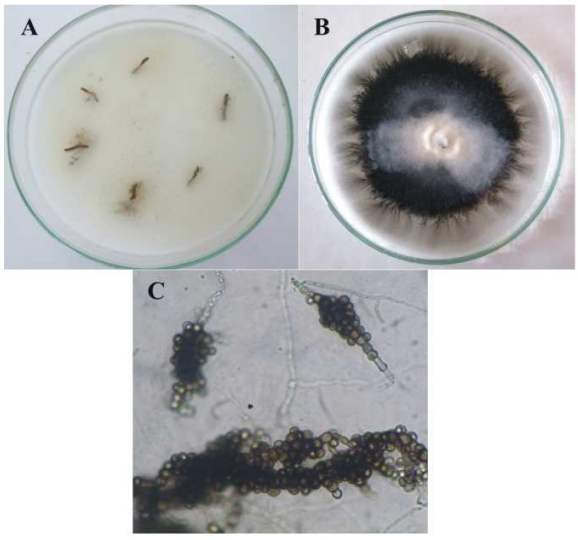
	Dahbia			Haouzia		
Different part of the olive plants	**C (%)**	**P (%)**	**V (%)**	**C (%)**	**P (%)**	**V (%)**
R.P	0^{b}	95^{a}	0^{b}	0^{b}	83^{a}	0^{b}
S.P	0^{b}	43^{a}	0^{b}	0^{b}	56^{a}	0^{b}
Pe.P	0^{b}	33.33^{a}	0^{b}	0^{b}	22^{a}	0^{b}
S.D.P	0^{b}	76^{a}	0^{b}	0^{b}	68.66^{a}	0^{b}
R.V	0^{b}	18^{a}	0^{b}	0^{b}	22.22^{a}	0^{b}
S.V	0^{b}	0^{b}	0^{ab}	0^{b}	0^{b}	0^{b}
Pe.V	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}
S.D.V	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}	0^{b}

Numbers in the same line followed by the same letter (a, b) are significantly different at the 5% level of significance. (C): Control; (V): *Verticillium dahliae*; (P): *Phytophthora palmivora*; (R): Roots; (S): Stem; (Pe): Petiole; (S.D) : Stems debris.

After measuring all these parameters, olive plants were cut on the level of the stems, Dark brown xylem discoloration in transverse section of a trunk from the olive plants inoculated with *P. palmivora* and *V. dahliae* (Figure 7).

Fig.7 Dark brown xylem discoloration in transverse section of a trunk from an inoculated olive plant by *P. palmivora* (A); xylem of the control

Fig. 8 *Verticillium dahliae* isolated from the roots of the inoculated olive plants (A); *V. dahliae* colony on the PSA plate; microsclerotia of *V. dahliae* (A)



Taking into consideration all the scoring criteria, it seems that it is the isolate of *Phytophthora palmivora* which induced more symptoms on the inoculated olive plants judging by the incubation period, leaves number, height, aerial and roots fresh weight and by the reisolation percentage. Likewise, all the olive plants of the two varieties (Haouzia and Dahbia) inoculated with P. palmivora and Verticillium dahliae have shown the decline symptoms.

DISCUSSIONS AND CONCLUSION

The obtained results indicate that both of *V. dahliae* and *P. palmivora* provoke defoliation and decline symptoms, there were some other symptoms like dwarfing on the olive plants inoculated with *V. dahliae* and root rot on the olive plants inoculated with *P. palmivora.* These obtained symptoms appeared quickly on the olive plants inoculated with *P. palmivora* than those inoculated with *V. dahliae* no matter the inoculation technique was.

Both of the tested isolates of *Phytophthora palmivora* and *V. dahliae* were pathogens on olive plants of Dahbia and Haouzia varieties. But, *P. palmivora* isolate affected the growth of olive plants more than the isolate of *V. dahliae*. It disrupts more the foliage (number of the formed leaves) as well as the root formation (estimated with the fresh weight) on the olive plants of the tested varieties.

The isolation of both pathogens was isolated two years after inoculation. The reisolation results of *V. dahliae* were positive only in the roots of the inoculated plants with this pathogen. However, *P. palmivora* was reisolated equally from the roots, stems, petiole and from vegetative debris of plants attacked with *P. palmivora*.

The olive-infecting *V. dahliae* pathotypes have been classified as defoliating (D) and non defoliating (ND) according to their ability to defoliate the tree[31]. Fallen leaves of the olive trees attacked by *V. dahliae* were found to harbour the fungus indicating their importance as a source of inoculum and in the build up of the pathogen in the soil[32]. Also, Rijkers et al.[16] *V. dahliae* formed microsclerotia in petioles of infected ash trees that could be a source of contamination of other plants. In our results *V. dahliae* was only isolated from the roots of the inoculated olive plants in the form of microsclerotia. Microsclerotia, the resting structure of *V. dahliae*, constitute the main potential infective inoculum of the pathogen in the field and persist in the soil for more than 20 years[33].

As with all cryptogamic diseases, field dissemination of olive wilting due to *P. palmivora* depends on the extent of the sites where the parasite survives or multiplies, on the abundance of primary and secondary inoculum in these sites, on factors which ensure their effectiveness as sources of contamination, and on vectors of the reproductive organs of the parasite[34,28] , The pathogen is disseminated through rain splash, insects and human activity into the canopy of trees, where symptoms appear. Secondary inoculum spreads rapidly through wind and rain splash, contact and vector activity in humid weather[35]. Muller[34] reported that the secondary inoculum of cocoa black pod caused by *Phytophthora palmivora* is triggered once the wet season begins. In the case of the olive trees, zoospores may scattering on to healthy olive roots through the impact of rain drops falling on diseased olive trees or on the ground which acts as a reservoir of the parasite[28].

Phytophthora palmivora was isolated from petiole and from the attacked olive plants debris; this results demonstrate that *P. palmivora* was able to persist two years in the debris of olive plants. This might be a source of a secondary inoculum of olive plant in the favorable season.

As with other *Verticillium* wilt diseases, xylem browning can be seen on longitudinal and transverse sections of twigs and branches affected by either of the disease syndromes[36]. The obtained results showed that *P. palmivora* was able to provoke the xylem necrosis. The vascular browning index of the infested plants was not used as an evaluation criterion of the pathogenic potential. The vascular browning was very irregular even in the most aggressive isolate[37]. Therefore, we have not been able to apply the scale of Subarao *et al.*[38], usually used to estimate the importance of the vascular browning due to the *Verticillium* wilt in other host plants.

It seems that the growth reduction, the perturbation of the leaves formation and reduction of the plants weight may constitute some discriminate criteria to distingue the aggressive, little or not aggressive isolates of *V. dahliae* and of *P. palmivora* on the olive tree. Consequently, they can be used to choice the isolates for varietal selection studies.

Acknowledgments

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