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RESEARCH PAPERS

Vegetative compatibility and pathogenicity of *Verticillium dahliae* isolates from olive (*Olea europea*) in Morocco

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Summary. Vegetative compatibility, determined by pairing auxotrophic mutants that are unable to utilize mineral nitrogen, was studied by means of 44 isolates of *Verticillium dahliae* Kleb. Thirty-seven isolates were collected from olive in the main areas in Morocco, 3 from Algerian olives, 2 from tomato, 1 from aubergine and 1 from olive-grove soil where Verticillium wilt was present. Approximately 475 mutants were used, allowing assignment of 34 isolates to 3 vegetative compatibility groups (VCGs). The VCGs of the remaining 10 isolates were not defined. The pathogenicity of ten isolates was assessed by inoculating olive plants with suspensions of conidia at 10^5 conidia ml^{-1}. Highly significant differences in the pathogenic ability of the ten isolates were recorded. No relationship was found between VCGs and pathogenicity of isolates.

Key words: VCG, heterokaryon, pathogenicity.

Introduction

Verticillium wilt threatens olive-growing in several Mediterranean Basin countries (Saydam and Copcu, 1972; Vigouroux, 1975; Caballero *et al*., 1980; Ligoxigakis and Vakalounakis, 1994; Tosi and Zazzerini, 1998). In Morocco, the disease was first noted in the region of Beni Mellal in 1979 (Benjamaa, personal communication). It is now widespread in the main olive-growing belt, where it causes serious damage (Serghini and Zeroual, 1995; Lachqer and Sedra, 1996). *Verticillium dahliae* is a major vascular-wilt pathogen. It attacks more than 160 woody and herbaceous plant species (Schnathorst, 1981). Apart from a few isolates which exhibit strong host specificity (Puhalla and Bell, 1981), most isolates are adapted to numerous host plants.

The lack of host specificity among *V. dahliae* isolates (Schnathorst, 1981) makes their classification into sub-species or *formae speciales* difficult. Moreover, characterization of pathotypes by means of pathogenicity tests is problematic. It must be complemented with other criteria such as optimal temperature of mycelium growth, germination of conidia, microsclerotia and conidia production (Willie and Devoy, 1970). In the case of olive, the greenhouse tests of pathogenicity are considered "delicate" and time consuming (Vigouroux, 1975). Therefore, other techniques such as vegetative compatibility (VC)

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are preferred. Based on heterokaryon formation among compatible isolates, VC is commonly used to study the population dynamics of both pathogenic and non-pathogenic fungi (Corell *et al*., 1987 and 1988; Joaquim and Rowe, 1990 and 1991; Dossa *et al*.**,** 1991; Elias, 1991; Strausbaugh, 1993; Chen, 1994; Daayf, 1995; Katan and Shabi, 1996; Bao *et al*., 1998; Van Heerden and Wingfield, 1998; Elena, 2000). Within the *Fusarium* species, for example, vegetative compatibility groups (VCGs) allow characterization of *formae speciales* and pathogenicity groups, thereby facilitating study of genetic diversity (Puhalla, 1985; Jakobson and Gordon, 1988; Elmer and Stephens, 1989; Djerbi, 1990; Tantaoui and Boisson, 1991; Fernandez *et al*., 1994; Katan *et al*., 1996). As regards *V. dahliae,* numerous studies have shown that vegetative compatibility analysis may serve as a useful tool to differentiate the strains (Puhalla, 1979; Puhalla and Hummel, 1983; Strausbaugh, 1993; Bao *et al*., 1998; Elena and Paplomatas, 1998). Nevertheless, it is not easy to evaluate relationships among the isolates. Previous studies on this issue gave variable results. The first study on vegetative compatibility using morphological mutants led to classifying 19 isolates into 4 VCGs (Puhalla, 1979). Puhalla and Hummel (1983) subsequently identified 16 VCGs within 86 isolates from different hosts collected in various countries. Joaquim and Rowe (1990), using non mineral nitrogen assimilating mutants (*nit*), found only 4 VCGs within 22 isolates. In contrast, while Puhalla and Hummel (1983) claimed to have identified 15 VCGs, Strausbaugh (1993) showed that VCG 4 of Joaquim and Rowe (1990) was divided into 9 sub-groups.

However, the vegetative compatibility of olive isolates has not been studied. Thus the object of the present study was to investigate genetic diversity in a population of *V. dahliae* isolated mainly from olive, and to explore relations between pathogenicity and the VCGs, with the aim of devising an alternative to greenhouse pathogenicity tests.

Materials and methods

Isolate origin

The study was based on 44 isolates of which 37 came from olive in the main olive-belt in Morocco, 2 from tomato, 1 from aubergine, 1 from soil and 3 from Algerian olive (Table 1). One monoconidial culture was prepared from each isolate and used in this study.

Selection of mutants for mineral nitrogen assimilation (*nit* mutants)

Nit mutants were obtained according to the technique developed by Cove (1976) for *Aspergillus nidulans* and adopted by Puhalla (1985) for *Fusarium oxysporum* and by Joaquim and Rowe (1990) for *V. dahliae*. For each isolate, 10 fragments of the mycelium from young colonies growing on potato-dextrose agar (PDA) were transferred to the Correll *et al*. (1987) minimal medium amended with potassium chlorate (MMC). The potassium chlorate ($KClO_3$) concentration was enhanced (from 20 to 35 and 45 g l^{-1}) each time refractory isolates were detected, in order to produce *nit* mutants. Isolates that did not yield *nit* mutants despite the highest dose of $KClO_3$ were cultured on Czapek-Dox medium amended with 50 mg l^{-1} of rose bengal and 30 g l^{-1} of $KClO_3$ (Elias and Cotty, 1995). Cultures were incubated at 25°C in a dark room. The mutants were selected after three-week incubation. Two selection methods were compared using 2 different isolates drawn at random among 38 isolates at the start. In the first method, mycelium fragments (about 2 mm^3) from chlorate-resistant sectors were transferred to minimal medium (MM; Puhalla, 1985). Three fragments were placed on each plate. In the second method, conidia were used instead of mycelium fragments. The suspension of conidia was obtained by shaking cultures in sterile water, followed by adjusting with a hemacytometer to 10^2 conidia ml^{-1}. One hundred µl of this suspension was plated in a Petri dish containing MM. Three replicates of 10 plates each were used for each method. The test was repeated twice. Results were submitted to analysis of variance. Conidia and fragments that produced short and hyaline mycelium were identified as *nit* mutants.

Characterization of *nit* mutants

Roughly 2 mm^3 pieces from *nit* mutants were plated on MM medium with either sodium nitrate at 2 g l^{-1}, sodium nitrite at 0.5 g l^{-1}, or hypoxanthine at 0.2 g l^{-1} as nitrogen source (Correll *et al*., 1987). After a week, the *nit* mutants phenotype *nit*1, *nit*3 and *nit*M were determined according to their reaction on the 3 media (Correll *et al*., 1987 and 1988). At the end of the experiment, isolates that did not give *nit*M mutants were also cultured twice on MMC at 45 g l^{-1} of $KClO_3$ and on the medium of Elias and Cotty (1995) to increase the chances of obtaining these mutants.

Table 1. Isolates of *Verticillium dahliae* used in this study, their host and geographic origin, year of isolation and number of different nitrate-nonutilizing (*nit*) mutants generated.

Isolate	Site	Country	Host	Year of isolation	*nit*1 mutant	*nit*3 mutant	*nit*M mutant	«crun»
V57	Tamellalet (El Kelâa of Sraghna)	Morocco	Olive	1994	14	3	0	1
V72	„	„	„	1995	10	0	0	8
V84	„	„	„	1995	2	0	0	2
V105	„	„	„	1996	6	2	0	3
V122	„	„	„	„	0	0	0	0
V156	„	„	„	„	0	0	0	0
VS	Ataouia (El Kelâa of Sraghna)	„	„	„	7	0	2	9
94	„	„	„	„	0	0	0	0
44	„	„	„	„	1	0	0	3
74	„	„	„	„	10	2	2	4
65	„	„	„	„	6	0	2	0
81	„	„	„	1996	0	0	0	0
VB2	„	„	„	„	6	0	3	2
147	Mhamdia (Marrakesh)	„	„	„	0	0	0	0
M4	Souihla (Marrakesh)	„	„	1995	2	0	0	1
S33	„	„	„	„	10	0	0	9
S41	„	„	„	1997	8	0	0	3
V111	„	„	„	„	4	0	0	6
V62	„	„	„	„	7	2	0	3
V92	„	„	„	„	1	0	0	2
R11	„	„	„	„	4	0	2	3
R21	„	„	„	„	7	1	2	3
R41	„	„	„	„	5	0	0	1
R32	Aghmat (Marrakesh)	„	„	1997	4	0	0	3
Br22	„	„	„	„	9	0	0	0
Br57	„	„	„	„	8	1	3	5
RK12	„	„	„	„	10	2	0	2
RK24	Chouitre (Marrakesh)	„	„	„	8	0	0	2
Ar4	Marrakesh city	„	„	„	4	0	3	4
A2	Tamzegleft (Marrakesh)	„	„	1995	5	0	2	10
TZ1	Marrakesh city	„	„	1996	3	0	2	0
C3	Mghilia (Beni Mellal)	„	„	1995	6	0	3	10
MG3	Oulad Ayag (Beni Mellal)	„	„	1997	4	0	0	4
B32	„	„	„	„	6	0	0	2
B14	Aïn Taoujdat (Meknes)	„	„	1995	8	1	3	8
AT16	„	„	„	„	9	1	2	7
AT21	„	„	„	„	9	3	0	3
AT32	Algeria	Algeria	„	1996	14	0	0	5
V11	„	Algeria	„	„	6	3	0	8
V21	„	Algeria	„	„	10	2	2	4
V34	Soil of Tamellalet	Morocco	Soil	„	0	0	0	0
P32	Laboratory of Pathology, University Mohamed V, Rabat	„	Tomato		11	0	4	3
D2 (R44)	Laboratory of Botany, University Iben Tofail, Kenitra	„	„		8	0	3	5
225	„	„	Aubergine		10	1	0	1

Pairings of mutants

To determine the VCGs, 556 pairings between *nit* mutants were carried out. The pairings occurred between *nit*1 and *nit*M. Mutants *nit*3 were not used. *Nit* mutants derived from each isolate were paired in Petri dishes containing MM (Puhalla, 1985; Correll *et al*., 1987). Conditions of incubation were the same as mentioned above. The results were evaluated based on the presence or the absence of heterokaryons in the contact zone of mutants.

Evaluation of isolate pathogenicity

For this study, 10 of the 44 isolates were chosen at random: V57, V62, V72, V84, V156, MG3, AT16, P32, TZ1 and S33. Six-month-old Moroccan Picholine olive trees were used. The plants were carefully uprooted and immersed for about 3 minutes in a suspension of conidia adjusted to 10^5 conidia ml^{-1}. The plants were then potted in plastics and placed in a greenhouse with a 12-hour day and temperatures ranging from 25 to 30°C. The roots of control plants were dipped in sterile water. For each isolate, 24 plants divided into 3 groups of 8 plants were used. Isolate aggressiveness was assessed as the percentage of plant mortality over three months. Results were processed with analysis of variance and the mean values compared using the Newman and Keuls test at 5%.

Results

Selection of *nit* mutants

No mutants were obtained on MMC amended with 20 g l^{-1} of $KClO_3$. Twenty-seven isolates developed mutants on MMC with 35 g l^{-1}, and 9 among the 17 resting on MMC at 45 g l^{-1}. Only 2 isolates out of the 8 remaining yielded mutants in presence of rose bengal. The 6 isolates (44, V122, V156, VB2,VS and M4) that did not produce mutants on these media were not used in this experiment.

Mycelium fragments from chlorate-resistant sectors were more effective than single conidia culture. About 63% of the mycelium fragments grown on MM yielded short and hyaline mycelium characteristic of *nit* mutants, while only 11% of the conidia gave *nit* mutants. The second method was subsequently abandoned.

Approximately 475 mutants were obtained from the first method. Their characterization revealed that 55.1% were *nit*1, 5% were *nit*3 and 8.4 *nit*M. The remaining 31.3% were "crun" types. Mutant number varied from 1 to 20 according to the isolates (Table 1).

VCG characterization

There is vegetative compatibility between two isolates when a thick aerial mycelium forms in the contact zone between them, indicating heterokaryon formation. This appeared, in general, after 5 days of incubation. In this study only 3 VCGs (001, 002 and 003) were identified among 34 isolates. The vast majority (31 isolates) were assigned to VCG001 including 27 isolates from Moroccan olive, 2 from Algerian olive, 2 from tomato, 1 from aubergine and 1 from infected soil. Isolates V105 and C3, from Moroccan olive, were assigned to VCG002 while AT16, also from Moroccan olive, was the only representative of VCG003 (Table 2). These VCGs were interlinked by intermediate isolates: VCG001 was linked to VCG002 by isolate A2 and to VCG003 by isolate Tz1. The VCGs of 4 isolates (V57, V21, S41 and RK24) were not determined because isolates S41, RK24 and V21 did not pair with any others. Heterokaryons were thin and poorly developed with some V21 pairings and absent for all RK24 and S41 pairings. Mycelium of V57 reverted to its initial aspect (thick and cottony) on MM, making it impossible to distinguish any heterokaryon.

Assessment of pathogenicity

All isolates tested were found to be pathogenic, at various levels of aggressiveness to olive. All but one (S33) isolates induced leaf browning followed by withering and finally leaf fall. Ultimately, some of the withered plants died.

A continuum of aggressiveness ranging from slight (0%) to high (100%) mortality was observed among isolates (Fig. 1). Classification of the mean values according to the Newman-Keuls test at 5% enabled at least four homogenic isolate groups to be distinguished (Fig. 1).

Isolate S33 inoculated plants showed partial defoliation without either previous browning or withering and subsequent weak bearing. All plants remained alive. It should be noted that in the field, plants infected by isolate S33 exhibited only leaf yellowing and a reduction of foliage density in contrast to plants infected with the other fungal iso-

Table 2. Results of pairing on a minimal medium between nitrate-nonutilizing (*nit*) mutants of *Verticillium dahliae*.

VCG[a]	Isolate	VCG001														VCG 002	VCG 003
		94	MG	147	D2	P32	R21	81	R41	AT21	RK12	A2[b]	65	Tz1[c]	V34	C3	AT16
VCG001	94	+	+	.	.	.	.	.	.	.	.	-	-	.	-	-	-
	MG	+	+	.	.	.	.	.	.	.	.	-	-	.	.	-	-
	147	+	+	+	.	.	.	.	.	.	.	-	-	.	.	-	-
	Tz1	+	+	+	+	+	-	-	-	.	-	-	-	**+**	-	-	**+**[b]
	D2	+	+	+	+	.	.	.	.	.	.	+	-	+	.	-	-
	P32	+	+	+	+	+	.	.	+	.	.	+	-	+	.	-	-
	R21	+	+	+	+	+	+	+/-	.	-	.	-	.	-	-	-	-
	81	+	+	+	+	+	+/-	+	.	.	.	-	+	-	.	-	-
	R41	+	+	+	+	+	+	+	+	.	.	-	-	-	-	-	-
	AT21	+	+	+	+	+	-	+	+	+	.	-	-	+	-	-	-
	RK12	+	+	+	+	+	+	+	+	+	+	-	-	-	.	-	-
	AT32	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
	Br22	+	+	+	+	-	+	+	+	-	+	-	-	+	-	-	-
	Br57	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	R11	+	-	+	+	-	+	+	+	+	+	-	-	+	-	-	-
	B32	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	B14	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	S33	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-	-
	V62	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	V92	+	-	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	V72	+	+	-	+	+	-	+	+	+	+	-	-	+	+	-	-
	V84	+	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-
	R32	+	+	+	+	+	+	-	+	+	+	-	-	+	+	-	-
	Ar4	-	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	74	+	+	+	+	+	+	+	+	+	+	-	-	+	-	-	-
	V111	+	+	+	+	+	+	+	+	+	-	-	+	+	-	-	-
	225	+	+	+	+	+	+/-	-	-	+	-	-	-	+	-	-	-
	V11	-	+	+	+	+	-	+	-	+	+/-	-	-	-	+	-	-
	V34	-	+	+	+	+	-	+	-	+	+	-	-	-	+	-	-
	65	-	-	-	-	-	+	+	-	-	-	-	+	-	-	-	-
	A2[c]	-	-	-	+	+	-	-	-	-	-	**+**	-	-	-	**+**	-
VCG002	C3	-	-	-	-	-	-	-	-	-	-	**+**	-	-	-	**+**	-
	V105	-	-	-	-	-	-	-	-	-	-	**+**	-	-	-	**+**	-
VCG003	AT16	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	+
?	V21	+/-	-	-	-	+/-	-	-	-	+/-	-	-	-	-	-	-	-
?	S41	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
?	RK24	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
?	V57	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

[a] Vegetative-compatibility group.
[b] Intermediate isolate linking VCG001 with VCG002.
[c] Intermediate isolate linking VCG001 with VCG003.
?, Not assigned to a VCG.
+, strong reaction; +/-, weak reaction; -, no visual reaction observed; ·, not paired.
*, reversion to an initial mycelium aspect dense and cottony.

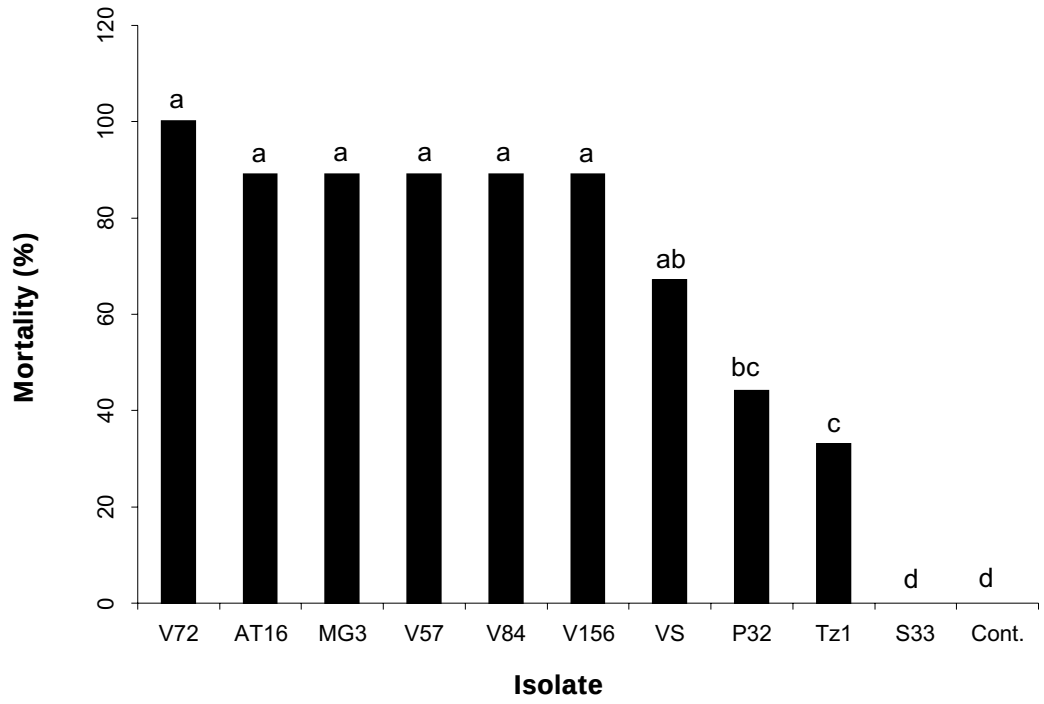

Fig. 1. Comparative pathogenicity of nine isolates of *Verticillium dahliae* isolated from olive in comparison to a reference strain (P32). Mean percentages of olive plant mortality showing the same letter were not significantly different (P=0.001), Newman and Keuls test. Cont., control.

lates, which showed the characteristic symptoms of the disease mentioned above.

Discussion

Vegetative compatibility analysis using *nit* mutants yielded interesting results on genetic diversity and pathogenicity within the *V. dahliae* population analysed in this study. This population was characterised by only 3 VCGs, which were not entirely distinct from each other and were linked through intermediate isolates. Moreover, not all isolates within a VCG were complementary to one another, and some formed weak heterokaryons with the unclassified isolates V21 (V21*94; V21*R21 and V21*RK1). These findings can be interpreted in two ways: 1, the Moroccan *V. dahliae* isolates have different origins, but a common ancestor or genetically close ancestors in which a given genotype was dominant; 2, all the isolates may have a common ancestor but they diverged either by mutation or by parasexual recombinations (Hartsie, 1964). Alternatively, they could still be in transient genetic evolution. Weak heterokaryons between different VCGs isolates could be attributed to synthesis of some functional nitrate reductase in cytoplasm of the anastomosed cells, which are not entirely killed. Hence, a transitory heterokaryon forms before vegetative compatibility occurs (Joaquim and Rowe, 1991). It is also known that weak reactions among mutants occur when isolates differ slightly in their incompatibility genes (Anagnostakis *et al.,* 1986). Previous VCG studies also showed that assignment of strains to a VCG subdivision in *V. dahliae* is problematic (Joaquim and Rowe, 1990 and 1991; Strausbaugh, 1993; Chen, 1994). A limited diversity of *V. dahliae* VCGs with the dominance of one of the latter was also reported for isolates from potato (Joaquim and Rowe, 1991; Strausbaugh, 1993), cotton (Qingji and Chiyi, 1990; Daayf *et al.,* 1995) ornamental woody plants (Chen, 1994), watermelon (Elena, 2000) and from different hosts (Elena and Paplomatas, 1998). The elevated genetic flexibility of *V.*

dahliae was also found in whiting cotton isolates (Daayf *et al*. 1995). In the same connection, genomic profiles of this parasite have also shown intermediate strains between the distinct groups (Okoli and Carder, 1993).

V. dahliae isolates varied widely in their ability to generate *nit* mutants (1 to 20). Klittich and Leslie (1988) showed that sectoring frequency was under polygenic control with additive effect and by transposable elements. The recovery of phenotypic classes of *nit* mutants showed the dominance of *nit*1 mutants (55.1%) and a low proportion of *nit*3 mutants (5%). In general, recovery of *nit*3 mutants from *V. dahliae* has been more difficult (Daayf *et al*., 1995; Elena, 2000). Others studies showed that *nit*3 mutants were never recovered (Joaquim and Rowe, 1991; Chen, 1994; Korolev and Katan, 1997).

The reversion of mutants of isolate V57 to the wild type in the pairing medium made it impossible to classify this isolate. Such behaviour was also observed by Elena (2000) and Chen (1994) who reported that the *nit*1 mutants had a much higher frequency of reverting to wild type than did *nit*M mutants.

Even though a small number of non-olive isolates were examined, the results showed that the olive (Oleaceae) isolates were vegetatively compatible with those recovered from tomato and aubergine (Solanaceae) and were classified in the same VCG (VCG001). Thus it seems there is no preferential host for most *V. dahliae* isolates between these plant species. It is possible that Verticillium wilt was transferred to olive through the usual associated cultures (tomato, aubergine, lucerne, potato…). In fact, the disease was observed in tomato, for example, in the 1960s (Lahlou, 1974) and it was not widespread in olive until the 1990s (Serghini, 1992). It has now become clear that current confrontations between *Verticillium* isolates and unusual or even resistant hosts lead to a rise in fungus pathogenicity towards the new host (Douira, 1989; El Aissami, 1990). Nevertheless, it would be advisable to validate this hypothesis by examining more isolates from different hosts and several locations and additional genetic markers. The vegetative compatibility between *V. dahliae* isolates from olive and those from other different hosts was recently reported (Bao *et al*., 1998).

It is interesting to notice that isolates from northern, southern or central Moroccan regions as well as Algerian isolates were in the same VCG. Despite the limited geographic diversity of our collection, no relation between geographic origin and the VCGs was observed in this study. Our results are in agreement with other works using *nit* mutants which demonstrated that *Verticillium* VCGs were not correlated to the geographic origin of isolates (Correll *et al*., 1988; Joaquim and Rowe, 1991; Daayf *et al*., 1995).

Greenhouse pathogenicity tests showed that soil, tomato and olive isolates were all pathogenic to olive. Nevertheless, the rate of dead plants and the severity of the disease showed differences in virulence among isolates. This is in line with Ashworth's hypothesis (1983) on a "continuum" of virulence in the soilborne population of *V. dahliae*, with all levels from weakly to highly virulent isolates. The high variability of pathogenicity among *V. dahliae* isolates was observed even in the case of descendants of the same clone (Lahlou and Boisson, 1984; Daayf *et al*. 1998).

Our work also showed that isolates characterized by different levels of virulence were in the same VCG. Thus, it seems that there is no relationship between the VCG and level of virulence. Although the defoliating and non-defoliating isolates from cotton were differentiated into different VCGs (Daayf *et al*. 1995; Elena, 1999), Daayf *et al*. (1998) reported that the two sub-clones V72, weakly virulent, and V77, highly virulent, belong to the same VCG. In other studies, a strong correlation between virulence of *V. dahliae* isolates and VCGs was found (Joaquim and Rowe, 1991; Strausbaugh, 1993).

In conclusion, we showed that the population of *V. dahliae* from olive studied presented a lack of VCG diversity. It was characterized by a limited number of subpopulations that were not entirely separate genetically. Most isolates were found in one VCG (VCG001) with 31 out of 34 isolates. In contrast, this population presented a great diversity of virulence and we noted the lack of relationship between virulence and VCGs.

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