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Short Communication

Departamento de Agronomía, Universidad de Córdoba, Campus Universitario de Rabanales, Córdoba, Spain

Correlation Between Virulence and Morphological Characteristics of Microsclerotia of *Verticillium dahliae* Isolates Infecting Olive

Francisco Javier López-Escudero[1], José Manuel Roca[1], Antonio Valverde-Corredor[2] and Jesús Mercado-Blanco[2]

Authors' addressses: [1]Departamento de Agronomía, Universidad de Córdoba, Campus Universitario de Rabanales, 14071 Córdoba, Spain; [2]Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (CSIC), Apartado 4084, 14080 Córdoba, Spain (correspondence to F. J. López-Escudero. E-mail: ag2loesj@uco.es)

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Abstract

The spreading of highly virulent isolates of *Verticillium dahliae*, causing Verticillium wilt of olive, is one of the most threatening concerns for olive cultivation. Using an isolate collection from infected olive trees in southern Spain, the morphology of microsclerotia (MS) produced on water agar was correlated with their molecular characteristics by a PCR-based pathotyping. Defoliating isolates (D) produced MS with a significantly higher length/width ratio than non-defoliating (ND) ones. These parameters were correlated using the logistic model $\log(y/1-y) = 3.73L/W - 6.95$, when the pathotype was regressed on length/width ratio of the propagules. Inflection point of the logistic curve corresponded to length/width = 1.86. This morphological differentiation of virulence groups could be a simple and useful tool at commercial laboratories for the assignation of the pathotype of *V. dahliae* isolates during routine microbiological-based diagnosis.

Introduction

Verticillium wilt of olive (VWO) is an important concern for olive cultivation in the Mediterranean Basin. In some regions, the presence of defoliating (D) isolates of the pathogen is significantly higher than non-defoliating (ND) ones (Dervis et al. 2010; López-Escudero et al. 2010; López-Escudero and Mercado-Blanco 2011). Several studies have proposed that morphological differences of *Verticillium dahliae* microsclerotia (MS) might be correlated with the differential virulence of a given isolate. Thus, cotton D isolates produced colonies with a mixture of elongated and rounded MS, whereas ND isolates only produced rounded MS when grown on water agar (WA; Bejarano-Alcázar 1990) or on a modified sodium polypectate agar (López-Escudero and Blanco-López 2005). This study aimed to assess such a correlation in isolates from olive, testing the hypothesis whether differential virulence displayed by these pathotypes, which can also be molecularly characterized by a PCR-based approach, could be correlated with a different morphology of their resting structures. This practical approximation could be a usable and very cost-effective tool for preliminary identification of virulent (D) *V. dahliae* isolates.

Materials and Methods

Collection of *Verticillium dahliae* isolates

Verticillium dahliae collection was obtained from VWO-diseased olive trees from 90 olive orchards in southern Spain (López-Escudero et al. 2010). Pathogen was isolated from three 20-cm-long and 1–2 cm in diameter stem pieces taken from wilted shoots from each of three affected olive trees, chosen at random per inspected field (López-Escudero et al. 2010). Forty-one *V. dahliae* isolates from this collection were processed as follows.

PCR-based pathotyping of *V. dahliae* isolates

Pathotype identification (D or ND) of isolates was performed by specific PCR assays. Active cultures of isolates were obtained on PDA plates incubated at 24°C in the dark for 5–6 days. The HotSHOT method (Truett et al. 2000) for rapid, small-scale DNA extraction was carried out. PCR conditions and primer pair combinations used in this study are described by Collado-Romero et al. (2006). PCR assays were repeated at least once, using freshly obtained lysates from a new HotSHOT round and always included control samples from D (V138I) and ND (V176I) *V. dahliae* representatives (Mercado-Blanco et al. 2003), as well

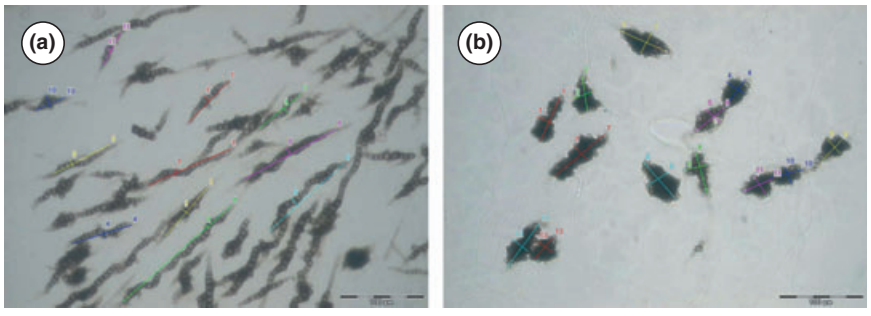

Fig. 1 Details of morphology (length and width) of microsclerotia of *Verticillium dahliae* isolates growing on water agar, recovered from olive plants affected by Verticillium wilt of olive: defoliating pathotype (AN-4-2-3) (a) and non-defoliating pathotype (LP-5-1-2) (b). Scale bars represent 100 μm in both images; colour bars represent the measured length and width as determined by the imaging software

as a negative control (no template DNA). Results of PCR assays were visualized by agarose gel electrophoresis according to standard procedures.

Morphological characterization of *Verticillium dahliae* microsclerotia

Length (*L*) and width (*W*) of MS produced on WA of the *Verticillium dahliae* isolates were determined. Pure cultures of each isolate were incubated on WA during 14 days at 24°C in the dark until MS formation. Two microscope slides per colony (isolate) were prepared with acidified fuchsine acid in lactophenol by taking pieces of agar from the centre of the colonies. *L* and *W* data from 200 MS of each isolate were scored (65–70 MS per colony), and the *L/W* ratio was calculated using the analysis software 'AnalySIS' (Soft Imaging System Gmbh 2000, Münster, Germany), connected to a Nikon Optiphot-200 microscope equipped with a video camera (Kappa; CF 20/4 DX, K-Vision BV, Huizen, Germany) (Fig. 1).

Analysis of results

Statistic analyses were performed for detecting morphological differences of MS formed on WA between isolates belonging to the D or ND populations determined as described previously. A logistic regression analysis of the isolate pathotype (binary dependent variable) and MS *L/W* ratio of isolates (independent variable) were performed by STATISTIX 9.0 for Windows (Analytical Software, Tallahassee, FL, USA).

Results and Discussion

Morphology of MS produced on WA by *V. dahliae* isolates recovered from diseased olive trees in surveyed plots varied considerably (Table 1, Fig. 1). Mean *L/W* ratio of MS was 2.62, ranging from 5.30 (the most elongated) to 1.49 (the most spherical and smallest). In some cases, morphological differences among MS formed by isolates recovered from the same field were also detected. For instance, isolates originating from a field located in Benacazón municipality showed *L/W* ratios ranging from 2.26 (AL-2-1-2) to 1.67 (AL-2-3-1; Table 1), the former being more elongated than the latter. PCR-based analysis characterized isolate Al-2-1-2 as D pathotype, whereas isolate AL-2-3-1 was molecu-

Table 1
Pathotype and length/width ratio of microsclerotia formed on water agar of *Verticillium dahliae* isolates from olive orchards affected by Verticillium wilt of olive

Isolate code	Province-Municipality	Isolate patothype[a]	Length/width ratio of isolate microsclerotia[b]
ARA-2-1-3	Seville-Carmona	D	5.30
AN-4-2-3	Jaén-Higuera de Arjona	D	4.49
CA-8-1-2	Córdoba-Montilla	D	4.71
CA-1-3-2	Córdoba-Fernán Nuñez	D	4.34
SE-3-1-1	Seville-Marinaleda	D	4.13
ARA-6-2-1	Seville-Écija	D	4.08
CA-8-1-3	Córdoba-Montilla	D	3.82
BA-6-1-1	Córdoba-Espejo	D	3.40
CA-7-1-3	Córdoba-Nueva Carteya	D	3.29
TORR-1-1-1	Jaén-Torredelcampo	D	3.20
LP-3-1-3	Seville-Utrera	D	3.19
LP-4-3-2	Seville-Alcalá de Guadaira	D	3.05
BENZ-1-1-2	Seville-Benacazón	D	2.76
SE-6-1-2	Córdoba-Santaella	D	2.63
SE-3-1-2	Seville-Marinaleda	D	2.61
SE-2-3-2	Seville-Écija	D	2.58
SE-4-2-1	Seville-Herrera	D	2.39
SE-6-1-1	Córdoba-Santaella	D	2.34
SE-5-1-1	Córdoba-Puente Genil	D	2.23
AL-2-1-2	Seville-Benacazón	D	2.26
ARA-5-2-1	Seville-Marchena	D	2.25
ARA-3-1-3	Seville-Arahal	D	2.24
ARA-4-2-1	Seville-Arahal	D	2.20
CO-1-2-1	Córdoba-Villa del Río	D	2.17
LP-5-1-2	Seville-Arahal	ND	2.16
AN-6-3-1	Jaén-Arjona	ND	2.16
ARA-4-1-3	Seville-Arahal	D	1.98
ARA-1-2-3	Seville-Carmona	D	1.97
ARA-4-3-1	Seville-Arahal	D	1.97
AN-2-3-3	Jaén-Andújar	ND	1.89
AN-1-1-2	Jaén-Andújar	D	1.85
ARA-3-2-1	Seville-Arahal	D	1.85
ARA-2-3-2	Seville-Carmona	D	1.83
CO-1-1-3	Córdoba-Villa de Río	ND	1.80
LP-1-2-3	Seville-Dos-Hermanas	D	1.78
ARA-1-3-2	Seville-Carmona	ND	1.76
AL 2-3-1	Seville-Benacazón	ND	1.67
BA-2-1-2	Córdoba-Luque	ND	1.54
CO-1-3-3	Córdoba-Villa del Río	ND	1.52
PO-3-2-2	Córdoba-Almodóvar	ND	1.49
BENZ-2-1-2	Seville-Aznalcázar	ND	1.49

[a]D, defoliating; ND, non-defoliating; characterized by PCR-based analysis.
[b]Logistic regression analysis of the isolates; pathotype and microsclerotia length/width ratio classified isolates as D when the *L/W* ratio was ≤1.86 and ND when the *L/W* ratio was ≥1.86. The fit of the model was high (P-value of the deviance = 0.8642).

larly assessed as ND pathotype. Thus, presence of different *V. dahliae* pathotypes in infected trees of the same orchard can be estimated by morphological analysis of their MS. This result confirmed previous findings about the coexistence of different *V. dahliae* genetic/virulence groups in a same field and even in the same infected tree (Mercado-Blanco et al. 2003; López-Escudero et al. 2010).

Isolates classified as D pathotype by molecular analysis produced MS with significantly higher *L/W* ratio than ND ones, according to the model log $(y/1 - y) = 3.73L/W - 6.95$ (P for *L/W* ratio = 0.0078 and P for the constant = 0.0132) when the binary variable (y = D or ND pathotype) was regressed on *L/W* ratio of MS. Although the logistic equation was not completely accurate, the fit of the model was high (P-value of the deviance = 0.864) and served as the dividing point between D and ND isolates. Inflection point of the logistic curve ($y = 0.5$) corresponded to a value $L/W = 1.86$. Some of the pathogen isolates included in either virulence group, with *L/W* ratio close to this point, could not be discriminated by the model. Nevertheless, the overall proportion of isolates correctly classified was higher than 80%.

Therefore, a simple morphological measure of MS of *V. dahliae* colonies formed on WA of isolates can be an effective tool at commercial laboratories for the assignation of the pathotype at the time of doing ordinary isolation of the pathogen from affected olive trees and infested soil samples in routine microbiological-based diagnosis.

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