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RESEARCH PAPERS

Identifying resistance to Verticillium wilt in local Spanish olive cultivars

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Summary. The resistance of 42 Spanish olive cultivars to *Verticillium dahliae* was evaluated in two experiments carried out in two consecutive years, conducted under greenhouse conditions. In both experiments, bare root systems of 5-month-old plants were inoculated with a semisolid mass of a mixture of culture medium and conidia and mycelium of the fungus. The cultivars Frantoio and Picual were used, respectively, as resistant and susceptible reference cultivars. All cultivars were evaluated on the basis of final values of the area under the disease progress curve, mean severity of symptoms and percentage of dead plants. Most of the tested cultivars were susceptible to Verticilium wilt. However, eight genotypes ('Cornezuelo de Jaén', 'Verdial de Badajoz', 'Jaropo', 'Negrillo de Estepa', 'Jabaluna', 'Ocal de Alburquerque', 'Asnal' and 'Racimal') exhibited resistance to the disease.

Key words: *Olea europaea*, *Verticillium dahliae*, defoliating pathotype, root-dip inoculation.

Introduction

Spain is the largest producer of olive oil and table olives in the world, producing about 44% of the total world production, with a cultivated area of olive orchards close to 2.5 million ha (Barranco *et al*., 2010). Verticillium wilt of olive (VWO), caused by the soilborne fungus *Verticillium dahliae* Kleb., is currently the most threatening disease for olive crops in Spain. In major areas of production, such as Guadalquivir Valley in Andalucía, the pathogen regularly causes the death of many infected trees (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al*., 2012; Trapero *et al*., 2013b). Recent studies conducted in the three main olive producing provinces within this valley have revealed an average disease incidence of 20%, from extensive surveys that comprised 90 affected olive orchards (López-Escudero *et al*., 2010).

In a necessary control strategy for ameliorating disease losses in high-risk areas, the use of resistant olive cultivars is considered as one of the most effective means of control, being the main disease management strategy at the time of planting (López-Escudero and Mercado-Blanco, 2011; Trapero *et al*., 2013b). The use of resistant or tolerant cultivars is likely the most economically effective and environmentally friendly control measure to be implemented (Hiemstra and Harris, 1998; Antoniou *et al*., 2008; Colella *et al*., 2008; Bubici and Cirulli, 2011; Erten and Yildiz, 2011; Tsror, 2011; Jiménez-Díaz *et al*., 2012). The development of new VWO-resistant genotypes is currently a major objective for olive breeding programmes (Rallo *et al*., 2007; Trapero *et al*., 2015). The ongoing programme of evaluation for resistance of olive genotypes to VWO [a joint project between the Government of Andalucía, the University of Córdoba, and the Center for Research, Training and Food of Andalucía (IFAPA)], established in 1994 in the Department of Agronomy, University of Córdoba, has focused on finding resistance in the Spanish and for-

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eign olive cultivars of agricultural and commercial interest preserved in the World Olive Germplasm Bank of Córdoba (WOGB) (Caballero *et al*., 2006).

In this evaluation programme, early studies revealed high resistance levels in 'Frantoio', 'Changlot Real' and 'Empeltre' (López-Escudero *et al*., 2004; 2007; Martos-Moreno *et al*., 2006). In addition, these studies revealed that olive tree cultivars were usually susceptible to infection by the pathogen, irrespective of the artificial inoculation method used (root-dipping using mycelium and/or conidial suspensions, or stem injection). Although more resistant cultivars have been identified in later research (García-Ruiz *et al*., 2014a; 2014c), the general susceptibility of olive cultivars has been corroborated (López-Escudero and Mercado-Blanco, 2011). Nearly 140 cultivars from the WOGB have been evaluated (Trapero *et al*., 2013b); however, an important number of accessions still remains to be evaluated, particularly many of the Spanish accessions. In Spain, 272 olive cultivars have been described, which have been classified in four categories according to their importance or area of distribution (Barranco, 2010). Of these, 24 are major cultivars, which are extensively grown and are dominant in at least one district. Another 24 are secondary cultivars, not dominant in any district but regularly used in olive orchards. Another 50 disseminated and 174 local cultivars are found as isolated trees in some or only one district (Barranco, 2010). Local cultivars are those confined to small geographical areas, and have been traditionally selected by farmers due to their adaptation to local environmental conditions. Because of their diffusion, the exchange of genetic material within *Olea europaea* genotypes from the Western Mediterranean Basin is limited (Besnard *et al*., 2001; 2013). These genotypes are likely to be products of local selection processes, and this genetic variability could be very important for identifying resistance sources against *V. dahliae* (Rallo, 2005).

The aim of the research described in this paper was to identify sources of resistance against *V. dahliae*, using artificial inoculation experiments, in a wide range of local olive genotypes confined in geographically isolated areas in Spain.

Materials and methods

Plant material

Self-rooted plants of 42 olive cultivars from the WOGB were used (Tables 1 and 2). These plants were propagated from soft-wood cuttings and hardened for 5 months in a greenhouse, following the methodology described by Caballero and Del Río (2010). Two experiments were conducted, in 2012 (Experiment I) and 2013 (Experiment II), in which, respectively, 27 and 16 Spanish local olive cultivars were evaluated (Tables 1 and 2).

Plant inoculation

The plants were inoculated with a highly virulent cotton-defoliating *Verticillium dahliae* isolate (V117), from the isolate collection of the Laboratory of Plant Pathology of the Department of Agronomy, University of Córdoba (Blanco-López *et al*., 1989). The inoculum, a semisolid mass consisted on culture medium, mycelium and conidia (5×10^8 conidia mL^{-1}), was prepared by homogenizing 6-day-old potato dextrose agar (PDA) Petri plate cultures of the pathogen in a kitchen blender, following the methodology described by García-Ruiz *et al*. (2014a).

Inoculations were performed by dipping the bare root system of each plant into the inoculum for 1 min, assuring that the roots were homogeneously impregnated with the semisolid mass of homogenized agar cultures. The plants were then transplanted individually into sterile plastic pots containing sterile peat and moved to a greenhouse. Non-inoculated control plants were subjected to the same process described above, but were treated with a mixture of distilled sterile water and PDA without the pathogen.

Incubation and experimental design

The experiments were performed from March to July in a 95 m^2 greenhouse, with a range of temperatures varying from 18±5 (March 2012) to 28±5ºC (July 2012) during the incubation period in Experiment I, and from 20±5 (March 2013) to 25.5±5ºC (July 2013) in Experiment II. Temperature and relative humidity were recorded in the greenhouse using measurement probes connected to Synopta 2.7.5.1 software (HortiMaX B.V., Pijnacker).

The plants were arranged on greenhouse benches according to a randomized block design with four blocks, using three (Experiment I) or two (Experiment II) plants of each cultivar per block. The cultivar 'Picual' (highly susceptible to the defoliating pathotype of *V. dahliae*) and 'Frantoio' (moderately resistant) (López-Escudero *et al*., 2004) were includ-

Table 1. Mean disease parameters assessed in the olive cultivars inoculated with the defoliating isolate of *Verticillium dahliae* in Experiment I (2012).

Cultivar[a]	R[b]	Importance[c]	AUDPCP[d]	FMS[e]	PDP[f]	R.L.[g]
'Gordal de Velez Rubio'	781	Local	89.1 a	3.9	91.7	ES
'Gordal de Granada'	761	Secondary	85.5 a	3.8	90.9	ES
'Alameño de Montilla'	299	Disseminated	65.4 ab	3.3	75.0	ES
'Sevillano de Jumilla'	593	Local	63.7 ab	3.1	41.7	ES
'Ocal'	354	Disseminated	57.0 ab	3.0	66.7	ES
'Cerezuela'	331	Local	54.8 ab	3.1	66.7	ES
'Habichuelero de Grazalema'	366	Local	54.2 ab	2.8	60.0	ES
'Manzanilla de Sevilla'	127	Major	52.2 ab	2.4	58.3	ES
'Picual'	9	Major	50.0 ab	2.8	36.4	S
'Nevado Rizado'	773	Local	48.5 ab	2.8	66.7	ES
'Ojo de Liebre'	413	Local	48.0 ab	2.5	27.3	S
'Chorruo'	29	Disseminated	48.3 ab	3.0	66.7	ES
'Cañivano Negro'	256	Disseminated	47.5 ab	2.7	50.0	S
'Habichuelero de Baena'	295	Local	46.3 ab	2.3	16.7	S
'Limoncillo'	35	Disseminated	42.8 ab	1.8	33.3	S
'Alameño de Cabra'	285	Disseminated	40.0 ab	2.0	33.3	S
'Manzanilla de Agua'	406	Local	38.3 ab	1.6	25.0	MS
'Morona'	376	Secondary	35.3 ab	2.1	45.5	S
'Pico Limon'	273	Secondary	32.0 bc	1.9	25.0	MS
'Nevado Basto'	305	Local	31.1 bc	1.9	27.3	MS
'Olivo de Mancha Real'	797	Local	26.3 bcd	2.2	25.0	MS
'Rechino'	372	Local	25.1 bcde	1.4	16.7	MS
'Cornezuelo de Jaén'	20	Disseminated	18.0 cdef	1.2	0.0	R
'Verdial de Badajoz'	400	Major	17.8 def	0.8	0.0	R
'Nevadillo Blanco de Lucena'	58	Local	14.1 f	1.0	25.0	MS
'Frantoio'	80	Major	10.9 ef	0.5	0.0	R
'Jaropo'	23	Local	10.3 f	0.9	0.0	R
'Negrillo de Estepa'	301	Disseminated	9.5 f	0.6	0.0	R
'Jabaluna'	392	Disseminated	6.9 f	0.4	0.0	R

[a] Cultivar identities were confirmed by Trujillo *et al.* (2013).
[b] Accession number in the World Olive Germplasm Bank.
[c] Cultivar importance: Major, Secondary, Disseminated, Local (Barranco, 2010).
[d] AUDPCP: Area under the disease progress curve estimated as the percentage with regard to the maximum potential value. Values in columns followed by the same letter are not significantly different (P =0.05) according to Fishers protected least significant difference test, after data transformation of Ln (ABCPEP+1).
[e] FMS: final mean severity of symptoms.
[f] PDP: percentage of dead plants.
[g] R.L. Resistance level of each cultivar as concluded from the AUDPCP, FMS and PDP values (Table 3). ES = extremely susceptible; S = susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant.

Table 2. Mean disease parameters assessed in the olive cultivars inoculated with the defoliating isolate of *Verticillium dahliae* in Experiment II (2013).

Cultivar[a]	R[b]	Importance[c]	AUDPCP[d]	FMS[e]	PDP[f]	R.L.[g]
'Corbella'	645	Disseminated	96.8 a	3.9	87.5	ES
'Carrasqueño de la Sierra'	286	Secondary	87.7 ab	3.9	87.5	ES
'Picual de Almería'	798	Disseminated	85.8 ab	3.7	85.7	ES
'Picual'	9	Major	83.0 ab	3.4	71.4	ES
'Cornicabra de Mérida'	522	Local	76.3 abc	3.5	50.0	ES
'Cerezuela'	349	Local	66.9 bcd	3.1	57.1	ES
'Lentisca'	384	Local	61.5 bcd	2.9	66.7	ES
'Caballo'	333	Local	54.8 cde	3.1	57.1	ES
'Sabatera'	665	Local	41.7 def	2.1	16.7	MS
'Loaime'	414	Secondary	33.1 ef	1.8	12.5	MS
'Sollana'	871	Disseminated	32.2 ef	1.3	12.5	MS
'Chorreao de Montefrío'	402	Local	29.6 ef	1.4	16.7	MS
'Datilero'	403	Local	27.4 f	1.6	0.0	MS
'Ocal de Alburquerque'	427	Local	23.3 f	0.8	0.0	R
'Ocal-CJ'	25	Local	22.3 f	1.1	16.7	MS
'Asnal'	437	Local	19.4 f	1.1	0.0	R
'Frantoio'	80	Major	18.7 f	0.3	0.0	R
'Racimal'	418	Local	17.9 f	1.3	0.0	R

[a] Cultivar identities were confirmed by Trujillo *et al*. (2013).
[b] Accession number in the World Olive Germplasm Bank.
[c] Cultivar importance: Major, Secondary, Disseminated, Local (Barranco, 2010).
[d] AUDPCP: Area under the disease progress curve estimated as the percentage with regard to the maximum potential value. Values in columns followed by the same letter are not significantly different ($P = 0.05$) according to Fisher's protected least significant difference test.
[e] FMS: final mean severity of symptoms.
[f] PDP: percentage of dead plants.
[g] R.L. Resistance level of each cultivar as concluded from the AUDPCP, FMS and PDP values (Table 3). ES = extremely susceptible; S = susceptible; MS = moderately susceptible; R = resistant; HR = highly resistant.

ed as reference cultivars in both experiments. Plants were sprinkle irrigated for 5 min, three times each day and fertilized every 2 weeks with 'Bolikel Fe' (base solution: 9.4 g L^{-1} of water) and 'HAKAPHOS green 15.20.15 2MgO' (base solution: 15 kg hL^{-1}).

Disease assessments

Disease severity was assessed weekly from 6 till 17 weeks after the inoculation in both experiments. Disease symptoms were evaluated using a severity scale from 0 (healthy plant or plant without symptoms) to 4 (dead plant) based on the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis and/or defoliation (López-Escudero *et al*., 2004). The area under the disease progress curve (AUDPCP) was calculated for each cultivar considering its percentage with regard to the maximum possible value that could be reached in the period of assessment, based on the calculation formula of Campbell and Madden (1990):

$$AUDPCP = [t/2 \cdot (S_2 + 2 \cdot S_3 + 2 \cdot S_4 + \ldots + S_i)/4 \cdot n] \cdot 100;$$

Table 3. Resistance categories, and disease parameters, for reactions of olive cultivars to the defoliating isolate V117 of *Verticillium dahliae* (by López-Escudero *et al*., 2007)

Resistance category	AUDPCP[a]	FMS[b]	PDP[c]
Highly resistant	0–10	0.0–1.5	0
Resistant	11–30	0.0–1.5	0
Moderately resistant	31–50	1.5–2.5	0–30
Susceptible	51–70	2.5–3.0	31–50
Extremely suscetibile	71–100	3.0–4.0	51–100

[a] AUDPCP, Area under the disease progress curve.
[b] FMS, final mean severity of symptoms.
[c] PDP, percentage of dead plants.

where t = days between observations; S_i = final mean severity; 4 = maximum disease rating; and n = number of observations.

Final mean severity (FMS) and percentage of dead plants (PDP) were also determined. To classify the reactions of the cultivars, resistance to Verticillium olive wilt was categorized considering the AUDPCP, FMS and PDP values according to López-Escudero *et al*. (2004; 2007), giving priority the most disadvantageous value (Table 3).

Pathogen re-isolation

Plant tissues from inoculated and symptomatic plants were cultured to confirm infection. Samples from affected woody tissue or leaf petioles were washed in running tap water, the bark was removed and the surface tissue was disinfected in 0.5% sodium hypochorite for 1 min. Wood chips were placed on PDA plates and incubated at 24ºC in the dark for 6 d.

Data analysis

Data were subjected to an analysis of variance (ANOVA) for a randomized block design, using the Statistix 9.0 program (Analytical Software). In Experiment I, the AUDPCP values were modified by the data transformation Ln(ABCPEP+1); in the case of Experiment II, data transformation was not required. Mean values were compared using the Fisher protected LSD at P=0.05.

Results

Disease symptoms

Injury due to the inoculation and transplanting processes during the first 4 weeks after the inoculation caused plant mortality of approx. 10% of plants. Non-inoculated plants did not exhibit any disease symptoms and started to grow from the fifth week after inoculation.

In both experiments, chlorosis was the most common disease symptom on moderately susceptible cultivars. In some genotypes, chlorosis affected whole plants and produced slight defoliation. The reduction and/or delay of plant growth when compared with non-inoculated control plants was normally observed in symptomatic plants, but was also common in inoculated plants that apparently did not exhibit leaf symptoms.

Defoliation of green leaves and apoplexy were the most common symptoms exhibited by the susceptible or extremely susceptible cultivars, occasionally followed by plant death. In some of the plants, defoliation developed abruptly, causing the fall of more than 70% of green leaves. In other cultivars, defoliation and apoplexy affected only some parts of the plants that exhibited severe symptoms in some shoots or branches.

Disease progress and resistance levels

Inoculated plants exhibited first VWO symptoms from the sixth week after inoculation. The susceptible control cultivar 'Picual' developed symptoms in 100% of plants in both experiments, being considered susceptible in Experiment I due to values of 50.0% AUDPCP, 2.8 FMS and 36.4% PDP (Tables 1 and 3). However, in Experiment II, 'Picual' was classified as extremely susceptible, with AUDPCP (83.0%), FMS (3.4) and PDP (71.4%) values much greater than in Experiment I (Tables 2 and 3). On the other hand, 'Frantoio' (moderately resistant control) showed a resistant reaction in both experiments. The disease values for this cultivar in Experiment I were AUDPCP, 10.9%; FMS, 0.5 and PDP, 0.0% (Tables 1 and 3). In Experiment II, the value of AUDPCP for 'Frantoio' was slightly greater (18.7%) than in Experiment I, and was similar for FMS (0.3) (Tables 2 and 3). In both experiments, values of AUDPCP were significantly greater for 'Picual' than 'Frantoio' (Tables 1 and 2).

In Experiment I, a group of ten cultivars (among them, 'Gordal de Velez Rubio', 'Sevillano de Jumilla' and 'Ocal') was considered extremely susceptible, with values of AUDPCP greater than 48.0% and FMS greater than 2.8, and significantly different compared to the resistant control 'Frantoio' (Tables 1 and 3). Six additional cultivars (among them 'Cañivano Negro', 'Limoncillo' and 'Alameño de Cabra') were susceptible, exhibiting values of PDP that ranged from 16.7 to 50.0% (Tables 1 and 3). The PDP value of another group of six cultivars (including 'Pico Limón', 'Rechino' and 'Nevado Basto') ranged from 16.7 to 27.3%, and these cultivars were therefore moderately susceptible. The cultivars 'Cornezuelo de Jaén', 'Verdial de Badajoz', 'Jaropo', 'Negrillo de Estepa' and 'Jabaluna' were considered resistant, with values of AUDPCP less than 18.0% and FMS less than 1.2. These values were significantly different from the susceptible control 'Picual' (Tables 1 and 3).

In the Experiment II, seven cultivars (including 'Corbella', 'Picual de Almería' and 'Lentisca') were classified as extremely susceptible, with values of AUDPCP greater than 54.8%, FMS more than 2.9, and PDP greater than 50.0%. These values were significantly different from those for 'Frantoio' (Tables 2 and 3). Another group of six cultivars (among them, 'Sabatera', 'Loaime' and 'Datilero') was considered moderately susceptible, with FMS values from 1.1 to 2.1 (Tables 2 and 3). The cultivars 'Ocal de Alburquerque', 'Asnal' and 'Racimal' exhibited resistant reactions, with values of AUDPCP less than 23.3% and FMS less than 1.3, which were significantly different from the susceptible control cultivar 'Picual' (Tables 2 and 3).

Verticillium dahliae was recovered (through re-isolation) from 82% of diseased plants in Experiment I and 79% of diseased plants in Experiment II.

Discussion

The inoculation method of root dipping in a semisolid mass of culture medium containing mycelium and conidia of the pathogen has been effective in developing consistent infections and symptoms on inoculated plants. As previously reported by García-Ruiz *et al*. (2014a), using this methodology, the inoculum mass probably provides continuous infections in the roots over several days; the fungus is not significantly affected by manipulations and *V. dahliae* propagules remain viable (Tjamos, 1981), allowing successful and effective inoculation. In addition, this inoculation method provides improved screening efficiency for VWO, using greenhouses instead of growth chambers. Moreover, the decreased time of inoculation procedure compared to the previous time-consuming methods (1 min of exposure in a semisolid mass versus 30 min when dipping roots in conidial suspensions) (López-Escudero *et al*., 2004), is valuable for rapid and accurate screening experiments. However, disease onset occurred in the sixth week after inoculation, representing a slight delay of the disease symptoms (2–3 weeks) compared to growth chamber experiments (López-Escudero *et al*., 2004; Martos-Moreno *et al*., 2006). This confirms results obtained in previous greenhouse assays (Trapero *et al*., 2013a; García-Ruiz *et al*., 2014a).

The physiological condition of plants during the inoculation processes is one of the factors that likely affected their susceptibility to the VWO, causing the differences of symptom expression observed in the two experiments. In previous studies, greater symptom expression was observed in plants that were actively growing, as a result of pathogen distribution and increased fungal biomass along the host xylem vessels (Báidez *et al*., 2007; Antoniou *et al*., 2008; Markakis *et al*., 2009; Prieto *et al*., 2009). On the other hand, the environmental conditions of the greenhouse, mainly temperature and light, could have played important roles in disease progress in Experiment I. Cloudy days and temperatures much greater or less than the optimum range of 20–25 ºC are reported to have negative influences on the disease progress (Soesanto and Termorshuizen, 2001; López-Escudero *et al*., 2004; Xu *et al*., 2012). The temperatures recorded during the incubation period in Experiment II ranged from 20±5 (March) to 25.5±5 ºC (July), which encouraged the disease development from the first weeks of evaluation. In Experiment I these temperatures were lower. The cultivar 'Cerezuela', which was included in the two experiments, exhibited an extremely susceptible reaction in both experiments with slightly greater values of AUDPCP in Experiment II, confirming the trend demonstrated for 'Picual' and 'Frantoio'.

The results of these experiments indicate the susceptibility of most olive cultivars to the defoliating pathotype of *V. dahliae*, and these results confirm previous studies (López-Escudero *et al*., 2004; 2007; Martos-Moreno *et al*., 2006). Nevertheless, eight of 42 assessed genotypes ('Cornezuelo de Jaén', 'Verdial de Badajoz', 'Jaropo', 'Negrillo de Estepa', 'Jabaluna',

'Ocal de Alburquerque', 'Asnal' and 'Racimal') exhibited resistant or tolerant reactions, showing slight symptoms only. Despite this, all identified resistant or potentially resistant cultivars should be tested in different experiments with several incubation conditions to confirm their resistance levels, because the present evaluation is the first assessment for most cultivars.

Olive genotypes probably have a quantitative polygenic resistance to *V. dahliae* and show a wide range of useful genetic variability for finding resistance to VWO (Wilhelm and Taylor, 1965; López-Escudero and Mercado-Blanco, 2011; Trapero *et al*., 2015). This variability is partially represented by local cultivars in the WOGB. Local cultivars are the most numerous, with more than 170 Spanish olive local genotypes recorded in the WOGB of Cordoba; major cultivars include only 24 out of 272 Spanish cultivars documented (Barranco, 2010). As these local cultivars were selected in, and confined to, local geographical areas of diffusion, they could have discriminative genetic composition which could be very important for identifying resistance sources against *V. dahliae* (Besnard *et al*., 2001; 2013; Rallo, 2005). Most of the resistant cultivars identified so far are local. Therefore, the assessment of accessions of this WOGB collection should be continued in order to maximize the number of resistant genotypes with different genetic background that can be included in a breeding programme to develop new resistant cultivars for the olive producing industry.

The cultivar 'Verdial de Badajoz' is a major cultivar that exhibited resistant or tolerant reactions in previous experiments in our Department (García-Ruiz *et al*., 2014b), and in the present experiments. This cultivar could therefore be a substitute for susceptible cultivars, and is being considered for future experiments in naturally infested fields.

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