TESIS DOCTORAL

Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre

Autora:
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TITULO: *Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre.*

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UNIVERSIDAD DE CÓRDOBA

Tesis Doctoral

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Doctoranda
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Área de Mejora y Biotecnología de cultivos y Área de Protección de cultivos.
Instituto de Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA),
Centro "Alameda del Obispo" de Córdoba

Memoria de Tesis presentada para la obtención del Título de Doctor
con mención internacional por la Universidad de Córdoba

Córdoba, 2015

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TÍTULO DE LA TESIS:
Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre

DOCTORANDO/A:
Rocío Arias Calderón

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS:
(se hará mención a la evolución y desarrollo de la Tesis, así como a trabajos y publicaciones derivados de la misma).

El trabajo titulado "Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre", realizado por Rocío Arias Calderón, se considera finalizado y reúne los requisitos necesarios para su exposición y defensa como Tesis Doctoral.

El trabajo se ha realizado en el Centro "Alameda del Obispo" de Córdoba perteneciente al Instituto de Investigación y Formación Agraria, Pesquera y Alimentaria (IFAPA), dentro de sus Áreas de Mejora y Biotecnología de cultivos y de Protección de cultivos. Durante el mismo se ha evaluado la resistencia a la Verticilosis en materiales de diverso origen y se han identificado un alto número de genotipos resistentes al desarrollo de síntomas de enfermedad que en trabajos futuros pueden proporcionar nuevas variedades de interés para el sector. Asimismo se ha avanzado en el conocimiento sobre diferentes aspectos acerca de la herencia y los mecanismos de defensa que operan en el patosistema olivo-*V. dahliae*.

Todo ello ha quedado reflejado en varias contribuciones científicas de diferentes ámbitos, incluyendo cuatro artículos en revistas incluidas en los dos primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por el Journal Citation Reports (JCR), un artículo en SCImago Journal Rank (SJR), tres artículos científico-técnicos en revistas de divulgación, siete comunicaciones a congresos internacionales y cinco a congresos nacionales. La doctoranda también ha participado en otras actividades de extensión y divulgación de los resultados obtenidos y ha realizado estancias en centros internacionales de prestigio de gran utilidad para completar su proceso formativo.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, 18 de mayo de 2015.

Firma del/de los director/es:

Fdo.: D. Lorenzo León Moreno.

Fdo.: D. ª Dolores Rodríguez Jurado.

TÍTULO DE LA TESIS:
Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre.

DOCTORANDO/A:
Rocío Arias Calderón.

ESCRITO RAZONADO DEL RESPONSABLE DE LA LÍNEA DE INVESTIGACIÓN

D. Antonio Martín Muñoz, com responsable de línea de investigación Biociencias y Ciencias Agroalimentarias y tutor de la Tesis Doctoral titulada "Mejora de olivo para resistencia a la Verticilosis: evaluación de progenies y material silvestre" por la doctoranda Rocío Arias Calderón, ratifica el informe favorable dado por los directores de la Tesis D. Lorenzo León Moreno y D.ª Dolores Rodríguez Jurado para su exposición y defensa.

Por todo ello, se autoriza la presentación de la Tesis Doctoral

Córdoba, 18 de mayo de 2015.
Firma del responsable de línea de investigación:

Fdo.: D. Antonio Martín Muñoz.

Mención internacional del título de doctor

Esta Tesis cumple con las directrices establecidas según RD 99/2011 para la obtención de Título de Doctor con mención internacional por la Universidad de Córdoba:

Estancia internacional predoctoral de 3 meses (1 septiembre 2014 - 29 noviembre 2014). Agronomy Department, Biotechnical Faculty, Ljubljana (Slovenia), bajo la supervisión de Prof. Dr. Branka Javornik, Head of Biotechnology Studies.

Parte de la Tesis Doctoral se ha redactado y será presentada en castellano e inglés.

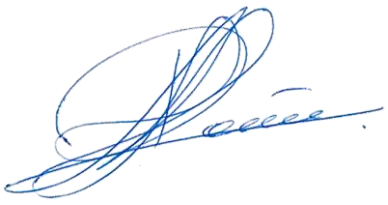
La Tesis haya sido informada por dos expertos doctores pertenecientes a instituciones de investigación no españolas.

- Dr. Bouchaib Khadari. Associate researcher Institut National de la Recherche Agronomique (INRA). Department UMR Amélioration Génétique et Adaptation des Plantes (UMR 1334 AGAP). INRA/ Montpellier SupAgro/ CBNMed, Montpellier (Francia).
- Dr. Biljana Kiprovski. Research Associate. Departmet of Field and Vegetable Crops Institution: Faculty of Agriculture, University of Novi Sad (Serbia).

Un experto doctor perteneciente a un centro de investigación no español, y distinto del responsable de la estancia mencionada formará parte del tribunal evaluador de la Tesis.

- Dr. Luciana Baldoni. Research area: Plant Genomics. Consiglio Nazionale delle Ricerche, Institute of Biosciences and BioResources, Perugia (Italia).

La doctoranda:



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Tesis como compendio de publicaciones

La Tesis Doctoral está constituida por el conjunto de trabajos publicados. Constituido por 4 artículos publicados en revistas incluidas en los dos primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por el Journal Citation Reports (SCI y/o SSCI). Se adjunta informe con el factor de impacto y cuartil del Journal Citation Reports (SCI y/o SSCI) o de las bases de datos de referencia del área en el que se encuentran las publicaciones presentadas.

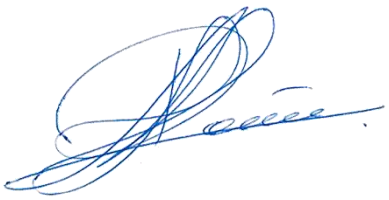
Arias-Calderón, R., Rouiss, H., Rodríguez-Jurado, D., de la Rosa, R., León, L., 2014. Variability and heritability of fruit characters in olive progenies from open pollination. Scientia Horticulturae 169, 94-98. Datos de 2013 (JCR): índice de impacto 1.504, posición 9/33 y segundo cuartil (Q2) en el área temática de Horticulture.

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., De la Rosa, R., Rodríguez-Jurado, D., 2015. Resistance to Verticillium wilt in olive progenies from open-pollination. Scientia Horticulturae 185, 34-42. Datos de 2013 (JCR): índice de impacto 1.504, posición 9/33 y segundo cuartil (Q2) en el área temática de Horticulture.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A. de la Rosa, R., León. L., 2015. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica DOI 10.1007/s10681-015-1463-7. Datos de 2013 (JCR): índice de impacto 1.692, posición 7/33 y primer cuartil (Q1) en el área temática de Horticulture.

Arias-Calderón, R., Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., Belaj. A., 2015. Pre-breeding for resistance to Verticillium wilt in olive: fishing in the crop wild relative gene pool. Crop Protection 75, 25-33. Datos de 2013 (JCR): índice de impacto 1.539, posición 25/72 y segundo cuartil (Q2) en el área temática de Agronomy.

La doctoranda:



Fdo.: Rocío Arias Calderón.

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Resumen

La Verticilosis del olivo (*Olea europaea* L.) causada por el hongo de suelo *Verticillium dahliae* Kleb., representa en la actualidad el mayor problema fitosanitario del olivar en muchas zonas de cultivo a nivel global. Para el control de la Verticilosis del olivo se recomienda un manejo integral de medidas preventivas y/o paliativas, de entre ellas, la más recomendada es el uso de variedades resistentes a la enfermedad. Sin embargo, un número muy reducido de variedades han exhibido cierto nivel de resistencia al desarrollo de síntomas ante la colonización de *V.dahliae*. Por este motivo, una nueva línea de trabajo se incorporó en el programa de mejora de olivo con el objetivo de obtener nuevas variedades que combinen buenas características agronómicas y altos niveles de resistencia al desarrollo de síntomas de la enfermedad.

El primer objetivo de este trabajo fue obtener información sobre la heredabilidad de algunos caracteres agronómicos de interés en olivo con el fin de alcanzar una estrategia combinada de selección a la enfermedad. De este modo, 26 caracteres morfológicos y agronómicos fueron evaluados en progenies de polinización libre de olivo procedentes de 17 variedades españolas conservadas en la Colección Mundial de Variedades de Olivo del Centro IFAPA de Córdoba, España (Junta de Andalucía, CAP; Universidad de Córdoba, UCO; Instituto Andaluz de Formación Agraria y Pesquera, IFAPA), así como de los cultivares parentales. Diferencias significativas entre progenies fueron observadas en todos los caracteres evaluados y diferente nivel de heredabilidad fue obtenido para diferentes caracteres.

La evaluación de la resistencia a la Verticilosis realizada en esta Tesis constituye, por tanto, una etapa avanzada dentro del programa de mejora de olivo del citado centro. Así, la evaluación inicial comentada antes permitió seleccionar 52 genotipos en base a sus características agronómicas interesantes. Las evaluaciones de la resistencia a la Verticilosis se realizaron además en 38 genotipos procedentes de tres cruzamientos de variedades conocidas de olivo: 'Changlot Real' x 'Dolce Agogia', 'Frantoio' x 'Arbosana' y 'Koroneiki' x 'Empeltre', presentando todas ellas al menos un parental categorizado en trabajos previos como resistente a la Verticilosis. Dichos genotipos se seleccionaron igualmente a partir de la evaluación agronómica de las progenies iniciales.

Con el objetivo de diversificar el origen del material evaluado, se han incluído 64 genotipos con diferente origen genético: olivos silvestres o acebuches (*Olea europaea* subsp. *europaea* var. *sylvestris*), nuevos genotipos procedentes de subspecies afines de *Olea* (*Olea europaea* subsp. *guanchica*) y genotipos procedentes de cruzamientos entre la variedad 'Picual' y dos acebuches seleccionados.

Las plantas se inocularon por inmersión de las raíces con un aislado altamente virulento del patotipo Defoliante de *V. dahliae* y después crecieron en condiciones controladas para evaluar la resistencia de los genotipos. Las variedades 'Picual' y 'Frantoio' se incluyeron como controles de enfermedad susceptible y resistente respectivamente, en los experimentos realizados. La reacción a la enfermedad también se evaluó en todos los parentales conocidos de los genotipos evaluados. Se ha desarrollado un Índice de Susceptibilidad Relativa (ISR o RSI en inglés) que aúna diferentes parámetros de enfermedad comúnmente utilizados. La colonización de la planta por el hongo se estimó mediante aislamientos de tejido aéreo y radicular. Entre todos los genotipos evaluados, ocho procedentes de polinización libre, diez

genotipos procedentes de los cruzamientos dirigidos de olivo cultivado y trece de los procedentes de genotipos silvestre y subsp. *guanchica*, mostraron un nivel de resistencia al desarrollo de síntomas similar que 'Frantoio' (cultivar control resistente).

El nivel de colonización de los genotipos por el hongo sugiere la implicación de posibles mecanismos de tolerancia ya que se han encontrado genotipos categorizados como resistentes similarmente colonizados que genotipos susceptibles. La asociación entre resistencia al desarrollo de síntomas y elevada colonización vascular no se halló entre los genotipos silvestres y se reprodujo sólo para una parte de los genotipos resistentes provenientes de cruzamientos dirigidos y polinización libre.

El material evaluado en este trabajo se encuentra en crecimiento en fincas experimentales aunando diferentes objetivos: posibles futuras propagaciones de los genotipos de interés, continuar las evaluaciones paralelas del programa de mejora, usar estos genotipos como posibles genitores y/o patrones y aplicar metodologías moleculares si se corrobora su resistencia. Evaluar el nivel de resistencia a la enfermedad en campo naturalmente infestado por *V. dahliae* permitirá en el futuro continuar los trabajos en el programa de mejora de olivo hasta el posible registro de nuevas variedades resistentes a la enfermedad.

Summary

The Verticillium wilt of olive (*Olea europaea* L.), caused by the soilborne fungus *Verticillium dahliae* Kleb., constitutes currently the major cultivation constraint in many olive growing areas. An integrated disease management strategy is recommended for Verticillium wilt of olive with preventive and/or palliative control including the use of resistant cultivars as one of the most efficient control measures. However, only a few traditional cultivars have showed high levels of disease resistance. For this reason, a new area of work in the olive breeding program was initiated aiming at obtaining new cultivars combining high levels of resistance to the development of disease symptoms and good agronomic characteristics.

The first target in this work was to obtain information about the inheritance of the main agronomic traits in olive in order to make a subsequent combined strategy for selection to the disease. For that, 26 morphological descriptors and agronomic traits were evaluated in progenies coming from open-pollination of 17 Spanish cultivars of the World Olive Germplasm Bank (WOGP) of Andalusian Institute of Agricultural Research and Training (IFAPA) of Córdoba (Spain) (Andalusian Regional Government, CAP; University of Córdoba, UCO; Andalusian Institute of Agricultural Research and Training, IFAPA) and in their corresponding parents. Significant differences among progenies were observed for all the evaluated traits and different level of heritability estimated for different characters.

Resistance of olive genotypes to Verticillium wilt was, therefore, evaluated in this work at an advanced phase into the olive breeding program. Thus, the above mentioned evaluation allowed to select 52 genotypes coming from open-pollination on the basis of their favorable agronomic performance. In addition, resistance to Verticillium wilt was also evaluated in 38 genotypes from three crosses: 'Changlot Real' x 'Dolce Agogia', 'Frantoio' x 'Arbosana' and 'Koroneiki' x 'Empeltre', including all of them as parent at least one cultivar previously categorized as resistant to the disease. These genotypes were also previously selected from the agronomic evaluation of the initial progenies.

The potential of wild olives tree could represent new sources of resistance to Verticillium wilt in olive. We evaluated 64 genotypes from different genetic origin: wild olive trees (*Olea europaea* subsp. *europaea* var. *sylvestris*), genotypes belonging to related subspecie (*Olea europaea* subsp. *guanchica*) and genotypes coming from crosses between 'Picual' and two selected wild olive trees.

Plants were inoculated by dipping roots in a highly virulent defoliating pathotype isolate of *V. dahliae* and then screened under controlled conditions to test the resistance of the genotypes. 'Picual' and 'Frantoio' cultivars were included as susceptible and resistant controls respectively in the experiments. The reaction to the disease of known parent cultivars was also evaluated. A Relative Susceptibility Index (RSI), which summarized several usual disease parameters, was developed. Plant colonization by the fungus was also assessed in root and stem by isolates.

Among all olive evaluated genotypes, eight from open-pollination, ten genotypes from the three selected crosses of cultivated olive and thirteen genotypes from wild or related subspecie, showed a resistance level to external development of symptoms similar than 'Frantoio' (the control resistant cultivar).

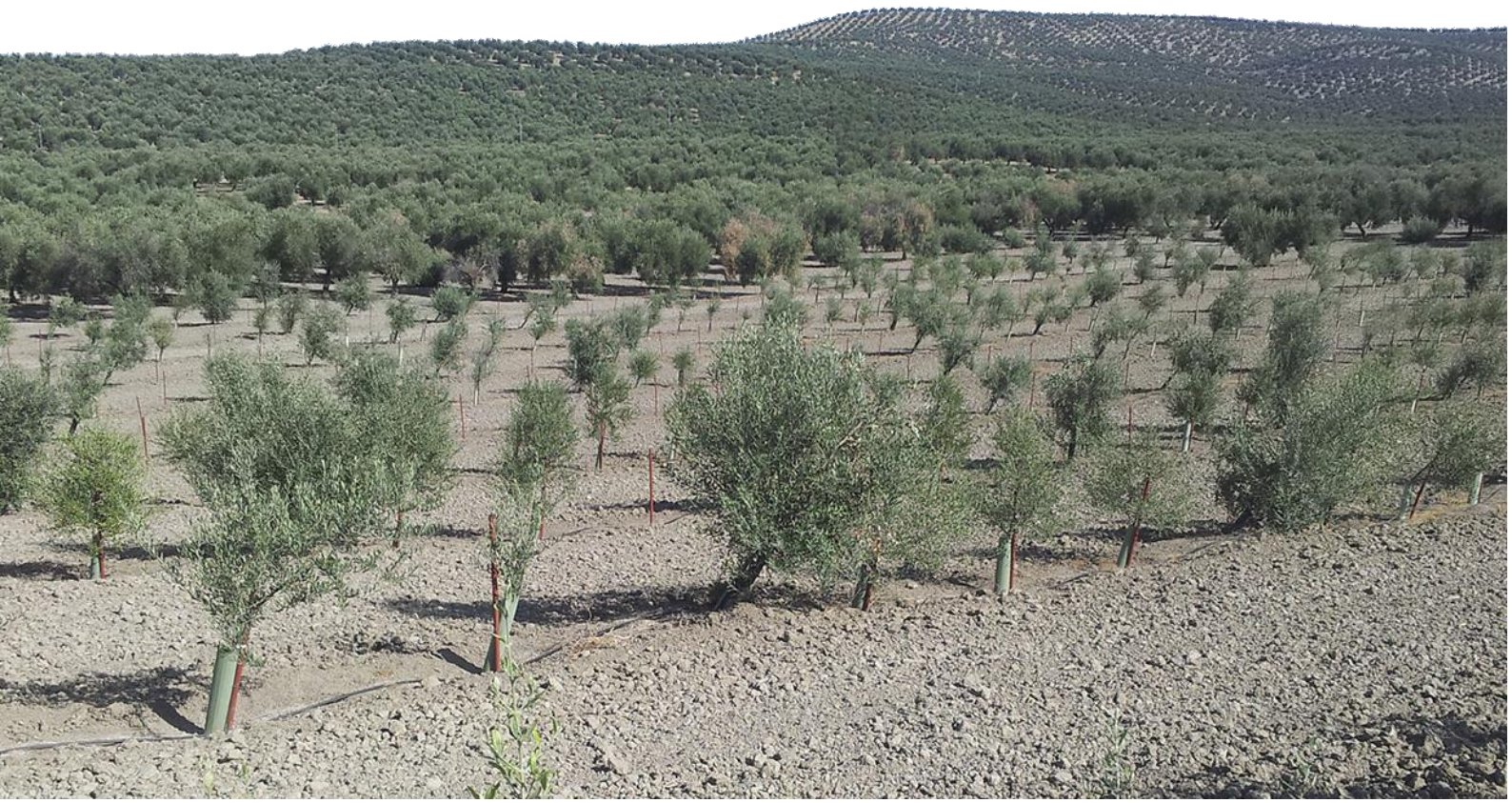
Genotypes colonization by the fungus led to suggest the possible involvement of tolerance mechanisms because some genotypes categorized as susceptible and resistant to development symptoms were colonized similarly. The association between resistance to development of symptoms and high vascular colonization was not found among the wild genotypes. This association was observed in some of the resistant genotypes from directed crosses and open-pollination.

Plant materials evaluated in this work are currently growing in experimental field trials with differents aims: possible future propagations of the interesting genotypes, to complete agronomic evaluations of the breeding program, to use these genotypes as potential genitors in future breeding cycles and/or rootstock, and application of molecular methods if their resistance would be confirmed. Assess the disease´s level in field naturally infested by *V. dahliae* would allow in the future to continue the works on the olive breeding program up to the possible registration new cultivars resistant to the disease.

Abreviaturas

CPDW	Content Pulp Dry Weight
CWA	Chlorotetracycline Water Agar
D	Defoliating pathotype
DFP	Disease-Free Period
DII	Disease Intensity Index
Far	Fruit Area
FCir	Fruit Circularity
FDI	Final Disease Incidence
FDII	Final Disease Intensity Index
FDPI	Final Dead Plants Incidence
FDW	Fruit Dry Weight
FFW	Fruit Fresh Weight
FM	Fruit Moisture
FMD	Fruit Maximum Diameter
FPer	Fruit Perimeter
MS	Moderately Susceptible cultivar/genotype
ND	Non Defoliating pathotype
NMR	Nuclear Magnetic Resonance
OCFDW	Oil Content Fruit Dry Weight
OCFFW	Oil Content Fruit Fresh Weight
OCPFW	Oil Content Pulp Fresh Weight
P/SDW	Pulp/Stone Ratio Both Dry Weight
P/SFW	Pulp/Stone Ratio Both Fresh Weight
PDA	Potato Dextrose Agar
PDW	Pulp Dried Weighed
PFW	Pulp Fresh Weight
PM	Pulp Moisture
R	Resistant cultivar/genotype
RCI	Root Colonization Index
RDFP	Relative Disease-Free Period
RI	Fruit Ripening Index
RSI	Relative Susceptibility Index
S	Susceptible cultivar/genotype
SAr	Stone Area
SAUDPC	Standardized Area Under the Disease Progress Curve
SCI	Stem Colonization Index
SCir	Stone Circularity
SDW	Stone Dry Weight
SFW	Stone Fresh Weight
SM	Stone Moisture
SMD	Stone Maximum Diameter
SP	Susceptibility in the reference susceptible cultivar ‘Picual’
SPer	Stone Perimeter
WOGBC	World Olive Germplasm Bank of Córdoba

Capítulo 1. Introducción general



1.1. Material vegetal de olivo

El olivo (*Olea europaea* L.) pertenece a la familia *Oleaceae*, compuesta por 29 géneros y más de 600 especies distribuidas en regiones de clima tropical y templado (Besnard et al. 2009). Según la morfología y distribución geográfica, se han identificado seis subspecies (Green, 2002) centrándose en dos de ellas las evaluaciones que recoge este trabajo. La primera subespecie es *europaea* y es propia del área del Mediterráneo, la cual se divide en dos grandes grupos: variedad *europaea,* que engloba a los olivos cultivados y variedad *sylvestris* que incluye los olivos silvestres (acebuches). La segunda subespecie es *guanchica*, endémica de las Islas Canarias.

Según el Comité Oleícola Internacional (COI) el olivar mundial se cifra en torno a 850 millones de árboles que ocupan una superficie de más de 10 millones de hectáreas. De éstas, más de un millón se dedica a la producción de aceitunas de mesa. En la actualidad, España cuenta con 2.593.523 hectáreas de olivar, concentrándose el 83,4% de la producción en Andalucía (MAGRAMA, 2014). Desde que el agricultor conociera la propagación vegetativa por estaquillado semileñoso, se comenzó a seleccionar las primeras variedades de olivo. Dicho filtrado de variedades correspondía posiblemente por su productividad, tamaño de fruto y adaptación al medio, transfiriéndose estas características interesantes por propagación vegetativa (Kaniewski et al. 2012). El germoplasma de olivo en el mundo contiene más de 2600 variedades diferentes (Muzzalupo et al. 2010). Para el caso de España, se han catalogado 276 variedades de olivo, pero sólo 24 de ellas se consideran variedades principalmente cultivadas (Barranco et al. 2008).

En 1971 se estableció el primer Banco de Germoplasma Mundial de Olivo (BMGO) ubicado en lo que hoy en día se conoce por IFAPA, Centro "Alameda del Obispo" de Córdoba. El BMGO respondía a la necesidad de conservar las diferentes variedades que históricamente se describían a nivel mundial con el principal objetivo de mantener la biodiversidad genética del cultivo. En este sentido en la Colección Mundial de Variedades de Olivo del Centro IFAPA "Alameda del Obispo" del BGMO (CAP, UCO, IFAPA) se conservan actualmente alrededor de 885 variedades de 24 países (Belaj et al. 2013). Con el tiempo se comprobó la necesidad de establecer un Banco de Germoplasma de Acebuches (BGA) en el mismo centro de investigación suscitado por la gran variabilidad genética *in situ* de las poblaciones de acebuches conocidas a nivel morfológico, agronómico y molecular (Belaj et al. 2007; Díez et al. 2011). El BGA alberga una colección de olivos silvestres y subespecies afines procedentes de diferentes zonas en España (Andalucía, Baleares, Canarias y Extremadura) y en Portugal (Islas Madeira).

1.2. *Verticillium dahliae:* variabilidad patogénica

Verticillium es un género de hongos fitopatógenos que pertenece a la división Ascomycota, clase Sordariomycetes y de la familia *Incertae sedis*, que causa enfermedades conocidas como Verticilosis sobre más de 500 plantas huéspedes según estudios filogenéticos recientes (Inderbitzin et al. 2011). El género comprende diez especies entre las cuales *Verticillium dahliae* Kleb. (Klebahn, 1913) es la más importante por presentar más de 200 especies como huéspedes en todo el mundo (Inderbitzin and Subbarao, 2014). Entre ellas se han descrito pérdidas de plantas de más del 50% en especies de importancia económica como

algodón (Friebertshauser and DeVay, 1982), lechuga (Atallah et al. 2011), olivo (López-Escudero and Mercado-Blanco, 2011), lúpulo (Radišek et al. 2006), patata (Rowe and Powelson, 2002) o fresa (Shaw et al. 2010).

En líneas generales, los aislados de *Verticillium dahliae* no presentan patogenicidad huésped específica en sentido estricto y aquellos que proceden de un huésped concreto poseen una graduación en virulencia, es decir causan síntomas que oscilan desde leves o muy leves hasta el extremo de originar la muerte en plantas del huésped de procedencia. Se ha demostrado virulencia cruzada entre aislados de olivo y algodonero (Rodríguez-Jurado, 1993; Bejarano-Alcázar et al. 1995). Tjamos (1981) estudió aislados de *V. dahliae* obtenidos de diferentes cultivos herbáceos y leñosos como almendro, pistacho, olivo, rosa y sandía. Él observó ausencia de especialización entre aislados y diferenció la virulencia entre ellos según el tipo de cultivo, monocultivo o cultivos diversificados donde los aislados presentaban mayor virulencia. Así, apuntó precaución para la práctica de rotación de cultivos como medida de control, ya que aislados de *V. dahliae* podrían incrementar la densidad de inóculo del hongo en el suelo al infectar y colonizar el siguiente cultivo huésped.

Las poblaciones de *V. dahliae* obtenidas de plantas de algodonero y olivo en España se han caracterizado a nivel de planta, genético y molecular, en dos grandes grupos o patotipos denominados Defoliante (D) y No Defoliante (ND) (Rodríguez-Jurado et al. 1993; Bejarano-Alcázar et al. 1995; Mercado-Blanco et al. 2003; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012). Sin embargo, los aislados ND presentan mayor heterogeneidad genética y molecular que los del patotipo D en Andalucía, los cuales se agrupan en un solo VCG (Grupo de Compatibilidad Vegetativa) (Korolev et al. 2001; Jiménez-Díaz et al. 2011). Algunos autores apuntan la recombinación genética (Heale et al. 1988) o una reorganización del genoma del hongo (de Jonge et al. 2013) como generadores de heterogeneidad. Recientemente, Milgroom et al. (2014) sugieren la posible existencia de una fase reproductiva sexual más que una presión de selección y adaptación al medio del hongo para explicar la variabilidad molecular y genética.

1.3 Ciclo de vida de *V. dahliae* y síntomas de la enfermedad

Las estructuras de resistencia característica de *V. dahliae* se conocen con el nombre de microesclerocios. Los microesclerocios son estructuras melanizadas mayoritariamente de 125 a 35 ó 38 µm formadas por septación sucesivas de las células que constituyen las hifas de hongo (Asworth et al. 1972; Smith and Rowe, 1984; López-Escudero et al. 2003). El carácter melanizado de los microesclerocios ofrece la posibilidad de mantener al hongo en estado latente en el suelo durante al menos 14 años (Wilhelm, 1955). López-Escudero et al. (2012), a partir de plantas de olivo infectadas con *V. dahliae*, señalan una tendencia morfológica más elongada que ovalada de los microesclerocios de aislados que pertenecen al patotipo D.

Los microesclerocios que se encuentran en el suelo libres o asociados a restos de plantas infectadas pueden comenzar a germinar estimulados por los exudados de las raíces e iniciar un ciclo de infección (*V. dahliae* es un patógeno monocíclico, Figura 1.1). Hay que destacar que es a través de las raíces por las que el hongo penetra en la planta habiéndose sugerido que la penetración en olivo es a través de roturas en las raíces más que mediada por alguna actividad enzimática atribuida al patógeno (Prieto et al. 2009). El hongo avanza por el córtex

de la raíz de olivo principalmente de manera intracelular hasta llegar al sistema vascular de la planta (Prieto et al. 2009).

Eventualmente, la esporulación y el transporte de conidias (esporas asexuales) originadas exógenamente en una hifa (fiálida) o estructura (conidióforo) específica ocurre a través del flujo xilemático. Se resalta que las conidias liberadas son transportan por el flujo favorecidas por el proceso de transpiración del olivo, lo que permite la colonización en longitud de la planta (Rodríguez-Jurado, 1993; Baídez et al. 2007). El xilema es colonizado también en sentido transversal mediante hifas del patógeno que pasan a través de los poros que comunican los elementos traqueales entre sí. Los síntomas de Verticilosis se manifiestan durante la colonización en longitud y transversal de los elementos traqueales por el patógeno (Rodríguez-Jurado, 1993; Baídez et al. 2007; Jiménez-Díaz et al. 2011).

La Verticilosis del olivo (VO) se desarrolla en campo en base a dos síndromes denominados Apoplejía y Decaimiento Lento descritos en España por Blanco-López et al. (1984). La Apoplejía se muestra principalmente entre invierno y primavera afectando de forma rápida a hojas, brotes y ramas con necrosis. Las hojas pueden abarquillarse y, generalmente, se secan manteniéndose en las ramas. Es un síndrome que con frecuencia provoca la muerte del árbol. Blanco-López et al. (1984) señalaron que estos síntomas pueden ir acompañados por una coloración rojiza en el exterior de la planta que ha sido colonizada, mostrando en el interior de la planta una coloración característica castaña oscura. El Decaimiento Lento normalmente tiene más impacto en la primavera y principio de verano, caracterizándose por la defoliación parcial de las ramas afectadas, y/o la necrosis de inflorescencias y frutos. Más recientemente se ha indicado en Andalucía que la enfermedad en árboles jóvenes establecidos en regadío e infectados por el patotipo D puede manifestarse por una parcial o total defoliación de hojas verdes o verde mate que tiene lugar principalmente desde finales de otoño a principios de primavera y que puede ocasionar la muerte de árboles (Navas-Cortés et al. 2008). Los árboles afectados por cualquiera de los síndromes descritos pueden recuperarse a largo plazo de la enfermedad siempre que ésta no afecte completamente a la copa de árbol y que tengan lugar circunstancias que no favorezcan la enfermedad (por ejemplo altas temperaturas) pero que permitan que el árbol reanude su crecimiento natural. Este fenómeno se ha denominado recuperación natural o remisión de síntomas y se ha descrito asociado a olivo en campo en distintos países (Wilhelm and Taylor, 1965; Thanassoulopoulos et al. 1979; Blanco-López at al. 1984; Tjamos et al. 1991; López-Escudero and Blanco-López, 2005a; Levin et al. 2003; Sesli et al. 2010; Bubici and Cirulli, 2014) y en condiciones de inoculación artificial (Trapero et al. 2015).

V. dahliae puede formar microesclerocios en el xilema y los tejidos extraxilemáticos de las partes del árbol severamente afectadas (Rodríguez-Jurado, 1993; Trapero et al. 2011). La caída al suelo de hojas, flores, frutos y ramas afectadas, devolverá el hongo al suelo.

La sintomatología descrita puede ser similar a la provocada por otras amenazas como la bacteria *Xylella fastidiosa,* que en la actualidad está causando altas pérdidas en olivo por ataque en Italia (Saponari et al. 2013), enfermedades causadas por otros hongos de suelo como *Phytophthora* spp. o por alteraciones climáticas o nutricionales, entre otros. Por tanto, el diagnóstico de la enfermedad resulta clave para el correcto manejo de la misma.

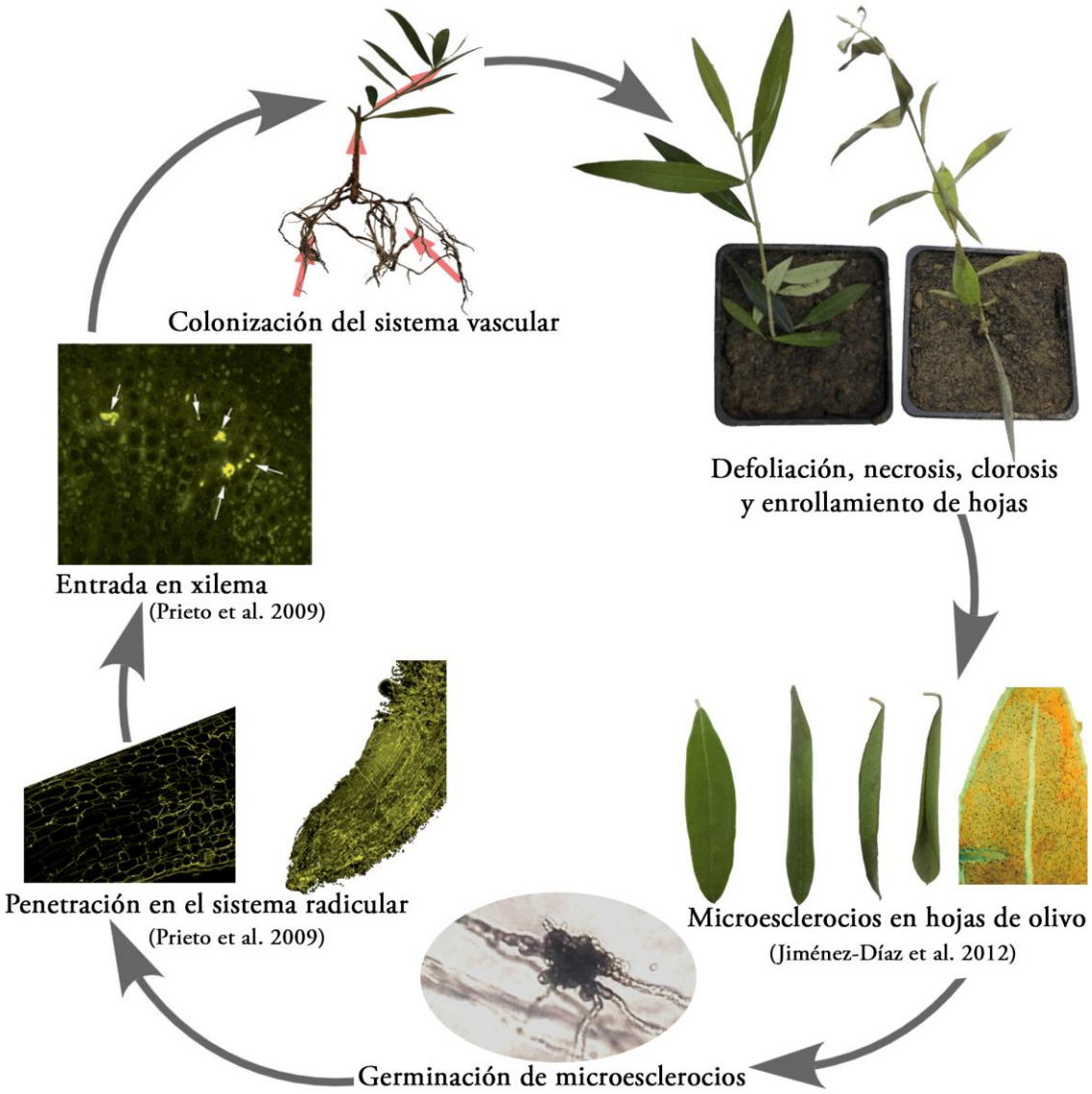


Figura 1.1. Adaptación del ciclo de vida de *Verticillium dahliae* descrito por Hiemstra and Harris (1998).

1.4. Antecedentes de la enfermedad

La VO se diagnosticó por primera vez en España en la década de 1980 en el trabajo de Blanco-López et al. (1984), desde que en 1946 Rugieri describiera la enfermedad por primera vez en Italia. En los últimos 30 años la VO se ha expandido en los principales países productores de olivo (España, Italia, Grecia, Turquía, etc.) previsiblemente por la expansión del cultivo con variedades que son susceptibles al ataque del patógeno y por los cambios en las prácticas de cultivo que han favorecido la presencia de aislados D asociados a olivo en países como España, Turquía y Túnez (Rodríguez et al. 2008; López-Escudero et al. 2010; Jiménez-Díaz et al. 2011; Triki et al. 2011; Gharbi et al. 2015). La situación es grave en España, concretamente en Andalucía, debido a la prevalencia del patotipo D altamente virulento (Moraño-Moreno et al. 2008; López-Escudero et al. 2010; Jiménez-Díaz et al. 2011), el cual ocasiona las epidemias más rápidas y severas conocidas (Navas-Cortés et al. 2008). El patotipo D fue identificado por primera vez en Andalucía infectando olivos entre

1996 y 1998 en la provincia de Sevilla (López-Escudero et al. 2001) y desde entonces se ha aislado de árboles en otras cuatro provincias andaluzas (López-Escudero et al. 2010; Jiménez-Díaz et al. 2012). A ello han contribuido los diversos medios de dispersión del hongo como el suelo, el agua de riego, las inflorescencias, las semillas y la multiplicación de plantas infectadas asintomáticas (López-Escudero and Mercado Blanco, 2011).

La amplia dispersión y la virulencia del patotipo D sumadas a la naturaleza vascular de la Verticilosis en un cultivo perenne han dificultado el control de la enfermedad. Hoy en día no existe fungicida disponible como medida de control de la VO, por lo que la resistencia varietal, es decir utilizar variedades de olivo que presenten resistencia al desarrollo de síntomas y a la colonización vascular, junto con el manejo adecuado del cultivo que impidan o reduzcan la dispersión del hongo y su incremento en el suelo, supone la estrategia más recomendable para el control de la VO en nuevas plantaciones y para replantación. La resistencia varietal es una medida de control de las enfermedades reconocida por ser más eficaz que otras medidas, económica y respetuosa con el medio ambiente.

1.5. Reacciones a la interacción variedad de olivo-patotipo de *V. dahliae*

En condiciones controladas los aislados del patotipo ND causan síntomas de severidad leve a moderada en la parte aérea de plantas de olivo, consistentes en clorosis foliar, nula a moderada defoliación y acortamiento de los entrenudos contrastando con los severos síntomas originados por los aislados D, caracterizados principalmente por ocasionar defoliación de hojas verdes, marchitez de hojas y muerte de plantas (Rodríguez-Jurado, 1993; López-Escudero et al. 2005b; Rodríguez-Jurado et al. 2007). Los síntomas se pueden iniciar o no en las hojas de la base del tallo y se desarrollan acrópetamente. La coloración castaño oscura no es frecuente en el interior de la planta (López-Escudero et al. 2004).

Los trabajos de evaluación de resistencia a la Verticilosis en condiciones controladas han demostrado que los aislados del patotipo ND son menos virulentos que los aislados del patotipo D sobre todas las variedades de olivo evaluadas en diferentes países (López-Escudero et al. 2004; Colella et al. 2008; Markakis et al. 2010; Sanei and Razavi, 2011; Triki et al. 2011; Calderón et al. 2014). La mayoría de las variedades tradicionales en España son extremadamente susceptibles al patotipo D del hongo. Algunas de estas variedades son las de mayor difusión en la actualidad, como 'Arbequina', 'Cornicabra', 'Hojiblanca', 'Manzanilla de Sevilla' y 'Picual'. Variedades de otros orígenes geográficos como 'Amfissis', 'Ascolana', 'Kalamon', 'Konservolia' y 'Leccino' muestran similar reacción al patotipo D. Sólo un número muy reducido de variedades, como 'Frantoio', 'Changlot Real', 'Dolce Agogia', 'Empeltre', 'Koroneiki', 'Sevillenca' y 'Oblonga', poseen niveles de resistencia moderados o considerables al desarrollo de síntomas causados por el patotipo D, aunque en algunos casos dichas variedades presentan alguna característica agronómica negativa que limita su uso (Paplomatas and Elena, 2001; López-Escudero et al. 2004; 2007; Martos-Moreno et al. 2006; García-Ruiz et al. 2014). 'Frantoio', 'Changlot Real' y 'Empeltre' son también resistentes en suelos naturalmente infestados bajo alta presión (21 microesclerocio/g suelo) de inóculo del patotipo D mientras que variedades como 'Arbequina' presentan resistencia moderada en dichas condiciones (Trapero et al. 2013a). Muchos de los cultivares ampliamente comercializados son susceptibles a la Verticilosis bajo condiciones de campo aunque no en todos los estudios se indicó el patotipo o la densidad de inóculo del hongo en

el suelo (López-Escudero et al. 2011). La recuperación de los síntomas (crecimiento de la planta después de un periodo sintomático) es en general más frecuente en plantones inoculados con el patotipo ND que con el D altamente virulento en los cultivares estudiados (Rodríguez-Jurado et al. 1993; López-Escudero et al. 2010) aunque el factor varietal también influye en dicha recuperación (López-Escudero et al. 2005). Todo ello indica la necesidad de disponer de fuentes de resistencia al patotipo D más virulento.

Todas las variedades o genotipos estudiados hasta la fecha son vascularmente infectados (Cicaresse et al. 2002; López-Escudero et al. 2004, 2005, 2007; Martos-Moreno et al. 2006; Colella et al. 2008; Dervis et al. 2010; Trapero et al. 2011, 2013b, 2015; Erten and Yildiz, 2011; García-Ruiz et al. 2014; Chliyeh et al. 2014). Los síntomas de Verticilosis en olivo se inician con posterioridad a la presencia del patógeno en la parte aérea de la planta (Rodríguez-Jurado, 1993; Mercado-Blanco et al. 2003; Markakis et al. 2010; Gramaje et al. 2013). El porcentaje más alto de aislamientos del hongo a partir de tejidos de la parte aérea se obtiene con anterioridad a la máxima expresión de la enfermedad en reacciones extremadamente susceptibles, o durante la fase asintomática cuando la reacción es de susceptibilidad moderada o resistente al desarrollo de síntomas (Rodríguez-Jurado, 1993). Mediante aislamientos a partir de tejidos, Rodríguez-Jurado (1993) indicó que una mayor continuidad de la colonización en longitud diferenció la reacción a la enfermedad de 'Picual' (síntomas severos) y 'Oblonga' (síntomas moderados) inoculados con el patotipo D.

En los últimos años se han desarrollado diferentes trabajos con el objetivo de aumentar el conocimiento de la interacción de *Verticillium* spp. con su amplia gama de huéspedes. Los términos resistencia y/o tolerancia se encuentran en la actualidad en debate para su aplicación a la VO. Diversos autores apoyan la hipótesis de resistencia verdadera a la enfermedad al haber encontrado que la colonización vascular es reducida en combinaciones genotipo-patotipo que expresan menor severidad de enfermedad comparadas con combinaciones susceptibles (Rodríguez-Jurado, 1993; Mercado-Blanco et al. 2003; López-Escudero and Blanco-López, 2005). Mercado-Blanco y colaboradores (2003) llevaron a cabo el primer estudio de cuantificación de ADN de *V. dahliae* en plantas de olivo mediante la reacción en cadena de la polimerasa en tiempo real (RT-PCR). La cantidad de ADN del hongo y la incidencia de enfermedad fueron correlacionadas en 'Arbequina' y 'Picual'. En este estudio, así como en otros realizados con diferentes genotipos o variedades (Markakis et al. 2009; Gramaje et al. 2013), la cuantificación de ADN del patógeno en el tallo fue mayor que en la raíz. Mercado-Blanco et al. (2003) encontraron que la colonización de la raíz (cantidad de ADN) fue similar en Acebuche-L y 'Arbequina', pero menor en 'Picual' mientras el hongo no fue cuantificado en la parte aérea de Acebuche-L, el genotipo menos afectado por la enfermedad. Gramaje y colaboradores (2013) contrastaron por medio de aislamientos y RT-PCR la presencia de *V. dahliae* en tejidos de plantas asintomáticas. Estos investigadores sugirieron que el cultivar 'Frantoio' debería ser considerado como tolerante en lugar de resistente a *V. dahliae* según el concepto de la tolerancia como la capacidad de un genotipo huésped para paliar la enfermedad habiendo sido colonizado por una cantidad de patógeno comparable a la de otro genotipo (Gao et al. 1995; Robb et al. 2007). Gramaje y colaboradores (2013) sugirieron también que el aislamiento del hongo de los tejidos y la RT-PCR podrían ser técnicas complementarias para una correcta cualificación de la interacción genotipo-aislado. Así por ejemplo, el hongo se ha aislado pero no ha podido ser cuantificado en la parte aérea de Acebuche-L (Mercado-Blanco et al. 2003). Los mecanismos de resistencia

y/o tolerancia del olivo a la Verticilosis deben ser dilucidados. Bubici y Cirulli (2012) defienden la resistencia a través de mecanismos bioquímicos de defensa, lo que les permitió el control de la Verticilosis con el uso de portainjertos, usando como tal un clon de la variedad 'Frantoio'. Por ello se sugerido que tales mecanismos se desarrollan principalmente en las raíces y/o pueden transferirse sistemáticamente, incluso con el uso de cultivares susceptibles como injertos. Con ello, se abre una posible opción de manejo de la enfermedad desde un programa de mejora de olivo.

1.5. Programa de mejora de olivo: línea de búsqueda de resistencia a la Verticilosis

En los últimos años el cultivo del olivo ha sufrido profundos cambios que han permitido el desarrollo de nuevas plantaciones con características muy diferentes al cultivo tradicional (mayores densidades de plantación, riego, mecanización de la recolección, alta calidad del producto, etc). Estos cambios han puesto de manifiesto la necesidad de disponer de nuevas variedades adaptadas a las nuevas técnicas de cultivo que ofrezcan nuevas alternativas a las variedades tradicionales que se cultivan en la actualidad.

En España, el primer programa de obtención de nuevas variedades de olivo se inició en Córdoba en el año 1992 en colaboración de la UCO e IFAPA) (Rallo, 1995). Los principales objetivos de interés agronómico en este programa son la precocidad y productividad, rendimiento graso, época de maduración, aptitud a la recolección mecánica, calidad del aceite y resistencia a factores limitantes. Desde su inicio se han realizado más de 80 cruzamientos diferentes empleando más de 30 variedades como genitores y se han generado más de 10.000 nuevos genotipos procedentes de estos cruzamientos. Como ejemplo del éxito del programa de mejora de olivo de Córdoba, cabe citar el trabajo de selección motivado por el cambio a sistemas de plantación de alta densidad con recolección mecanizada mediante cosechadoras cabalgadoras iniciado por el sector a mediados de los noventa (Tous et al. 2010). Siguiendo la metodología que se detallará a lo largo de este documento, las etapas en el programa de mejora dieron como resultado el registro de la primera nueva variedad de olivo adaptada a este nuevo tipo de plantaciones: Sikitita (Rallo et al. 2008).

La obtención de nuevas variedades de olivo en programas de mejora constituye un proceso muy largo que comienza con la realización de los cruzamientos y obtención de plantas de semilla y, termina con la selección definitiva de nuevas variedades en función de sus características agronómicas. El proceso completo incluye una serie de etapas consecutivas en función de los objetivos de mejora (Figura 1.2). A través de estas etapas va disminuyendo el número de genotipos en evaluación a la vez que se van ampliando las repeticiones de cada uno y las características objeto de evaluación.

En los últimos años se han puesto a punto diferentes técnicas que permiten simplificar en parte esta evaluación y mejorar la eficiencia de los trabajos. Un ejemplo de técnicas actuales con marcadores moleculares permiten realizar pruebas de paternidad y establecer con seguridad la identidad del genotipo polinizador, evitando contaminaciones (De la Rosa et al. 2004). Se ha conseguido acortar el periodo juvenil mediante el forzado del crecimiento, primero en invernadero y luego en campo (Lavee et al. 1996; Santos-Antunes et al. 2005; Moreno-Alías et al. 2010). Además, se han desarrollado criterios de selección precoz que permiten, en las primeras etapas del proceso, eliminar el mayor número de genotipos que no

van a presentar características interesantes y por tanto su mantenimiento supondría un coste innecesario.

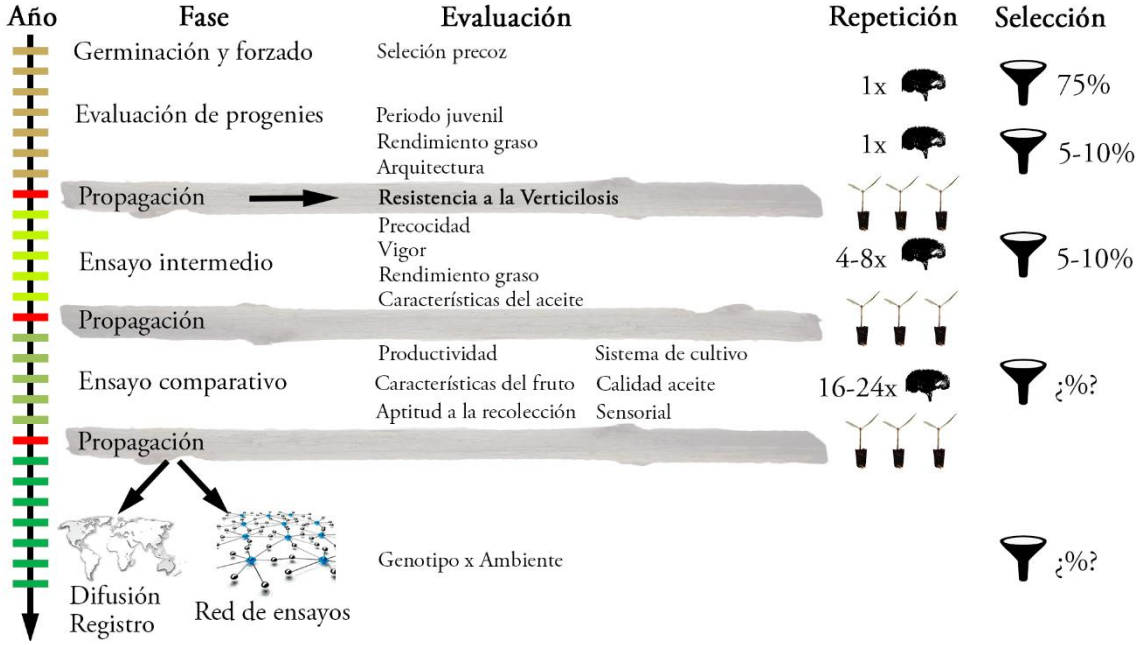


Figura 1.2. Etapas del programa de mejora de olivo (León, 2014).

Trabajos recientes han demostrado por ejemplo que es posible seleccionar para corto periodo juvenil en base a medidas de crecimiento y vigor en las etapas iniciales de crianza de las plantas de semilla (De la Rosa et al. 2006; Rallo, P. et al. 2008). Una vez superado el periodo juvenil, se produce la primera floración y fructificación y se inicia el proceso de evaluación para los caracteres de interés. Una observación común en los trabajos de evaluación de progenies son los amplios intervalos de variación para todas las características analizadas (Lavee, 1990; Bellini, 1993; León et al. 2004ab), en algunos casos iguales o superiores a los citados por algunos autores en colecciones varietales. Esta trasgresión de los límites de variabilidad de la generación previa es de gran utilidad en mejora de olivo debido a la posibilidad de conservar cualquier genotipo mediante propagación vegetativa. En la actualidad se empieza a disponer información acerca de la herencia de caracteres como el periodo juvenil, el vigor, el tamaño del fruto, el contenido de aceite y la capacidad de enraizamiento (León et al. 2004ab; Santos-Antunes et al. 2005; Zeinanloo et al. 2009; Lavee and Avidan, 2011, 2012).

La determinación de muchas de las características de interés se debe realizar principalmente en las etapas finales del proceso de mejora ya que su evaluación previa en las plantas de semilla iniciales es poco precisa al disponerse únicamente de una repetición (planta) por genotipo (León et al. 2007; De la Rosa et al. 2008). La propagación vegetativa de los genotipos seleccionados en cada etapa permite establecer ensayos de campo con suficiente número de repeticiones para evaluar las principales características de interés. Según los objetivos concretos de mejora y los recursos disponibles se pueden proponer diferentes esquemas alternativos de selección respecto al número de etapas, repeticiones y caracteres. Recientemente se ha propuesto la utilidad de una etapa intermedia de selección (Figura 1.2) que permite realizar una selección más precisa para caracteres de productividad, vigor y calidad del aceite (León et al. 2015).

La etapa final previa al registro de nuevas variedades consiste en el establecimiento de redes de ensayo con los genotipos más prometedores, usando algunas de las variedades más difundidas como 'Picual' y 'Arbequina' como testigos. De esta manera se puede analizar la interacción genotipo x ambiente ya que las condiciones ambientales pueden influir significativamente en caracteres de interés agronómico de las variedades. Finalmente se seleccionarán como nuevas variedades aquellos genotipos que hayan mostrado un mejor comportamiento, bien de un modo general en diferentes condiciones de cultivo o bien de modo específico para unas condiciones edafoclimáticas y de cultivo particulares.

El programa de mejora se encuentra en continuo cambio para satisfacer las demandas de un sector en evolución constante. Por ejemplo, la incorporación al programa de una nueva línea de investigación para encontrar resistencia a la Verticilosis, enfermedad respecto a la cual gira la presente Tesis Doctoral. El desarrollo de esta línea de investigación en el Centro IFAPA "Alameda del Obispo" en Córdoba contempla entre sus objetivos principales la obtención de nuevas variedades con resistencia a la Verticilosis a partir de la evaluación de plantas originarias de semilla procedentes de cruzamientos así como material de olivo silvestre (acebuche) de distinta procedencia. La evaluación de ambos tipos de materiales puede proporcionar a medio plazo nuevas variedades o patrones altamente resistentes a la enfermedad.

El esquema de selección propuesto para esta nueva línea se adapta, con modificaciones, al esquema general seguido en el programa de mejora. Así, el procedimiento de selección para resistencia a la Verticilosis se llevaría a cabo a partir de los genotipos seleccionados tras la evaluación inicial de progenies. Por tanto, esta etapa constituiría en esencia un tipo de ensayo intermedio centrado en la evaluación de resistencia a la Verticilosis como principal criterio de selección. Este esquema de selección ofrece una serie de ventajas respecto a la selección inicial en plantas de semilla realizada en otros trabajos de selección en olivo (Wilhelm and Taylor, 1965; Trapero et al. 2015):

- Trabajos previos han demostrado que se puede realizar una selección eficiente en plantas de semilla en base principalmente a sus características de corto periodo juvenil y alto rendimiento graso, manteniendo al mismo tiempo amplia variabilidad para otras características no consideradas (León et al. 2015).
- Esta selección inicial incrementa las posibilidades de selección final de genotipos que combinen altos niveles de resistencia y adecuadas características agronómicas, con una utilidad comercial equilibrada.
- La propagación vegetativa permite disponer de repeticiones de cada uno de los genotipos, lo que permite la realización de evaluaciones en condiciones controladas más precisas incluyendo tanto plantas inoculadas como no inoculadas (controles).
- Al final de la evaluación anterior se sigue disponiendo de material exento de infección, sin haber tenido contacto con el hongo, para posibles usos futuros si resulta de interés por su nivel de resistencia u otros caracteres paralelos del programa de mejora.

En el desarrollo de la presente Tesis Doctoral, se ha establecido un esquema homólogo para el programa de mejora procedente del material silvestre desde que se incorpora al BGA. En olivos silvestres o acebuches (*Olea europaea* subsp. *europaea* var. *sylvestris*) se ha demostrado la gran variabilidad genética de dichas poblaciones vegetales y su potencial uso

en programas de mejora genética de olivo (Baldoni and Belaj, 2009). Como se ha indicado con anterioridad, los acebuches son alógamos y de propagación por semillas por lo que presentan un nivel de heterocigosidad y de variabilidad genética superior al olivo cultivado (Lumaret et al. 2004). Las poblaciones silvestres de olivo podrían ser una importante fuente de genes de resistencia a estreses abióticos como frío (Makhzoumi and Pungetti, 2005), sequía (Lo Gullo et al. 2003) o salinidad (Therios, 2009) y a agentes bióticos como los que causan la Verticilosis (Colella et al. 2008) o el repilo (Díez et al. 2012).

1.6. Hipótesis y Objetivos

El desarrollo de la presente Tesis Doctoral está inmerso en un programa de mejora de olivo para seleccionar nuevas variedades adaptadas a una olivicultura moderna. La incorporación de la resistencia a la Verticilosis como objetivo de interés ha supuesto la creación de una nueva línea de investigación que persigue combinar unas deseadas características agronómicas de interés y la búsqueda de la resistencia a la enfermedad.

Por tanto y con los antecedentes comentados anteriormente, el objetivo general de la Tesis ha sido seleccionar genotipos procedentes de progenies de olivo y de material silvestre que presenten niveles elevados de resistencia a la Verticilosis y ampliar el conocimiento acerca de la herencia de dicha resistencia. Este objetivo general se ha plasmado en diferentes objetivos concretos en función del material vegetal utilizado en cada caso:

- Evaluación agronómica y selección de nuevos genotipos procedentes de polinización libre de variedades de interés y estimación de la heredabilidad de los caracteres agronómicos (Capítulo 2).
- Evaluación de la resistencia a la Verticilosis de genotipos procedentes de polinización libre de variedades previamente seleccionados por sus características agronómicas. (Capítulo 3).
- Evaluación de la resistencia a la Verticilosis de genotipos provenientes de cruzamientos dirigidos entre algunas de las variedades que han mostrado mayores niveles de resistencia en trabajos previos (Capítulo 4).
- Evaluación de la resistencia a la Verticilosis de genotipos de olivo silvestre y subespecies afines y de descendientes de cruzamientos cultivado x silvestre (Capítulo 5).

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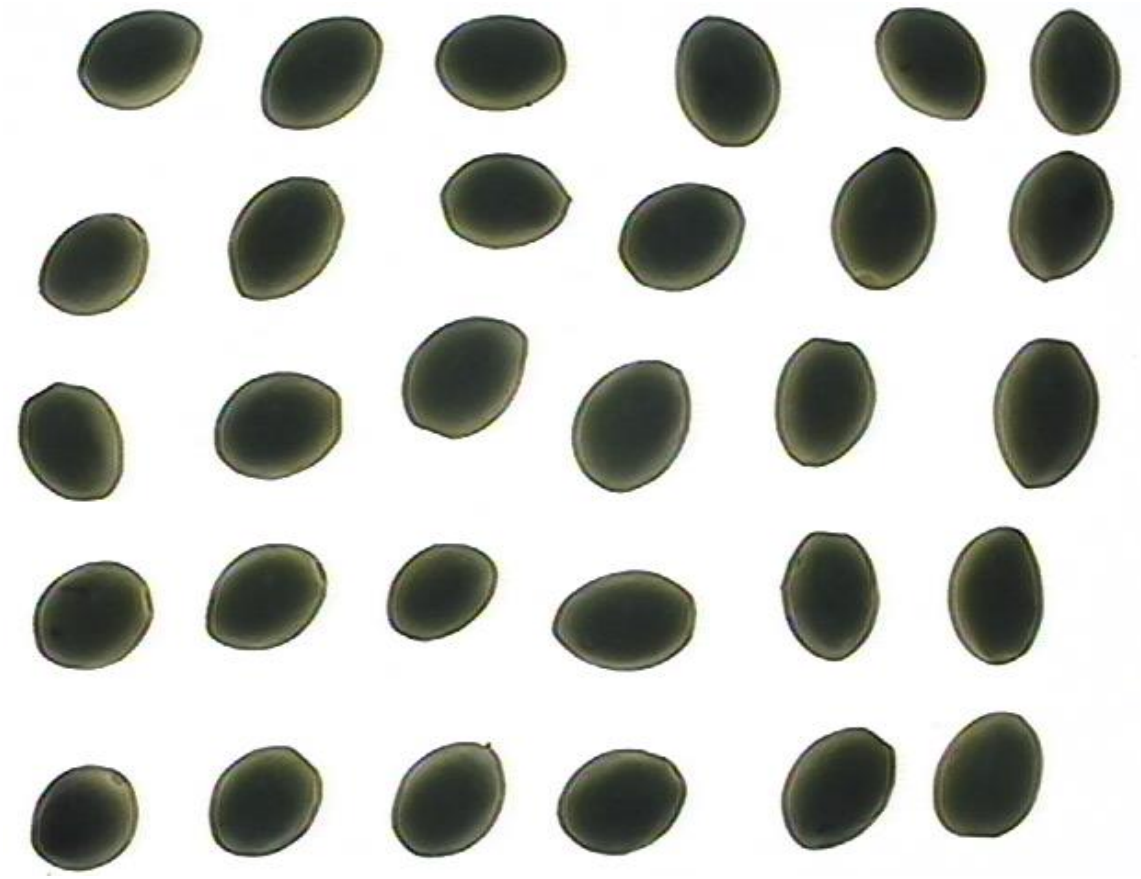
Capítulo 2. Characters in olive progenies from open-pollination



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2.1. Abstract

Several olive breeding programs have been initiated in the last years to obtain new olive cultivars adapted to modern olive growing techniques. However, up to now there is limited information about the inheritance of the main agronomic traits in olive (*Olea europaea* L.). In the present work, twenty six morphological descriptors and agronomic traits were evaluated in progenies coming from open-pollination of 17 Spanish cultivars of the World Olive Germplasm Bank of IFAPA Córdoba, Spain and in their corresponding parents. Significant differences among progenies were observed for all the evaluated traits. Heritability estimates showed low values for oil and moisture content, moderate for flesh/stone ratio and high for fruit size and shape. For those characters with high heritability, average values of the parents followed similar pattern than the observed in their corresponding open pollinated progenies. Implications of the heritability values on olive breeding are discussed.

2.2. Keywords

Agronomic traits, Breeding program, Inheritance, Morphological traits, *Olea europaea* L.

2.3. Introduction

Despite of the modernization of different aspects of olive growing, most of the cultivars today used are traditional and not specifically adapted to the new cultivation systems (Lavee and Avidan, 2012). Olive breeding is still limited, among other factors, by the scarce genetic information about the mode and level of inheritance of the characters of interest. Descriptive characterization of progenies from crosses has shown a wide variability for many morpho-agronomic traits in olive (Avidan et al. 2012; Klepo et al. 2013; León et al. 2004a). Dominant heredity has been observed for some characters in some specific crosses. For instance, Bellini (1992), showed the predominance of fruits with medium-small size and round shape. A dominant female heredity has also been reported for some vegetative characteristics including tree size, leaf and fruit shape (Lavee, 1990). However, the analysis of a wide range of characters in progenies from self, free and cross pollination questioned the overall dominance of female heredity (Lavee and Avidan, 2011). The possibility illicit pollination on the breeding crosses caused by airborne pollen might led to an underestimation of the male parent effect. In fact, the recent development of molecular markers allowing to confirm the parentage of seedlings from crosses has permitted the detection of pollen contamination in breeding crosses (Díaz et al. 2007; Biton et al. 2012).

Some attempts to provide appropriate estimation of heritability have also been reported. Heritability estimates can be obtained from the partitioning of phenotypic variance into genetic and environmental components or by studying the degree of resemblance between relatives by rather simple measurements made on the population (Falconer and Mackay, 1996). Estimates of genetic and environmental components of variation for oil content components were obtained from the evaluation of genotypes from a diallel cross among 'Arbequina', 'Frantoio' and 'Picual' over three consecutive harvest seasons (León et al. 2004a). The genetic determinism of the vegetative and reproductive traits was calculated from the evaluation of genotypes from the cross between 'Olivière' and 'Arbequina' (Ben

Sadok et al. 2013). Heritability estimates for some fruit characters were also obtained from the analysis of general and specific combining ability in a one-way six-parents diallel cross (Zeinanloo et al. 2009). However, only a limited number of parents and progenies size was used in these works.

In this work, performed in the framework of the olive breeding program of Córdoba, seedlings with a wide genetic base coming from open-pollination of 17 olive cultivars were used to study the variability of morphological descriptors and agronomic traits and the relationships among them. Heritability of these characters was also estimated according to the degree of resemblance between relatives.

2.4. Materials and Methods

2.4.1. Plant materials

Seedlings derived from open pollination coming from 17 Spanish cultivars of the World Olive Germplasm Bank of IFAPA Córdoba (WOGBC), in Southern Spain were used in this study: 'Arbequina' (Arb), 'Blanqueta' (Bla), 'Canetera' (Can), 'Changlot Real' (ChR), 'Chorrúo Castro del Río' (CCR), 'Empeltre' (Emp), 'Hojiblanca' (Hoj), 'Lechín de Granada' (LGr), 'Lechín de Sevilla' (LSe), 'Manzanilla del Piquito' (MPi), 'Manzanilla de Sevilla' (MSe), 'Morona' (Mor), 'Ocal' (Oca), 'Picudo' (Pdo), 'Picual' (Pic) and two previous selection of our breeding program (Sel1 and Sel2). These cultivars were selected according to their agronomic behaviour as potential interesting parents for olive breeding. As more than 360 accessions are included in the WOGBC orchard where the fruits were collected (Belaj et al. 2012), a wide variability of pollinators could be expected. Germination of seeds and forced growth of the seedlings in greenhouse were carried out according to the standard procedures used in the olive breeding program (Santos-Antunes et al. 2005). Plants of the cultivars used as parents were also obtained by vegetative propagation of adult semi-hardwood stem cuttings. Seedlings and parent plants were transplanted into the field at 4 m x 1.5 m spacing in 2008 and standard cultural practices were followed in the orchard to ensure tree growth. Morpho-agronomic evaluation was carried out when they reached the adult phase in 2012 in 10 seedlings per parent.

2.4.2 Traits evaluated

Fruit samples of 500 g were randomly collected for each seedling and parent. Fruit ripening index (RI) was calculated based on colour changes of peel and pulp classified into eight categories from green intense (0) to black peel and 100% purple pulp (7). From each sample, three subsamples of around 25 g were randomly selected to measure fruit fresh weight (FFW) and then the pulp was removed and the stones cleaned to calculate the pulp (PFW) and stone (SFW) fresh weight. Remaining pulp and stones were dried in a forced-air oven at 105ºC for 42 h, to ensure dehydration. Dried samples were weighed to determine pulp (PDW) and stone (SDW) dry weight and moisture content (PM and SM). Oil content was determined in the pulp subsamples using an NMR fat analyzer and expressed as a percentage on both fresh (OCPFW) and dry (OCPDW) basis. Other fruit characters were calculated on the basis of pulp and stone measurements: fruit dry weight (FDW), fruit moisture content (FM) and the oil content expressed as a percentage on both fruit fresh (OCFFW) and fruit

dry weight basis (OCFDW). The pulp/stone ratio both in fresh (P/SFW) and dry weight basis (P/SDW) were also calculated.

An additional set of 30 fruits per seedling was used for morphological characterization including measurements of perimeter, maximum diameter, circularity and area both for fruits (FPer, FMD, FCir, FAr) and stones (SPer, SMD, SCir, SAr). These measurements were taken using a computer based image analysis system (Skye Instruments Ltd, Powys, UK).

2.4.3. Data analysis

Variability histograms of the different morpho-agronomic traits were obtained for the whole dataset of progenies and parents. Principal components analysis (PCA) was used to investigate the relationship among traits. Analysis of variance was performed to determine between-progeny and within-progeny variation in those traits showing important loading weights in the PCA analysis. Narrow-sense heritability (h_n^2) was estimated from the variance components between-progenies (σ^2_{BP}) and within-progenies (σ^2_{WP}) according to a half-sib model (1) (Falconer and Mackay, 1996).

$$h_n^2 = 4\sigma^2_{BP}/(\sigma^2_{BP}+\sigma^2_{WP}) \quad (1)$$

2.5. Results and Discussion

A wide range of variation was found in all the 26 morpho-agronomic traits evaluated for the whole set of seedlings (Table 2.1). Similar or slightly higher ranges of variation were observed for the progenies than for the cultivars used as parents (Figure 2.1). These wide ranges of variation in the progenies have been previously reported in other progenies populations (León et al. 2004a; Avidan et al. 2012). Therefore, open pollination might be considered an interesting strategy in olive breeding as seedlings with high values for agronomic traits have been observed in the progenies. Variability for fruit shape has also been previously observed in olive progenies although, as in our work, spherical and oval forms were predominant, while elongated forms scarcely observed (Bellini, 1992, Klepo et al. 2013).

Table 2.1. Descriptive statistics for 26 morpho-agronomic traits evaluated in progenies from open pollination of 17 olive cultivars.

Variable	Mean	SD	Minimum	Maximum
RI	1.92	1.01	0.16	4.38
FFW	3.34	1.50	0.44	11.78
FDW	1.15	0.45	0.21	3.08
FM	64.44	4.46	52.74	76.32
OCFFW	14.00	3.16	2.88	24.50
OCFDW	39.33	6.99	7.80	54.99
PFW	2.88	1.37	0.25	10.68
PDW	0.78	0.35	0.07	2.12
PM	72.52	4.41	60.41	83.75
OCPFW	15.56	3.64	2.94	26.74
OCPDW	56.18	7.41	15.17	68.53
SFW	0.45	0.15	0.19	1.10

Variable	Mean	SD	Minimum	Maximum
SDW	0.37	0.12	0.13	0.95
SM	16.68	5.25	11.46	60.47
OCSFW	4.65	1.25	1.98	9.90
OCSDW	5.61	1.57	2.30	12.07
P/SFW	6.31	1.79	1.29	12.89
P/SDW	2.07	0.62	0.54	3.76
FPer	62.89	8.92	33.90	98.89
FMD	22.12	3.33	12.57	36.52
FCir	0.77	0.06	0.57	0.91
FAr	299.40	88.36	76.40	719.12
SPer	35.25	4.22	25.91	55.83
SMD	13.85	2.05	9.94	23.71
SCir	0.52	0.07	0.30	0.73
SAr	77.09	18.38	40.04	170.54

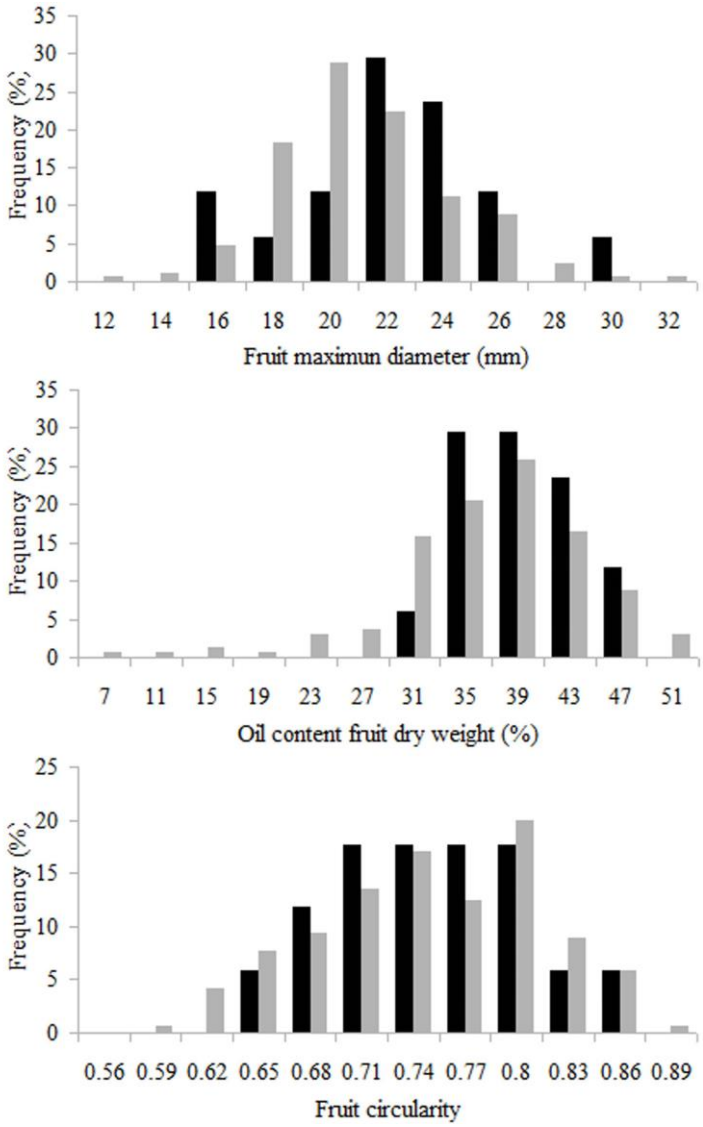

Figure 2.1. Frequency distributions (%) for morpho-agronomic traits evaluated in 17 olive cultivars (black) and progenies from open pollination (grey).

PCA was carried out to explore the relationships among the morpho-agronomic traits evaluated in the seedlings. The first three principal components explained 39.4, 19.7 and 12.2 % of the total variance respectively (Table 2.2). The first component was mainly positively associated to fruit weight and dimensions (FFW, FDW, FPer, FMD, Far), which were highly correlated among them. The second component was negatively associated with oil content traits (OCFFW, OCFDW, OCPFW, OCPDW) and P/SDW, and positively with moisture content (PM). The third principal component was mainly negatively associated with circularity measurements (Fcir, Scir) and moisture content (FM). Similar relationships among some of these characters have been previously found from the evaluation of cultivar collections (Fanizza, 1982), seedlings from crosses (León et al. 2004b) and wild olives (Belaj et al. 2011). No special grouping of seedlings in the score plots of principal components was observed according to the open pollination family (data not shown), which suggests a wide variability regardless of the parent used.

Table 2.2. Vector loadings of PCA with 26 morpho-agronomic traits evaluated in progenies from open pollination of 17 olive cultivars. Main loadings are indicated in bold.

	PC1 (39.4 %)*	**PC2 (19.7 %)**	**PC3 (12.2 %)**
FAr	0.29	0.01	-0.07
FCir	-0.02	-0.15	-0.44
FDW	0.29	-0.06	0.03
FFW	0.29	0.02	-0.08
FM	0.11	0.17	-0.38
FMD	0.29	0.03	0.05
FPer	0.29	0.00	-0.05
OCFDW	0.11	-0.38	-0.04
OCFFW	0.04	-0.41	0.16
OCPDW	0.09	-0.35	-0.01
OCPFW	0.04	-0.40	0.18
OCSDW	-0.12	-0.04	0.19
OCSFW	-0.12	-0.05	0.18
P/SDW	0.14	-0.32	-0.12
P/SFW	0.17	-0.18	-0.30
PDW	0.28	-0.12	0.00
PFW	0.29	0.00	-0.10
PM	0.03	0.32	-0.30
RI	0.09	0.01	-0.21
SAr	0.26	0.13	0.15
SCir	-0.07	-0.09	-0.33
SDW	0.25	0.12	0.11
SFW	0.25	0.12	0.12
SM	-0.02	0.03	0.07
SMD	0.23	0.14	0.26
SPer	0.25	0.14	0.21

* Explained variance in brackets.

Significant differences among progenies were observed for all the evaluated traits. In general, higher values were obtained for within-family than between-family variance (Table 2.3). However, the relative contribution of both sources of variation was different for each trait. As a consequence, heritability estimates showed low values for oil and moisture content, moderate for pulp/stone ratio and the highest values were obtained for fruit size and shape (Table 2.3). Zeinanloo et al. (2009) obtained high values of broad sense heritability, from 0.31 to 0.86 for nine quantitative fruit characters from the analysis of a diallel cross involving six parents, showing fruit length the highest value. Lower values were obtained for estimates of narrow sense heritability for these traits (0.17-0.36). A higher specific than general combining ability was observed for most of the traits, which suggests a higher influence of nonadditive than additive effects in the inheritance of these characters. Fanizza (1982) obtained broad sense heritability estimates of 0.6 for fruit weight and pulp/stone ratio based on the evaluation of 23 cultivars, over 2 years, using two plants per cultivar and 50 fruits per plant. Differences in heritability estimates in different works might be caused by the effect of different genetic backgrounds.

Table 2.3.Variance components between (VB) and within (VW) progenies, estimates of additive genetic variance (VA), phenotypic variance (VP) and narrow-sense heritability (h_n^2) for morpho-agronomic traits evaluated in progenies from open pollination of 17 Spanish olive cultivars.

Variance	RI	FFW	OCFFW	OCFDW	FM	P/SDW	FCir
VB	0.108	0.457	0.885	5.121	1.580	0.051	0.0010
VW	0.865	1.767	9.265	50.310	16.763	0.367	0.0032
VA	0.432	1.828	3.540	20.484	6.319	0.204	0.0039
VP	0.973	2.224	10.150	55.431	18.342	0.418	0.0042
h_n^2	0.444	0.822	0.349	0.370	0.344	0.488	0.930

For characters with high heritability such as fruit weight and circularity, a similar trend was observed for average values of the parents and their open pollinated progenies (Figure 2.2). However, no clear relationships were obtained for characters with low heritability such as fruit oil and moisture content. Some clear exceptions were observed for all characters, such as higher oil content and lower fruit moisture than expected in progenies from 'Lechín de Sevilla', higher values of circularity than expected in progenies from 'Changlot Real' or lower fruit weight than expected in progenies from 'Morona'. This lack of relationship was particularly evident in progenies from open pollination of cultivars showing extreme values for any character. A high negative linear correlation between the average value of the characteristic for the cultivar used as female parent and the percentage of its progeny showing higher values for characters such as fruit weight, oil content and stone/fruit ratio has been previously reported in olive in progenies from open pollination of cultivars different than the evaluated in this work (Lavee and Avidan, 2012). For instance, all the seedlings from open pollination of 'Koroneiki' bore fruits larger than those of the cultivar 'Koroneiki' (low fruit weight cultivar) and all the seedlings from open pollination of 'Kadesh' yielded higher oil content than those of the cultivar 'Kadesh' (low oil content cultivar).

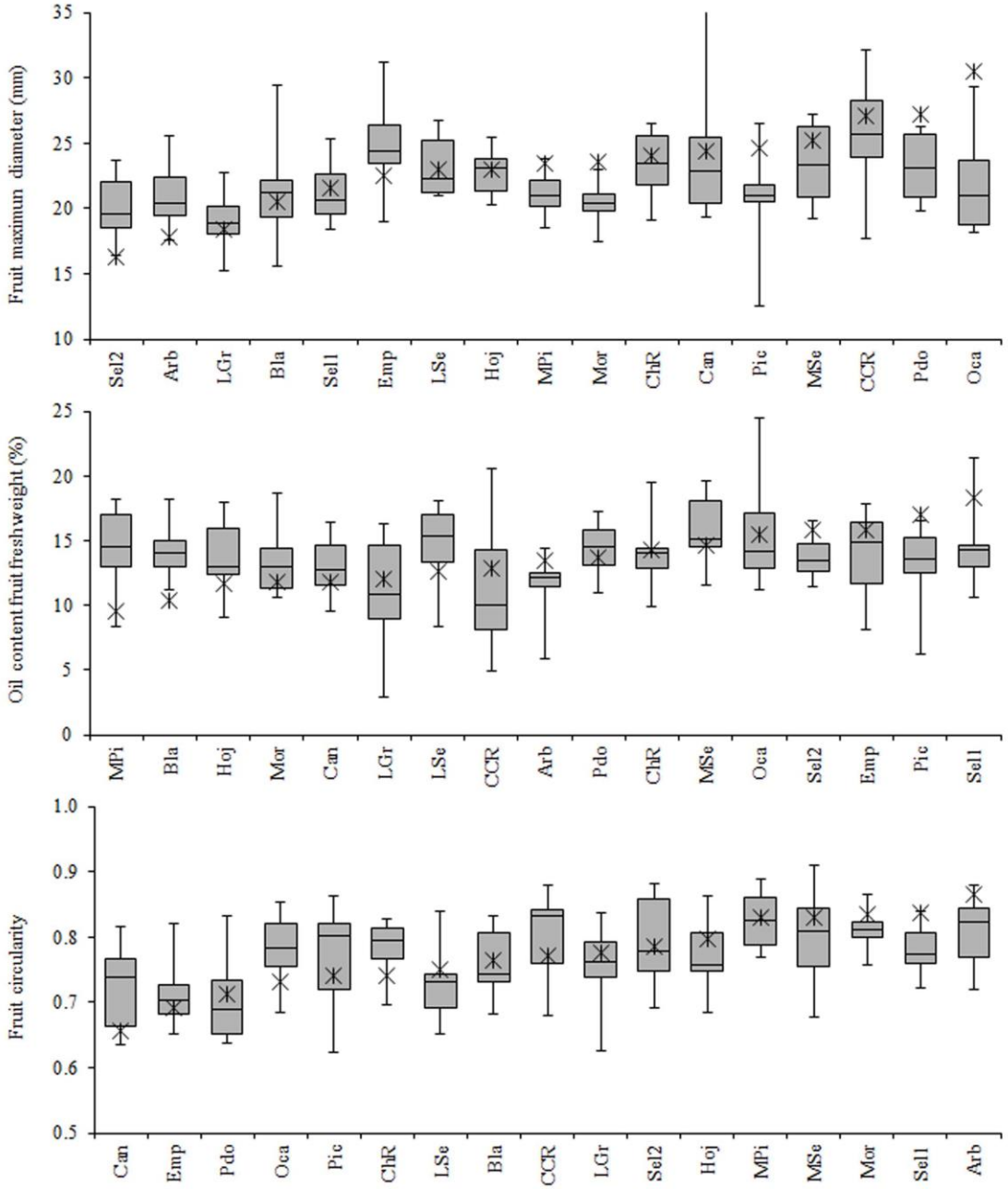

Figure 2.2. Box whisker plots for morpho-agronomic traits evaluated in progenies from open pollination. Progenies are shown in ascending order regarding the values of the cultivars used as parents (represented by stars).

Although it is not fully understood yet, the domestication of olive started by selection of the best trees from wild olive groves, being this process probably carried out simultaneously at different locations in the Mediterranean basin (Lavee and Zohary, 2011). In an evolutionary perspective, according to fruit tree productivity patterns and the genetic and physiological distance from their wild progenitors, olive tree is considered in an intermediate step between wild germplasm and full domestication (Goldschmidt, 2013). Initial selection was probably carried out on the basis of fruit traits as well as vegetative and productive characters and environment adaptation. Fruits of most wild olives are characterized by small size (FFW<1g, FMD<15 mm), long shaped (FCir<0.65), and low oil content (OCFDW<30%) (Belaj et al. 2011). For characters with high heritability, such as fruit size

and shape (FCir, FFW), these values typical of wild olives are not observed in the cultivars used as parents and hardly observed in their progenies. It seems that traditional cultivars were obtained from wild olives mainly on the basis of these characters after several cycles of selections, probably more than just a few generations as previously suggested (Lavee, 2013). In progenies from open pollination of a wild olive and open pollination of 'Picual', Klepo et al. (2013) observed almost no overlapping ranges for these characters. These characters are therefore highly fixed in current cultivars and transgression to the original low values of wild olives are hardly found on the seedlings from cultivated germplasm. On the contrary, for characters such as oil content, high values similar to the ones obtained for the cultivars used as parents were observed in the open pollinated progenies, but transgression to low values, typical of the wild olives, were also found. It seems, therefore, that several new cycles of selection may allow increasing oil content in breeding progenies. In fact, new cultivars with high oil content have been obtained in recent years as a result of breeding works (Lavee et al. 1986; De la Rosa et al. 2008). However, due to the low heritability of this character, low response to selection could be expected, and searching for specific cross combinations could be more efficient to achieve significant improvement for oil content (Hansche, 1983). Anyway, open pollination has proven to be useful to broaden the genetic base of fruit breeding programs and guarantee that enough variability is available for cultivar breeding in future generations (Kumar et al. 2010; Badenes et al. 2011).

2.6. Conclusion

In summary, open pollination of outstanding cultivars grown in a germplasm bank seems to be an interesting strategy for olive breeding, according to the high variability found for all the traits measured. However, the different heritability values found for the different characters should indicate that different breeding strategies would be convenient for selection of different traits as fruit size or oil content.

2.7. Acknowledgements

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Capítulo 3. Resistance to Verticillium wilt in olive progenies from open-pollination

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3.1. Abstract

Verticillium wilt, a vascular disease caused by the soilborne fungus *Verticillium dahliae* Kleb., constitutes currently the major cultivation constraint in many olive growing areas. An integrated disease management strategy is recommended for Verticillium wilt control including the use of resistant cultivars as one of the most efficient control measures. However, only a few traditional cultivars have showed high levels of disease resistance. For this reason, an olive breeding program was initiated aiming at obtaining new cultivars displaying both high levels of disease resistance and good agronomic characteristics. In this work, the resistance to the defoliating pathotype of *Verticillium dahliae* was evaluated in 64 genotypes that included 12 cultivars from the World Olive Germplasm Bank of Córdoba (Spain) and 52 genotypes coming from open-pollination of these cultivar plus three other cultivars. These genotypes were previously selected from a wider initial population on the basis of their favourable agronomic performance, mainly early crop (short juvenile period) and high oil content. Inoculation experiments were carried out under controlled conditions in a growth chamber by dipping the root system of plant cuttings in a conidial suspension of the pathogen. A Relative Susceptibility Index (RSI), which summarized several disease parameters, and plant colonization parameters were used to assess the resistance response. Eight of the evaluated genotypes showed similar resistance level to external development of symptoms than 'Frantoio' (the control resistant cultivar). Vascular colonization estimated by isolation of the fungus suggests that a tolerant more than resistant plant defence mechanism may be operating in some genotypes. The correlation between values for female parents and their progenies suggests a high heritability in some disease parameters.

3.2. Keywords

Defoliating pathotype, Olive breeding, *Olea europaea*, Vascular wilt, *Verticillium dahliae*

3.3. Introduction

Verticillium wilt caused by *Verticillium dahliae* represents one of the most important diseases in many olive (*Olea europaea* L.) growing areas for both its high incidence and difficulty of disease control (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012). High economic losses due to tree death and potential yield reduction together with the lack of effective eradication methods, collectively have a negative effect on farmer's outlook concerning future of olive production (Areal et al. 2014). Therefore, there is an urgent need to develop effective control measures for Verticillium wilt and their implementation in an integrated disease management strategy for olive (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012). In this context, the use of resistant plant material is considered one of the most economic and environmentally-friendly measures to control the disease.

However, evaluation of Verticillium wilt resistance has shown that most of the commercial cultivars are susceptible to D pathotype of the fungus, which is currently prevalent in most olive growing areas (Trapero et al. 2011). In Spain, extensive evaluations have been carried out in cultivars preserved at the World Olive Germplasm Bank of Córdoba

(WOGBC; Belaj et al. 2012). The results obtained indicate that all cultivars were more susceptible to highly virulent D than to less virulent ND pathotype of *V. dahliae,* and only a low number of them exhibited levels of resistance to development of symptoms caused by D pathotype (López-Escudero et al. 2004, 2007; Martos-Moreno et al. 2006; García-Ruiz et al. 2014). Furthermore, some of the cultivars classified as resistant have been shown to present some negative agronomic traits that limit their commercial use, such as late bearing, excessive vigour, frost susceptibility or low oil content (Rallo et al. 2005).

The importance of the disease encourage the development of breeding programs to select new cultivars with improved resistance levels. Breeding olive has been aimed at producing new cultivars with early bearing, high yield and oil content, suitability to different growing systems and oil quality (Bellini et al. 2008). The information currently available regarding breeding for Verticillium wilt in olive resistance is very scarce and only preliminary information about its inheritance is available. Initial works by Wilhelm and Taylor (1965) indicated that the cultivars 'Frantoio' and 'Arbequina' yielded more resistant seedlings than the susceptible cultivars tested, but it was doubtful whether resistant progenies from resistant cultivars were more resistant than those derived from susceptible. Recent works have been also carried out to test seedlings from different crosses before their evaluation under field conditions (Trapero et al. 2013a).

The whole selection process in olive breeding programs is carried out through several steps from crossing to release of new cultivars. In Trapero et al. (2013a), screening for Verticillium wilt resistance was carried out in the first step of selection at the seedling stage. Selection for Verticillium wilt at this stage carries a high risk of selection of genotypes that will probably show poor agronomic performance afterwards. Previous results in our breeding program indicate that more than 90 % of genotypes are usually discarded at the initial seedling stage on the basis of their long juvenile period (De la Rosa et al. 2006) and low oil content (León et al. 2015).

In this present work, the resistance to Verticillium wilt was evaluated in 52 genotypes coming from open-pollination of 15 cultivars and their corresponding female parents' cultivars. These cultivars come from different geographical origin and, therefore, a wide genetic base could be expected (Belaj et al. 2012). These genotypes were previously selected from a wider initial seedling population based on their agronomic performance. Plants obtained by vegetative propagation from each genotype were used for inoculation experiments carried out under controlled conditions. Disease and colonization parameters served to establish variation in disease resistance among genotypes. The aim of this work was to gain a deeper knowledge on the heritability for Verticillium wilt resistance in olive based on the degree of resemblance between relatives, the possible mechanisms involved and the implications to design efficient breeding strategies.

3.4. Materials and Methods

3.4.1. Plant materials

Genotypes evaluated in this study were selected from an initial seedling population derived from open-pollination from 13 cultivars of the WOGBC: 'Arbequina', 'Canetera', 'Chalkidiki', 'Changlot Real', 'Empeltre', 'Frantoio', 'Koroneiki', 'Leccino', 'Manzanilla de Sevilla', 'Ocal', 'Picual', 'Picudo', 'Toffahi', and two previous selections of our breeding

program, named as Sel1 and Sel2. These female parent cultivars were selected according to their agronomic behaviour as potential interesting parents for olive breeding.

Previous work was carried out for agronomic evaluation of the initial progenies coming from open pollination (Arias-Calderón et al. 2014). As a result, 52 genotypes (1-6 per parent) showing favourable agronomic performance were finally selected mainly on the basis of their early crop (short juvenile period) and high oil content. Plants of the selected genotypes as well as the cultivars used as parents were obtained by vegetative propagation of semi-hardwood stem cuttings for screening to Verticillium wilt resistance. Successful propagation was not possible in three of the female parent cultivars used and, therefore, screening of these three cultivars was not carried out due to the lack of plant materials.

3.4.2. Fungal inoculum and plant inoculation

Monosporic culture of *V. dahliae* isolate axenically stocked on plum extract agar, covered with liquid paraffin at 4ºC in the dark, was used. Isolate from affected olive trees in southern Spain was characterized in previous study as highly virulent D pathotype (D) (Rodríguez-Jurado et al. 2008). Small agar plugs of stored culture were placed on chlorotetracycline water agar plates (CWA, 20 g of agar and 0.03 g of chlorotetracycline per litter of distilled water) to obtain active culture which was subculture on Potato Dextrose Agar (PDA) (250 g of potato, 20 g of agar, and 20 g of glucose per litter of distilled water). For inoculum preparation, cultures on PDA were grown for 14 days at 24ºC in the darkness and agar plugs (0.3 x 0.3 mm) colonized by the fungus were transferred to flasks containing 100 mL of potato dextrose broth. Fungal culture incubated for 7 days at 24ºC on an orbital shaker at 125 rpm in the dark was filtered through double cheesecloth. The resultant suspension was assessed using a haemacytometer and it was adjusted to 10^7 conidia/mL with sterile distilled water.

Plants of six-month-old rooted cuttings grown in 0.25 L pots in a greenhouse were used. Plants (8-20 plants per genotype) were inoculated by dipping the root systems slightly trimmed in the conidia suspension for 15 minutes according to Rodríguez-Jurado et al. (2007). Inoculated plants of 'Picual' and 'Frantoio' cultivars were included as known reaction controls, susceptible and resistant respectively (López-Escudero et al. 2004). Non-inoculated control plants (6-10 plants per genotype) were immersed in sterile distilled water for the same time. Following inoculation, plants were transplanted to plastic pots (one per pot) with autoclaved twice soil (lime: peat, 2:1, v/v, at 121ºC for 75 min) and kept in the growth chamber at 22±2ºC. The relative humidity was 45-85 % and 14 h photoperiod light of 360 $\mu E\ m^{-2}\ s^{-1}$. Plants were watered as required and fertilized weekly with Hoagland´s nutrient solution.

3.4.3. Assessment of plant reaction to inoculation

Symptoms severity was assessed in each plant every three-four days for 112 days on a 0 to 4 scale according to the percentage of aerial part affected by defoliation, chlorosis, wilt and/or necrosis compared with control plants (0 = absence of symptoms; 1= 1-33 % aerial part affected; 2=34-66 % aerial part affected; 3=67-100 % aerial part affected; 4= dead plant) (Rodríguez-Jurado et al. 1993). Several disease parameters were calculated from this scale values.

Standardized Area Under the Disease Progress Curve (SAUDPC) adapted the expression proposed by Campbell and Madden (1990) (1):

$$SAUDPC = [\sum_{i=1}^{n}((S_i + S_{i-1})/2)\Delta t][100/(S_{max}T)] \quad (1)$$

where S_i=mean severity of the experimental unit in the observation i; Δt=the number of days between observation; S_{max}=maximum value of severity (=4); T= experimental period in days; n= number of observations.

Disease Intensity Index (DII) calculated for each observation as shown in equation (2):

$$DII = \sum_{x=1}^{n}((S_x N_x)/(4N_t))\,100 \quad (2)$$

where S_x= severity in an individual plant, N_x= number of plants with symptoms of severity S_x and N_t= total number of plants for each experimental unit. Disease Intensity Index (DII) at the end of the evaluation is called FDII.

Others disease parameters were: Final Disease Incidence (FDI) and Final Dead Plants Incidence (FDPI) representing, percentage of plants with disease symptoms and percentage of dead plants at the end of the experiment, respectively; Disease-Free Period (DFP) estimated as the number of days without appearance symptoms. Parameters were calculated for each experimental unit.

A Relative Susceptibility Index (RSI) was calculated summarizing all previous disease parameters weighted according the following coefficients: (3):

$$RSI = \left[\left(0.3SAUDPC + 0.3FDPI + 0.2FDII + 0.05FDI + 0.15(100 - RDFP)\right)/SP\right]100 \quad (3)$$

where *SP*=average susceptibility in the reference susceptible cultivar 'Picual', i.e. the value of numerator calculated for this cultivar; *RDFP*=Relative Disease-Free Period estimated as the number of days without appearance symptoms and expressed as percentage to the total experimental period in days.

Plant colonization by the fungus was also assessed in the root system and aerial part of all plants at the end of the experiments (symptomatic and asymptomatic) or dead plants by isolating the fungus on CWA. For each plant, several randomly chosen pieces of root and stem (from which the outer layer of bark had been previously removed) were thoroughly washed under running tap water for one hour. Then, ten 5-mm-long root (two per root next to stem) and ten pieces of debarked stem were surface disinfested in 0.5 % sodium hypochlorite for 1.5 min. (stems) or 2.0 min. (roots), rinsed with sterile water, plated onto the medium, and incubated at 24°C in the dark for 9 days (stem) to 21 days (root) according to Rodríguez-Jurado (1993). *V. dahliae* was identified by microscopic observations of verticillate conidiophores and microsclerotia on different layers in the medium plate. Result from root and stem isolations of the pathogen were used to calculate a Root and Stem Colonization Index (RCI and SCI, respectively) (4) for each experimental unit the number of root and stem pieces from which the fungus was isolated relative to the total number of root and stem pieces sampled, respectively, expressed as a percentage:

$$RCI\ or\ SCI = [\sum_{x=1}^{n}(P_x N_x)/(P_t N_t)]100 \quad (4)$$

where P_x= number of root (RCI) or stem (SCI) pieces from which fungus was isolated, N_x= number of plants with P_x; P_t= total number of root (RCI) or stem (SCI) pieces assessed for each experimental unit and N_t= total number of plants assessed for each experimental unit.

3.4.4. Data analysis

Cultivars used as female parents and their selected 52 genotypes from free-pollination were evaluated in four separate experiments which always included 'Picual' (susceptible) and 'Frantoio' (resistant) cultivars as disease reference controls. Each experiment was arranged according to a complete randomized block design with 8-20 inoculated plants per genotype distributed in four blocks with 2-5 plants per block, as well as 6-10 mock inoculated plants per genotype also distributed in the four blocks. Initial analyses found no differences among experiments for the various disease parameters of the two control cultivars. Therefore data were pooled over all experiments and ANOVAs were performed to test for significant differences in disease parameters among genotypes (P<0.05). The relative importance of genotype effect vs. experimental error was considered for selection of coefficients for RSI calculation. Means were compared between each genotype and those of susceptible 'Picual' and resistant 'Frantoio' reference controls by the Dunnett´s test at P=0.05. Parameters expressed as percentages were subjected to angular transformation before being statistically analysed. Pearson's correlation coefficient was calculated among average values for RSI, RCI and SCI in genotypes from open pollination progenies and their correspondent female parent. All analysis were carried out using Statistix 9.0 software (Analytical Software, Tallahassee, Florida, USA).

3.5. Results

Defoliation of green leaves, rolling leaves, chlorotic leaves and necrotic leaves and stems were the most common visual symptoms observed. The pattern of the symptoms´ progress over time showed a wide variability among the genotypes evaluated according to DII (Figure 3.1). In the cultivar 'Picual', used as a susceptible reference control, the first external disease symptoms started 14 days after inoculation, continued increasing at high rate until 49 days after inoculation and then more slowly until 84 days. In the resistant control 'Frantoio', the first disease symptoms appeared 18 days after inoculation, increasing until 50 days after inoculation and remained steady until the end of the experimental period. Among the evaluated genotypes, selected from open-pollination progenies, symptoms development started from the 7 to 50 days after inoculation.

Significant differences between genotypes were observed for all the disease parameters evaluated (Table 3.1). SAUDPC, FDPI and FDII values were significantly higher and DFP significantly lower in 'Picual' than in 'Frantoio' cultivars used as reference controls. Differences between 'Picual' and 'Frantoio' were not significant for FDI. Among the cultivars used as female parents, two of them ('Empeltre' and 'Changlot Real') showed significant differences with susceptible control 'Picual' for most of the disease parameters evaluated, seven of them showed significant differences with resistant control 'Frantoio', and one ('Koroneiki') was not significant different of any of the reference controls.

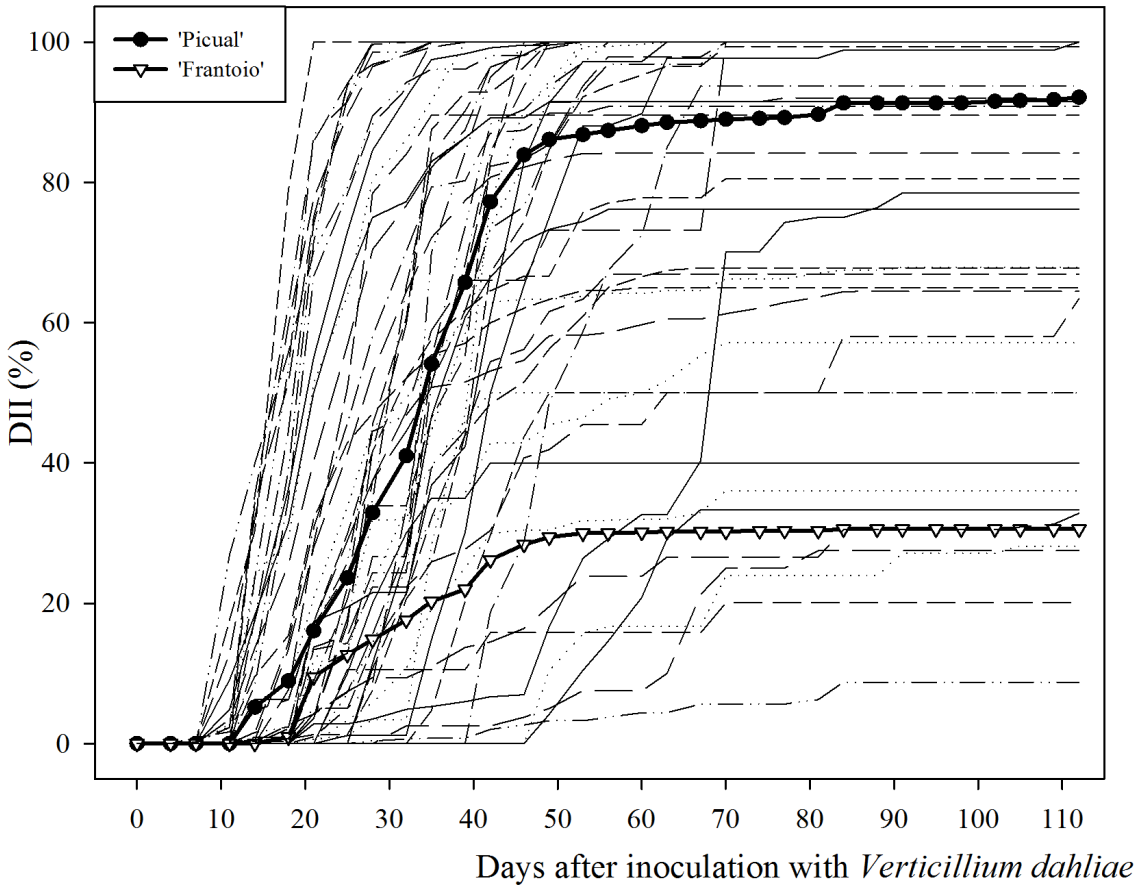

Figure 3.1. Progress of the Disease Intensity Index (DII) in olive genotypes selected from open-pollination progenies. Reference controls cultivars 'Picual' (susceptible) and 'Frantoio' (resistant) are indicated in bold.

For the selected 52 genotypes from free-pollination, a high percentage of them (90.4 %) were similarly grouped according to significant differences from controls by SAUDPC and FDPI values. Similar groupings were observed for FDII and FDPI in 86.5 % of genotypes, and for FDII and SAUDPC in 84.6 % of genotypes. Similar groupings were also observed for DFP and FDPI in 63.5 % of genotypes, and for DFP and SAUDPC in 71.1 % of genotypes. FDI provided the poorest separation among genotypes compared with the other disease parameters. The main differences in grouping occurred for genotypes that, according to Dunnett´s test, were not significantly different from 'Picual' and 'Frantoio' cultivars. These genotypes represent an intermediate group with different number of genotypes depending on the parameter: 19 genotypes for DFP or FDII values, 12 for FDPI and 11 for SAUDPC. These results show that each of these disease parameters did not equally classify the genotypes according to differences from the reference controls, despite them being highly correlated with one another (Table 3.2).

A Relative Susceptibility Index (RSI) was calculated in order to integrate the information of the five disease parameters to develop a novel and easy classification of genotypes according to their levels of resistance to Verticillium wilt. Significant differences (P<0.01) between genotypes were also observed for RSI (Table 3.1).

Table 3.1. Disease parameters estimated in olive cultivars used as female parents and genotypes selected from open-pollination progenies sorted from high to low RSI. Reference controls cultivars 'Picual' (susceptible) and 'Frantoio' (resistant) are indicated in bold.

Cultivar/ Genotype	Female parent[a]	Disease parameter[b]						Resistance category[c]
		SAUDPC	FDPI	FDII	FDI	DFP	RSI	
1	'Manzanilla de Sevilla'	86.55#[d]	100#	100#	100	12.55#	119.04#	S
2	Sel2	84.23#	100#	100#	100	12.37#	117.69#	S
3	'Arbequina'	84.12#	100#	100#	100	15.37#	116.82#	S
4	'Leccino'	78.33#	100#	100#	100	19.78#	116.41#	S
5	'Canetera'	82.96#	100#	100#	100	14.75#	116.30#	S
6	'Picual'	77.16#	100#	100#	100	20.26#	116.10#	S
7	'Leccino'	80.44#	100#	100#	100	21.12#	115.75#	S
8	Sel1	81.71#	100#	100#	100	17.36#	114.85#	S
9	Sel1	92.33#	100#	100#	100	23.04#	114.59#	S
10	'Arbequina'	87.75#	100#	100#	100	22.53#	113.47#	S
11	Sel1	67.93#	100#	100#	100	25.14#	112.27#	S
Sel1	-	76.36#d	100#	100#	100#	17.00#	111.74#	S
'Toffahi'	-	79.58#	100#	100#	100#	25.92#	111.29#	S
'Arbequina'	-	75.92#	100#	100#	100#	18.33#	111.12#	S
12	'Canetera'	73.33#	100#	100#	100	18.75#	109.46#	S
13	'Toffahi'	73.06#	100#	100#	100	20.12#	108.93#	S
Sel2	-	71.58#	100#	100#	100#	17.50#	108.75#	S
14	'Koroneiki'	73.43#	100#	100#	100	23.5#	108.25#	S
'Manzanilla de Sevilla'	-	84.00#	87.50#	88.3#	100#	32.50	107.42#	S
15	'Canetera'	73.05#	91.66#	92.7#	100	15.75#	107.14#	S
16	'Canetera'	71.61#	91.66#	92.18	100	24#	105.54#	S
17	'Toffahi'	71.01#	100#	100#	100	28.75	105.40#	S
18	'Chalkidiki'	69.34#	100#	100#	100	27.35#	104.78#	S
19	'Arbequina'	69.14#	100#	100#	100	27.37#	104.65#	S
20	'Ocal'	65.75#	100#	100#	100	29.88	103.98#	S
21	'Toffahi'	68.33#	100#	100#	100	30.45	103.34#	S
22	'Picual'	66.69#	100#	100#	100	31.26	102.14#	S
23	'Chalkidiki'	69.49#	87.5#	88.28	100	20.25#	102.01#	S
'Ocal'	-	74.48#	100#	100#	100#	51.25	101.46#	S
24	'Picual'	58.12#	100#	100#	100	35.64	101.46#	S
25	'Toffahi'	85.19#	100#	100#	100	38.52	101.38#	S
27	'Canetera'	65.14#	100#	100#	100	31.83	101.06#	S
26	'Ocal'	95.4#	83.33#	90.1	100	27.48#	101.06#	S
28	'Picual'	63.67#	95#	99.4#	100	28.85	100.70#	S
'Picual'	-	64.88#	84.16#	92.6#	98.33	23.8#	100#	S
29	'Toffahi'	68.99#	100#	100#	100	34.02	99.74#	S
30	'Toffahi'	60.47#	100#	100#	100	36.04	97.14#	S
'Picudo'	-	59.59#	100#	100#	100#	42.00	95.01#	S

Cultivar/ Genotype	Female parent[a]	Disease parameter[b]						Resistance category[c]
		SAUDPC	FDPI	FDII	FDI	DFP	RSI	
31	'Koroneiki'	75.78#	77.08#	83.33	100	26.55#	94.99#	S
32	'Chalkidiki'	46	62.5	62.5	62.5	44.62	87.01#	S
33	'Picudo'	55.28	79.16#	82.29	91.66	49.38	85.63	MS
34	'Empeltre'	49.86	50	92.18	100	45.5	84.84	MS
35	'Manzanilla de Sevilla'	78.08#	54.16	68.61	100	28.23	81.28	MS
36	'Changlot Real'	62.38	58.33	63.28	91.7	36.63	75.78	MS
37	'Leccino'	42.08	37.5	67.96	100	27.75#	75.18	MS
38	'Changlot Real'	53.81	75#	78.12	100	25.79#	74.21	MS
39	'Canetera'	47.7	63.33	65.31	85	42.51	73.68	MS
40	'Koroneiki'	36.07	45.83	77.6	100	42.08	71.65	MS
41	'Manzanilla de Sevilla'	45.12	65	67.81	80	38.5	70.95	MS
42	'Manzanilla de Sevilla'	39.7	50	58.59	75	42.5	66.12	MS
43	'Picudo'	39.02	62.5	62.5	62.5	64.5*	61.45	MS
'Koroneiki'	-	43.10	37.50	45.31	75.0	63.17*	57.25	MS
44	'Changlot Real'	31.87*	50	50	50	74.5*	49.42	MS
45	'Picudo'	23.84*	30*	36.03	60	64.27*	41.66*	R
'Frantoio'	-	22.09*	22.05*	30.16*	59.23	63.25*	38.49*	R
'Empeltre'	-	19.40*	25*	26.56*	37.50*	65.25*	34.91*	R
46	'Manzanilla de Sevilla'	18.71*	37.5	37.5	37.5*	88.87*	32.62*	R
47	'Picual'	15.42*	0*	32.81*	62.5	76.87*	31.79*	R
'Changlot Real'	-	15.93*	25*	25.31*	30.00*	69.20*	31.27*	R
48	'Koroneiki'	16.57*	22.5*	29.37*	46.25*	80.76*	30.18*	R
49	'Frantoio'	18.49*	25*	25*	25*	90.25*	27.05*	R
50	'Empeltre'	11.86*	12.5*	27.34*	50	91*	23.76*	R
51	'Arbequina'	13.7*	16.25*	20.31*	31.25*	92.41*	21.68*	R
52	'Koroneiki'	5.02*	0*	10.93*	50	83.25*	15.11*	R

[a] Name of the female parent cultivar including two previous selections of our breeding program (Sel1 and Sel2).

[b] SAUDPC: Standardized Area Under the Disease Progress Curve; FDPI: Final Dead Plants Incidence; FDII: Final Disease Intensity Index (DII); FDI: Final Disease Incidence; DFP: Disease-Free Period; RSI: Relative Susceptibility Index.

[c] Evaluated genotypes were classified by RSI values in three resistance categories to Verticillium wilt: resistant (R), significantly different from 'Picual'; susceptible (S), significantly different from 'Frantoio'; and moderately susceptible (MS), non-significantly different from 'Picual' and 'Frantoio'.

[d] *=significantly different from 'Picual'; #=significantly different from 'Frantoio'; without symbol=non significantly different from 'Picual' and 'Frantoio' according to Dunnett´s test at $P = 0.05$).

Table 3.2. Correlation coefficients among Verticillium wilt disease parameters. All values were significant at $P< 0.001$.

	DFP	FDI	FDII	FDPI
FDI	-0.93			
FDII	-0.90	0.92		
FDPI	-0.86	0.81	0.96	
SAUDPC	-0.91	0.86	0.90	0.89

For the whole set of genotypes, 32 (61.5 %), 12 (23.1 %) and 8 (15.4 %) of them were classified as Susceptible (S, significantly different from 'Frantoio', Moderately Susceptible (MS, non-significantly different from both 'Picual' and 'Frantoio') and Resistant (R, significantly different from 'Picual'), respectively. The D pathotype did not cause dead plants in two of eight R tested genotypes and RSI values was 60.7 % lower in one of them than in the resistant control 'Frantoio'.

Frequency distributions of resistant progenies according to RSI values were different depending on their correspondent female parent (Figure 3.2). As a general trend, parents that were resistant phenotypically to the disease such as 'Empeltre' and 'Frantoio' produced a higher proportion of R genotypes compared with parents classifies as susceptible. In fact, a significant correlation (r=0.79**) was found between the average values of RSI in parents and their progenies (Figure 3.3).

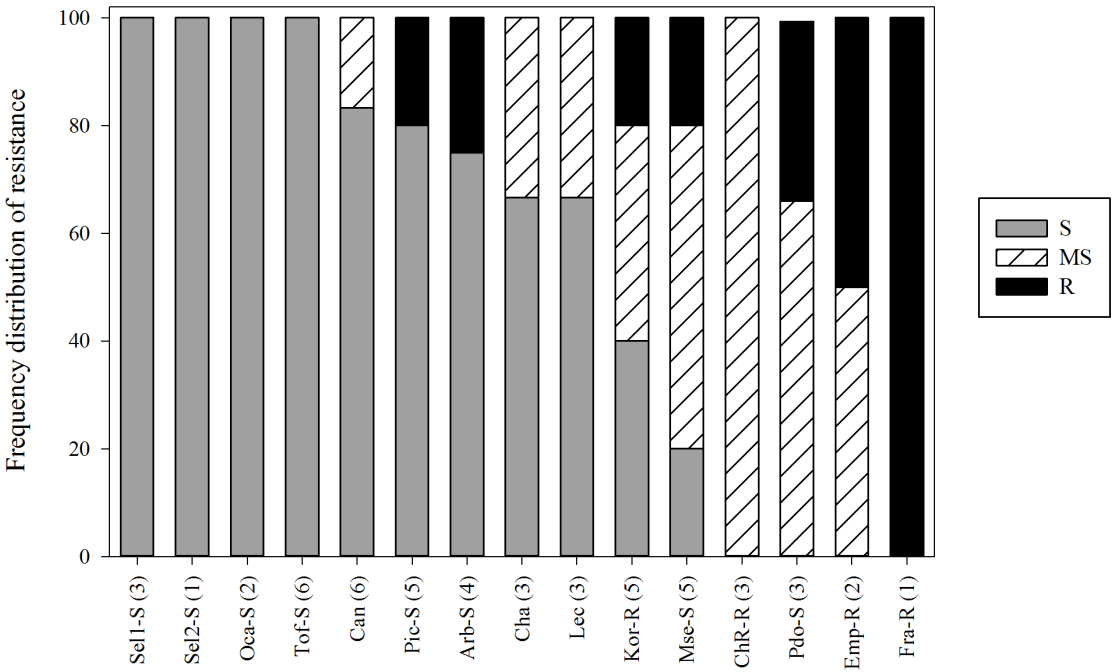

Figure 3.2. Correlation between average values of Relative Susceptibility Index (RSI) in genotypes from open-pollination progenies and their correspondent female parent (**, significant at P<0.01).

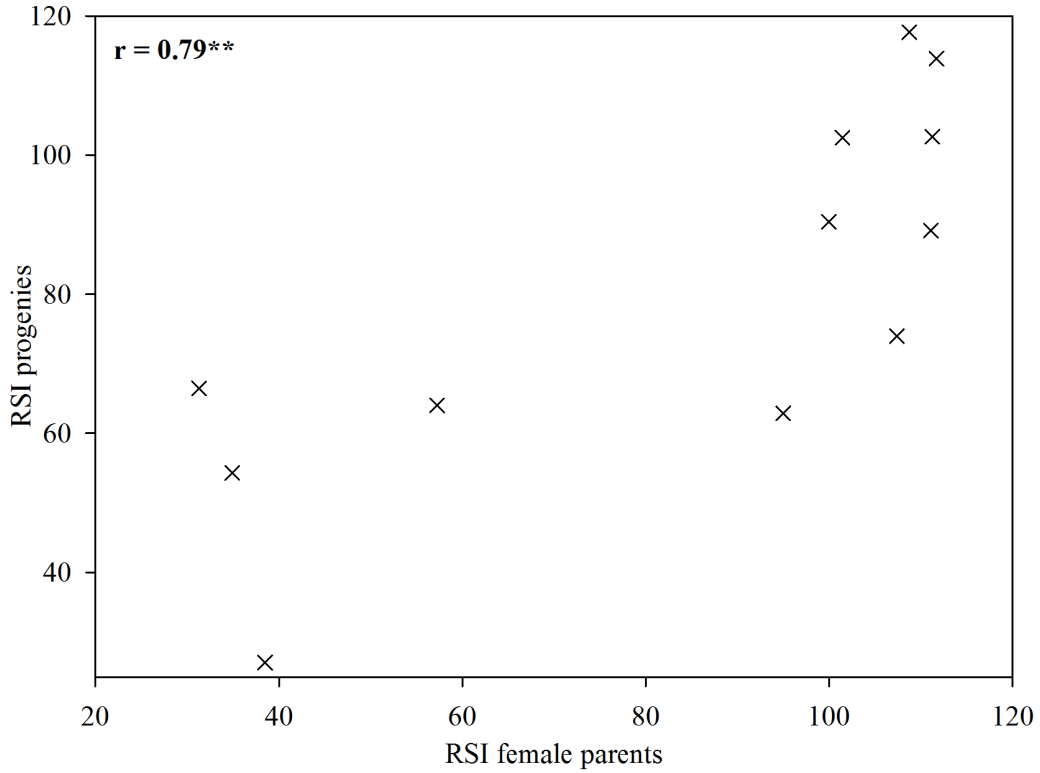

Figure 3.3. RSI progenies vs RSI female parents.

Plant colonization was verified by the isolation of the fungus at the end of the experiment. *V. dahliae* was isolated from root system and stem tissues of both reference cultivars, with lower RCI and SCI values for the resistant cultivar 'Frantoio', 8.0 and 13.0 % respectively, than the susceptible 'Picual', 50.3 and 58.7 % respectively (Figure 3.4). A wide variability was found for both RCI and SCI among the genotypes evaluated, although no clear differences were observed according to the different resistant categories. RCI and SCI values from 13.7 to 30.5 % and from 20.3 to 88.0 % respectively were observed in R genotypes, from 0 to 35.0 % and from 0 to 75.8 % in MS genotypes and from 0 to 43.8 % and from 0 to 77.3 % in S genotypes. Therefore, genotypes showing similar values for both colonization indexes showed quite different disease reactions, being classified by RSI as R, MS or S genotypes. Thus, two of the eight genotypes classified as R according to RSI displayed the highest SCI values among the evaluated genotypes and four of them reached SCI values as high as 'Picual'. For instance, one R genotype, coming from 'Koroneiki' open pollination, showed the lowest value for RSI (15.1 %) while at the same time presented some of the highest values for both SCI and RCI (88.0 and 27.5 %, respectively). On the contrary, S and MS reactions were observed in some genotypes for which isolation of the fungus was not possible from roots, stems or both. Significant correlation (P<0.01; r=0.80) was found between values of RCI for female parents and the average of their progenies, but not for SCI (P>0.05; r=0.07) (Figure 3.5).

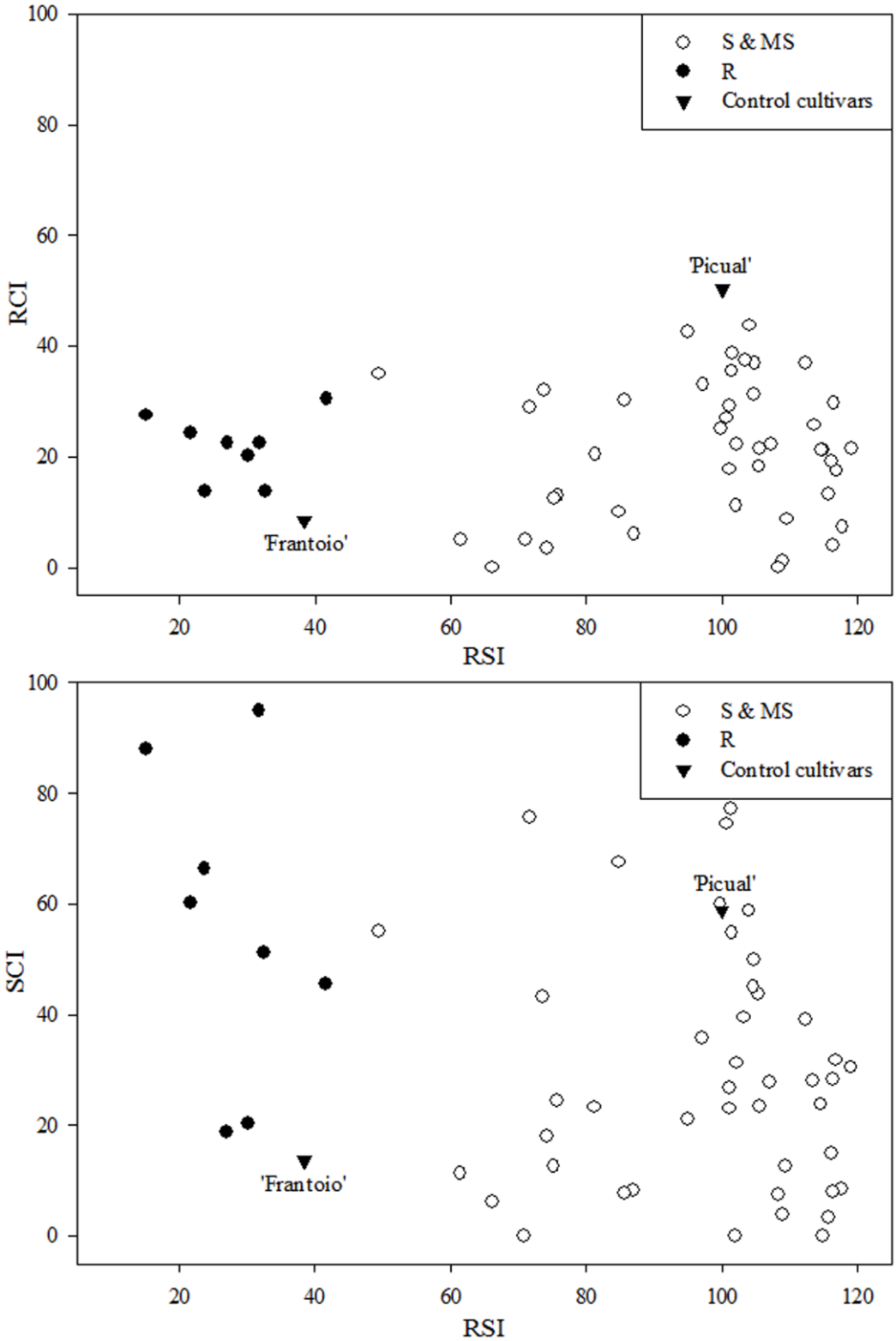

Figure 3.4. Scatter plots of Relative Susceptibility Index (RSI) vs. Root colonization index (RCI) and Stem Colonization index (SCI) in 52 genotypes selected from open-pollination seedlings. Genotypes classified as resistant according to RSI are indicated by black dots. Triangles represents values for reference control cultivars susceptible 'Picual' and resistant 'Frantoio'.

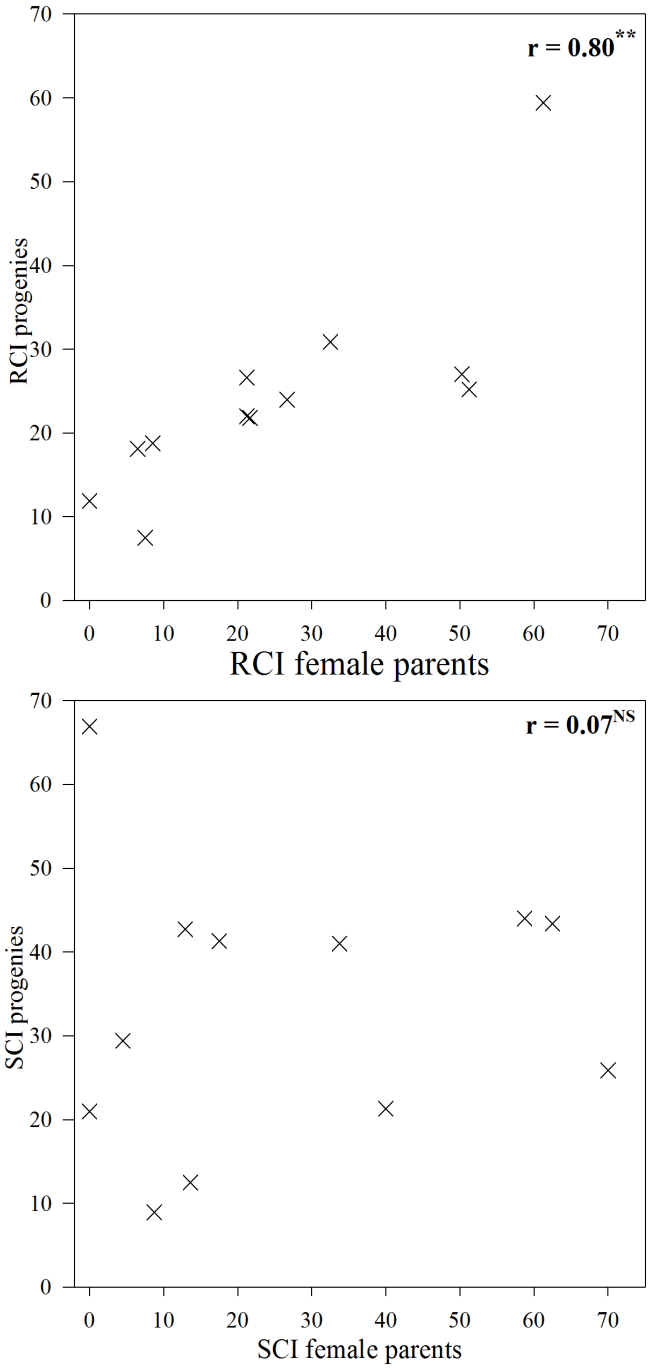

Figure 3.5. Correlation between average values for Root (RCI) and Stem Colonization Index (SCI) in genotypes from open-pollination progenies and their correspondent female parent (**, significant at $P<0.01$; NS, non-significant).

3.6. Discussion

Resistance to Verticillium wilt was evaluated in olive genotypes from open-pollination progenies, previously selected from a wider initial population due to its favourable agronomic performance regarding several fruit and tree characters. Female parents included 13 cultivars of the World Olive Germplasm Bank of Córdoba and two previous selections of our breeding program. Disease progress and resistance category of the cultivars used as parents was similar to previously reported (López-Escudero et al. 2004; 2007; Martos-Moreno et al. 2006), with

only slight differences in the absolute values of individual disease parameters. These differences from previous works could be attributed to differences on experimental procedures. Different inoculation methods, environments, age and type of plants have been used to screen olive for resistance to Verticillium wilt in olive (Rodríguez-Jurado et al. 1993, 2007; López-Escudero et al. 2004; Levin et al. 2007; Trapero et al. 2013b; García-Ruiz et al. 2014).

The development of disease symptoms showed wide variability among the genotypes evaluated, being in them slower than observed for the resistant control 'Frantoio'. Evaluation of Verticillium wilt resistance in inoculated olive materials have been usually based on disease parameters such as area under disease progress curve (AUDPC), percentage of dead plants and disease severity index (López-Escudero et al. 2004; Erten and Yıldız, 2011; Trapero et al. 2013b). In this work, a high correlation between different Verticillium wilt disease parameters was observed, as previously reported in other species such as cotton (Zhou et al. 2014). However, comparison among genotypes slightly varied according to the different disease parameters, which led us to develop a new index (Relative Susceptibility Index, RSI) for final classification of genotypes in three Verticillium wilt resistance categories: Resistant (R, significantly different from 'Picual'), Susceptible (S, significantly different from 'Frantoio') and Moderately Susceptible (MS, non-significantly different from both 'Picual' and 'Frantoio'). The results indicate that genotypes with a similar level of resistance to Verticillium wilt as 'Frantoio', which is currently considered one of the cultivars with higher level of resistance, can be obtained from open pollination progenies previously selected for agronomic traits of interest. A higher proportion of resistant genotypes was observed in progenies from open pollination of cultivars of known high resistance compared with those derived from susceptible cultivars, and significant correlation was found between the average values of RSI in parents and their progenies. However, it should be noted that these results come from a limited number of genotypes, only one to six genotypes were evaluate per parent. It is not clear either whether previous selection of genotypes for favourable agronomic performance could have affected the results. To the best of our knowledge, association between Verticillium Wilt resistance and other agronomic characters has not been previously reported in olive but it cannot be excluded and it must be checked in future works. Moreover, R genotypes were also obtained coming from parents of known susceptibility, such as 'Picual', 'Arbequina', 'Manzanilla de Sevilla' and 'Picudo'. Previous works reported that resistant cultivars such as 'Frantoio' yielded significantly more resistant seedlings than susceptible cultivars such as 'Manzanillo' and 'Mission'. However some resistant seedlings were obtained from all parents (Wilhelm and Taylor, 1965). Similarly, Trapero et al. (2011) found lower mean SAUDPC values in progenies from resistant cultivar 'Frantoio' (although not from 'Empeltre') than susceptible cultivars such as 'Picual', 'Arbequina', and 'Manzanilla de Sevilla'. Besides, resistant genotypes were selected from all progenies and no significant differences between cultivars were obtained for the percentages of plants of each progeny showing no symptoms.

The continuous distribution observed for disease parameters seems to indicate a quantitative inheritance of the resistance that might have a polygenic control. This has already been reported for other crops such as hop (Jakse et al. 2013), strawberry (Shaw et al. 1997), cotton (Yang et al. 2008) and tomato (Giotis et al. 2009). In cotton, high heritability values were obtained for different Verticillium wilt resistance traits, thus indicating that

variation in resistance is predominantly due to genetic factors (Aguado et al. 2008; Zhou et al. 2014). The high correlation between average RSI values in progenies and parents seems to indicate a high heritability of this character and, therefore, a high response to selection could be expected. On the other hand, transgressive resistant segregants from susceptible cultivars were also observed, which suggests that non-additive genetic effects could be of major importance. Therefore, seeking for specific cross combinations, with high specific combining ability, could be also efficient to achieve enhanced resistance levels. Future works will be carried out to confirm the mode of inheritance of resistance to Verticillium wilt in olive.

Considering the whole set of evaluated genotypes, no relationship among either RCI or SCI and any of the disease parameters was found. A significant correlation was found between values of RCI for female parents and the average of their progenies, which seems to indicate a higher heritability for the intensity of root colonization rather than stem colonization. Higher SCI than RCI values were obtained for most of the evaluated genotypes, which agree with a higher isolation of *V. dahliae* in samples from stems than roots previously reported for some cultivars (Mercado-Blanco et al. 2003; Gramaje et al. 2013). Extent (root and stem) and intensity (RCI and SCI) of plant vascular colonization estimated by isolation of fungus were not associated with to the resistance category to Verticillium wilt attributed to the different genotypes. No significant differences in the frequency of *V. dahliae* reisolation has been reported for cultivars with contrasting disease response by others authors (Bubici and Cirulli, 2012). However, correlation between plant colonization by pathogen, measured as percentage of positive isolation or fungal DNA amount, and resistance level to Verticillium wilt has also been reported from the evaluation of a limited number of cultivars (Mercado-Blanco et al. 2003; Antoniou et al. 2008). High vascular colonization found in genotypes classified here as R seems to indicate that the action of different resistance mechanisms could be more important in terms of disease reaction than the extent of fungus colonization itself (Fradin and Thomma, 2006). Histopathological and biotechnological methods have shown that true resistance (vascular colonization hindered) occurs in some olive cultivars inoculated with *V. dahliae* (Rodríguez-Jurado, 1993; Baídez et al. 2007; Markakis et al. 2009), although tolerance (defined "as a plant´s ability to sustain extensive systemic colonization but express few, if any, symptoms and provide a reasonable crop yield") rather than resistance has been also suggested (Gramaje et al. 2013). Extensive (root and stem) and intensive (RCI and SCI) colonization found in genotypes classified as R in this work support the hypothesis of tolerance for olive-*Verticillium dahliae* pathosystem.

3.7. Conclusions

Screening for Verticillium wilt resistance was carried out in 64 genotypes including 12 cultivars from the World Olive Germplasm Bank of Córdoba (Spain) and 52 genotypes coming from open-pollination of these cultivar plus three other cultivars. A wide variability in Verticillium wilt symptoms development and plant colonization was observed. Among the 52 genotypes from open pollination evaluated, eight of them (15.4 %) were classified as resistant (R) according to Relative Susceptibility Index (RSI) values calculated from five disease parameters. R genotypes were obtained from parents of different known disease reaction, from susceptible ('Picual') to resistant ('Frantoio'), although a high heritability was

inferred regarding the degree of resemblance. The level of resistance to symptoms development of the evaluated genotypes was not associated to the degree of plant colonization by the fungus based on isolation of the fungus from both root and stem, which suggests a possible tolerant plant defence mechanism in olive /*V. dahliae* pathosystem. Future works will be carried out to confirm the level of resistance and agronomic performance of these genotypes under field conditions and to elucidate the defence mechanisms involved in the observed resistance.

3.8. Acknowledgements

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Capítulo 4. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses

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4.1. Abstract

Verticillium wilt (VW) resistance was evaluated in genotypes from olive crosses including resistant cultivars as parents. Thirty-eight genotypes from three crosses were evaluated: 'Changlot Real' x 'Dolce Agogia' (16), 'Frantoio' x 'Arbosana' (13) and 'Koroneiki' x 'Empeltre' (9). These genotypes were previously selected for several agronomic traits from wider initial progenies populations. Several disease severity and plant colonization parameters were evaluated in inoculation experiments under controlled conditions by dipping roots cutting in a conidial suspension of a highly virulent defoliating isolate of *Verticillium dahliae*. Significant differences among the evaluated genotypes, including parents and selections from crosses, were obtained for all the disease parameters assessed. A wide variability in disease parameters was observed in the three cross combinations tested. Genotypes with lower Relative Susceptible Index (RSI) values than both parents were found in the three progenies tested and 10 out of 38 genotypes (26 %) were finally classified as resistant. The level of resistance of these genotypes will be confirmed in future studies under field conditions.

4.2. Keywords

Defoliating pathotype, inheritance, *Olea europaea* L., plant colonization, *Verticillium dahliae*.

4.3. Introduction

Verticillium wilt (VW), a vascular disease caused by the soilborne fungus *Verticillium dahliae* Kleb, is one of the main problems for olive (*Olea europaea* L.) growing in many areas worldwide. Currently, there are no available control measures which are sufficiently effective when applied singly. Therefore, an integrated disease management strategy, including different control methods before and after planting (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012), has been recommended. In this sense, the use of resistant plant materials is considered as the most economic and efficient control measure for the disease. Several sources of resistance to VW have been identified in olive, including both cultivated and wild germplasm. Considerable levels of resistance to development of symptoms caused by the highly virulent defoliating (D) pathotype of *V. dahliae* have been reported for a limited number of traditional cultivars such as 'Frantoio', 'Changlot Real' and 'Empeltre', both upon artificial inoculations and in naturally-infested soils (López-Escudero et al. 2004; Trapero et al. 2013). However, due to some disadvantages such as late bearing and excessive vigour, these cultivars may not represent the best agronomical choice for a modern olive growing and are not suitable for high-density orchards. Screening for Verticillium wilt resistance has also been carried out in wild olive materials, which allowed the selection of new sources of resistance to the disease (Mercado-Blanco et al. 2003; Colella et al. 2008; Jiménez-Díaz et al. 2012). The use of resistant materials as rootstocks for grafting of VW-susceptible olive cultivars has been successfully reported, providing significant reduction on the severity of the attacks (Porras-Soriano et al. 2003; Bubici and Cirulli, 2012). However, no source of total resistance has been found and all plant materials tested up to now have been infected by the pathogen, so that complete control of VW of olive by means

of resistant rootstock cannot be guaranteed, at least in soils heavily infested with *V. dahliae*. In olive, the mechanisms of infection and colonization by *V. dahliae* and plant defense response of the plant are not fully understood yet.

Resistant genotypes can be used as parents in breeding programs to obtain new cultivars combining both high disease resistance and appropriated agronomic characters. In olive, the wide dispersion of the disease and the increase importance of crop losses associated with the rapid extend of fungal pathotypes highly virulent, together with the reduced number of traditional cultivars showing a significant level of resistance to VW, have promoted the development of breeding programs to select new cultivars with improved resistance levels to the disease (Erten et al. 2011; Trapero et al. 2013; Arias-Calderón et al. 2015). However, even in progenies from crosses between cultivars of known merit, most of the seedlings obtained show poor agronomic performance and should be discarded as soon as possible. Thus, the selection process in conventional fruit breeding programs involves several selection steps in which the most promising individuals are retained for further clonal testing (León et al. 2015). Previous results in our olive breeding program selection indicate that selection, at the seedling stage, for early yield, fruit size and oil content can be efficiently performed in the cross progenies, while still retaining high variability for characters not previously considered (León et al. 2015). Therefore, screening of VW resistance after preselection for important agronomic traits at the seedling stage would increase the chances of final selection of genotypes combining both high disease resistance and appropriated agronomic characters. This strategy of multi-stage selection could be a superior alternative particularly in cross-fertilized crops for characters under polygenic control. For instance, multiple-trait selection for VW resistance and agronomic traits resulted in cultivars of balanced commercial utility in strawberry (Shaw et al. 2010) or cotton (Zhou et al. 2014).

In the present work, the resistance to Verticillium wilt was evaluated in 38 genotypes from three crosses including at least one parent cultivar previously categorized as resistant to the disease. These genotypes were previously selected for important agronomic traits from wider progenies populations. The aims of this work were to study the inheritance of Verticillium wilt resistance and to select the most outstanding genotypes displaying high levels of disease resistance and good agronomic characteristics for further evaluation tests. For that purpose, several disease and plant colonization parameters were assessed in inoculation experiments under controlled conditions.

4.4. Materials and Methods

4.4.1. Plant materials

VW resistance was evaluated in genotypes from three different crosses: 'Changlot Real' x 'Dolce Agogia', 'Frantoio' x 'Arbosana' and 'Koroneiki' x 'Empeltre'. Parents included some of the cultivars of the World Olive Collection of IFAPA Córdoba (WOGB, CAP-UCO-IFAPA), Spain, that have showed higher resistance response in previous works ('Changlot Real', 'Dolce Agogia', 'Empeltre' and 'Frantoio') as well as cultivars cataloged previously as moderately susceptible ('Koroneiki') and susceptible ('Arbosana') (López-Escudero et al. 2004; Martos-Moreno et al. 2006; Arias-Calderón et al. 2015).

Crosses, germination of seeds and forced growth of seedlings plants in a greenhouse were carried out according to the standard procedures used in the olive breeding program (Santos-

Antunes et al. 2005). Afterwards, seedlings plants were transplanted into the field at 4 m x 1.5 m spacing. Drip irrigation and standard cultural practices were followed in the orchard to ensure adequate tree growth. The three progenies were evaluated for length of the juvenile period and fruit characters (oil content and fruit size). As a result, 38 genotypes were selected from the initial populations: 16 from the cross 'Changlot Real' x 'Dolce Agogia', 13 from 'Frantoio' x 'Arbosana' and 9 from 'Koroneiki' x 'Empeltre'.

Plants of the selected genotypes and the cultivars used as parents were obtained by vegetative propagation of semi-hardwood stem cuttings to provide material for screening to VW resistance. 'Picual' and 'Frantoio' cultivars were also propagated as controls of known reaction, susceptible and resistant respectively.

4.4.2. Inoculation procedure and growth conditions

The applied monosporic *V. dahliae* isolate comes from affected olive trees in southern Spain and was characterized in previous study as highly virulent D pathotype (Rodríguez-Jurado et al. 2008). For inoculum preparation, cultures were grown on potato dextrose agar for 7-14 days at 24º C. Then it was translated to flasks containing 100 mL of potato dextrose broth. Fungal culture incubated for 7 days at 24ºC on an orbital shaker at 125 rpm in the darkness and it was filtered through double cheesecloth. The resultant suspension was assessed using a haemacytometer and it was adjusted to 10^7 conidia mL^{-1} with sterile distilled water.

Eight to twenty plants of six-month-old of each genotype and cultivar were inoculated by dipping the root system slightly trimmed in the conidial suspension for 15 minutes according to Rodríguez-Jurado et al. (2007). Six to ten non-inoculated control plants of each genotype and cultivar were immersed in sterile distilled water for the same time. Following inoculation, plants were transplanted to individual plastic pots with twice autoclaved soil (lime: peat, 2:1, v/v, 75 min at 121ºC) and kept in a controlled growth chamber at 22±2ºC, 45-85 % relative humidity and 14 h photoperiod light. Plants were watered as required and fertilized weekly with Hoagland´s nutrient solution.

4.4.3. Plant infection and symptom assessment

Symptoms severity was periodically assessed for 112 days according to the percentage of aerial part affected by chlorosis, curling leaves, necrosis, green defoliation and/or death of the plant using a severity scale from 0 to 4 (0 = absence of symptoms; 1= 1–33 % aerial part affected; 2=34–66 % aerial part affected; 3=67–100 % aerial part affected; 4= dead plant) (Rodríguez-Jurado et al. 1993). Several disease parameters were calculated:

Standardized Area Under the Disease Progress Curve (SAUDPC) that was implemented according to Campbell and Madden (1990) (1):

$$SAUDPC = [\sum_{i=1}^{n}((S_i + S_{i-1})/2)\Delta t][100/(S_{max}T)] \quad (1)$$

where S_i=mean severity of the experimental unit in the observation i; Δt=the number of days between observation; S_{max}=maximum value of severity (=4) ; T= experimental period in days; n=observation numbers.

Disease Intensity Index (DII) was calculated for each observation as shown in equation (2):

$$DII = \sum_{x=1}^{n}((S_x * N_x)/(4N_t))\,100 \quad (2)$$

Where S_x= severity in an individual plant, N_x= number of plants with symptoms of severity $S_x$and N_t= total number of plants for each experimental unit. Disease Intensity Index (DII) at the end of the evaluations is called FDII.

Others additional disease parameters were calculated: Final Disease Incidence (FDI) and Final Dead Plants Incidence (FDPI) that represent the percentage of plants with disease symptoms and the percentage of dead plants at the end of the experiment, respectively; Disease-Free Period (DFP) estimated as the number of days without appearance symptoms. All parameters were calculated for each experimental unit.

A Relative Susceptibility Index (RSI) was calculated summarizing all previous disease parameters weighted according the following coefficients (Arias-Calderón et al. 2015) (3):

$$RSI = \left[\left(0.3SAUDPC + 0.3FDPI + 0.2FDII + 0.05FDI + 0.15(100 - RDFP)\right)/SP\right]100 \quad (3)$$

where SP=average susceptibility in the reference susceptible cultivar 'Picual', i.e. the value of numerator calculated for this cultivar; RDFP=Relative Disease-Free Period estimated as the number of days without appearance symptoms and expressed as percentage to the total experimental period in days.

Plant colonization by the fungus was also assessed in root system and aerial part of each plant at the end of experiments (symptomatic and asymptomatic) or at dead plants by reisolation of the fungus on chlortetracycline water agar. Ten stem's pieces and ten from roots previously cleaned and disinfected, were plated onto the medium and incubated at 24°C in the darkness for 9 (stem) to 21 (root) days (Rodríguez-Jurado, 1993). Plant colonization by *V. dahliae* was microscopically characterized by the presence of hyaline conidiophores and conidia from the tips of phialides borne in whorls of erect conidiophores (namely verticils) and by the formation of typical melanized, resistant resting structures called microsclerotia (López-Escudero and Mercado-Blanco, 2011). The results from root and stem isolations of the pathogen were used to calculate a Root and Stem Colonization Index (RCI and SCI, respectively) for each experimental unit, as root and stem pieces of which the fungus was isolated relative to number total of root and stem pieces sampled, respectively.

4.4.4. Data analysis

Cultivars used as parents and the selected 38 genotypes were evaluated in two separate experiments that always included 'Picual' (susceptible) and 'Frantoio' (resistant) cultivars as disease reference controls. Initial analyses found no significant differences (P>0.05) among experiments for the various disease parameters assessed in the two control cultivars. Therefore data were pooled over two experiments. Each experiment was arranged according to a complete randomized block design with 8-20 inoculated plants per genotype distributed in four blocks with 2-5 plants per block, as well as 6-10 mock inoculated plants per genotype also distributed in the four blocks. ANOVAs were performed to test for significant differences among genotypes in disease parameters. Means were compared between each genotype and those of susceptible 'Picual' and resistant 'Frantoio' reference controls by the Dunnett´s test at P=0.05. Parameters expressed as percentages were subjected to angular transformation before being statistically analysed. Chi-square-test (χ^2) was used to compare the distribution

of genotypes among crosses according to the resistance categories established by RSI. Pearson's correlation coefficient was calculated among colonization parameters (RCI and SCI) and RSI, in the different crosses. All analyses were carried out using Statistix 9.0 software (Analytical Software, Tallahassee, Florida, USA).

4.5. Results

First foliar wilt symptoms after inoculation appeared on the susceptible control 'Picual' 14 days post inoculation (dpi), increased until 77 dpi up to a final 90.3% DII (Figure 4.1). No visual symptoms were observed on the resistant control 'Frantoio' until 28 dpi. Disease symptoms increased in this cultivar until 46 dpi and then remained steady for a final DII of 23.1 %. Among the genotypes evaluated, a wide variability in symptom progress was observed, irrespective of the progeny (Figure 4.1).

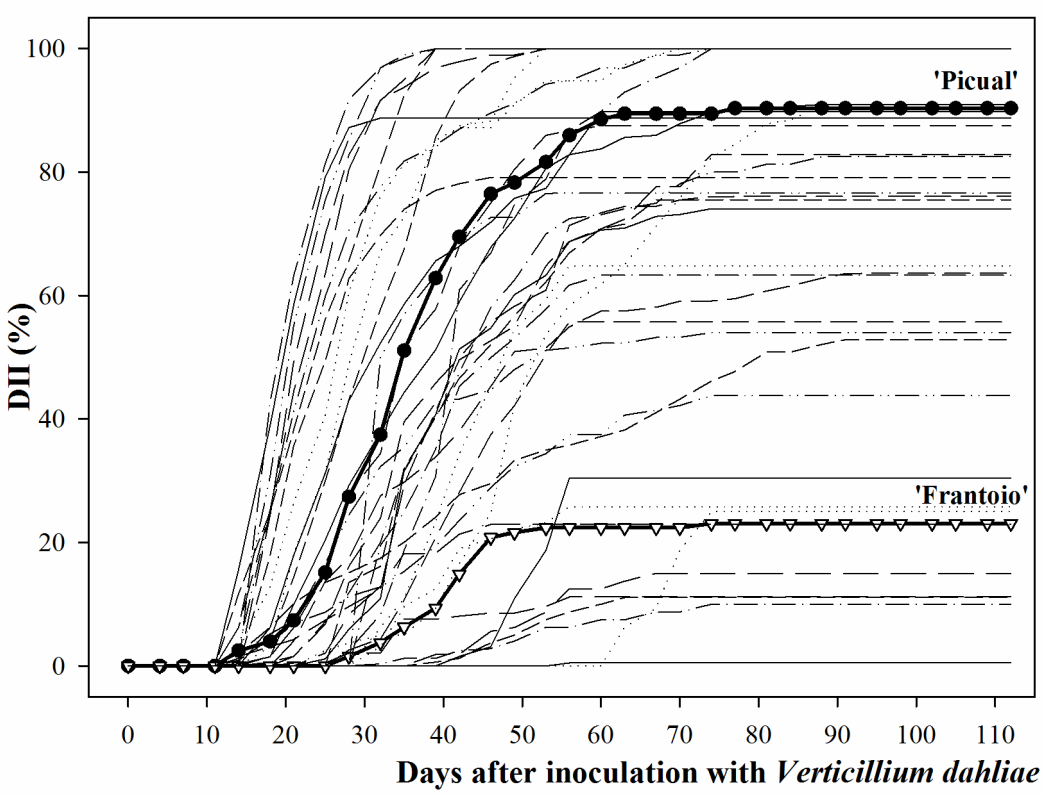

Figure 4.1. Progress of the Disease Intensity Index (DII) in olive genotypes from crosses. Reference control cultivars 'Picual' (susceptible) and 'Frantoio' (resistant) are indicated in bold.

Significant differences (P<0.05) among the evaluated genotypes, including parents and selections from their respective crosses, were obtained for all the disease parameters evaluated (Table 4.1). All disease parameters except DFP were highly correlated (data not shown), even though all of them did not provide equal grouping of genotypes when compared to the 'Picual' and 'Frantoio' reference controls according to Dunnett´s test. For instance, SAUDPC, FDPI and FDII, but not FDI and principally DFP, similarly grouped all the evaluated genotypes. Thus, three of the evaluated genotypes (CRxDA-11, FrxAr-11 and FrxAr-6) showed significant differences from control 'Picual' for all disease parameters.

To integrate the information of the different disease parameters, the Relative Susceptibility Index (RSI) was applied for final classification of genotypes according to their levels of resistance to Verticillium wilt (Table 4.1). Among the selections from crosses, 22 (58 %) of them were classified as Susceptible (S, significantly different from 'Frantoio'), 6 (16 %) as Moderately Susceptible (MS, non-significantly different from both 'Picual' and 'Frantoio') and 10 (26 %) as Resistant (R, significantly different from 'Picual'). All parent cultivars were classified as R except 'Arbosana' (S).

Table 4.1. Disease parameters calculated in genotypes from three olive crosses. Genotypes and parents are sorted in descendant order for RSI by crosses. Reference control cultivars 'Picual' (susceptible) and 'Frantoio' (resistant) are indicated in bold.

Cultivar/cross[a]	Disease parameter[b]						Resistance category[c]
	SAUDPC	FDPI	FDII	FDI	DFP	RSI	
'Picual'	62.12#[d]	88.3#	90.32#	100#	24.11#	100#	S
CRxDA-9	78.54#	100#	100#	100#	20.2#	113.77#	S
CRxDA-4	79.87#	100#	100#	100#	32.95#	111.87#	S
CRxDA-10	76.08#	100#	100#	100#	32.01#	110.62#	S
CRxDA-5	81.79#	100#	100#	100#	59.4	107.61#	S
CRxDA-1	74.96#	100#	100#	100#	77.01*	101.71#	S
CRxDA-3	80.99#	100#	100#	100#	93.01*	100.97#	S
CRxDA-12	60.83#	100#	100#	100#	61.39	99.33#	S
CRxDA-6	60.64#	87.5#	87.5#	87.5#	17.36#	98.92#	S
CRxDA-8	72.37#	100#	100#	100#	99.16*	96.56#	S
CRxDA-7	60.54#	90#	90.94#	95#	52.98	94.44#	S
CRxDA-2	62.59#	79.17#	79.17#	79.17	86.98*	80.77#	S
CRxDA-13	49.91#	75#	76.04#	91.67#	61.07	79.3#	S
CRxDA-14	47.58#	67.5#	74.06#	78.75	50.24	76.33#	S
'Dolce Agogia'	20.88*	30*	30.63*	40*	82.54*	32.68*	R
'Changlot Real'	15.93*	25*	25.31*	30*	58.3	3 1.54*	R
CRxDA-15	16.68*	20.83*	25.78*	62.5	81.38*	28.06*	R
CRxDA-16	6.15*	10*	11.09*	21.25*	47.97	20.02*	R
CRxDA-11	7.47*	8.33*	11.2*	29.17*	71.3*	16.02*	R
FrxAr-12	73#	100#	100#	100#	69.49	102.39#	S
FrxAr-1	73.71#	88.75#	88.75#	88.75#	31.95#	101.96#	S
FrxAr-9	51.41#	81.25#	82.81#	100#	23.06#	91.62#	S
FrxAr-2	47.32#	90#	90.94#	95#	52.83	89.49#	S
FrxAr-8	63.65#	87.5#	89.84#	100#	87.17*	88.27#	S
'Arbosana'	49.14#	70#	75.31#	90#	66.47	75.82#	S
FrxAr-7	41.01	55	63.59	82.5#	43.15	68.08	MS
FrxAr-4	38.55	45.83	54.04	91.67#	65.05	57.74	MS
FrxAr-3	31.79	49.58	52.89	79.58#	70.31	54.57	MS
FrxAr-13	28.06*	42.5	43.75*	52.5*	53.69	49.62*	R
FrxAr-5	16.45*	22.92*	22.92*	22.92*	57.94	29.97*	R
'Frantoio'	14.89*	19.3*	23.08*	34.67*	70.94*	26.35*	R
FrxAr-11	10*	25*	25*	25*	83.89*	24.08*	R

Cultivar/cross[a]	Disease parameter[b]						Resistance category[c]
	SAUDPC	FDPI	FDII	FDI	DFP	RSI	
FrxAr-10	0.27*	0*	0.52*	8.33*	22.93#	15.28*	R
FrxAr-6	5.36*	10*	10*	10*	76.25*	13.41*	R
KoxEm-1	79.41#	100#	100#	100#	72.86*	104.17#	S
KoxEm-3	53.74#	75#	76.56#	100#	46.13	84.22#	S
KoxEm-2	51.86#	80#	82.5#	95#	61.84	83.61#	S
KoxEm-9	48.23#	70.83#	75.52#	100#	52.23	79.16#	S
KoxEm-8	40.61	62.5	64.84	75	37.17#	71.73	MS
KoxEm-6	34.76	54.17	55.73	62.5	25.04#	65.59	MS
KoxEm-5	41.37	62.5	63.28	75	92.19*	61.25	MS
'Koroneiki'	14.24*	20.83*	22.79*	35.42*	23.4#	35.62*	R
'Empeltre'	19.4*	25*	26.56*	37.5*	50.11	35.18*	R
KoxEm-7	16.72*	25*	30.47*	62.5	82.81*	30.55*	R
KoxEm-4	8.27*	15*	15*	15*	69.49	19.24*	R

[a]CRxDA: 'Changlot Real' x 'Dolce Agogia', FrxAr: 'Frantoio' x 'Arbosana', KoxEm: 'Koroneiki' x 'Empeltre'.
[b]SAUDPC: Standardized Area Under the Disease Progress Curve; FDPI: Final Dead Plants Incidence; FDII: Final Disease Intensity Index; FDI: Final Disease Incidence; DFP: Disease-Free Period; RSI: Relative Susceptibility Index.
[c]Evaluated genotypes were classified by RSI values in three resistance categories to Verticillium wilt: resistant (R), significantly different from 'Picual'; susceptible (S), significantly different from 'Frantoio'; and moderately susceptible (MS), non-significantly different from 'Picual' and 'Frantoio'.
[d]*=significantly different from 'Picual'; #=significantly different from 'Frantoio'; without symbol=non significantly different from 'Picual' and 'Frantoio' according to Dunnett´s test at P=0.05.

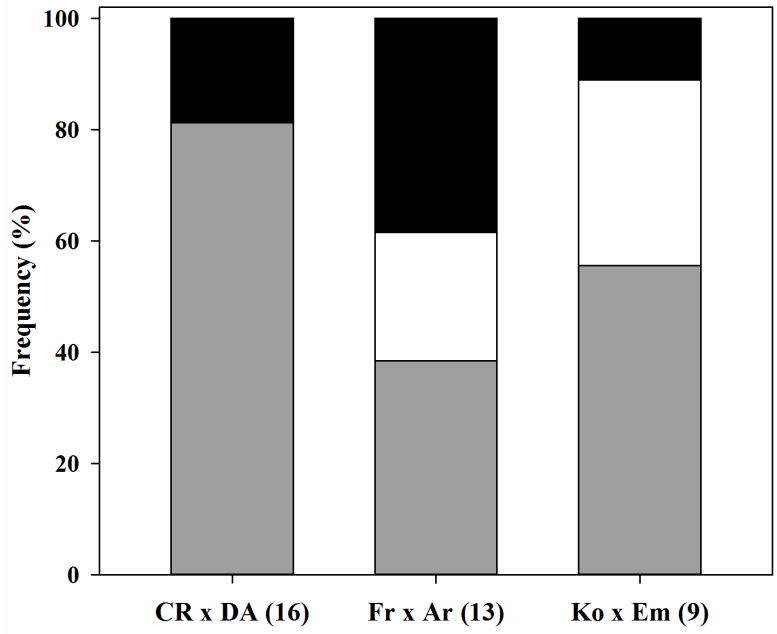

Figure 4.2. Frequency distribution of genotypes (%) by cross for resistance category classification according to Relative Susceptibility Index (RSI): resistant (black). susceptible (grey) and moderately susceptible (white). In brackets the total number of evaluated genotypes by cross.

A wide variability was obtained in all cross combinations: RSI values ranged from 13.41 to 102.39 in 'Frantoio' x 'Arbosana', 16.02 to 113.77 in 'Changlot Real' x 'Dolce Agogia' and 19.24 to 104.17 in 'Koroneiki' x 'Empeltre' (Table 4.1). In fact, genotypes with lower RSI values than both parents were found in the three progenies. However, the pattern of distribution of genotypes according to resistance categories established by RSI was significantly different among crosses (Figure 4.2, Table 4.2). This was mainly due to 'Changlot Real' x 'Dolce Agogia' cross, in which a higher proportion of S genotypes was obtained and none of the genotypes evaluated was classified as MS.

Table 4.2. Chi-square comparison between crosses for resistance category classification according to Relative Susceptibility Index (RSI).

Comparison	**df**	χ^2	**p**
'Changlot Real' x 'Dolce Agogia' vs. 'Frantoio' x 'Arbosana'	2	6.82	0.033
'Frantoio' x 'Arbosana' vs. 'Koroneiki' x 'Empeltre'	2	0.69	0.707
'Changlot Real' x 'Dolce Agogia' vs. 'Koroneiki' x 'Empeltre'	2	6.52	0.038
Overall	4	8.49	0.075

Scatter plots of RSI vs. colonization by the fungus in stem (SCI) and root (RCI) are shown in Figure 4.3. The relationships between these parameters varied according to the different crosses and tissues. The highest correlation was found in 'Changlot Real' x 'Dolce Agogia' for SCI (r=0.84, $P<0.001$), with all genotypes categorized as R showing low values of SCI that ranged from 2% to 9%. All genotypes categorized as resistant according to RSI showed low values of colonization in both root and stem tissues, with maximum values of 14% and 25% for RCI and SCI respectively. However, similar values of colonization was also found in some genotypes showing higher RSI values and, therefore, classified as MS or even S.

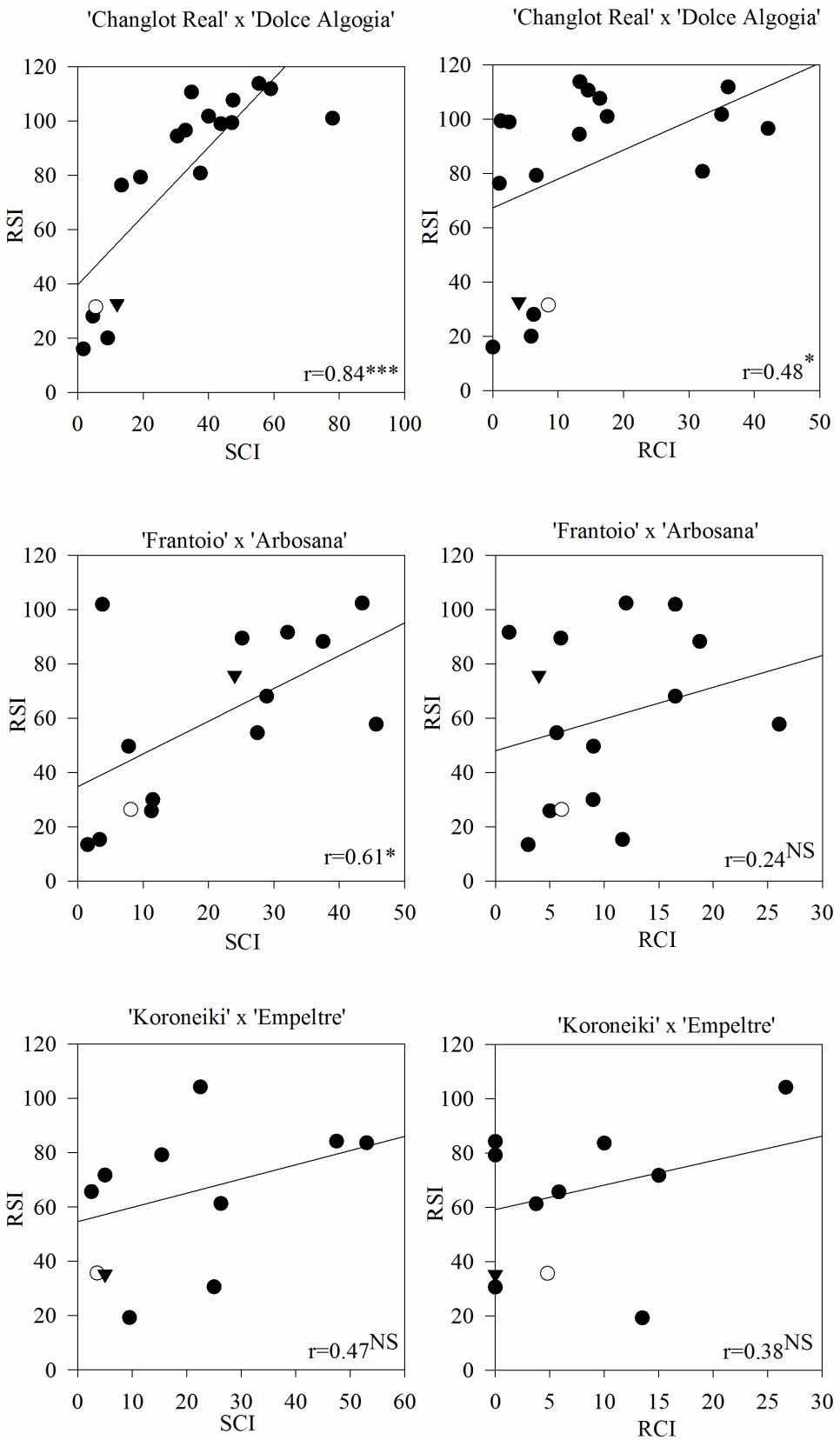

Figure 4.3. Scatter plots of Relative Susceptibility Index (RSI) vs. Root Colonization Index (RCI) and Stem Colonization Index (SCI) in 38 genotypes selected from three progenies. For each cross, white circle and triangle represent values for female and male parents, respectively.

4.6. Discussion

V. dahliae exhibits a broad host range including many economically-important herbaceous and woody crop species (Bhat and Subbarao 1999; Pegg and Brady, 2002; López-Escudero and Mercado-Blanco, 2011). For some of them, the importance of the disease and crop losses induced by this fungus has promoted the development of breeding programs to select new cultivars with improved resistance levels to the disease. Considering the limited number of traditional olive cultivars which have displayed a high level of VW resistance, development of olive breeding programs to obtain new cultivars with enhance resistance levels could be of paramount importance for a better control strategy of the disease (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al. 2012). In this work, artificial inoculation experiments under controlled conditions were carried out to evaluate the resistance to VW in seedlings from three olive breeding crosses and their respective parents. Some of the cultivars currently known to show higher levels of resistance to VW were used as parents. The assessment of the resistance to VW in the seedlings was preceded by their evaluation and selection for important agronomic traits as the general objective was to identify new genotypes combining good agronomic behavior with high level of VW resistance.

The disease reaction obtained for the cultivars used as parent of the crosses was similar than previously reported under similar experimental procedures (López-Escudero et al. 2004; Martos-Moreno et al. 2006; Arias-Calderón et al. 2015). Thus, R response was observed for 'Changlot Real', 'Dolce Agogia', 'Empeltre' and 'Frantoio' and S for 'Arbosana'. Slight difference was observed for 'Koroneiki', previously cataloged as MS ussing different inoculation methods (dipping root, soil infested and stem puncture) (López-Escudero et al. 2007; Markakis et al. 2010; Arias-Calderón et al. 2015) but categorized as R in this work. It should be noted that 'Koroneiki' cultivar has also showed high level of resistance under natural field conditions (Trapero et al. 2013).

A wide variability in the values of the different disease parameters calculated was observed in the three cross combinations tested and 10 out of 38 evaluated genotypes (26 %) were finally classified as R according to RSI values. These results represent a higher proportion of resistant genotypes than previously reported in olive progenies from open pollination, although resistant seedlings were selected in progenies of different cultivars including susceptible parents such as 'Arbequina', 'Manzanillo', 'Mission' and 'Picudo' (Wilhelm and Taylor, 1965; Arias-Calderón et al. 2015). Trapero et al. (2015) found approximately half the proportion of R genotypes obtained in our work in the evaluation of a high number of genotypes derived from open pollination and crosses involving different olive cultivars, wild olive genotypes and other *Olea* genus. In our case, however, a different strategy was followed as the seedling progenies were first evaluated for agronomic traits and only those genotypes showing good agronomic performance were included in the inoculation tests.

The proportion of genotypes classified in different resistance categories varied among crosses according chi-square comparison. In this sense, 'Frantoio' x 'Arbosana' showed the highest proportion of R genotypes (38 %) while Changlot Real' x 'Dolce Agogia' showed the highest proportion of S genotypes (81 %). These results are in concordance with previous studies (Wilhelm and Taylor, 1965; Trapero et al. 2015), which identified higher proportion of R genotypes in progenies from free pollination and different cross combinations including

'Frantoio' as parent. Different inheritance patterns against VW have been reported in other species such as cotton with no consensus on the genetic basis of resistance (Zhang et al. 2014). Several studies in cotton seems to indicate a quantitative inheritance of the resistance under polygenic control, predominantly due to additive genetic factors and, therefore, highly heritable (Aguado et al. 2008; Yang et al. 2008; Zhou et al. 2014). The high variability obtained from different crosses in our work seems to indicate similar pattern of quantitative inheritance under polygenic control. However, the higher proportion of R genotypes in seedlings from 'Frantoio' x 'Arbosana' cross (the only one involving one S parent) suggests that other than additive effects could be of major importance, indicating a likely complex mode of genetic control of VW resistance in olive. Therefore, searching for specific cross combinations producing high percentage of R genotypes could be the best strategy in olive breeding programs for this trait. New breeding cycles, involving as parents some of the R genotypes identified in this work, are currently underway. This will be useful to test whether a selection strategy based on increased level of parental resistance may result in increased percentage of resistant genotypes, as previously demonstrated in other species (Shaw et al. 2010).

In olive, previous studies have demonstrated that plant defense mechanisms against VW involve the activation of different physical and biochemical barriers to hamper the progress of *V. dahliae* from root to stem (Baídez et al. 2007; Markakis et al. 2010). In 'Arbequina' plants, early and profuse colonization of the root surface have been observed, followed by inter and intracellular hyphae growth within the root and rapid colonization of the plant by hyphae and conidia (Prieto et al. 2009). Plant colonization has been rarely compared to the level of resistance of olive cultivars. Mercado-Blanco et al. (2003) found a correlation of the level of susceptibility to VW of three cultivars ('Picual' > 'Arbequina' > 'Acebuche-L') with the amount of pathogen DNA quantified in roots and stems. However, in Greek cultivars with different susceptibility levels ('Amfissis' > 'Kalamon' and 'Koroneiki'), this correlation was clear in stems but not in roots (Markakis et al. 2009). In the present work, a high significant correlation between RSI and SCI and also between RSI and RCI were observed in the cross 'Changlot Real' x 'Dolce Agogia'. Similarly, significant correlation between RSI and SCI was observed in 'Frantoio' x 'Arbosana', the cross with the highest number of genotypes categorized as R. This might indicate the occurrence of a resistance mechanism to vascular level of the plant involved in the disease response. On the contrary, no correlation between RSI and plant colonization parameters was observed in 'Koroneiki' x 'Empeltre'. Thus, some genotypes with similar values of colonization (SCI, RCI) but different disease response (RSI) were found in this work, as previously reported also in genotypes from open pollination progenies (Arias-Calderón et al. 2015). All the above maybe suggests the existence of a tolerance or resistant response mechanism depending on the olive genotype (Robb, 2007), as previously reported in inoculation experiments in tomato isolines with different fungal isolates (Chen et al. 2004). Further investigations are needed to really elucidate the resistance or tolerance mechanisms operating in stems and roots on different olive cultivars/*V. dahliae* interactions.

4.7. Conclusions

The assessment of the resistance to VW in olive genotypes from crosses, which have previously displayed good agronomic performance, was carried out under controlled conditions in the present work. This procedure may represent some advantages compared to selection at the seedling stage previously reported (Wilhelm and Taylor, 1965; Trapero et al. 2015). On the one hand, the possibility of replication of plant materials, instead of one single plant per genotype, allows more accurate evaluation for disease resistance. Moreover, a balanced commercial utility could be expected in the genotypes finally selected. Among the evaluated genotypes, ten of them were classified as resistant, although future trials to confirm the disease reaction of these genotypes under field conditions are also needed. Also, the genotypes evaluated in the present study represent valuable material for future studies about pattern plant colonisation by the fungus and the resistance/tolerance mechanisms operating on different olive cultivars. Finally, the confirmation of the effect of the cultivar 'Frantoio' in conferring high VW resistance to its progeny, in addition to similar and previously reported effects in conferring high resistance to fungal aerial disease caused by *Spilocea oleagina* and *Colletotrichum acutatum* (Moral et al. 2015), reinforce its utility as a valuable parent in olive breeding programs for disease resistance.

4.8. Acknowledgements

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Capítulo 5. Pre-breeding for resistance to Verticillium wilt in olive: fishing in the crop wild relative gene pool

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5.1. Abstract

This study aimed to identify new sources of resistance to Verticillium wilt in olive. We evaluated various types of genotypes: wild olive trees (*Olea europaea* subsp. *europaea* var. *sylvestris*), genotypes belonging to related subspecie (*Olea europaea* subsp. *guanchica*) and genotypes coming from crosses between Picual cultivar and wild olive trees. Fifty-six genotypes were inoculated by dipping roots and then screened under controlled conditions to test their resistance to a highly virulent Defoliating isolate of *Verticillium dahliae*. Picual (susceptible) and Frantoio (resistant) were control cultivars. Wide variability and significant differences were obtained in the evaluated disease parameters. The Relative Susceptibility Index (RSI), summarizing the disease parameters, was used for final classification of genotypes. Thirteen genotypes were categorized as resistant: eight wild olives from different locations, two genotypes belonging to subsp. guanchica populations from Canary Islands and three genotypes obtained from one of the crosses Picual x wild. The identification of high levels of resistance to *V. dahliae* among wild olive genotypes may be helpful for the management of this disease. The resistant genotypes could be used as rootstocks for susceptible olive cultivars or parents in future breeding programs.

5.2. Keywords

Cross breeding, *Olea europaea* subsp. *europaea* var. *sylvestris*, *Olea europaea* subsp. *guanchica*, Rootstock, *Verticillium dahliae*, Wild olive.

5.3. Introduction

Cultivated olive (*Olea* europaea subsp. europaea var. europaea) exhibits wide genetic variability represented by thousands of cultivars coming from empirical selection carried out by growers and often restricted to their specific areas of origin (Belaj et al., 2012). However, most of the olive cultivars currently in use are susceptible to Verticillium wilt which is caused by the soil borne fungus Verticillium dahliae Kleb, particularly to the highly virulent Defoliating pathotype (D) of *V. dahliae*. Few cultivars show resistance to the development of symptoms upon inoculations under controlled conditions and in naturally-infested fields (López-Escudero and Mercado-Blanco, 2011; Bubici and Cirulli, 2012; Trapero et al. 2013). Verticillium wilt represents the main phytosanitary limitation in olive orchards for both its destructive potential and the lack of effective strategies for its control. Therefore, finding new sources of genetic resistance is of paramount importance for the continuity of olive production in many areas (Areal and Riesgo, 2014).

Wild olives (*Olea europaea* subsp. *europaea* var. *sylvestris*) represent another botanical variety of the subsp. *europaea*. Both cultivated and wild olives are spread throughout the whole Mediterranean basin. *Olea europaea* includes five other subsp. based on morphology and geographical distribution (Green, 2002), including subsp. *guanchica* (hereafter *guanchica*) restricted to the Canary Islands. Many studies have focused on evaluating the distribution of variability between cultivated and wild olives and on establishing the genetic relationships among the different *O. europaea* subspecies that are distributed beyond the Mediterranean area (Baldoni et al. 2006; Belaj et al. 2010; García-Verdugo et al. 2010). Olive

cultivars exhibit lower genetic diversity than their wild relatives, indicating that wild relatives could enrich the genetic basis of cultivated material (Lumaret et al. 2004). Therefore, wild olives may represent a useful source of genetic variability for some characters which are seldom found in cultivated material, such as Verticillium wilt resistance.

Wild relatives of extant crop plants can also be used as parents in breeding programs to improve crop performance for different agronomic traits. This strategy has been used by plant breeders of different crops for over a century and recent surveys indicate that over 80% of the beneficial traits conferred by wild relatives involved pest and disease resistance (Hajjar and Hodgkin, 2007), including Verticillium wilt (Diwan et al. 1999; Jansky et al. 2004). In olive, screening of wild germplasm from the Mediterranean region has been carried out in recent work, revealing its potential use as a new source of resistance to the disease (Mercado-Blanco et al. 2003; Colella et al. 2008; Jiménez-Díaz et al. 2012). Additionally, the possibility of hybridization between the cultivated olives and wild olives and related subspecies allows the introduction of genes from remote gene pools (Hannachi et al. 2009; Lavee and Zohary, 2011; Besnard et al. 2012). However, there remained limited information regarding the potential use of wild relatives as parents in olive breeding programs (Hannachi et al. 2009; Klepo et al. 2013, 2014).

In Spain, wild olives represent an important component of olive genetic heritage and Mediterranean flora. Many efforts have been directed toward the conservation, evaluation and usefulness of wild olive germplasm. Previous results indicate that wild olive genetic resources in Andalusia (Southern Spain) represent a differentiated gene pool from cultivars of the same area and those wild olive populations of other regions (Belaj et al. 2010, 2011). The presence of morphological and agronomical variability in wild olive populations of this area has been reported (Belaj et al. 2011). The high diversity of wild olive populations has also been reported in Northeast Spain as well as in Balearic Islands (Rubio de Casas et al. 2006; Belaj et al. 2010). In addition, the presence of the *guanchica* restricted to the Canary Islands represents another element of the Spanish genetic patrimony of wild olive genetic resources (García-Verdugo et al. 2010; Toumi 2013).

The aim of this work was to assess wild olives and related subspecies as new sources of resistance to Verticillium wilt in olive. The response to the disease was evaluated in genotypes representing heterogeneous ecological conditions, including wild olives maintained ex situ and collected from different locations in the Iberian Peninsula and Balearic Islands, genotypes from related subspecie (*guanchica*) from the Canary Islands and from two crosses between the Picual cultivar and wild genotypes.

5.4. Materials and Methods

5.4.1. Plant material

An ex situ collection of wild olives and related subsp. representing heterogeneous ecological conditions was established at IFAPA Centro 'Alameda del Obispo' Córdoba, Spain, as a result of prospective surveys (Belaj et al. 2010). This collection includes 185 different wild genotypes and the diversity is being evaluated for molecular and agro-morphological traits (De la Rosa et al. 2013; Toumi, 2013).

From this extensive collection, 45 wild olive genotypes (*Olea europaea* subsp. *europaea* var. *sylvestris*) collected from different populations in the Iberian Peninsula and Balearic

Islands and three genotypes belonging to *Olea europaea* subsp. *guanchica* from Canary Islands were evaluated in this work (Table 5.1). Previous examination of genetic diversity and morphological description confirmed that all genotypes could be cataloged as genuine wild and *guanchica* populations, i.e. not feral forms from crosses involving cultivated material (Belaj et al. 2011; Toumi, 2013). Original sampling sites were located in undisturbed habitats representing wide variability for geographic location and climatic conditions (Table 5.1, Figure 5.1). An *ex situ* collection of wild olives and related subsp. representing heterogeneous ecological conditions was established at IFAPA Centro 'Alameda del Obispo' Córdoba, Spain, as a result of prospective surveys (Belaj et al. 2010). This collection includes 185 different wild genotypes and the diversity is being evaluated molecular and agro-morphological traits (De la Rosa et al. 2013; Toumi, 2013).

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Table 5.1. Geographic location and climatic classification of sampling sites where wild olive analysed were collected[a].

Region	Province	Sampling sites	Population	N[b]	Latitude	Longitude	Altitude	Climatic Classification[c]
Balearic Islands	Menorca	Es Mercadal	1	1	40º0.73' N	4º7.75' E	39	Mediterranean Maritime
Balearic Islands	Menorca	Sant Climent	2	2	39º52.91' N	4º11.99' E	72	Mediterranean Maritime
Balearic Islands	Mallorca	Palma	3	1	39º34.04' N	2º37.22' E	90	Mediterranean Maritime
Balearic Islands	Mallorca	Llucmajor	4	1	39º23.89' N	2º55.21' E	46	Mediterranean Maritime
Andalusia	Almería	Tabernas	5	1	36º59.65' N	2º21.34' W	1225	Mediterranean Maritime
Andalusia	Jaén	Navas de San Juan	6	3	38º15.11' N	3º21.16' W	407	Subtropical Mediterranean
Andalusia	Jaén	Úbeda	7	1	38º6.17' N	3º22.34' W	517	Subtropical Mediterranean
Andalusia	Jaén	Carboneros	8	2	38º10.86' N	3º35.42' W	504	Subtropical Mediterranean
Andalusia	Jaén	Centenillo	9	1	38º20.61' N	3º43.06' W	657	Subtropical Mediterranean
Andalusia	Jaén	Martos	10	1	37º40.27' N	3º59.46' W	770	Subtropical Mediterranean
Andalusia	Jaén	Andújar	11	2	38º9.76' N	3º59.78' W	272	Subtropical Mediterranean
Andalusia	Jaén	Arjona	12	1	37º56.35' N	4º3.67' W	355	Subtropical Mediterranean
Andalusia	Jaén	Arjona	13	1	38º1.67' N	4º4.24' W	221	Subtropical Mediterranean

Region	Province	Sampling sites	Population	N[b]	Latitude	Longitude	Altitude	Climatic Classification[c]
Andalusia	Sevilla	Osuna	14	1	37º9.81' N	4º57.65' W	371	Subtropical Mediterranean
Andalusia	Sevilla	Osuna	15	1	37º4.67' N	4º59.92' W	597	Mediterranean Maritime
Andalusia	Sevilla	Osuna	16	2	37º10.84' N	5º6.79' W	388	Subtropical Mediterranean
Andalusia	Sevilla	Marchena	17	3	37º20.92' N	5º18.13' W	119	Subtropical Mediterranean
Andalusia	Córdoba	Palma del Río	18	1	37º37.35' N	5º20.73' W	55	Subtropical Mediterranean
Andalusia	Cádiz	Jerez de la Frontera	19	1	36º34.91' N	5º36.23' W	312	Mediterranean Maritime
Andalusia	Cádiz	Alcalá de los Gazules	20	6	36º30.75' N	5º44.63' W	205	Mediterranean Maritime
Andalusia	Cádiz	San José del Valle	21	4	36º39.71' N	5º47.19' W	149	Subtropical Mediterranean
Andalusia	Cádiz	Medina Sidonia	22	3	36º19.56' N	5º51.02' W	46	Mediterranean Maritime
Andalusia	Sevilla	Los Palacios	23	2	37º13.93' N	5º58.77' W	18	Subtropical Mediterranean
Extremadura	Cáceres	Cáceres	24	2	39º13.64' N	6º25.49' W	420	Subtropical Mediterranean
Extremadura	Badajoz	Burguillos del Cerro	25	1	38º22.46' N	6º37.42' W	382	Subtropical Mediterranean
Canary Islands	Tenerife	Valle Brosque	26	2	28º31.49' N	16º13.64' W	307	Tropical Mediterranean
Canary Islands	Tenerife	Guimar	27	1	28º17.65' N	16º24.64' W	379	Tropical Mediterranean

[a] Wild genotypes were collected in sites located in different provinces within four Spanish regions. Various populations were collected in some sampled sites as Arjona and Osuna. Genotypes collected from Canary Islands belong to *Olea europaea* subsp. *guanchica*, and genotypes from other regions belog to *Olea europaea* subsp. *europaea* var. *sylvestris*.

[b] Number of genotypes collected from each sampled site.

[c] Climatic classification (J. Papadakis) was obtained from Spanish Ministry of Agriculture, Food and Environment.

Eight additional genotypes from crosses between the Picual cultivar and two of the wild genotypes (W2, W3) were also evaluated. These two wild olive trees (W2, W3) used as male parents of the crosses were selected among the genotypes and included in the collection based on abundant flowering and fruiting (Belaj et al. 2011; Toumi, 2013). Germination of seeds, forced growth of the seedlings in a greenhouse, and growth in the field were carried out according to standard procedures (Santos-Antunes et al. 2005), and fruit characters were evaluated once they reached the adult phase (Klepo et al. 2014). As a result, five genotypes from Picual x W2 and three genotypes from Picual x W3 were selected from wider populations on the basis of their early crop (short juvenile period) and relatively high fruit size and oil content.

Plants of the selected genotypes, including control cultivars: Picual (reference susceptible cultivar) and Frantoio (reference resistant cultivar), were obtained by vegetative propagation of semi-hardwood stem cuttings for screening for Verticillium wilt resistance. Resistance levels of Picual and Frantoio were confirmed in previous work (Rodríguez-Jurado, 1993; López-Escudero et al. 2004; Martos-Moreno et al. 2006; Trapero et al. 2013).

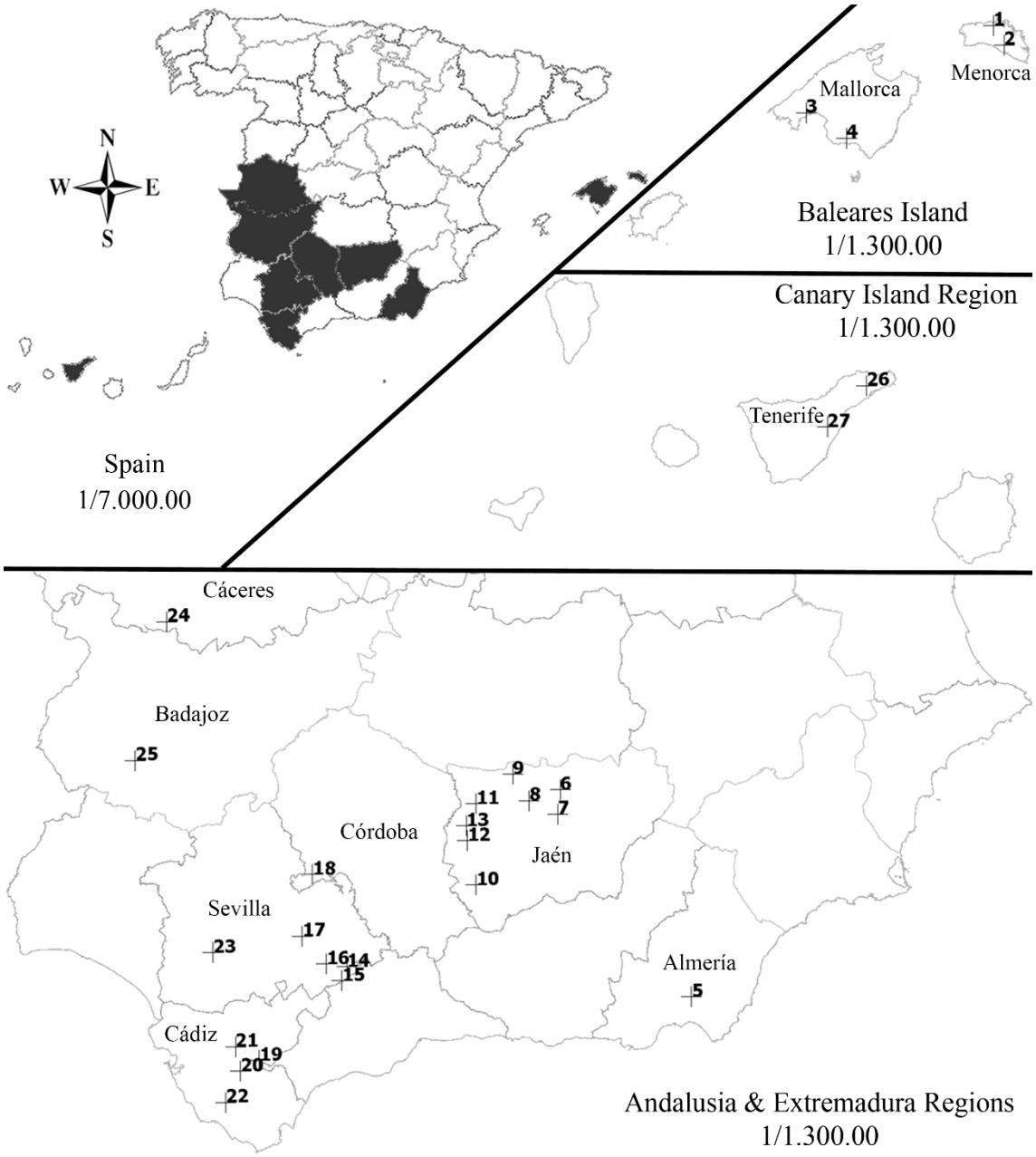

Figure 5.1. Locations of wild olive populations used in this study. Population sites where located in ten Spanish provinces (Almería, Badajoz, Cáceres, Cádiz, Córdoba, Jaén, Mallorca, Menorca, Sevilla and Tenerife) within four Spanish regions (Andalusia, Baleares, Canary and Extremadura).

5.4.2. Fungal cultures and plant inoculations

A monosporic culture of an axenically stocked isolate of *V. dahliae* was used for all experiments. This isolate was obtained from affected olive trees in southern Spain and was characterized as the highly virulent D pathotype in a previous study (Rodríguez-Jurado et al. 2008). Small agar plugs of stored culture were placed on water agar amended with chlorotetracycline (20 g of agar and 0.03 g of chlorotetracycline per liter of distilled water) (CWA) to obtain an active culture which was subcultured on potato dextrose agar (PDA) (200 g of potato, 20 g of agar and 15 g of glucose per liter of distilled water). For inoculum preparation, cultures on PDA were grown for 7-14 days at 24º C in the dark and agar plugs

(0.3 x 0.3 mm) colonized by the fungus were transferred to flasks containing 100 mL of potato dextrose broth (200 g of potato and 15 g of glucose per liter of distilled water). After being incubated for seven days at 24ºC on an orbital shaker at 125 rpm in the dark, the fungal culture was filtered through double sterile gauze. The resultant conidial concentration was quantified using a haemacytometer (Neubauer chamber) and ajusted to 107 conidia mL-1 with sterile distilled water.

Plants of six-month-old rooted cuttings were grown in 0.25 L pots in a greenhouse. Plants were grown in the greenhouse located in Cordoba (southern Spain) for six months, inoculated and kept in the growth chamber for resistance evaluations. During the growth period of the plants in the greenhouse, average temperature was 19.1 ºC. Photoperiod was supplemented with sodium vapour lights and irrigation was applied by drip on alternate days according to the weather. Plants (8-20 plants per genotype) were inoculated by dipping slightly trimmed root system in the conidia suspension for 15 minutes according to Rodríguez-Jurado (1993). Six to ten mock-inoculated control plants of each genotype and for reference control cultivars were immersed in sterile distilled water for the same length of time. Following inoculation, plants were individually transplanted to plastic pots with soil that had been autoclaved twice (lime: peat, 2:1, v/v, autoclaved at 121ºC for 75 min) and kept in the growth chamber at 22±2ºC, 45-85% relative humidity and 14 h photoperiod light of 360 µE m-2 s-1. Plants were watered with tap water as required and fertilized weekly with Hoagland´s nutrient solution (each nutrient solution was made in distilled water, stored at 4ºC and mixed with tap water at watering).

5.4.3. Assessment of plant reaction to inoculation

Symptom severity was assessed in each plant every three to four days after inoculation for 112 days on a 0-4 scale according to the percentage of aerial part affected by defoliation, chlorosis, wilt and/or necrosis compared with control plants (0 = absence of symptoms; 1= 1-33% aerial part affected; 2=34-66% aerial part affected; 3=67-100% aerial part affected; 4= dead plant).

Several disease parameters were calculated from these data:

a) Standardized Area Under the Disease Progress Curve (SAUDPC) calculated according to Campbell and Madden (1990) (1):

$$SAUDPC = [\sum_{i=1}^{n}((S_i + S_{i-1})/2)\Delta t][100/(S_{max}T)] \quad (1)$$

where Si=mean severity of the experimental unit in the observation i; Δt=the number of days between observations; Smax =maximum value of severity (=4); T= experimental period in days; n= number of observations.

b) Disease Intensity Index (DII) was calculated for each observation as shown in equation (2):

$$DII = \sum_{x=1}^{n}((S_x * N_x)/(4N_t))\,100 \quad (2)$$

where Sx= severity in an individual plant, Nx= number of plants with symptoms of severity Sx and Nt= total number of plants for each experimental unit.

Disease Intensity Index (DII) at the end of the evaluation is called Final Disease Intensity Index (FDII).

c) Final Disease Incidence (FDI) and Final Dead Plants Incidence (FDPI) representing, the percentage of plants with disease symptoms and the percentage of dead plants at the end of the experiment, respectively.

d) Disease-Free Period (DFP), estimated as the number of days without appearance symptoms, was also calculated.

Susceptibility in the reference susceptible Picual cultivar (SP) was estimated summarizing all previous disease parameters according Arias-Calderón et al. (2015). For all genotypes, a Relative Susceptibility Index (RSI) was calculated for each experimental unit relative to the average of SP, according to (3):

$$RSI = \left[\left(0.3SAUDPC + 0.3FDPI + 0.2FDII + 0.05FDI + 0.15(100 - RDFP)\right)/SP\right]100 \quad (3)$$

where SP= average susceptibility in the reference susceptible Picual cultivar, i.e. the value of numerator calculated for this cultivar; RDFP=Relative Disease-Free Period estimated as the number of days without appearance of symptoms and expressed as percentage of the total experimental period in days.

Plant colonization by the fungus was also assessed in the root system and aerial part of plants by isolating the fungus on CWA. Ten pieces of root and ten pieces of stem (without the outer layer of the bark) were thoroughly washed under running tap water for one hour. Five mm long root pieces (two per root next to stem) and ten pieces of the stem were surface disinfested in 0.5% sodium hypochlorite for 1.5 min. (stems) or 2 min. (roots), rinsed with sterile water, plated on CWA, and incubated at 24°C in the dark for 9 (for stem) to 21 days (for root) (Rodríguez-Jurado, 1993). *V. dahliae* was identified by microscopic observations. Results from isolations of the pathogen were used to calculate a Root and Stem Colonization Index (RCI and SCI, respectively) for each experimental unit as a percentage of root and stem pieces from which the fungus was isolated, relative to the number total of root and stem pieces sampled.

5.4.4. Experimental design and data analysis

Forty-eight genotypes maintained ex situ coming from diverse origins and eight genotypes coming from crosses were evaluated in four separate experiments, including Picual (susceptible) and Frantoio (resistant) cultivars as disease reference controls. Experiments were carried out in a growth chamber under the controlled conditions indicated above. Each experiment in the growth chamber was arranged according to a randomized complete block design with 8-20 plants per genotype (four blocks with 2-5 plants per block). Previous ANOVAs from data of disease parameters in reference control cultivars (included in all experiments) were performed with the experiment as a factor and significant differences were not detected between experiments. Therefore data were pooled over experiments for final analysis and presentation. Means were compared between each genotype and those of susceptible Picual and resistant Frantoio reference controls by the Dunnett´s test at P=0.05. The parameters were expressed as percentages and subjected to angular transformation before statistical analysis. Pearson's correlation coefficient was calculated among average values for

RSI, RCI and SCI in genotypes from wild olive trees. All analyses were carried out using Statistix 9.0 software (Analytical Software, Tallahassee, Florida, USA).

5.5. Results

The observation of symptom progression in inoculated plants revealed wide variability of DII among the genotypes evaluated (Figure 5.2). All genotypes were symptomatic after inoculation with *V. dahliae*. The most common symptoms included defoliation of green leaves, inwards rolled leaves that remained on the branches or fell as well as chlorotic and necrotic leaves (Figure 5.3). The first external disease symptoms appeared simultaneously, 14 days after inoculation, in the susceptible (Picual) and resistant (Frantoio) controls. For the Picual cultivar, such symptoms rapidly increased through seven weeks after inoculation and less rapidly in the second half of the experimental period. In Frantoio the symptoms increased during the first five weeks after inoculation, and then remained steady until the end of the experimental period. Thirteen of the genotypes evaluated showed disease symptoms before both the control cultivars and twenty genotypes remained without symptoms for one more week than Picual and Frantoio. The DII remained lower in those genotypes with later starting of symptom development.

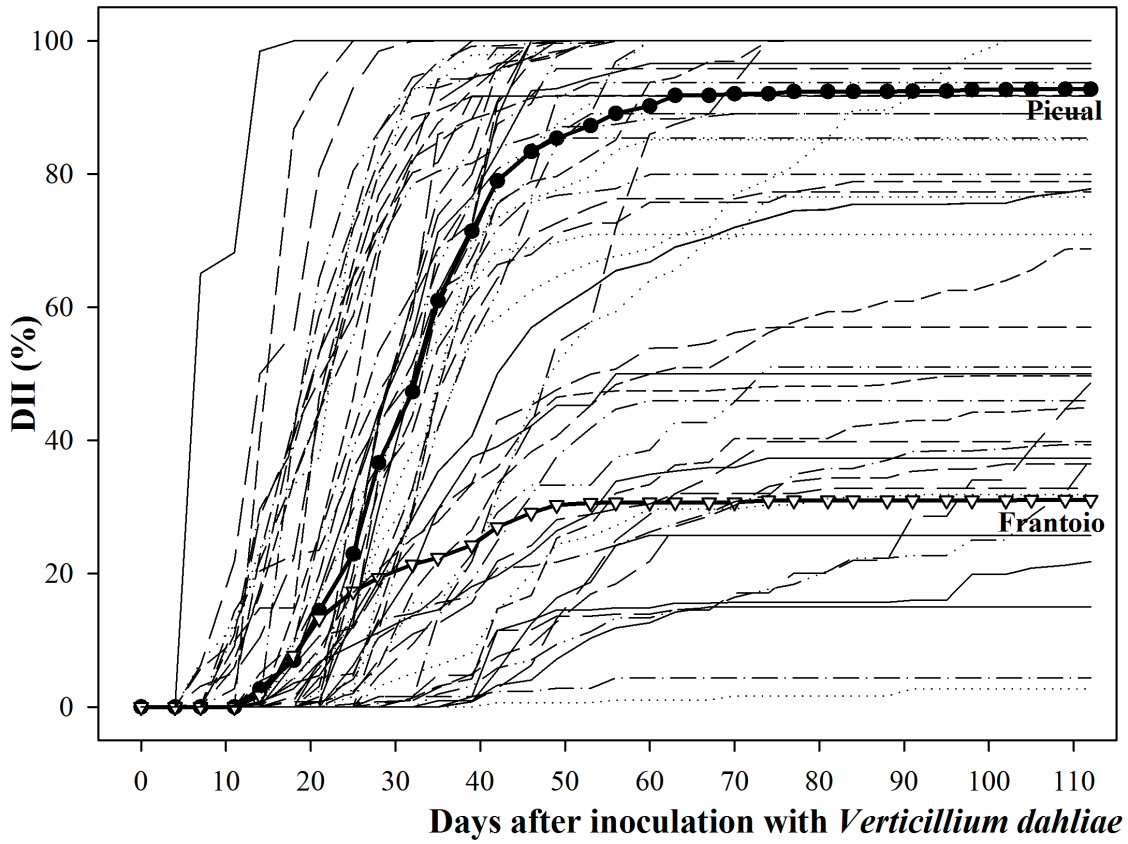

Figure 5.2. Progress of the Disease Intensity Index (DII) evaluated in 48 wild olive genotypes from different sampling sites and eight additional genotypes from crosses between the Picual cultivar and two wild genotypes. Reference control cultivars Picual (susceptible) and Frantoio (resistant) are indicated in bold.

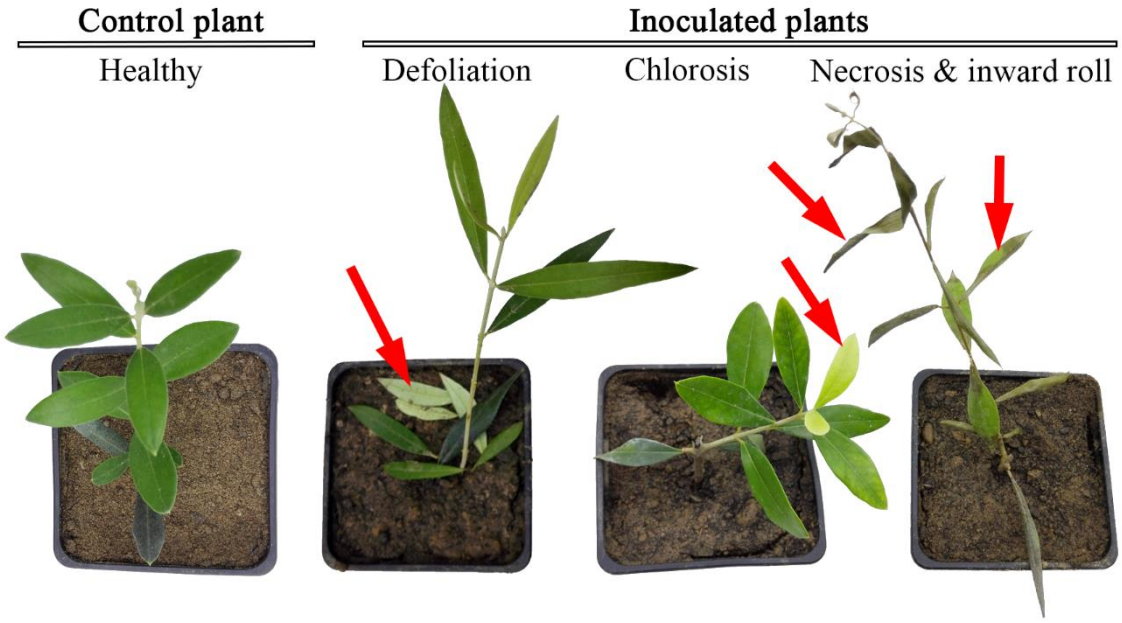

Figure 5.3. Verticillium wilt symptoms. A mock-inoculated control plant (left) with healthly leaves. On inoculated plants (right), arrows show four representative disease symptoms: green defoliation, chlorosis, necrosis and inwards rolled leaves, respectively.

Significant differences ($P<0.01$) in the disease levels were observed among the 48 wild olive genotypes derived from different locations and progenies from crosses between the Picual cultivar and wild genotypes (Table 5.2). A very high percentage of the evaluated genotypes (43 genotypes, 76.8%) was similarly grouped according to significant differences from controls by SAUDPC and FDPI values. Among the evaluated genotypes, all disease parameters were related according to Pearson´s correlation ($P < 0.001$) (Table 5.3). The RSI, which integrates the different disease parameters, was used for final classification of the genotypes according to their levels of resistance to Verticillium wilt. Significant differences among the genotypes were also observed for RSI (Table 5.2), which allows their classification in three different resistance categories: 32 (57.1%) of them were classified as Susceptible (S, significantly different from Frantoio), 11 (19.6%) as Moderately Susceptible (MS, not-significantly different from both Picual and Frantoio) and 13 (23.2%) as Resistant (R, significantly different from Picual). Among the wild olives classified as R, eight of them (W19, W12, W6, W35, W5, W15, W13, W16; sorted by descending order for RSI) were wild olives from the Andalusian villages of Marchena and Osuna (Seville), Alcalá de los Gazules, Medina Sidonia and Jerez de la Frontera (Cádiz), Navas de San Juan and Martos (Jaén). And two R wild olive genotypes (G2 and G1; higher to lower RSI) belonged to *guanchica* and were collected in Tenerife island, Valle Brosque and Guimar, respectively (Figure 5.1, Table 5.2). The genotypes W13 and W16 showed RSI values 77% and 86% lower than the resistant control Frantoio and no incidence of dead plants during the evaluation period.

Table 5.2. Disease and colonization parameters in genotypes from wild olives and progenies from crosses between Picual x wild genotypes. Names G or W indicate the genotypes belonging to *guanchica* and to the wilds, respectively. Reference control cultivars Picual (susceptible) and Frantoio (resistant) are shown in bold. Genotypes are sorted in descending order for Relative Susceptibility Index (RSI).

Geno-type	Population[a]	Disease parameter[b]						Resistance category[c]	Colonization[d]	
		SAUDPC	FDPI	FDII	FDI	DFP	RSI		RCI	SCI
G3	26	92,96#[e]	100#	100#	100#	6.47#	119.35#	S	27.29	13.75
W31	3	85.91#	100#	100#	100#	11.16#	115.70#	S	53.75#	26.25
W33	20	86.37#	100#	100#	100#	13.39#	115.44#	S	47.50#	23.75
W26	23	81.78#	100#	100#	100#	13.49#	113.64#	S	55.85	25.47
W25	21	80.77#	100#	100#	100#	12.11#	113.51#	S	25.00	31.46
W28	1	80.93#	100#	100#	100#	13.76#	113.25#	S	23.33	7.50
W32	2	81.27#	100#	100#	100#	15.07#	113.13#	S	26.25	17.50
W27	4	79.86#	100#	100#	100#	12.44#	113.09#	S	36.04	26.25
W30	11	81.45#	100#	100#	100#	15.85#	113.05#	S	41.67	16.04
PW2-3	Picual xW2	76.57#	100#	100#	100#	16.24#	111.07#	S	58.75#	52.29
W39	8	74.84#	100#	100#	100#	16.61#	110.33#	S	9.63	26.00
W44	9	73.36#	100#	100#	100#	22.32#	108.64#	S	5.00*	50.00
W38	5	72.56#	100#	100#	100#	22.19#	108.35#	S	8.33	37.50
W18	20	72.03#	100#	100#	100#	22.21#	108.14#	S	28.75	56.25
W43	24	71.75#	100#	100#	100#	22.21#	108.03#	S	2.50*	6.25
W41	20	70.59#	100#	100#	100#	23.1#	107.40#	S	6.67	42.50
W20	21	69.06#	100#	100#	100#	22.77#	106.88#	S	25.00	37.50
PW2-5	Picual xW2	75.78#	91.67#	95.83#	100#	15.63#	106.56#	S	19.17	36.67
W9	2	67.65#	100#	100#	100#	24.11#	106.07#	S	16.25	41.25
W10	21	65.27#	100#	100#	100#	24.55#	105.05#	S	47.50#	61.25
W34	25	70.39#	95.00#	96.56#	95.00#	21.38#	104.82#	S	28.50	18.00
W29	21	74.41#	91.67#	91.67#	91.67#	21.58#	103.78#	S	17.92	8.33
W24	8	73.90#	87.50#	91.80#	100.00#	14.3#	103.41#	S	32.88	33.75
PW2-4	PicualxW2	69.23#	93.75#	93.75#	93.75#	21.24#	103.18#	S	20.63	35.83
W1	16	57.95#	100#	100#	100#	25.6#	102.00#	S	56.67#	73.33#
W2	20	71.31#	87.50#	89.06#	100.00#	12.78#	101.99#	S	35.00	23.13
Picual	Control S	65.94#	89.16#	92.73#	95.09#	20.46#	100.00#	S	35.08	45.15
W21	13	56.52#	100#	100#	100#	34.37	99.73#	S	32.50	42.50
W45	24	63.33#	87.50#	89.06#	87.50	23.44#	96.80#	S	8.75	6.25
W37	14	64.14#	85.42#	85.42#	85.42	31.47	93.79#	S	19.58	17.08
W42	7	58.52#	79.17#	85.16#	79.17	27.49	89.88#	S	6.25*	21.67
W3	19	54.42#	79.17#	79.95#	79.17	33.3	85.80#	S	20.83	11.25
W14	22	55.02#	75.00	78.91#	100.#	27.01#	85.37#	S	42.50	46.25
W40	6	53.98#	68.75	77.34#	68.75	26.26#	82.26	MS	19.38	26.67
W23	17	45.36	75.00	76.56#	100.00#	32.37	79.96	MS	45.00	62.50
PW2-2	Picual x W2	53.06#	65.00#	70.94#	93.75#	21.95#	79.63	MS	27.88	19.5
PW2-1	Picual x W2	40.16	70.83#	74.58#	100#	38.36	74.64	MS	46.08	33.13
W4	11	35.46	63.75	66.91	85.00	53.65	65.07	MS	25.75	27.63
W17	6	37.28	50.00	68.75	75.00	51.67	61.28	MS	22.50	30.00
W7	20	38.92	50.00	57.03	75.00	40.74	61.01	MS	17.50	32.50
W8	23	34.02	50.00	52.60	66.67	51.6	55.83	MS	26.25	41.25
W22	18	34.36*	50.00	50.00	50.00	62.72*	53.11	MS	35.00	50.00
W11	22	30.89*	50.00	51.04	66.67	57.29	53.10	MS	35.00	36.67
W36	12	34.62	40.00	49.69	40.00	44.33	52.81	MS	15.50	51.00
PW3-3	PicualxW3	28.89*	34.58*	45.99*	50.83*	49.62	46.49*	R	16.13	31
G2	26	14.63*	38.33	48.59	86.67	54.9	42.07*	R	12.75	31.58

Geno-type	Population[a]	Disease parameter[b]						Resistance category[c]	Colonization[d]	
		SAUDPC	FDPI	FDII	FDI	DFP	RSI		RCI	SCI
W19	17	20.47*	37.50	39.84*	75.00	60.71*	40.61*	R	15.00	28.75
W12	20	21.33*	25.00*	32.81*	87.50	40.85	38.13*	R	17.50	38.75
W6	15	22.44*	25.00*	39.38*	65.00	62.1*	36.11*	R	23.00	43.00
Frantoio	Control R	23.52*	27.06*	31.08*	45.89	58.86*	35.80*	R	18.16	25.72
PW3-1	PicualxW3	18.27*	29.17*	36.46*	75.00	60.57*	35.66*	R	25.42	40
W35	16	19.15*	25.00*	31.88*	30.00	69.91*	31.34*	R	3.00*	1.50*
W5	22	12.03*	18.75*	37.19*	93.75#	63.56*	28.79*	R	33.75	38.75
W15	17	14.87*	25.00*	25.78*	37.50	77.79*	26.56*	R	17.50	18.75
G1	27	10.44*	14.58*	21.80*	62.50	77.44*	19.83*	R	12.67	21.67
PW3-2	PicualxW3	8.34*	0.00*	15.00*	62.50	60.60*	14.85*	R	26.25	20
W13	6	2.76*	0.00*	4.38*	50.00	69.53*	8.18*	R	8.75	26.25
W16	10	1.06*	0.00*	2.71*	50.00	80.21*	5.00*	R	10.00	11.67

[a]See Table 5.1 for geographic location and climatic classification of the sampling sites where wild genotypes were collected.

[b]Disease parameters: SAUDPC: Standardized Area Under the Disease Progress Curve; FDPI: Final Dead Plants Incidence; FDII: Final Disease Intensity Index (DII); FDI: Final Disease Incidence; DFP: Disease-Free Period; RSI: Relative Susceptibility Index.

[c]Genotypes were classified in three resistance categories according to RSI values: resistant (R), significantly different from Picual; susceptible (S), significantly different from Frantoio; and moderately susceptible (MS), non-significantly different from Picual and Frantoio, according to Dunnett´s test at P=0.05.

[d]Colonization parameters: Root and Stem Colonization Index (RCI and SCI, respectively).

[e]Symbols beside values indicate: *= significantly different from Picual and non significantly different from Frantoio; #=significantly different from Frantoio and non significantly different from Picual; without symbol=non significantly different from Picual and Frantoio, according to Dunnett´s test (P=0.05).

Table 5.3. Correlation coefficients among Verticillium wilt disease parameters. All values were significant at $P < 0.001$.

	DFP[a]	FDI	FDII	FDPI
FDI	-0.82			
FDII	-0.93	0.82		
FDPI	-0.92	0.81	0.99	
SAUDPC	-0.96	0.77	0.96	0.96

[a]Disease parameters: DFP: Disease-Free Period; FDI: Final Disease Incidence; FDII: Final Disease Intensity Index; FDPI: Final Dead Plants Incidence; SAUDPC: Standardized Area Under the Disease Progress Curve.

RSI varied between the two progenies which included genotypes from Picual x W2 (PW2-1, PW2-2, PW2-3, PW2-4 and PW2-5) and Picual x W3 (PW3-1, PW3-2 and PW3-3) (Table 5.2), even though both wild parents showed similar values for disease parameters corresponding to the susceptible category. In fact, three genotypes (PW2-3, PW2-4 and PW2-5) from Picual x W2 were classified as S and two of them (PW2-1, PW2-2) as MS whereas all three genotypes from Picual x W3 were classified as R.

Isolation of the fungus from colonized root system and stem tissues revealed significant lower RCI and SCI values for the resistant Frantoio cultivar, 18.2 and 25.7% respectively, than the susceptible Picual, 35.1 and 45.1% respectively (Table 5.2). However, no clear relationship was observed among these colonization parameters and RSI in the evaluated genotypes although RSI values were more related to RCI than SCI (Figure 5.4). A wide variability was found for both RCI and SCI values in all categories (S, significantly different

from Frantoio; MS, not-significantly different from both Picual and Frantoio; R, significantly different from Picual). For instance, a wide range of RCI (3% to 33.7%) and SCI (1.5% to 43%) values were observed in wild olive genotypes classified as R according to RSI values (Table 5.2). Similarly, RCI and SCI values ranging from 15.5 to 45% and from 26.7 to 62.5%, respectively, were obtained in MS wild genotypes; in S genotypes, the same parameters ranged from 2.5% to 56.7% and from 6.2% to 73.3%, respectively. Wide ranges of RCI (19.2 to 58.7%) and SCI (19.5 to 52.3%) values were observed also in genotypes from Picual x W2, while in R genotypes from Picual x W3 RCI values ranged from 16.1 to 26.2% and SCI values ranged from 20.0 to 40.0%. None of the genotypes evaluated was devoid of fungal colonization in the root and stem tissues.

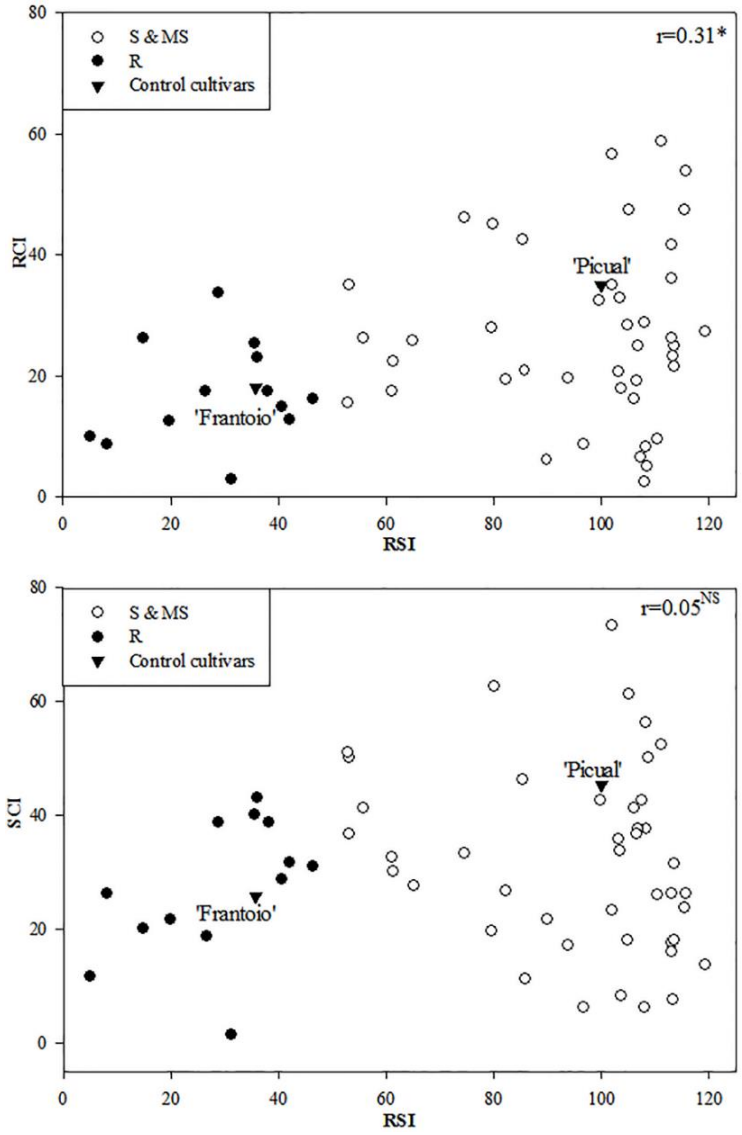

Figure 5.4. Scatter plots of Relative Susceptibility Index (RSI) vs. Root Colonization Index (RCI) and Stem Colonization Index (SCI) in 56 wild olives genotypes (*, significant at P<0.05; NS non-significant; according Pearson´s correlation test). Genotypes classified as resistant (R) are indicated by black dots. Triangles represent values for reference control cultivars Picual (susceptible) and Frantoio (resistant). The rest of genotypes susceptible (S) and moderately susceptible (MS) are represented by circles.

5.6. Discussion

In the framework of an integrated disease management strategy for Verticillium wilt in olive, the use of resistant cultivars represents a useful tool due to its low cost and environmental friendliness. There are currently few olive cultivars that exhibit some resistance to the defoliating (D) pathotype of *V. dahliae* (López-Escudero and Mercado Blanco, 2011). Therefore, wild olives and related subspecies were screened in this work as new possible sources of resistance to Verticillium wilt.

Disease symptoms in the control cultivars Picual (susceptible) and Frantoio (resistant) were comparable to previously reported results under similar experimental conditions (López-Escudero et al. 2004, Martos-Moreno et al. 2006). A high variability in the response to the disease has been observed among the wild genotypes evaluated. The susceptible control olive plants typically developed symptoms more quickly and these symptoms progressed more rapidly than in the resistant control. Resistant responses according to RSI were found in 10 wild genotypes (20.8%) from different geographic locations of Andalusia (Southern Spain) and Canary Islands, representing a wide variability in soil and climatic conditions. Similar to the results presented herein, a high variability in resistance to Verticillium wilt was previously observed in wild olives collected from different areas in Italy and Spain (Colella et al. 2008), and no association among resistance level and area of origin was reported. However, Colella et al. (2008) analysed 57 seedlings, 13-months-old from different wild olive populations and found that only 3 (5.7%) were resistant to D and ND pathotypes, whereas in this work a higher percentage of genotypes resistant (23.2%, 13 out of 56) to the D patothype was obtained by using clonally propagated replicated plants. Some of the genotypes evaluated in our work were placed in nearby areas with high Verticillium wilt incidence, while accessions randomly collected from the Mediterranean basin were assessed by Colella et al. (2008).

In spite of the small number (three) of *guanchica* genotypes evaluated, two of them were characterized as R. To the best of our knowledge, the response to Verticillium wilt in *guanchica* populations has not been reported. These olive populations, growing in isolated geographical regions under rather different edaphoclimatic conditions than the Mediterranean ones, may represent greater genetic diversity and, therefore, potential interesting genetic resources in breeding programs (Lavee and Zohary, 2011). This subspecies can be found throughout the islands as small evergreen trees, usually with shrub like appearance. Genetic isolation between populations of *guanchica* from different islands of the Canary Islands has been observed (García-Verdugo et al. 2010). However, only genotypes from Tenerife Island were evaluated in this work, which underlines the need for future testing of *guanchica* populations from different islands.

The finding of new sources, either in wild olive germplasm and related subspecies, of high levels of resistance to Verticillium wilt is of paramount importance for the olive industry as it provides tools for the management of this severe disease. Direct cultivation of these wild materials is not possible for commercial purposes due to their poor agronomic performance; particularly small fruit size and oil content (Belaj et al. 2011). Therefore, as previously reported for other vegetables and fruit tree species (Epstein et al. 2004, King et al. 2008), the most straightforward application must be attempted as rootstocks for grafting of susceptible cultivars. In fact, several wild and cultivated olive genotypes with high levels of resistance to

Verticillium wilt have been selected for testing rootstocks for susceptible olive cultivars (Porras-Soriano et al. 2003; Colella et al. 2008; Bubici and Cirulli, 2012; Jiménez-Díaz et al. 2012). In olive, it is not clear whether effective control of the disease can be obtained by using potentially resistant rootstocks. Initial work carried out by Hartmann et al. (1971) led to the selection of the cultivar Oblonga as an olive rootstock resistant to Verticillium wilt. This was based on the negative isolation of the fungus from plants, which suggested low probability of transmission of the pathogen from resistant Oblonga to a grafted susceptible scion. Subsequent studies showed frequent positive infections of Oblonga plants by the D and non-defoliating *V. dahliae* pathotypes (Rodríguez-Jurado, 1993; López-Escudero et al. 2004). Indeed, similarly to our results, a complete absence of root and stem colonization by the fungus is not usually found in resistant genotypes (Rodríguez-Jurado, 1993; López-Escudero et al. 2004; Martos-Moreno et al. 2006). However, inoculation experiments under controlled conditions suggest that the control of Verticillium wilt in susceptible olive cultivars can be achieved by using resistant genotypes as rootstocks. Significant reduction in the severity of the symptoms was observed in the highly susceptible cultivar Cornicabra grafted on resistant Frantoio (Porras-Soriano et al. 2003). Bubici and Cirulli (2012) indicated that grafting of the Leccino and Coratina cultivars onto resistant Frantoio rootstock provided an excellent control of Verticillium wilt, although the frequency of *V. dahliae* reisolation from the trunk among inoculated grafted plants of the three cultivars was not significantly different.

The results obtained encourage the use of wild resistant genotypes as parents in breeding programs. In fact, R genotypes were obtained from a cross between cultivated and wild olive genotypes (Picual x W3). However, only susceptible or moderately susceptible genotypes were obtained from the cross Picual x W2, even though both wild male parents showed similar susceptible reaction. The evaluated genotypes were previously selected from wider initial populations on the basis of their early crop (short juvenile period) and relatively higher fruit size and oil content than their correspondent wild parents. On average, the genotypes selected from Picual x W3 progeny showed shorter juvenile period and higher initial vigor at the seedling stage than the ones from Picual x W2 progeny. The presence of transgressive resistant segregants from crosses between susceptible parents indicates that certain cross combinations, with high specific combining ability, allow the expression of enhanced resistance levels. The use of wild genotypes as parents may represent additional advantages in olive breeding programs, such as transmission of shorter juvenile period to their progenies (Klepo et al. 2014). On the negative side, several additional backcross generations will be probably required to select new resistant genotypes with adequate agronomic performance.

Results from Verticillium wilt bioassays, either on wild olive genotypes, related subspecies or progenies from crosses between cultivated and wild genotypes, must be always corroborated under field conditions. Some discrepancies between results obtained under controlled and field conditions have been reported in olive by Trapero et al. (2013). These differences could be attributable to the effect of different environmental conditions, inoculum density, and crop management practices. Therefore, long-term field observations including different rootstock/scion combinations must be carried out before rootstock recommendation (Bubici and Cirulli, 2012).

In summary, this research identified wild olive genotypes and progenies from crosses between the Picual cultivar and wild genotypes, with high level of resistance to the D

pathotype of *V. dahliae*. Moreover, this is the first characterization of resistant *guanchica* genotypes opening possibilities in the search for new sources of resistance to Verticillium wilt disease. The genotypes characterized as resistant in this research will be propagated for future trials to confirm their disease reaction under field conditions and to test their possible use as rootstocks for grafting susceptible cultivars and as parents in breeding programs.

5.7. Acknowledgements

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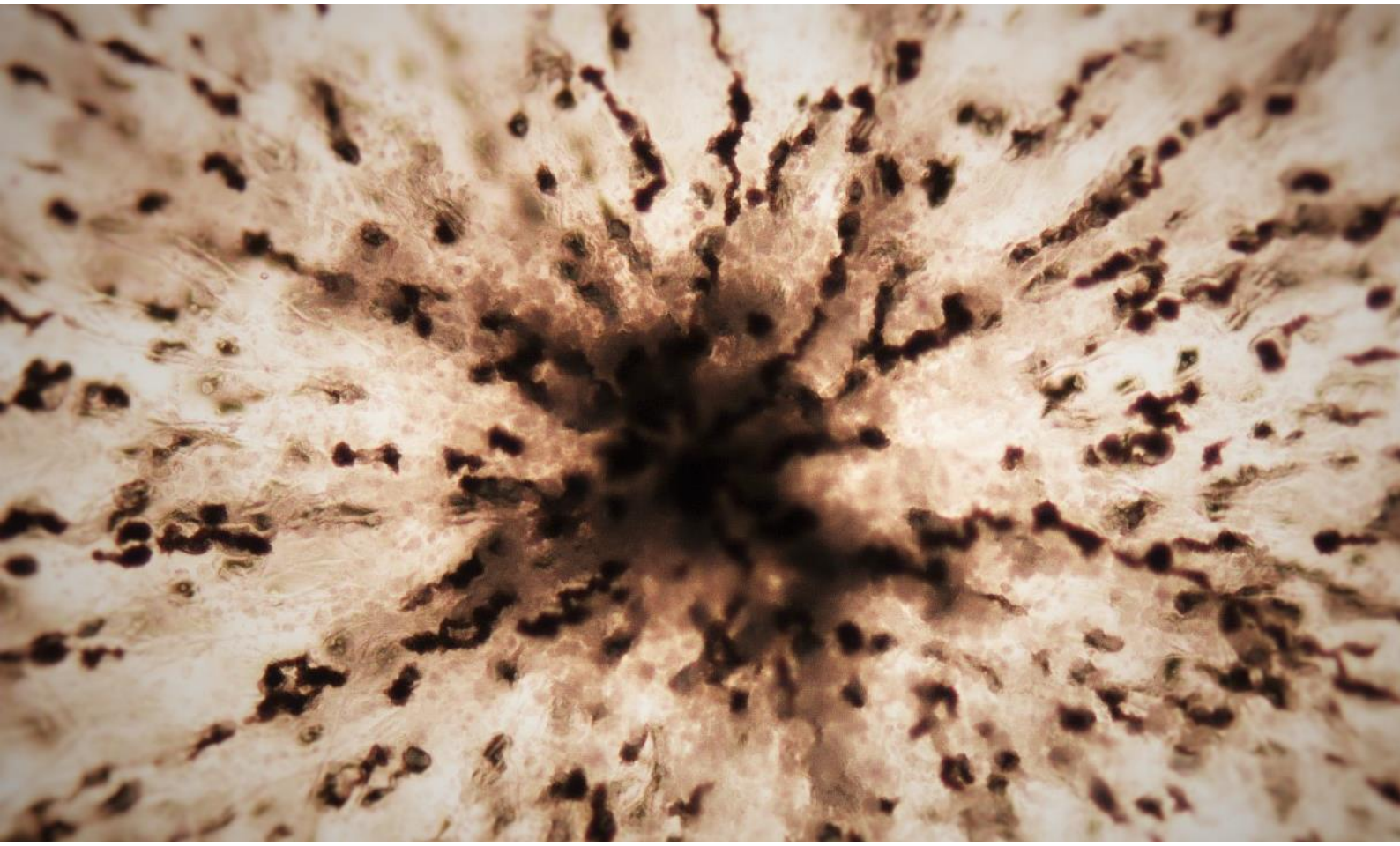
Capítulo 6: Discusión general



La investigación que se desarrolla en la presente Tesis Doctoral se enmarca dentro del Programa de mejora de olivo del centro IFAPA "Alameda del Obispo" en su línea de trabajo para la selección de material vegetal resistente a la Verticilosis.

En los últimos años, la extensión de la Verticilosis del olivo (VO) en zonas productoras de olivar ha estado favorecida por la distribución varietal constituida principalmente por variedades altamente susceptibles a la enfermedad. Así, según el reciente Plan Director del Olivar en Andalucía, el 59.6% de la superficie es ocupada por 'Picual', 17.8% por Hojiblanca y 4.9% por 'Manzanilla de Sevilla', siendo las tres variedades descritas como susceptibles a la Verticilosis por López-Escudero y colaboradores (2004). Esta situación es especialmente grave por la extensa distribución y prevalencia de la variante patogénica defoliante (patotipo D) del hongo que es la más virulenta (Navas-Cortés et al. 2008; López-Escudero et al. 2010; Jiménez-Díaz et al. 2011; Moraño-Moreno et al. 2011; Trapero et al. 2011).

Distintas evaluaciones de la reacción a la infección por *V. dahliae* han mostrado que todas las variedades de olivo testadas son más susceptibles al patotipo D que ND, pero no al contrario (López-Escudero et al. 2004; 2007; Trapero et al. 2013). La primera evaluación de un alto número de variedades en condiciones controladas mostró una elevada incidencia de la enfermedad en la variedad 'Picual', con el 95% de plantas muertas por el patotipo D frente al 22% de plantas enfermas y 17.5% de plantas muertas por el patotipo ND (López-Escudero et al. 2004). Además, la recuperación de los síntomas (crecimiento de la planta después de un periodo sintomático) es en general más frecuente en plantones inoculados con el patotipo ND que con el D altamente virulento en las variedades estudiadas aunque depende también del cultivar (López-Escudero et al. 2004; 2010).

Como se ha indicado, la línea de búsqueda de resistencia a la VO se ha desarrollado en el marco del Programa de mejora de olivo. El objetivo general de este programa es la obtención de nuevas variedades para producción de aceite de oliva, productivas, con alto rendimiento graso, calidad del aceite y adaptadas a diferentes sistemas de cultivo (León et al. 2015). Dichos caracteres se tienen en cuenta asimismo en la selección para resistencia a la enfermedad estudiada, de manera que las posibles nuevas variedades aúnen alto nivel de resistencia y caracteres agronómicos de interés.

Los caracteres de fruto relativos al tamaño, rendimiento graso y relación pulpa/hueso son claves en olivo puesto que determinan la productividad final en rendimiento de aceite. Por ello, un estudio de partida para la selección del material vegetal se ha desarrollado en el Capítulo 2. En este trabajo se evaluaron 26 caracteres agronómicos en progenies generadas por polinización libre de 17 variedades españolas. Se ha observado una amplia variabilidad en las progenies de polinización libre para todos los caracteres agronómicos evaluados, como se había descrito previamente en otros trabajos (León et al. 2004; Avidan et al. 2012). En todos los caracteres se observó una mayor varianza dentro de las progenies que entre las diferentes familias. El análisis del parecido entre parientes parece indicar una alta heredabilidad para caracteres relacionados con el tamaño y forma del fruto y valores más bajos para otros caracteres como el contenido de aceite, lo cual puede dificultar la selección de posibles nuevas variedades con alto contenido de aceite. En estos casos, puede ser más interesante la búsqueda de combinaciones específicas de cruzamientos que favorezcan un incremento mayor del contenido graso. De hecho, se han obtenido algunas selecciones de nuevos genotipos con mejoras en cuanto a este carácter (Lavee et al, 1996; De la Rosa et al, 2008). En cualquier caso, la amplia variabilidad observada en caracteres agronómicos en

progenies producidas por variedades en polinización libre hace de esta estrategia un método de discriminación en el programa de mejora, permitiendo seleccionar para algunos caracteres en función de sus valores de heredabilidad y, al mismo tiempo, retener amplia variabilidad para futuras generaciones.

La combinación de caracteres de resistencia a la enfermedad y presencia de características agronómicas de interés en algunas especies y para otros estreses abióticos pueden aparecer tan ligados que hacen al mejorador priorizar la selección sobre uno de los dos caracteres. Esta selección combinada puede resultar incompatible cuando se alcanza una etapa avanzada de selección, mostrando a veces correlación entre ellas como el caso de maíz para la tolerancia al frío o resistencia al vuelco (Poehlman, 1987). Sin embargo, Bae et al. (2009) demostraron que en la interacción patata/*V. dahliae* era posible obtener selecciones interesantes mediante una estrategia de caracteres combinados para los citados objetivos perseguidos. Desde esta perspectiva se ha afrontado la investigación llevada a cabo en la presente Tesis Doctoral: evaluar las características agronómicas en progenies, seleccionar las de mayor interés agronómico y posteriormente evaluar la resistencia a la Verticilosis en aquellos genotipos considerados.

Se ha utilizado la metodología de inoculación en condiciones controladas, puesta a punto en plantones de olivo por Rodríguez-Jurado (1993) y adaptada a plantas de menor edad (Rodríguez-Jurado et al. 2007). La propagación por estaquillado semileñoso de los genotipos seleccionados por sus características agronómicas originó repeticiones de plantas que se inocularon por inmersión de raíces ligeramente heridas en una suspensión de conidias de un aislado D durante 15 min. Se evaluó la resistencia al patotipo más virulento que infecta olivo, coincidiendo con evaluaciones de resistencia que se han venido haciendo en otros países de la cuenca Mediterránea como Egipto, Grecia, Irán, Italia, Jordania, Túnez , o Turquía, a pesar de que en algunos de ellos el patotipo D no se haya encontrado asociado a olivo (Karajeh and Masoud, 2006; Cirulli et al. 2008; Markakis et al. 2009; Dervis et al. 2010; Erten and Yiliz, 2010; Sanei and Razavi, 2011; Triki et al. 2011; El Said et al. 2012). En los experimentos realizados en esta Tesis se incluyeron las variedades 'Picual' (susceptible) y 'Frantoio' (resistente) por ser conocida su reacción a la enfermedad en cuanto al diferencial desarrollo de síntomas e infección vascular. Los valores obtenidos para estas variedades permitieron comparar experimentos entre sí, estimar el RSI en base a la reacción de la variedad más susceptible ('Picual') y buscar, entre otros, genotipos iguales o más resistentes que 'Frantoio' según el RSI y/o los índices de colonización vascular.

Para cada genotipo evaluado, el número de plantas inoculadas utilizadas fue entre 8 y 20 y para las plantas control no inoculadas entre 6 y 10. Esta variación ha dependido de la disponibilidad de plantas de cada genotipo, generadas según la capacidad de enraizamiento propia de cada genotipo (Santos-Macedo et al. 2012). El enraizamiento en olivo varía entre variedades, destacando entre las evaluadas la variedad 'Arbequina' como ejemplo de elevado porcentaje de enraizamiento, proceso en el que parecen estar involucrados diferentes genes, como el altamente inducido AOX2 (Denaxa et al. 2014). Así, el escaso potencial para enraizar de variedades resistentes a la enfermedad como 'Empeltre', ha limitado la evaluación de su descendencia en nuestros experimentos.

En algunos genotipos tuvieron lugar pérdidas no significativas de plantas inoculadas y no inoculadas entre 7-17 días después de la inoculación. Esta pérdida se atribuye al estrés sufrido por las plantas a causa del trasplante más que al efecto directo del hongo porque el número

de plantas perdidas por genotipo fue similar entre inoculadas y no inoculadas y el hongo no siempre fue aislado de las plantas inoculadas en el tiempo antes indicado. Las plantas no inoculadas fueron de gran utilidad ya que los síntomas por trasplante no se distinguieron siempre de los causados por el patógeno, particularmente en los procedentes de olivos silvestres. De la misma manera, la ausencia de infección en plantas inoculadas y no inoculadas con similar sintomatología poco tiempo después de la inoculación, llevó a descartar que los síntomas fuesen causados por el hongo.

Las dos variedades de reacción conocida a la enfermedad 'Picual' y 'Frantoio', susceptible y resistente respectivamente, han reproducido con consistencia los niveles de enfermedad, lo que ha permitido aunar para el análisis estadístico los datos de los experimentos realizados con igual diseño experimental y material de la misma naturaleza. La enfermedad en la variedad de referencia susceptible 'Picual' comenzó a los 14 días, alcanzando valores máximos de DII (Disease Intensity Index) entre los 70 y los 84 días después de la inoculación con *V. dahliae* en los experimentos (Figuras 3.1, 4.1 y 5.2). La variedad 'Frantoio', referente resistente a la enfermedad, mostró una reacción más homogénea, comenzando a desarrollar síntomas entre los 14 y 50 días después de la inoculación según el experimento. Por tanto, como se ha demostrado en otros trabajos, la metodología de inoculación y las dos variedades de referencia han sido útiles para evaluar un elevado número de genotipos al mismo tiempo por proporcionar síntomas consistentes y repetibles en los experimentos (Rodríguez-Jurado, 1993, López-Escudero et al. 2004; Raya-Ortega, 2005; Martos-Moreno et al. 2006; Rodríguez-Jurado et al. 2007; Trapero et al. 2013; García-Ruiz et al. 2014).

Los análisis estadísticos indicaron variabilidad para los parámetros de enfermedad (DFP, FDI, FDII, FDPI, SAUDPC) debida al genotipo en material vegetal de distinta procedencia (polinización libre, cruzamientos dirigidos y material de olivo silvestre). FDI (Final Disease Incidence; Incidencia de enfermedad final) y DFP (Disease-Free Period; periodo libre de enfermedad) diferenciaron menos a los genotipos que el SAUDPC, la FDPI y el FDII (Standardized Area Under the Disease Progress Curve, Final Dead Plants Incidence y Final Disease Intensity Index, respectivamente; en castellano Área bajo la curva del progreso de enfermedad estandarizada, Incidencia final de plantas miertas e Índice de intensidad de enfermedad final). Entre los tres últimos parámetros, el SAUDPC y la FDPI fueron los que más coincidieron en agrupar a los genotipos según el test de Dunnett. Ambos parámetros han venido siendo utilizados para caracterizar la resistencia a la Verticilosis de las variedades de olivo desde que López-Escudero et al. (2004) los pusiera en valor por primera vez. Distintas evaluaciones han indicado valores del SAUDPC de 43.8-66.6% y 7.3-40.3% para 'Picual' y 'Frantoio' respectivamente, y una FDPI de 33.3-95.0% y 0-12.0% para las mismas variedades en el orden indicado (López-Escudero et al. 2004; Martos-Moreno et al. 2006; García-Ruiz et al. 2014). En los Capítulos 3-5 de esta Tesis el SAUDPC y la FDPI variaron de 62.1-65.9% y 90.3-92.7% respectivamente, para 'Picual' y, 14.8-23.5% y 23.0-31.1% según el orden anterior para 'Frantoio'. Los análisis estadísticos preliminares de los valores de los parámetros de enfermedad obtenidos durante la Tesis ponen de manifiesto que parámetros como el SAUDPC y el FDII no clasifican siempre de igual manera a los genotipos y que el DFP o FDI en casos concretos pueden variar la clasificación. Por ejemplo, el genotipo 35 procedente de polinización libre (parental femenino 'Manzanilla de Sevilla') se mostró susceptible según el SAUDPC pero resistente de acuerdo a los restantes parámetros, en particular el DFP; los genotipos de olivos silvestres W11 y W22 fueron resistentes según el

SAUDPC aunque moderadamente resistentes según los restantes parámetros, incluidos la FDI y el DFP. En consideración a lo anterior se desarrolló un índice de susceptibilidad relativa (RSI) que integra los parámetros de enfermedad, permitiendo una categorización más completa en base a la comparación de los valores de dicho índice en los genotipos evaluados con los valores en las variedades de referencia de enfermedad 'Picual' y 'Frantoio'.

El diferente origen del material vegetal usado en esta Tesis ha permitido hacer un análisis de heredabilidad en base al parecido entre parientes. En los genotipos procedentes de polinización libre de diferentes variedades se ha observado una alta correlación positiva (r=0.79**) entre los valores del RSI obtenidos para los parentales femeninos y los valores medios de sus respectivas progenies. Ello indica, en términos generales, que se obtuvo una mayor proporción de genotipos resistentes procedentes de variedades categorizadas como resistentes, destacando 'Empeltre' y 'Frantoio', tal como se había señalado en trabajos previos (Wilhelm and Taylor, 1965; Trapero et al. 2011). Además de esta tendencia general, se han seleccionado genotipos resistentes procedentes de variedades categorizadas como susceptibles ('Picual', 'Abequina' y 'Manzanilla de Sevilla'). La situación inversa también se ha encontrado, es decir, descendencias de variedades categorizadas como resistentes resultaron susceptibles al desarrollo de síntomas de la enfermedad, como es el caso de 'Koroneiki'. Dada la alta correlación para el RSI entre la progenie y genitor/es, cabría esperar una alta respuesta a la selección en cruzamientos dirigidos entre variedades de interés resistentes a la Verticilosis. No obstante, los problemas de compatibilidad entre variedades suponen una dificultad añadida para el desarrollo de esta línea de trabajo. Trabajos previos han mostrado incompatibilidad entre algunas de las variedades conocidas con mayor nivel de resistencia como 'Frantoio', 'Empeltre' y 'Changlot Real' lo que impide la realización de cruzamientos entre ellas (Belaj et al. 2012).

Por tanto, en el Capítulo 4 de la Tesis se ha abordado la evaluación de genotipos seleccionados en progenies procedentes de cruzamientos entre algunas de las variedades que habían sido caracterizadas en otros trabajos con mayor resistencia a la enfermedad y otras de interés agronómico. Los genotipos fueron previamente seleccionados por sus interesantes características agronómicas y se propagaron para su evaluación a la enfermedad. Así, se incluyeron genotipos procedentes de 'Changlot Real' x 'Dolce Agogia', 'Frantoio' x 'Arbosana' y 'Koroneiki' x 'Empeltre'. Esta estrategia posibilitó la selección de 10 genotipos resistentes a la enfermedad de los 38 evaluados, que representó una alta proporción (26%) de genotipos resistentes. Cabe destacar que la progenie 'Frantoio' x 'Arbosana' fue la que generó en su descendencia el mayor porcentaje de genotipos categorizamos como resistentes aunque el parental masculino de dicho cruzamiento era susceptible a la enfermedad, lo que confirma el interés de la variedad 'Frantoio' como parental (Wilhelm and Taylor, 1965; Trapero et al. 2015). Nuestros resultados son coherentes con los publicados recientemente por Trapero y colaboradores (2015), quienes destacan el interés de 'Frantoio' al transferir alto nivel de resistencia (20.5%) a su descendencia generada por polinización libre. La proporción de genotipos resistentes entre la descendencia de otras variedades resistentes como 'Changlot Real' y 'Empeltre' en polinización libre fue aproximadamente la mitad de la obtenida para 'Frantoio' en el mismo estudio (Trapero et al. 2015). El nivel de resistencia transferido por 'Frantoio' a la progenie fue también alto en los cruzamientos dirigidos con Picual en los que 'Frantoio' fue el progenitor femenino (23.7%) pero más bajo cuando se utilizó como parental masculino en cruzamientos con 'Picual' (11.4%) y 'Arbosana' (12.4%) (Trapero et al. 2015).

El cruzamiento 'Frantoio' x 'Arbosana' mostró mayor descendencia (23.1%) de genotipos resistentes en la evaluación desarrollada en el Capítulo 4. Los resultados obtenidos en los Capítulos 3 y 4 de esta Tesis, apuntan el interés del parental 'Frantoio' en cuanto a la transferencia del nivel de resistencia a su progenie, a pesar de intervenir en el cruzamiento un parental categorizado como susceptible a la enfermedad. Si bien los resultados coinciden con lo sugerido por otros autores, hay que destacar que mientras en otros trabajos se han evaluado plántulas de progenies procedentes de semilla, en este trabajo se han evaluado plantas obtenidas por estaquillado semileñoso.

El interés de 'Frantoio' como variedad no sólo es destacable por ser resistente a la Verticilosis, ha resultado también de interés como parental en líneas de investigación enfocadas a la resistencia a *Spilocea Oleagina* y *Colletotrichum acutatum* (Moral et al. 2015). A través del programa de mejora de olivo, la generación de alto número de nuevas posibles variedades que permitan unir características agronómicas de interés y resistencia a la VO, dotan la estrategia seguida de importancia para la mejora.

Por otro lado, los parientes silvestres de muchas especies se han utilizado en la mejora con el objetivo de transmitir características conservadas en dicha subespecies como por ejemplo de calidad y resistencia a agentes bióticos y abióticos (Hajjar y Hodgkin, 2007). La aplicación más importante ha sido para la mejora de resistencia a plagas y enfermedades y en algunas especies para resistencia a la Verticilosis (Diwan et al. 1999). En olivo, trabajos recientes sobre la evaluación de material silvestre (*Olea europaea* var. *sylvestris*) han puesto en evidencia su posible empleo como nueva fuente de resistencia a la enfermedad señalando su posible uso como patrón o genitor en programas de mejora (Mercado-Blanco et al. 2003; Colella et al. 2008;). Para comprobar la utilidad de estas posibles nuevas fuentes de resistencia, en el Capítulo 5 se han evaluado genotipos de material silvestre incluyendo acebuches *(Olea europaea* subsp. *europaea* var. *sylvestris*) recolectados de distintas zonas de España, genotipos pertenecientes a la subespecie *guanchica* procedentes de las Islas Canarias y dos progenies descendencia entre la variedad 'Picual' y dos genotipos de origen silvestre.

Los resultados de esta línea confirman una amplia variabilidad en el alto número de genotipos evaluados para los diferentes parámetros estudiados y la posibilidad de seleccionar genotipos con alto nivel de resistencia a la enfermedad. Así, de los 48 genotipos evaluados de diferente origen, 10 de ellos (20.8%) se han categorizado como resistentes al desarrollo de síntomas de la enfermedad, que incluyen 8 acebuches y 2 genotipos de la subespecie *guanchica*.

En una primera aproximación para conocer la transmisión de la herencia a la resistencia a la VO desde genotipos de origen silvestre, se han evaluado genotipos procedentes de dos cruzamientos entre 'Picual' y dos de los acebuches evaluados. Los resultados fueron diferentes para ambas progenies a pesar de presentar ambos parentales reacciones de susceptibilidad similares al desarrollo de síntomas de la enfermedad. Los cinco genotipos de uno de los cruzamientos (Picual x W2) se mostraron susceptibles o moderadamente susceptibles mientras que fueron resistentes los tres genotipos evaluados del otro cruzamiento evaluado (Picual x W3). El limitado número de genotipos evaluados y la ausencia de trabajos previos de evaluación de material procedente de cruzamientos de olivo cultivado x silvestre dificultan la interpretación de estos resultados. Además, trabajos previos desarrollados en el programa de mejora, permitieron señalar diferencias no significativas para caracteres morfo-agronómicos entre W2 y W3. No obstante, hay que señalar que la aplicación práctica de

dichos materiales es limitada porque suelen presentar pequeño tamaño de fruto y bajo contenido de aceite, lo que impide su uso como cultivo comercial (Belaj et al. 2011). Así, la aplicación más directa del material seleccionado consistiría en su uso como patrón para injertar variedades susceptibles, una vía de interés sugerida previamente en olivo (Porras-Soriano et al. 2003; Colella et al. 2008; Bubici and Cirulli 2012; Jiménez-Díaz et al. 2012). Además, se podría recomendar su uso como genitor en futuros ciclos de mejora. En ese caso, se aprovecharían también algunas ventajas adicionales observadas en trabajos previos como la transmisión de corto periodo juvenil a las descendencias, que permitiría reducir la duración de los ciclos de selección (Klepo et al. 2014). En cualquier caso, parece demostrarse la utilidad de materiales de origen silvestre como fuentes de resistencia a la enfermedad, las cuales podrían transmitir de diferente forma su resistencia.

Los resultados obtenidos del conjunto de materiales procedentes de progenies evaluadas no permiten establecer conclusiones definitivas acerca del mecanismo de herencia de resistencia a la enfermedad y, por consiguiente, la estrategia de selección más adecuada. Por un lado, la amplia variabilidad observada y la alta correlación progenie/genitor en variedades en polinización libre parecen indicar un mecanismo de herencia cuantitativa bajo control poligénico con alta heredabilidad. Seguida esta estrategia de selección en el programa de mejora clásica de olivo, cabría esperar una alta respuesta a la selección en cruzamientos dirigidos entre variedades de interés caracterizadas por su resistencia a la enfermedad. Sin embargo, la selección de genotipos resistentes a partir de genitores susceptibles indica que ciertos cruzamientos con alta aptitud combinatoria específica pueden permitir obtener descendencias con mayores niveles de resistencia. La generación y evaluación de nuevas descendencias de segunda generación incluyendo algunos de los genotipos seleccionados en este trabajo, en marcha en la actualidad, permitirá a medio plazo ampliar conocimientos acerca de la herencia de la resistencia a la enfermedad.

La colonización de los genotipos por el patógeno se ha considerado en las diferentes evaluaciones recogidas en esta Tesis con el objetivo de identificar genotipos no colonizados sistémicamente y estudiar la correlación de los niveles de enfermedad representados por el RSI y la colonización de la planta. Se han obtenido para cada genotipo dos índices de colonización según la parte evaluada de la planta: raíz y tallo. Ambos tejidos son de importancia para nuestras evaluaciones por la implicación que cada uno puede generar como elemento de resistencia, ya que el patógeno podría quedar restringido a la raíz y la planta no mostrar síntomas o, la planta podría ser colonizada sistémicamente sin mostrar síntomas.

El sistema radicular es el órgano de la planta por el que el hongo accede a la misma. Diferencias entre sistemas radiculares se han descrito entre variedades de mayor y menor resistencia a la enfermedad como 'Oblonga' y 'Picual' (Rodríguez-Jurado, 1993). Molina-Molina (2010) sugirió que las diferentes anatomías estructurales de los sistemas radiculares de determinadas variedades podrían ser parcialmente responsables de la colonización de la planta por el hongo. En el citado trabajo desarrollado con plantas sin inocular para evitar una posible respuesta de inducción por el hongo, se señaló que 'Frantoio' (R) presenta una mayor área conductora que 'Picual' (S), debido a un mayor número de vasos y superficie que podría explicar en parte la resistencia de 'Frantoio' y la recuperación natural a la enfermedad, lo cual ha sido recientemente respaldado por Bubici y Cirulli (2014).

En la interacción sintomática a la infección del olivo por *V. dahliae* parece existir una vertiente en la que prevalece el concepto "resistencia", en particular en los estudios revisados

de evaluaciones a la enfermedad. *A priori*, la variedad perfecta que parece interesar al mejorador es aquella que no presente síntomas de enfermedad ni colonización de la planta por el hongo. Según Niks, et al. (1993) la resistencia es la capacidad de la planta para reducir el crecimiento y desarrollo del patógeno después del contacto entre el huésped y el patógeno o después de iniciado su desarrollo o cuando esté establecido. El interés de genotipos con bajo nivel de enfermedad y de colonización, además de para su uso como posibles futuras variedades y genitores en el programa de mejora, puede ser para un posible uso como patrones sobre los que injertar las variedades tradicionales, pudiendo el agricultor mantener una producción homogénea y un porte similar al de las variedades deseadas. La utilidad de 'Frantoio' como patrón ha sido indicada por distintos investigadores (Porras-Soriano et al. 2003; Bubici and Cirulli, 2012).

Para algunas progenies evaluadas en este documento se ha encontrado correlación positiva y altamente significativa entre el RSI y los índices de colonización de la parte aérea y/o radicular. El mayor valor de coeficiente de correlación de Pearson fue obtenido en la progenie de 'Changlot Real' x 'Doce Agogia' al correlacionar colonización en tallo y RSI (r=0.84; P<0.001). La colonización del tallo por el hongo en esta progenie también correlacionó de forma positiva y significativa con RSI (r=0.48; P<0.05). Un valor intermedio de correlación positiva y significativa fue obtenido entre el RSI y la colonización del tallo para la progenie 'Frantoio' x 'Arbosana' (r=0.61; P<0.05). Para el resto de progenies evaluadas procedentes de olivo cultivado, estas correlaciones con colonización de la planta han resultado no significativas. Genotipos con similares valores de colonización mostraron reacciones diferentes a la enfermedad siendo clasificados como resistentes, moderadamente resistentes o susceptibles según el RSI. La presencia de genotipos resistentes al desarrollo de síntomas de enfermedad con similar colonización vascular que genotipos susceptibles apoya la idea del concepto de tolerancia más que de resistencia para la interacción olivo/*V. dahliae*. Para el caso de los genotipos de origen silvestre y subsp. *guanchica* evaluados, es destacable que a pesar de la elevada resistencia al desarrollo de síntomas mostrada por alguno de ellos, se encuentra una amplia variabilidad para la colonización en raíz y tallo. En las evaluaciones de la presente investigación, muchos de los genotipos evaluados con valores bajos de RSI han sido altamente colonizados por el hongo. Así, nuestros resultados parecen sugerir que la relación entre los síntomas mostrados y la colonización vascular de la planta por el hongo dependen del genotipo evaluado.

El concepto de tolerancia que Clarke (1986) propuso posee tres diferentes connotaciones, todas ellas referidas al nivel comparado con los daños que podrían darse en condiciones similares en otras plantas. Desde el punto de vista del patógeno, es la capacidad de una planta para soportar los efectos de los niveles de infección del hongo. Desde la perspectiva de la enfermedad, es la capacidad de una planta para soportar los efectos del nivel de la enfermedad. Desde la combinación de las anteriores, es la capacidad de una planta para soportar los efectos de los niveles de infección por el patógeno y por la enfermedad. La tolerancia es un concepto relativo que debe usarse tras la cuantificación al menos de la enfermedad y de la colonización por el hongo (Robb, 2007). En esta Tesis no se ha realizado una cuantificación estricta de la biomasa del hongo en la planta pero se han obtenido índices de colonización que han sido utilizados para estudiar la correlación entre síntomas y colonización. Estos datos sugieren que quizás sea conveniente adoptar los términos de resistencia o tolerancia dependiendo del

genotipo, lo que podría estar asociado a la expresión de mecanismos de defensa de la planta distintos para genotipos resistentes y tolerantes.

Mejorar el conocimiento de los mecanismos que se activan ante la infección por el patógeno a través de la Biotecnología es la vía más rápida y probablemente la más eficaz para el manejo de la enfermedad (Fradin and Thomma, 2006). El hallazgo de genes que sean inducidos o expresados solo, antes o en mayor cantidad en reacciones asintomáticas, no infectadas o infectadas vascularmente que en reacciones con diferente nivel de síntomas, podría llevar a seleccionar con rapidez nuevos genotipos en el programa de mejora de olivo para resistencia a la Verticilosis.

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Capítulo 7: Conclusions

- Different strategies have been established in the olive breeding program for simultaneous selection for agronomics traits and resistance to Verticillium wilt (Chapters 2 to 5).

- Open pollination has proved to be useful to broaden the genetic base in olive breeding programs, allowing selection for agronomic characters of interest while maintaining high variability for characters not previously considered at the seedling step, such as resistance to Verticillium wilt (Chapter 2 & 3).

- Inoculation experiments carried out under controlled conditions in growth chamber using root dipping methodology and the development of a Relative Susceptibility Index (RSI), which summarized several disease parameters, have been useful for accurate screening for Verticillium wilt resistance and classification of new genotypes from breeding programs and wild olive relatives. The possibility of plant materials replication for inoculation experiments, instead of one single plant per genotype form initial seedlings populations, allows more accurate evaluation for disease resistance (Chapters 3 to 5).

- A wide variability in Verticillium wilt symptoms development has been observed in all plant materials tested. A high heritability for Verticillium wilt resistance was inferred regarding the degree of resemblance between relatives. Although the presence of transgressive resistant segregants from crosses including susceptible parents indicates also that certain cross combinations, with high specific combining ability, may allow the expression of enhanced resistance levels (Chapters 3 to 5).

- New genotypes with similar level of resistance to Verticillium wilt than 'Frantoio', which is currently considered one of the cultivars with higher level of resistance, can be obtained from open pollination and crossing progenies previously selected for agronomic traits of interest. Eight genotypes from open pollination (15.4% of tested) and ten genotypes from crosses including at least one resistant parent (26.3% of tested) were classified as resistant (Chapters 3 & 4).

- Resistant genotypes were also selected from wild olive genotypes, related subspecies and progenies from crosses between 'Picual' and wild genotypes. The first characterization of resistant *guanchica* genotypes opens new possibilities in the search for sources of resistance to Verticillium wilt disease (Chapter 5).

- Among the evaluated genotypes, different responses were found between the level of resistance to symptoms development and the degree of plant colonization based on isolation of the fungus from both root and stem, from high correlation to absence of association. This suggests that, at least in some materials, a possible tolerant plant defence mechanism could be operating in olive-*V. dahliae* pathosystem (Chapters 3 to 5).

- Future works will be carried out to confirm the level of resistance and agronomic performance of the selected genotypes under natural field conditions. Furthermore, these genotypes represent valuable materials for future studies about the pattern of plant colonisation by the fungus and the resistance/tolerance mechanisms operating on different olive materials (Chapters 3 to 5).

La Verticilosis del olivo (Olea europaea L.) causada por el hongo de suelo Verticillium dahliae Kleb., representa en la actualidad el mayor problema fitosanitario del olivar en muchas zonas de cultivo a nivel global. Para el control de la Verticilosis del olivo se recomienda un manejo integrado de medidas preventivas y/o paliativas. El uso de variedades resistentes a la enfermedad es la medida más esperada por el sector del olivar, la más respetuosa con el medio ambiente y, posiblemente, la mejor puede combinar con otras medidas de control. Por ello, se ha evaluado la resistencia al patotipo Defoliante, el más virulento y extendido en Andalucía (España), de nuevos genotipos en una etapa avanzada dentro del programa de mejora de olivo del centro IFAPA "Alameda del Obispo" de Córdoba. Una selección inicial de Olea europaea subsp. europaea basada en características agronómicas interesantes, permitió seleccionar genotipos procedentes de variedades en polinización libre y de tres cruzamientos dirigidos. Además, se han evaluado genotipos de diferente origen genético: olivos silvestres o acebuches (Olea europaea subsp. europaea var. sylvestris), nuevos genotipos procedentes de subspecies afines de Olea (Olea europaea subsp. guanchica) y genotipos procedentes de cruzamientos entre la variedad 'Picual' y dos acebuches seleccionados. Al menos un 15% de cada material evaluado, ha mostrado un alto nivel de resistencia al desarrollo de síntomas causados por V. dahliae. La colonización de los genotipos por el hongo sugiere que la adopción de los términos resistencia o tolerancia podría variar con el genotipo. El comportamiento agronómico y nivel de resistencia de estos genotipos se confirmará en trabajos futuros en condiciones de campo.

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