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Isolation and Characterization of Verticillium Wilt of Olive Trees in Algeria

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Abstract: Verticillium wilt of olive trees caused by the fungus *Verticilium dahliae Kleb* is one of the main vascular diseases that could cause a considerable loss of yield. However, the employed struggle methods against fungus remain limited due to its morphological diversity. The aim of the present work is the study of the morphological diversity and the identification of the pathogen fungi by a statistical principal component analysis (PCA). In order to protect the agricultural crop campaign of the olive groves during the 2010-2011 years from the region of Sidi-Bel-Abbes and Mascara, located in the north-western Algeria, has been used by the isolation of twenty isolates of *Verticilium dahlia* on Potato Dextrose agar culture medium and selected according to their morphological, cultural and biometric characteristics.Furthermore, the morphological variability between isolates was recorded through the microsclerotia density, the colour of mycelia and the appearance of aerial mycelium, which was giving an equivalent PCA of three analytical morphotypes (M1, M2 and M3), represented by seven isolates, nine isolates and four isolates, respectively.The ratio of the morphological variability by the study of three characters, such as olive variety, geography and biometric characteristics indicated the presence any distinctive relationship.

Key words: Olive trees · *Verticillium dahliae* · Morphological type · Variety · Biometric characteristics · Geography · Relationship

INTRODUCTION

Verticillium wilt caused by the soil fungi such as *Verticilium dahliae* [1] is one of the most vascular diseases causing considerable worldwide losses in the olive groves [2]. In Algeria, *Verticillium* wilt has expanded in several olive-growing regions [3-4].

The control of *Verticilium dahliae* by the using of several resistant varieties of olive trees constitutes one of the best solutions depending on its vascular nature. The establishment of such variety indicated the morphological diversity of the pathogenic fungus, where the control received more difficulties. The study of the morphological diversity was used as a simple parameter for the construction of the morphotypes [5-8] where, a statistical approach was used as a principal component analysis (PCA).

The main aim of the present work was the study of the morphocultural and biometric characteristics (dimensions of spores) of the isolated *Verticilium dahliae* from olive trees of two varieties (Chemlal and Sigo¿se varieties), planted in the region of Sidi-Bel-Abbes and Mascara. Furthermore, the different morphotypes isolates of *Verticilium dahliae* was selected by the using of the PCA similarity. Finally, the study was completed by the discovery of the relationship between the morphotype, the spores dimensions and characteristics of the fungus according to the olive trees variety and geographical origin.

MATERIALS AND METHODS

Description of the Study Area: The present study was performed in the period between March and May 2011, by two olive groves, located in the region of northwest Algeria, where an intensive olive groves has been planted and as characterized semi-arid climate and an annual rainfall about 200-410 mm (Table 1).

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Table 1: Presentation of climatic parameters of two region (Sidi-Bel-Abbes and Mascara) olive groves during 2011.

	Mascara				Sidi-Bel-Abbes			
Month	$T°_{min}$	$T°_{max}$	$T°_{mean}$	rainfall	$T°_{min}$	$T°_{max}$	$T°_{mean}$	rainfall
February	02.3	15.3	8.8	25.1	01.2	15.5	8.35	16.1
March	06.5	19.7	11.6	18.5	04.1	19.5	11.8	16.6
April	10.7	25.8	18.3	88.8	08.6	25.1	16.85	67.8
May	13.6	26.9	20.25	50.6	12.2	26.5	19.35	61.7
June	15.8	31.7	23.75	49.3	14.5	32.0	22.25	18.0

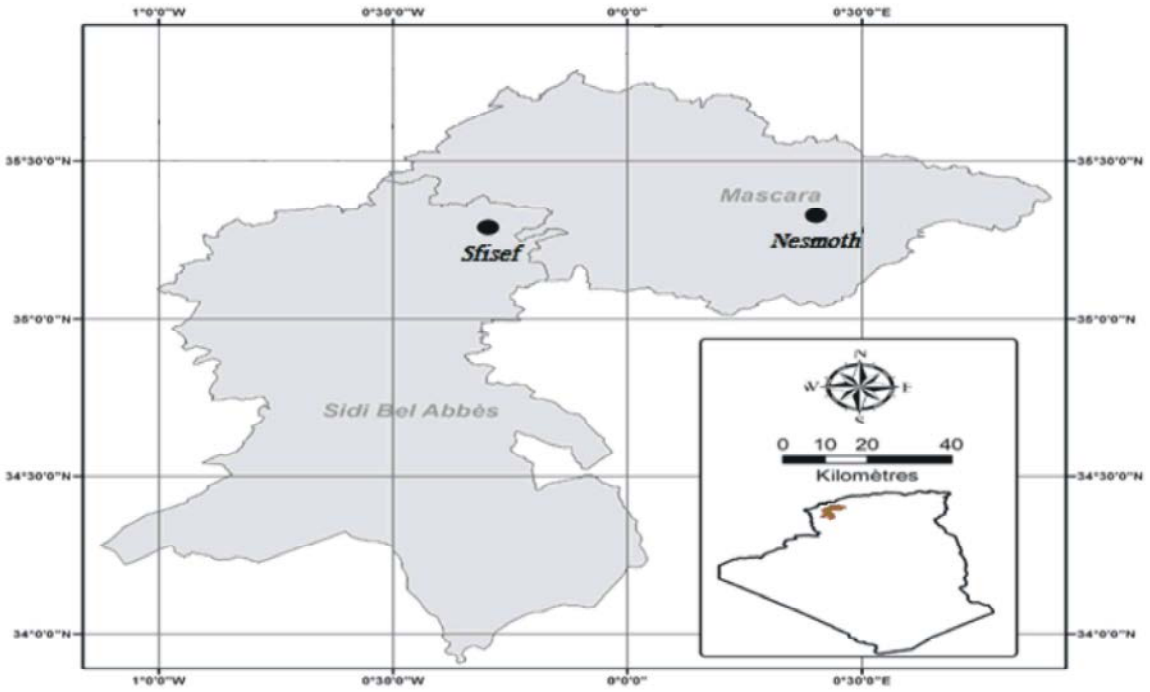

Fig. 1: Presentation of geographical location of the two prospected olive groves (Sidi-Bel-Abbes and Mascara).

The first olive grove studied was located in the region of Sfisef (Sidi Bel Abbès, Algeria), which was characterized with an altitude of 548 meters (35° 14' 04" Nord, 0° 14' 36" West). The old 6 years olive grove located on an area of 9 hectares, included 500 trees/ha (4x5m) of the planted *Chemlal* variety. The second olive grove located in the Municipality of Nesmoth region (35 km in the Southeast region of Mascara, characterized with an altitude of 1200 meters (35° 15' 00" North, 0° 23' 00" East) and covers an area of 2 hectares with old 3 years 625 trees/ha (4 x 4 m) of the planted Sigo¿se variety (Figure 1).

The investigation was based on some observation such as a symptomatic diagnostic in the presence of the disease. The branch samples were collected randomly from the infested olive trees and kept at a temperature of 4°C for 24 hours. The Isolation of *Verticillium dahliae Kleb* was used by the cutting of the xylem into small pieces; immersed in a solution containing 5% sodium hypochlorite for three minutes and the branches were rinsed for three times with sterile distilled water. The rinsed branches were dried on a sterile paper filter, where six pieces were filed on PDA culture medium containing the following components: 200 g potato, 20 g glucose, 15 g agar in the Petri boxes containing streptomycin, incubated at temperature of 25°C for 15 days in the absence of light [9]. The monitoring of the cultural variations of thalli derived from primary isolates through the obtained homogeneous isolates by monospore culture indicated that the study of morphological characters showed as the most suitable technique [10]. For this purpose, a spore suspension was streaked onto a thin layer of water agar in a Petri dish, incubated at temperature of 25°C for 4 days. The obtained thalli from the sporulation of a single spore were harvested under sterile conditions and deposited separately on PDA

culture medium. The obtained isolates was inoculated on PDA culture medium, overlayed with sterile mineral oil and kept at temperature of 4°C, whereas the obtained spore culture by transplanting of the mycelium on PDA culture medium was kept in the dark at 25°C for 15 days.

Cultural Characteristics: In order to explore the growth characteristics of the fungus, a disc of 5 mm diameter was taken from the periphery of active culture and placed in the centre of a Petri dish containing 20 ml of PDA culture medium. After that, each isolate was transplanted on the surface of solid PDA culture medium in four Petri dishes. The determination of the cultural characteristics of fungus was monitored for two weeks according to the following criteria such as radial growth and the outline of the colony, the appearance of the aerial mycelium and the production of the pigmentation and the presence of micro-sclerotia.

Microscopic Characteristics: The microscopic observation of the fungus and their micro-cultivation on a thin layer of PDA culture medium was carried out aseptically on a slide covered with a cover. For this purpose, the fungus was placed on the edges of the four sides of the cover, incubated in humid sterile room at temperature of 25°C for two to three days [11]. The micro-culture allowed the microscopic observation of spatial arrangements of conidia on conidiophores.

Determination of Biometric Characteristics: In order to explore the biometric characteristics of the fungus, a fragment was prepared from the cultivated culture, with high density of conidia, which was previously observed with microscopic. Furthermore, the fragment of the cultivated fungus was treated with drops of lactophenol solution and observed with a light microscope at 100 x objective in the presence of oil immersion. The biometric characteristic was determined by the microscopic observation of the shape, the length and the width of conidia of a sample of 20 conidia.

Identification of *Verticilium dahliae kleb*: The used micro-morphological and macro-morphological parameters for the identification of single spore of the isolated *Verticilium dahlia*, cultivated on the PDA culture medium has been established according to the described, proposed protocol by Menzies [12-14].

Statistical Analysis: The comparison of the average diameters of spores such as width and length of the isolated *Verticilium dahliae* was performed by using of the the Student *t* test ($p<0.05$). The principal component analysis used in this study was the description of the cultural characteristics such as mycelia colour, aerial mycelium and the density of micro-sclerotia).

RESULTS

The prospection of the agricultural crop campaign of the olive groves from the region of Sidi-Bel-Abbes and Mascara, located in the north-western Algeria, showed the apparition of some wilting in young olive trees, fading branches with a brown-purple bark, defoliation and shoots of young branches, browning and gutter rolling.

The obtained indicated the of twenty isolates *Verticilium dahlia* on Potato Dextrose agar culture medium and selected according to their morphological, cultural and biometric characteristics, where some difficulties by the isolation of *Verticilium dahliae* has been presented, followed by the reduction of isolation rate. The isolation *Verticilium dahlia* on PDA culture medium after seven days incubation revealed the development of white subaerial cottony mycelia. However, the produced white color of mycelium was developed after two weeks in black color, due the appearance of micro-sclerotia and the formation of a small black particle in the center of the colony. The isolated twenty single spore isolates on PDA culture medium from the collected sample from the olive trees of both region of Sfisef and Nesmoth revealed the presence of a circular colonies with an aerial mycelium with a variable micro-sclera density and a black pigmentation of mycelial (Table 2).

The use of the initial classification according to the described basis indicated the presence four types of colonies, where seven isolates appeared milky white with abundant aerial mycelium, thick and dense with smooth cottony aspect and the contour of the colony was regular. The micro-sclerotia of the isolates were produced in late phase very weakly.

A four grayish white isolates with flaky and relatively dense aerial mycelium and irregular contour has been showed, where the isolates was produced micro-sclerotia in late phase.

Furthermore, three irregularly-shaped black and white isolates with cottony aspect characterized by the presence of the white and dense aerial mycelium in the center of the colony due to the intense production of micro-sclerotia. At last, six greyish black isolates, with aerial mycelium, melanised by the abundant production of micro-sclerotia, characterised by the presence of an irregular front growth.

Table 2: Determination of cultural and biometric characters of twenty isolates *Verticilium dahliae* collected from the olive trees of Algerian

Isolates	Cultural traits			Spore dimensions in microns	
Code	Colour of the mycelium	Aerial mycelium	Density of microsclerotia	Length	Width
Vdm 1	milky white	Abundant	Sparse	4, 38	2, 15
Vdm 2	greyish black	Ras	Dense	4, 49	2, 11
Vdm 3	greyish black	Ras	Dense	4, 95	2, 3
Vdm 4	milky white	Abundant	Sparse	3, 76	1, 56
Vdm5	white and black	Abundant	Medium	4, 56	2, 01
Vdm 6	greyish black	Ras	Dense	3, 50	1, 66
Vdm 7	milky white	Abundant	Sparse	4, 50	2, 20
Vdm 8	greyish white	Medium	Sparse	4, 85	2, 00
Vdm 9	milky white	Abundant	Sparse	4, 30	2, 12
Vdm 10	milky white	Abundant	Sparse	4, 38	2, 04
Vds 1	greyish white	Medium	Sparse	4, 96	2, 40
Vds 2	white and black	Abundant	Medium	4, 50	1, 88
Vds 3	greyish black	Ras	Dense	4, 15	2, 05
Vds4	milky white	Abundant	Sparse	3, 85	1, 80
Vds 5	greyish black	Ras	Dense	3, 91	1, 90
Vds 6	greyish white	Medium	Sparse	4, 32	2, 01
Vds 7	white and black	Abundant	Medium	4, 95	2, 25
Vds 8	greyish white	Medium	Sparse	4, 50	2, 24
Vds 9	greyish black	Ras	Dense	4, 45	2, 2
Vds 10	milky white	Abundant	Sparse	4, 20	1, 90

(Isolates Code: Vdm: *Verticilium dahliae* isolated from Mascara, Vds: *Verticilium dahliae* isolated from Sidi-Bel-Abbes)

The microscopic observation showed that all isolates produce whorled conidiophores, bearing in turn whorled phialides, inserted by three to four with rounded conidia, which mucilage globules (spherule). Furthermore, conidia showed hyaline, unicellular, with length and width varied from 3.76-4.96 and 1.56-2.40 µm respectively (Table 2).

The microscopic observation of the old culture indicated the presence of enlargement and a partitioning of some parts of the mycelium, followed by an apparent swelling, a thickening of the walls with impregnated blackish pigment (melanin). Furthermore, a new hyaline cell mass was budded and the formed dark brown pigmentation was transformed into micro-sclerotia.

The observation on the regional organization and west contingency of the isolated *Verticilium dahlia* through crosses cultural characters such as aerial mycelium, contour, density, micro-sclerotia density and mycelium color was presented in Table 3.

The investigation of the aerial mycelium color indicated the presence of abundant color for WB isolates, intermediate for GW, crew for GB isolates and plentiful for MW isolates.

Furthermore, the formed micro-sclerotia was dense for GB, intermediate for WB isolates and sparse for MW and GW isolates. Where, the contour shape is regular for WB, GW and MW isolates and irregular for GB isolates (Table 3). Therefore, the irregular contour of GB isolates indicated the variability of the mycelium colours. The combination of two cultural characters such as aerial mycelium and micro-sclerotic density, demonstrated that high aerial mycelium density of isolates was associated with a low density of micro-sclerotia (Table 4). Whereas, the observations of the regional organization of contingency that crosses cultural characters such as aerial mycelium, contour, density, density of micro-sclerotia and mycelium colour showed that an important correlation matrices between the regional organization and West (Nesmoth or Sfisef) (Table 4).

The strongest correlations on the west map concerns the WG and MW isolates (0.93), especially at the regional level, where Vds of GW isolate and Vdm/Vds of MW were (0.98/0.86) respectively. Furthermore, the GB isolate manifested a significant negative correlation coefficients with the WB (-0.6), with MW (-0.67) and with BG (-0.59).

The correlations of the regional organization were significantly positive for the same mycelial color of the isolated *Verticilium dahliae* in Nesmoth (Vdm) and Sfisef (Vds): WB (1.00), MW (0.94), BG (0.74) and GB (0.98) respectively. Simultaneous illustration of all relationships can only be carried out through correspondence analysis.

The total West inertia (64.07+30.36 = 94.43) is higher than the total regional inertia (60.19+28.22=88.41). Thus, variability and the level of similarity is best expressed if isolates are grouped together regardless of their isolation website (West: Nesmot and Sfisef).

Table 3: Regional organisation and west contingency of the isolated *Verticilium dahlia*

		Colour of the mycelium											
		Regional organization											
		WB		MW		GW		GB NG		West organization			
		Vdm BN	Vds BN	Vdm BL	Vds BL	Vdm BG	Vds BG	Vdm NG	Vds NG	BN	BL	BG	NG
aerial mycelium	Abundant	1	2	1	1	0	00	0	0	3	2	0	0
	Medium	0	0	4	1	1	3	0	0	0	5	4	0
	Ras	0	0	0	0	0	0	3	3	0	0	0	6
Shape	Regular	1	2	5	2	1	3	0	0	3	7	4	0
	Irregular	0	0	0	0	0	0	3	3	0	0	0	6
Density of microsclerotia	Dense	0	0	0	0	0	0	3	3	0	0	0	7
	Medium	1	2	0	0	0	0	0	0	3	0	0	0
	Sparse	0	0	5	2	1	3	0	0	0	7	4	0

(Mycelial colour code: White and Black (WB BN); greyish white (GW BG) Milky white (MW BL); greyish Black (GB NG)

Table 4: Regional and west contingency of *Verticilium dahliae* isolates.

	Regional organization								West organization				
	Vdm BN	Vds BN	Vdm BL	Vds BL	Vdm BG	Vds BG	Vdm NG	Vds NG		BN	BL	BG	NG
Vdm BN	1, 00								BN	1, 00			
Vds BN	1, 00	1, 00							BL	0, 1	1, 00		
Vdm BL	0, 04	0, 04	1, 00						BG	-0, 02	0, 93	1, 00	
Vds BL	0, 23	0, 23	0, 94	1, 00					NG	-0, 6	-0, 67	-0, 59	1, 00
Vdm BG	0, 15	0, 15	0, 69	0, 52	1, 00								
Vds BG	-0, 07	-0, 07	0, 98	0, 86	0, 74	1, 00							
Vdm NG	-0, 59	-0, 59	-0, 65	-0, 69	-0, 44	-0, 59	1, 00						
Vds NG	-0, 60	-0, 60	-0, 66	-0, 70	0, 45	-0, 60	0, 98	1, 00					

(Mycelial colour code: White and Black (BN); greyish white (BG) Milky white (BL); greyish Black (NG)), bold correlations are significant at $p<0.05$

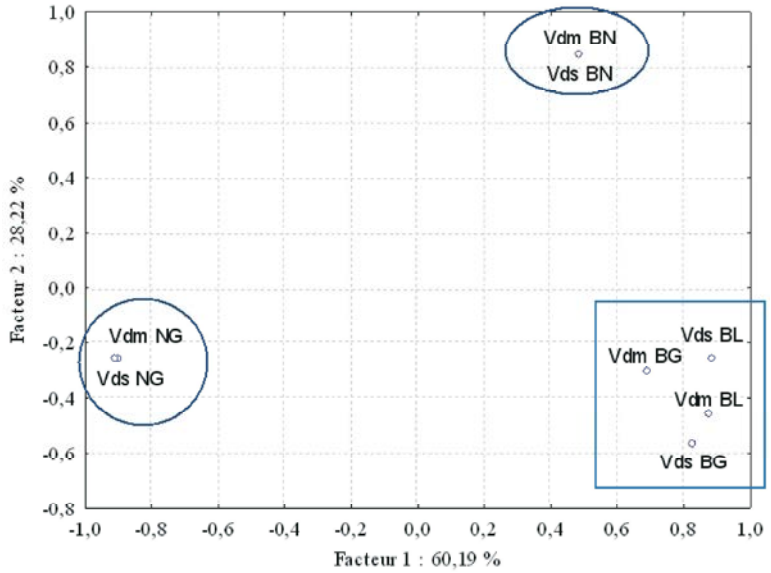

Fig. 2: Analysis of regional correspondence of *Verticilium dahliae* isolates

The obtained results by the using of the geographical location did not indicate at best the morphotypical distribution, which joined the descriptive results of the regional profile discussed previously. Furthermore, the analysis of the western correspondence, which was divided into three "sub-cloud" corresponding to three groups of dissimilar isolates showed two main axes represented by 64, 07 % and 30, 36 % of the total inertia.

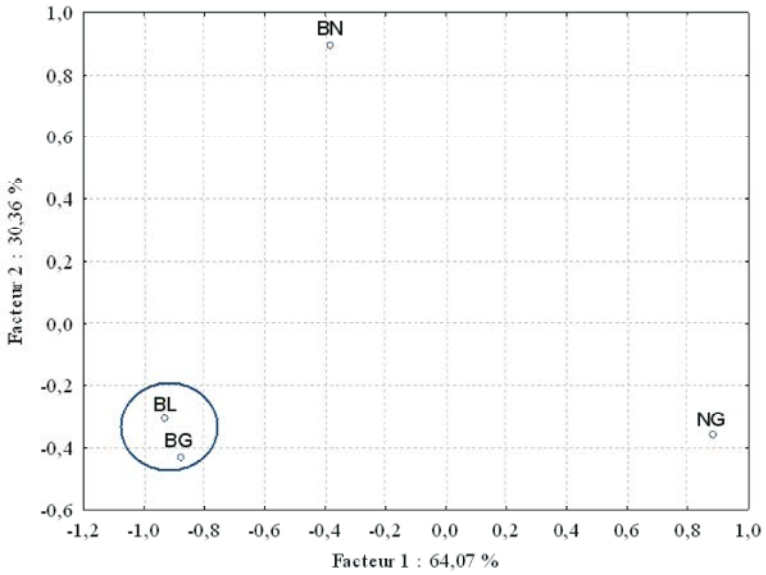

Fig. 3: Analysis of west correspondence of *Verticilium dahliae*

Where, the first axis of greyish black isolates (GB) opposed to the rest of the mycelium colors (negative correlation coefficient).

The second axis opposed the black and white isolates (WB) of the milky white group (MW) and greyish white (GW) (correlation coefficient nearer zero: respectively, 0.1 and 0.02). According to the correspondence analysis, the study of the morphotypical of the twenty isolated *Verticilium dahlia* was identified three analytical morphotypes, which presented several common features. The Morphotype class 1 showed a greyish black mycelium isolates (NG) and characterized by production of a dense micro-sclerotia and irregular contour. The second class of Morphotype 2 presented a black and white mycelium isolates (BN) and characterized by the production of an intermediate micro-sclerotic density and abundant aerial mycelium. The third class of Morphotype 3 presented a milky white mycelium (BL) and greyish white (BG) isolates and characterized by the production of a sparse micro-sclerotic density. Finally, the morphotypical profile statement described the presence of three morphotype isolates in both olive groves of Nesmoth and Sfisef and the same for the two varieties (*Chemlal* and *Sigo¿se*). The Student *t* test compared the averages of measurements (width and length) of the isolates did not show any significant difference ((*p*=0.01).

DISCUSSION

The study of Verticillium wilt of olive trees in Algeria caused by the fungus *Verticilium dahliae* is of crucial importance in the Algerian arboriculture. The in vitro morphological variability of this fungus has been the subject of intense research for several decades [15].

Although a huge progress has been done for the comprehension of the morphocultural characters of the fungus, the interpretation of the molecular mechanisms of the morphological instability remained unclear [16].

The obtained results by the prospection of the olive groves in Sfisef and Nesmoth "Northwest Algeria" revealed the presence of typical symptoms of Verticillium wilt; such as withering, defoliation, browning and leaf drying, with clearly noticeable symptoms during the spring season, when the soil moisture augmented and the temperature decreased and the germination of fungal micro-sclerotia in the olive tree roots.

In early study, Benchabane, Bellahcene and co-workers has reported the same symptoms on olive trees in Algeria [3-4]. Furthermore, the isolation this fungus from diseased plant tissue was often encountered [17-18]. The encountered difficulty could be explained by the environmental conditions such as hot weather during spring season, the type of vascularization of olive variety (Sigoïse and Chemlal) and the density of the pathogen in the xylem of the tree [2, 19-20].

The obtained results of the cultural, morphological and biometric characteristics indicated the typical morphocultural description of *Verticilium dahlia* [21-22]. The use of macro-morphological parameter for the identification of *Verticilium dahlia* indicated a variability in micro-sclerotia density (sparse, intermediate and plentiful), in the mycelial color (milky, grayish-white, white

and black, grayish black), in the appearance of the aerial mycelium (ras, intermediate and plentiful) and in contour (regular and irregular). Korolev and co-workers have observed the presence of the cultural differences [23-26]. Furthermore, Pegg and co-workers [6] have reported the distribution of the isolated *Verticilium dahlia* by several analytical morphotype PCA and described two *Verticilium dahlia* morphotypes, the black and the white form. Furthermore, Bellahcene has described three *Verticilium dahlia* morphotypes: hyaline, wild and intermediate. Jabnoun and co-workers has described four morphotypes [7]. The presence of the three isolated *Verticilium dahlia* isolated morphotypes in both Nesmoth and Sfisef olive groves indicated that the geographical location was not necessary for the morphotypical determination of the fungus.

The obtained results suggested a random morphological distribution of *Verticilium dahlia* in the Algerian orchard, where, the distribution is probably due to a genetic homogeneity of the isolates of the neighbouring localities, the spread of the pathogen via the micro-sclerotia (Sfisef) induced the initiation of new infection in the others localities (Nesmoth). The presence of three isolated *Verticilium dahlia* morphotypes despite the varietal olive *Chemlal* in Nesmoth and *Sigo¿se* in Sfisef, showed that the morphotype of fungus was not associated with the olive variety.

Bellahcene and co-workers [5] has reported the absence of such relationship on the isolated *Verticilium dahlia* from the same Algerian olive varieties. Jabnoun and co-workers [7] has reported that the same isolated morphotypes from different host plants might show varying morphology and aggressiveness against the same plants. Carder & Barbara [27] has also reported the absence of the correlation between *Verticilium dahlia* and host plants.

The micro-morphological identification of the isolated *Verticilium dahlia* revealed the presence of conidia with dimension ranges (length and width) [4, 8, 26].

The homogeneous spores' dimensions detected in the three isolated *Verticilium dahlia* morphotypes revealed that the spore size does not characterize the fungerus morpho-type.

Darai and co-workers [26] has reported the absence of morphotypes for the spore dimensions ratio, where no significant difference by the measure of width and length of the spore and and the size of the fungus micro-sclerotia.

The obtained results from the geographical olive grove, the variety of olive tree and the size of spores do not provide any indication about the morphotypical identity of the *Verticilium dahlia*. However, Lopez and co-workers [2] *et al.* (2005) has reported that the virulence has a relationship with the morphology of *Verticilium dahlia,* on culture medium and in particular with micro-sclerotia morphology [28, 2]. Furthermore, Lopez and co-workers [2] has reported a considerable morphological variation of *Verticilium dahlia* micro-sclerotia isolated from the same soil sample. According to the described study reported by Tolmsoff [15], the micro-sclerotia was the headquarters of the morphological conservation and variable production that induced the morphological variables in the *Verticillium* genus.

Darai and co-workers has reported [26] the presence of a polymorphism in the micro-sclerotic DNA extracted from the isolated *Verticilium dahlia* and induced by a variable activation of gene for pigment synthesis (melanogenic) and micro-sclerogenic.

From the present study, it was emerged that *Verticilium dahlia* has some variations that could affect the morphogenesis characteristics such as thalli growth and sclerogenic, where the molecular mechanisms has explained that the variability was not clear [29]. The asexual mode of reproduction by the isolated of *Verticilium dahlia*, the spontaneous mutations could be a source of genetic variation and further morphological differences [30]. The mitotic recombination via the para-sexual cycle or transposable genetic elements observed by the isolated of *Verticilium dahlia* could also lead to variations [31, 32, 30]. Nevertheless, artificial mutagenesis or the transformation by the insertion of DNA fragments could be induced a probable genetic variation [33]. The take in consideration of the followed probable explanations could be predicted that the isolated of *Verticilium dahlia* from olive trees in Algeria presented a common origin with a homogeneous genetic and then evolved as under-heterogeneous population as reported by Bellahcene [8]. The limitation of the present study lies in the reduced number of the isolated fungi (20 isolates) collected from two olive groves. Furthermore, the present result may give a good support for further investigation such as studying physiological study, pathogenesis and molecular aspects of the isolated *Verticilium dahlia* populations from Algerian olive groves and olive varieties.

CONCLUSION

A population of twenty isolates of *Verticilium dahlia* in olive groves from Algeria was identified based on morphological, cultural and biometric characters. However, joining the morphocultural identification to the molecular identification is excessively necessary.

The morphological heterogeneity within this *Verticilium dahlia* population has resulted in three PCA analytical morphotypes clearly dissimilar. The distribution of these morphotypes was not connected to the geography of the olive grove, to the host olive variety and to the spore's size. The morphological study of *Verticilium dahlia* brings up some relatively individualized morphology isolates in the third morphotype, which gives way to the emergence of new morphotype not previously reported. The Algerian population of *Verticilium dahlia* could serve as preliminary fungal material for variety selection programs.

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