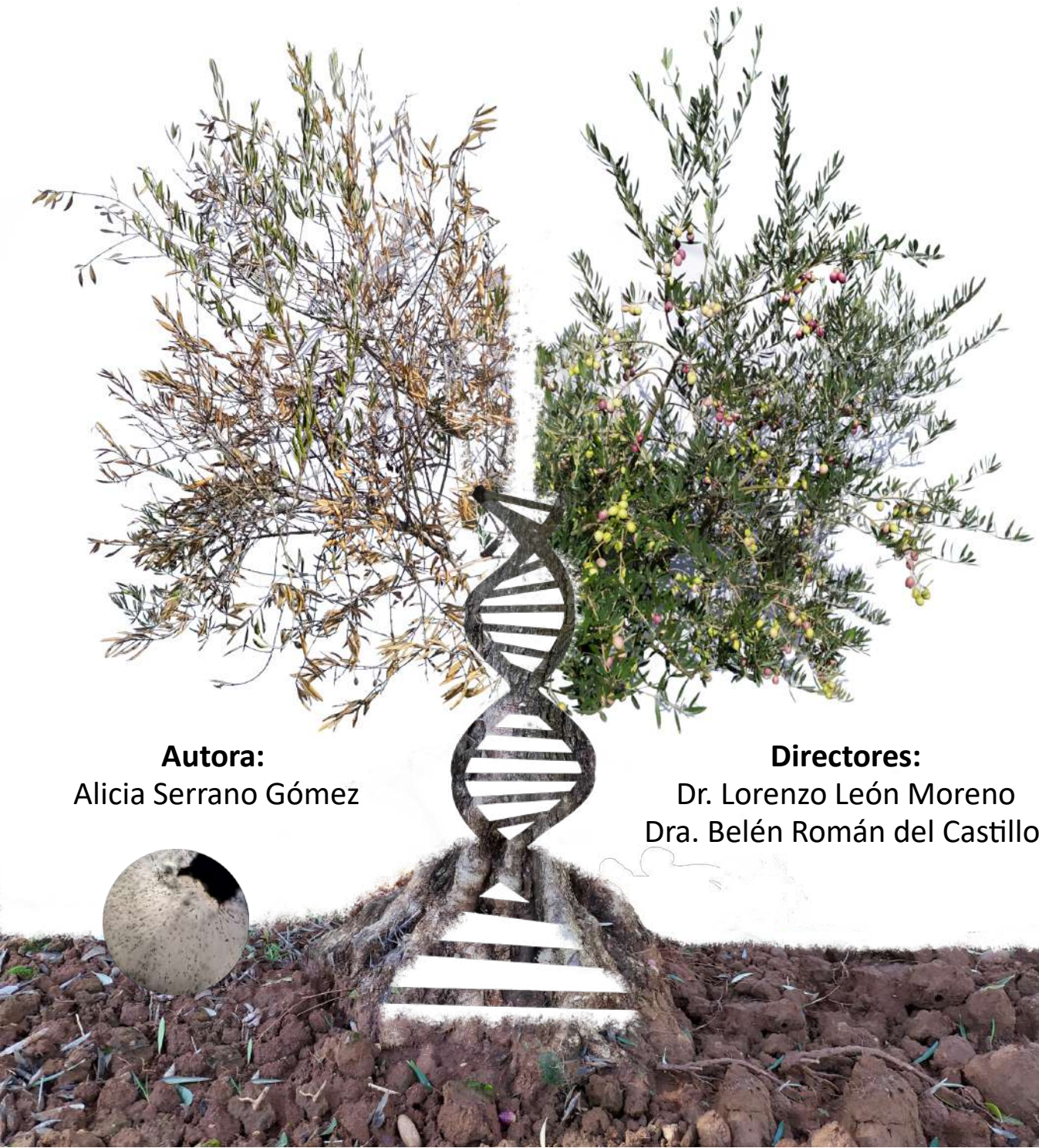
Tesis Doctoral

Caracterización Agronómica y Genómica de Selecciones Obtenidas en un Programa de Mejora de Olivo para Resistencia a la Verticilosis



Autora:
Alicia Serrano Gómez

Directores:
Dr. Lorenzo León Moreno
Dra. Belén Román del Castillo

TITULO: *Agronomic and genomic characterization of selections obtained from an olive breeding program for verticillium wilt resistance*

AUTOR: *Alicia Serrano Gómez*

Campus de Rabanales
Ctra. Nacional IV, Km. 396 A
14071 Córdoba

https://www.uco.es/ucopress/index.php/es/
ucopress@uco.es

UNIVERSIDAD DE CÓRDOBA

Programa de doctorado en Ingeniería Agraria, Alimentaria, Forestal y del Desarrollo Rural Sostenible

Caracterización agronómica y genómica de selecciones obtenidas en un programa de mejora de olivo para resistencia a la Verticilosis

Agronomic and genomic characterization of selections obtained from an olive breeding program for verticillium wilt resistance

Tesis Doctoral presentada por:

Alicia Serrano Gómez

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

Directores:

Dr. Lorenzo León Moreno
Dra. Belén Román Del Castillo

Área de genómica y biotecnología
Instituto de Investigación y Formación Agraria y Pesquera de Andalucía
Centro IFAPA "Alameda del Obispo"

Córdoba, 2021

Mención de Doctorado Internacional

Esta tesis cumple los requisitos establecidos por la Universidad de Córdoba para la obtención del Título de Doctor con Mención Internacional:

- Estancia internacional predoctoral de 3 meses (11 mayo 2018 – 10 agosto 2018) realizada en Istituto per la Protezione Sostenibile delle Piante del Consiglio Nazionale delle Ricerche (Bari, Italia), bajo la supervisión de Dra. Maria Saponari, y Dr. Pasquale Saldarelli.
- Parte de la Tesis Doctoral se ha redactado y se presentará en dos idiomas, castellano e inglés.
- La tesis cuenta con un informe previo de dos doctores con experiencia acreditada pertenecientes a instituciones de educación superior o institutos de investigación distintos de España:
  - Dr. Luciana Baldoni. Senior Researcher. Research area: Plant genomics. Institution: National Research Council of Italy, Institute of Biosciences and Bioresources of Perugia.
  - Dr. Arnon Dag. Senior Researcher. Research area: Fruit tree sciences. Institution: Gilat Research Center, Agricultural Research Organization, Ministry of Agriculture, Israel.
- Un doctor o doctora perteneciente a una institución de educación superior o centro de investigación no español forma parte del tribunal evaluador de la tesis:
  - Dr. Wassim Habib. Research area: Plant protection. Institution: Lebanese Agricultural Research Institute, Laboratory of Mycology, Fanar, Lebanon.

La doctoranda:

Fdo.: Alicia Serrano Gómez

Tesis como Compendio de Publicaciones:

Esta tesis se presenta como compendio de publicaciones, cumpliendo con los requisitos establecidos por la Universidad de Córdoba, ya que incluye cuatro capítulos correspondientes a 4 artículos publicados en revistas incluidas en los tres primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por el Journal Citations Reports (SCI):

- **Serrano, A.**, Rodríguez-Jurado, D., Román, B., Bejarano-Alcázar, J., De la Rosa, R., León, L., 2020. Verticillium wilt evaluation of olive breeding selections under semi-controlled conditions. Plant Disease n/a. Datos de 2020 (JRC): Índice de impacto 3.809, posición 31/234, primer cuartil (Q1) en el área temática de Plant Sciences.
- **Serrano, A.**, De la Rosa, R., Sánchez-Ortiz, A., Cano, J., Pérez, A.G., Sanz, C., Arias-Calderón, R., Velasco, L., León, L., 2021. Chemical components influencing oxidative stability and sensorial properties of extra virgin olive oil and effect of genotype and location on their expression. LWT-Food science and technology 136 (1), 110257-110266. Datos de 2020 (JRC): Índice de impacto 4.006, posición 28/139, primer cuartil (Q1) en el área temática de Food Science and Technology.
- **Serrano, A.**, De la Rosa, R., Sánchez-Ortiz, A., León, L., 2020. Genetic and Environmental Effect on Volatile Composition of Extra Virgin Olive Oil. European Journal of Lipid Science and Technology 122, 2000162. Datos de 2020 (JCR): Índice de impacto 2.056, posición 71/139, tercer cuartil (Q3) en el área temática de Food Science and Technology.
- **Serrano, A.**, León, L., Belaj, A., Román, B., 2020. Nucleotide diversity analysis of candidate genes for Verticillium wilt resistance in olive. Scientia Horticulturae 274: 109653-109665. Datos de 2020 (JRC): Índice de impacto 2.769, posición 5/36, primer cuartil (Q1) en el área temática de Horticulture.

La doctoranda:

Fdo.: Alicia Serrano Gómez

Financiación

La presente Tesis Doctoral ha sido realizada gracias al contrato predoctoral concedido a la doctoranda Alicia Serrano Gómez, dentro del programa del Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA) para la formación de personal investigador (FPI-INIA), convocado por Resolución de 13 de abril de 2015 (BOE-A-2015-4777) y financiado por el INIA y el Ministerio de Economía y Competitividad de España (actualmente Ministerio de Ciencia, Innovación y Universidades).

Asimismo, los trabajos experimentales que han permitido el desarrollo de la investigación se han realizado al amparo de un proyecto INIA: "Mejora de olivo para resistencia a Verticilosis" (PR.INI.RTA201300.3); y dos proyectos del Instituto de Investigación y Formación Agraria y Pesquera de Andalucía (IFAPA) "Investigación e innovación en el sector del olivar" (PP.AVA.AVA201601.2), e "Investigación e innovación en mejora genética de olivo" (PP.AVA.AVA2019.027), todos ellos cofinanciados con fondos FEDER de la Unión Europea.

TÍTULO DE LA TESIS:

Caracterización agronómica y genómica de selecciones obtenidas en un programa de mejora de olivo para resistencia a la Verticilosis.

DOCTORANDO/A:

Alicia Serrano Gómez

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El trabajo titulado "Caracterización agronómica y genómica de selecciones obtenidas en un programa de mejora de olivo para resistencia a la Verticilosis", realizado por la doctoranda Alicia Serrano Gómez, reúne todos los objetivos planteados inicialmente y se considera finalizado.

El trabajo se ha realizado en el Centro IFAPA "Alameda del Obispo" dentro del área de Genómica y Biotecnología. A lo largo de su investigación, se han abarcado distintos aspectos importantes en el desarrollo de nuevas variedades de olivo. Por un lado, se ha evaluado, en condiciones de campo, la resistencia a la Verticilosis de genotipos del programa de mejora, y se ha analizado la composición de los aceites de aquellos genotipos con mayor nivel de resistencia a la enfermedad. Por otro lado, se ha realizado un estudio de genes candidatos para la resistencia, con el fin de identificar polimorfismos en una amplia colección material de diverso origen. Estos trabajos han permitido la selección definitiva de tres genotipos por sus características de resistencia y calidad de sus aceites. Asimismo, se ha avanzado en el conocimiento de aspectos moleculares de los mecanismos de resistencia del olivo.

Todo ello ha quedado reflejado en varias contribuciones científicas de diferentes ámbitos incluyendo cuatro artículos en revistas de prestigio científico indexadas en la base de datos JCR, tres artículos científico-técnicos en revistas de divulgación, 6 comunicaciones a congresos internacionales y 6 a congresos nacionales, además de la participación en otras actividades de extensión y divulgación de los resultados.

La doctoranda ha realizado dos estancias internacionales de 3 meses para formase en el estudio de enfermedades vasculares del olivo. Una primera estancia en el "Istitute per la Protezione Sostenible delle Piante" en Bari (Italia) bajo la cotutela de la Dra. Maria Saponari y el Dr. Pascuale Saldarelli, en el que abordó trabajos de evaluación de resistencia y expresión génica en olivo para *Xylella fastidiosa*. Una segunda estancia en el "Deparment of microbiology and plant pathology of Riverside (University of California), donde ha estudiado mecanismos de virulencia de *Xylella fastidiosa*.

Además, durante este periodo, la doctoranda ha sido codirectora de un proyecto de fin de grado y un proyecto fin de máster, lo que le ha permitido transmitir sus conocimientos a otros estudiantes en formación.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 25 de enero de 2021

Firma de los directores

Fdo.: Lorenzo León Moreno

Fdo.: Belén Román del Castillo

TÍTULO DE LA TESIS:

Caracterización agronómica y genómica de selecciones obtenidas en un programa de mejora de olivo para resistencia a la Verticilosis.

DOCTORANDO/A:

Alicia Serrano Gómez

INFORME RAZONADO DEL TUTOR

El trabajo desarrollado en esta Tesis Doctoral es de suma actualidad y se une a las investigaciones realizadas por otros grupos para solventar el control de la enfermedad más grave que afecta hasta el momento al cultivo estratégico del olivar. Es un trabajo bien organizado y redactado con los objetivos bien justificados. La metodología empleada es correcta y ha permitido obtener resultados significativos que, sin duda, en un próximo futuro, podrán satisfacer al sector con nuevas variedades agronómicamente rentables y con un alto grado de resistencia a la Verticilosis del olivo.

Por todo ello, se autoriza la presentación de la tesis doctoral.

Córdoba, 19 de enero de 2021

Firma del tutor

LOPEZ ESCUDERO FRANCISCO JAVIER - 30524833S

Firmado digitalmente por LOPEZ ESCUDERO FRANCISCO JAVIER - 30524833S
Fecha: 2021.01.19 14:28:29 +01'00'

Fdo.: Francisco Javier López Escudero

AGRADECIMIENTOS

Hace unos años, entre muchas dudas y la gran indecisión que me caracteriza, inicié un nuevo camino que me ha sido imposible de recoger en este manuscrito porque hay muchos aspectos que han hecho de esta una experiencia inolvidable. En realidad, lo mejor de caminar en esta dirección han sido las personas que he tenido a mi lado, que han hecho posible alcanzar esta meta y que me han enriquecido tanto en lo profesional como en lo personal.

En primer lugar, agradecer a mis directores Lorenzo León y Belén Román, toda su dedicación y la confianza depositada en mí. Asimismo, gracias por haberme transmitido todo vuestro entusiasmo y pasión por la investigación. A Lorenzo, quien siempre ha tenido las palabras adecuadas en los momentos más oportunos, transmitiendo la energía necesaria para seguir adelante. A Belén, por transmitirme siempre su positividad y su visión molecular de la relación "olivo-verticillium".

Me gustaría agradecer también el apoyo que me ha brindado Lola Rodríguez, quien se ha encargado de enseñarme la parte más fitopatólogica de esta tesis y siempre ha estado dispuesta a ayudarme, incluso en mis ausencias.

Junto a ellos, Raúl y Angjelina, complementan la buena sintonía y organización que hay en este grupo de investigación, y quienes también han contribuido a mi formación investigadora. A Angjelina le agradezco sus consejos y esas visitas que amenizan las mañanas de encierro en el despacho.

A Lola Madrid por su actitud alegre y entusiasmo por las secuencias, no podría haber tenido mejor maestra de laboratorio y amiga. Gracias también a Juan Carlos y Javier Hidalgo, por contar conmigo en las actividades de divulgación y por dejarme participar en las apasionantes catas de aceite.

Gracias a todos los compañeros del grupo de olivicultura que habéis colaborado para que esta tesis haya sido posible. Sin olvidar a los que han pasado de forma temporal y me han acompañado en las visitas de campo, pasando buenos momentos tanto dentro como fuera del IFAPA, especialmente a Maria Kosma, Matteo, Carine y Faten.

Toda esta aventura se la debo a Juan Cano y a Mamen, quienes me animaron a emprender esta etapa investigadora y auguraron que sería un camino largo y duro, pero del que no me iba a arrepentir.

Gracias a Javier y Esteban por vuestra disposición y por enseñarme el aspecto más fisiológico del olivo.

Agradecer a Paco Luque y a Jorge su acogida en el Departamento de Biología Experimental de la Universidad de Jaén y su total disposición y entusiasmo en una estancia corta pero muy intensa, que permitió mi "Hola mundo" en la bioinformática. También, agradecer a Paco su colaboración para un tercer conato de estancia internacional, proyecto que se vio interrumpido por la pandemia y que ojalá en un futuro podamos retomar.

Thanks to Maria Saponari and Pasquale Saldarelli for receiving me in your Institute and teaching me new olive diseases. It has been a pleasure working with you and each of the lab members.

I would also like to thank Dr. Caroline Roper for receiving me in her lab at UCR (California) and teaching me new research approaches. Especially to Claudia, Flavia and Barbara who supported me during my stay in the lab.

Llegar hasta aquí tampoco habría sido posible sin el apoyo incondicional de mis amigos y familia. A Aida, con quien he compartido penas y alegrías desde que inicié la vida universitaria, gracias por esas horas de charlas interminables, por tus consejos y por apoyarme siempre. A Silvia y Antonio, por seguir mis discursos incluso en sus momentos más difíciles. A Víctor por apoyarme en esta etapa y hacerme ver siempre la parte lógica de las cosas. A mis padres por su confianza y apoyo permanente en todos los aspectos de mi vida, y por haber vivido cada uno de mis pasos con la misma inquietud que yo.

LIST OF CONTENT

List of Tables

List of Figures

ABBREVIATIONS

C x E: Cultivar by location interaction

D: Defoliating pathotype of *V. dahliae*

DI: Disease Incidence

E: Environment

EVOO: Extra Virgin Oive Oil

FDI: Final disease incidence

FDII: Final disease intensity index

FDPI: Final death plant incidence

G x E: Genotype by environment interaction

IFAPA: Institute of Agriculture and Fisheries Research and Training of Andalusia

IT: Induction time

LA: Linoleic acid

LnA: Linolenic acid

MAP: Month after planting

MP: Microplots

ND: Non-defoliating pathotype of *V. dahliae*

OAV: Odor activity value

PDA: Potato dextrose agar

PLS: Partial least squares

RAUDPC: Relative area under disease progress curve

RDFP: Relative disease-free period

RSI: Relative susceptibility index

SNP: Single Nucleotide Polimorphism

VWO: Verticillium wilt of olive

RESUMEN

En las últimas décadas la olivicultura ha experimentado grandes cambios dirigidos a incrementar la rentabilidad y a reducir los costes de manejo del cultivo del olivo. Estos cambios han consistido principalmente en la intensificación de los olivares tradicionales y en el aumento de la superficie cultivada en todo el mundo. Además, los avances científicos sobre las propiedades saludables de los compuestos del aceite de oliva han desencadenado un aumento de la demanda mundial y el interés por los aceites de oliva vírgenes extra (AOVEs) de alta calidad. No obstante, han surgido también ciertos inconvenientes, como la aparición o propagación de enfermedades como la Verticilosis (VO) y la falta de variedades adaptadas a plantaciones intensivas o a una recolección mecanizada.

En base a lo anterior, el programa de mejora del olivo desarrollado en el Centro 'Alameda del Obispo' del Instituto de Investigación y Formación Agraria y Pesquera de Andalucía (IFAPA) tiene como uno de sus objetivos la obtención de nuevas variedades resistentes a la VO, con características agronómicas mejoradas y adecuada calidad del aceite. En este sentido, como resultado de las etapas previas del programa de mejora, se ha preseleccionado un conjunto de genotipos potencialmente resistentes y altamente productivos, pero es necesaria una evaluación a largo plazo en ensayos comparativos de campo antes de la selección definitiva.

Por tanto, el primer objetivo de este trabajo consistió en evaluar 20 preselecciones frente al patotipo defoliante de *Verticillium dahliae* en condiciones semicontroladas. Para esta evaluación se realizó un experimento en microparcelas, en el que se plantaron los genotipos seleccionados tanto en suelo libre de inóculo como en suelo infestado artificialmente con microesclerocios de *V. dahliae*. Los resultados mostraron discrepancias en comparación con la respuesta a la enfermedad obtenida en evaluaciones bajo condiciones controladas, ya que 11 genotipos resultaron susceptibles. Además, las plantas que crecieron sobre el suelo infestado mostraron una reducción del crecimiento y un retraso en la entrada en producción, independientemente del desarrollo de síntomas de la enfermedad.

Estos genotipos también fueron evaluados en ensayos comparativos instalados en fincas de agricultores distribuidas por la provincia de Jaén (Andalucía, España). Estas fincas estaban infestadas por *V. dahliae*, por lo que la resistencia a la enfermedad fue reevaluada en condiciones naturales de cultivo. Como resultado de estos ensayos, se seleccionaron tres genotipos por su mayor resistencia a la enfermedad (IFAPA111-2, IFAPA117-117 e IFAPA117-120), y se ha estudiado la composición de sus aceites y contrastado con la variedad de referencia 'Picual'.

Un primer análisis consistió en evaluar la variabilidad de 66 compuestos químicos, la estabilidad oxidativa y los atributos sensoriales en aceites de 4 genotipos diferentes cultivados en 4 localidades (Córdoba, Úbeda, Begíjar y Arjona). El análisis estadístico aplicado ha revelado la posibilidad de predecir de forma precisa la estabilidad del AOVE en función de su composición, influyendo positivamente el contenido de ácido oleico y 3,4-DHPEA-EA. Por otra parte, para los atributos sensoriales no se ha observado una clara

correlación con la composición química del AOVE y, tampoco, un efecto significativo del genotipo o el ambiente de cultivo.

El segundo análisis se centró en determinar de forma más específica el efecto de los factores genético (G) y ambiental (A), así como la interacción de ambos (G x A), en la composición volátil de los AOVEs, un tipo de estudio que no se había realizado previamente en olivo. Los resultados revelaron que el factor genético influye principalmente en el perfil volátil y el factor ambiental afecta al contenido total, mientras que el efecto G x A sólo se ha observado en los compuestos C5/LA y ésteres. Atendiendo a la fracción volátil, las selecciones del programa de mejora se diferenciaron de la variedad 'Picual', por lo que dichas selecciones podrían ser una alternativa para la diversificación de variedades y ampliar la oferta comercial de los AOVEs.

Por último, se llevó a cabo un estudio a nivel genético para analizar polimorfismos entre 77 genotipos con diferente nivel de resistencia a la enfermedad, incluyendo variedades del Banco Mundial de Germoplasma de Olivo de IFAPA, acebuches y subespecies afines (*cerasiformis* y *guanchica*) de la Colección de olivo silvestre y progenies del programa de mejora. Concretamente, se han estudiado polimorfismos en fragmentos de 7 genes asociados a diferentes mecanismos de respuesta a la Verticilosis. En total se han identificado 92 SNPs y 2 InDels. Los SNPs detectados en el gen TLP1 podrían ser de utilidad para distinguir entre subespecies y una delección en el gen PFN2 podría estar relacionada con la respuesta de resistencia. Estas diferencias nucleotídicas podrían contribuir al establecimiento de marcadores para la gestión de colecciones de germoplasma y la selección en programas de mejora genética de olivo.

Como resultado de todos estos trabajos de caracterización agronómica y de resistencia se ha solicitado el registro de tres nuevas variedades que serán adecuadas para su plantación en zonas donde haya riesgo de incidencia de Verticilosis y con unas características diferenciadas de sus aceites. Estas nuevas variedades continúan su evaluación en la actualidad en diferentes ensayos para comprobar también su adaptación a diferentes condiciones de cultivo. Al igual que se profundizará en los trabajos a nivel molecular, para dilucidar las diferencias genéticas que podrían ser responsables de la resistencia o susceptibilidad en olivo.

SUMMARY

In the last decades olive growing has undergone great changes focused on increasing the profitability as well as reducing the olive crop management costs. These changes have mainly consisted of the intensification of the traditional olive orchards and the expansion of the cultivated area all around the world. Additionally, the scientific advances about the healthy properties of olive oil compounds have led to an increase in the worldwide demand and the interest in high quality extra virgin olive oils (EVOOs). Nonetheless, certain drawbacks aroused, such as the emergence or spread of diseases as verticillium wilt (VWO) and the lack of cultivars suitable for intensive plantations or mechanized harvesting.

On the basis of that, one of the main objective of the olive breeding program developed at the Andalusian Institute of Agricultural and Fisheries Research and Training (IFAPA) center 'Alameda del Obispo' is to obtain new bred cultivars combining high resistance to VWO and improved agronomic characteristics and oil quality. In this sense, a set of genotypes potentially resistant and highly productive has been preselected in previous stages of the breeding program. However, long-term experimentation and comparative field trials are necessary before the final selection.

The objective of this work consisted in evaluating 20 preselections against the defoliant pathotype of *Verticillium dahliae* under semi-controlled conditions. For this assessment an experiment was installed in microplots, in which the selected genotypes were planted both on inoculum-free soil and on soil artificially infested with *V. dahliae* micro-sclerotia. The results showed discrepancies comparing with the disease response obtained in previous evaluations under controlled conditions, since 11 genotypes out of the total were susceptible. Moreover, all plants growing in infested soils showed a limitation in growth and delayed bearing, regardless of the disease symptoms development.

These genotypes were also evaluated in comparative field trials installed in olive farms distributed throughout Jaén province (Andalusia, Spain). *V. dahliae* was detected in soils of these farms, so the genotypes were re-evaluated for the disease resistance under natural growing environments. Moreover, as a result to them, the three most promising highly resistant genotypes ('IFAPA111-2', 'IFAPA117-117' and 'IFAPA117-120') and 'Picual' cultivar were selected for oil extraction and composition analysis.

The first approach consisted of evaluating the variability of 66 chemical compounds, oxidative stability and sensory attributes in EVOO from 4 genotypes grown in 4 locations (Córdoba, Úbeda, Begíjar and Arjona). The statistical analysis accurately predicted the stability of EVOO based on the composition with high positive influence of oleic acid and 3,4-DHPEA-EA. However, cultivar and location showed limited effect on the sensory properties of EVOO, which did not show significant correlation with the chemical composition either.

A specific study, was focused on determining the effect of genetic (G) and environmental (E) factors, as well as their interaction (G x E), on EVOO's volatile composition. The genetic factor mainly influenced the volatile profile and the environmental factor affected the total

content of volatile compounds, while significant G x E interaction was observed only for C5/LA compounds and esters. Considering the volatile fraction, these breeding selections were differentiated from 'Picual' cultivar, so they could be an alternative for olive cultivars and the commercial diversification of EVOOs.

Finally, genetic differences were analyzed among 77 genotypes with different VWO resistance response, including cultivars from the World Olive Germplasm Bank of IFAPA, wild olives and related subspecies (*cerasiformis* and *guanchica*) from the Wild Olive Collection and progenies from the breeding program of IFAPA. Specifically, 7 genes associated with different VWO response mechanisms were studied to identify DNA polymorphisms. In total, 92 SNPs and 2 InDels have been identified. SNPs detected in the TLP1 gene could be useful to distinguish between subspecies and the deletion in PFN2 gene could be related to the resistance response. These nucleotide differences could contribute to establish a set of markers for the management of olive germplasm collections and assist breeders in the screening processes.

As a result of all these agronomic works and resistance characterization, three the new cultivars, which will be suitable for planting in *V. dahliae* infested areas and whose EVOOs has different characteristics to 'Picual' cultivar, have been sent for registration. Besides, several new trials have been established to evaluate their suitability to different growing conditions. Additional works at molecular level will be also carried out to elucidate the genetic differences involved in resistant or susceptible response to *V. dahliae* infection in olive trees

1. INTRODUCTION

1.1. Olive growing and olive oil production in the world

Olive (*Olea europaea* L.) is the most economically significant oil-producing crop in temperate areas, mainly in the Mediterranean countries, where olive has also important social and environmental considerations. Currently, olive crop is present over the world, occupying 10.5 million hectares spread in 58 countries on five continents (FAO, 2018). The five countries with largest olive-growing area are Spain (2.6 Mha), Tunisia (1.5 Mha), Italy (1.2 Mha), Morocco (1.1 Mha) and Greece (1.0 Mha). In the last years, this surface is constantly increasing and expanding to different countries which are introducing olive orchards as new crop. In the last three decades, the introduction of irrigation systems have increase tree density optimizing the use of land and facilitating the mechanization of crop management techniques. Traditional orchards with wide plating patterns (< 200 trees/ha) represent the 73.9% of the world olive-growing area, 21.3% are high-density or intensive plantations (200-800 trees/ha) and hedgerow (> 800 trees/ha) olive orchards occupy 4.8% of the total surface (Vilar and Pereira, 2018). In this sense, most of traditional orchards are in countries where olive is originally cultivated. For instance, in Spain 71.2% of the total olive groves are older than 50 years (MAPA, 2017), and 51.9% correspond to traditional orchards, whereas hedgerow plantations are only 2.5% (Vilar and Pereira, 2018).

The world olive fruit production reached 21.1 Mt in 2018 (FAO, 2018), of which only 2.6 Mt were destinated to table olive (EC, 2020). So, most of the olive fruit production is destinated to olive oil extraction. In the last three crop years, the average of world olive oil production was 3.2 Mt (EC, 2020). European Union countries produced 66% of the total olive oil, mainly Spain (1.39 Mt), Italy (0.32 Mt) and Greece (0.27 Mt). In addition, other non-EU countries have remarkable production as Tunisia (0.26 Mt), Turkey (0.23 Mt) or Morocco (0.16 Mt). This production is demanded in 179 countries all around the world (Vilar and Pereira, 2018). Among producing countries, Italy and Spain are also the largest consumers. The main import markets of olive oil are United States, Brazil, Japan, Canada, China, Australia and Russia along with non-producers EU countries (i.e. Germany or UK) (IOC, 2020). The olive oil consumption is increasing around the world thanks to proving the healthy benefits of olive oil in the diet (Jimenez-López et al., 2020; Ros et al., 2014; Sacchi et al., 2014).

1.2. Challenges and perspectives

Olive oil production and consumption is increasing all around the world, which is implying changes in both growing systems and consumer habits.

On the one hand, the expansion of olive groves outside traditional olive-growing regions and the introduction of high-density plantation models (intensive and hedgerow) have increased the competitivity of the productive system (Rodríguez-Cohard et al., 2020). The new production techniques in olive growing improve the agronomic conditions and crop practices, increasing the productivity and achieving a cost-effective management of olive orchards (Fernández-Escobar et al., 2013). However, the mechanization of traditional olive orchards has sometimes difficulties due to the high age of trees, the sloping land, lack of water for irrigation or the small farm dimension and fragmentation. In these cases, olive production is unprofitable and low competitive in comparison to other growing systems

(Duarte et al., 2008; Perujo-Villanueva and Colombo, 2017; Rodríguez-Cohard et al., 2020). Therefore, the conversion to organic farming could be an alternative for the survival of traditional olive farms getting higher selling prices of their productions (Fernández-Escobar et al., 2013). At the same time, modern farms of monovarietal cultivars are reducing the olive diversity because of few cultivars have been shown to be adapted to hedgerow plantations ('Arbequina', 'Arbosana', 'Koroneiki' and 'Sikitita'). These monocultures are also introduced in areas where it is unknown the suitability for olive orchards (Duarte et al., 2008; Fernández-Escobar et al., 2013; Torres et al., 2017). Thus, the study of the adaptability of olive cultivars to different growing areas and the effect of environmental conditions on olive fruit productivity is necessary to ensure the long-term survival of olive crop, as well as the development of new cultivars in breeding programs for olive cultivar diversification in new plantations.

On the other hand, the olive oil consumption pattern is changing due to the rising interest in healthy food and healthier cooking methods, which could be associated to the Mediterranean diet using olive oil (Ros et al., 2014; Rodríguez-Cohard et al., 2020). At the same time, olive oil consumers are also better informed about the differences between product categories on the market. Thus, the consumers trends to increase the extra virgin olive oil demand. Analysis of consumers preferences revealed that they highly appreciated the certified products by geographical origin or organic farming (Pagliuca and Scarpato, 2014). Nonetheless, the cultivar is also an important factor contributing to the consumer preferences, like mono-varietal differentiated olive oils (Jiménez-Guerrero et al., 2012; Pagliuca and Scarpato, 2014). In this sense, the development of new cultivars adapted to the new growing systems is also necessary for the diversification of olive oil on markets. Similarly, more studies on the environmental conditions influence and the effect of the genetic by environmental interaction on the olive oils differentiation are demanded.

1.3. Genetic resources and olive breeding

Olive tree is a perennial crop belonging to the *Olea europaea* specie, which include six wild subspecies mainly differentiated by morphological and geographical aspects. Subsp. *maroccana* and *cerasiformis* are polyploid and endemic from Morocco and Madeira Island, respectively (Besnard et al., 2008). The other subspecies are diploid and considered the primary genetic resource for cultivated olive through natural hybridization (Besnard et al., 2014, 2013). Some subspecies are non-Mediterranean, like subsp. *laperrinei* from Saharan massifs, subsp. *cuspidata* spread from South Africa to south-west China and subsp. *guanchica* endemic in the Canary Islands. However, the Mediterranean wild olive (*O. europaea* subsp. *europaea* var. *sylvestris*) has been revealed as the main ancestor of the cultivated olive (*O. europaea* subsp. *europaea* var. *europaea*) (Belaj et al., 2007; Besnard et al., 2018; Diez et al., 2015). Olive was one the first fruit tree to be domesticated in the Middle East, where was cultivated since 6,000 years ago (Breton et al., 2009; Diez et al., 2015; Besnard et al., 2018). Human migrations spread cultivated olives from East to West of the Mediterranean basin, entailing probably several domestication events by admixtures with local wild olives (Julca et al., 2020; Besnard et al., 2018; Diez et al., 2015; Belaj et al., 2007). The result of this domestication process was the development of several local cultivars linked to specific growing areas. Farmers selected some of them based on their agronomic

characteristics and were clonally propagated to cultivate at large scale (Belaj et al., 2010; Breton et al., 2009; Khadari and El Bakkali, 2018). The cultivated germplasm is estimated to include more than 1,200 olive cultivars. A large number of them are preserved in worldwide olive germplasm banks as well as other regional and national germplasm collections (Belaj et al., 2016; El Bakkali et al., 2019). Additionally, Mediterranean forests include a rich diversity of wild olives as well (Belaj et al., 2010, 2007).

The domestication process marked differences at genome level between cultivated and wild olives affecting mainly the gene expression (Gros-Balthazard et al., 2019). In this sense, the annotation of a wild olive genome revealed differences in some genes involved in fatty acid biosynthesis pathway, which were duplicated in cultivated olive affecting the oleic and linoleic acids accumulation (Unver et al., 2017). The main differences among genomes revealed the activation of transposons in cultivated genotypes which could be involved in process with agronomic interest, stress response or developmental processes (Jiménez-Ruiz et al., 2020; Julca et al., 2020; Mariotti et al., 2020). As results, the genomes of cultivated olives are larger and contains more genes than wild genomes (Jiménez-Ruiz et al., 2020). For instance, the wild olive genome has 1.48 Gb and 50,684 protein-coding genes (Unver et al., 2017), 'Picual' genome size has been estimated to 1.81 Gb containing 79,667 genes (78,079 protein-coding genes), but the sequenced length of 'Farga' genome was 1.31 Gb with 56,349 coding genes annotated (Cruz et al., 2016).

Understanding the domestication and diversification processes contribute to the efficient management of germplasm collections and breeding programs (Besnard et al., 2018; Khadari and El Bakkali, 2018). Olive crop has an unvaluable heritage of genetic variability, but the current olive-growing techniques are increasing the risk of genetic erosion (Besnard et al., 2018; Rallo et al., 2013). Therefore, the identification and maintenance of olive genetic diversity is needed for future breeding programs, even more important in the current globalization context.

Olive breeding initiatives have been developed in several countries around the world (Argentina, Australia, Croatia, France, Greece, Iran, Israel, Italy, Jordan, Lebanon, Montenegro, Morocco, Portugal, Spain, Tunisia, Turkey, Uruguay and USA) (Rallo et al., 2018). However, few cultivars have been registered for olive oil production up to date. In Spain, the breeding program of IFAPA and University of Córdoba released 'Sikitita' (Rallo et al., 2008), a new cultivar suitable for hedgerow plantations. 'Sikitita2' and 'Sikitita3' are in registration process (L. León, personal communication). 'Barnea' and 'FS-17' have been registered among others by breeding programs developed in Israel and Italy, respectively. Both of them are well adapted to high density orchards (Fernández-Escobar et al., 2013). Furthermore, breeding programs are focused on improve other agronomic traits such as early bearing (León et al., 2007), high productivity, oil content and composition (Dıraman et al., 2020; De la Rosa et al., 2016; León et al., 2008). Tolerance to abiotic and biotic stress like *Verticillium dahliae* (Arias-Calderón et al., 2015b; Trapero et al., 2015; Colella et al., 2008) or even the emergent *Xylella fastidiosa* (Sion et al., 2019) are also important objectives for olive breeding programs.

The strategy followed by the olive breeding program carried out in Cordoba, Spain, is based on crossing between cultivars (or wild olives) of known merit and subsequent evaluations of

the new generated genotypes in several steps (León et al., 2015; Rallo et al., 2018). Firstly, seedlings from crosses or open pollinations are forced to growth in greenhouse and transplanted into the field after 18 months, where are evaluated for early bearing, fruit size and oil content at the third or fourth year after planting (Arias-Calderón et al., 2014; León et al., 2015; De la Rosa et al., 2016). Selected genotypes are vegetatively propagated for intermediate evaluation trials with several replications per genotype. The selection criteria in this step are productivity, tree vigor, oil content and composition (León et al., 2015), but also could depend on the specific objectives as disease resistance (Arias-Calderón et al., 2015b, 2015a). In the last step, the assessment is performed in several comparative trials including few genotypes and increasing the replications per genotype (Rallo et al., 2018). This final selection step is of paramount importance for accurate characterization of the breeding selections under different environments and commercial growing conditions (De la Rosa et al., 2013; León et al., 2011, 2008). As above mentioned, few genotypes reach the final stage and are eventually released as new cultivars. Nonetheless, the works in progress by different breeding programs will enlarge the diversity on modern commercial olive orchard ensuring the sustainability of the crop.

In the future, the development of molecular techniques and the advanced knowledge of olive genome could allow the identification of genes related to interesting agronomical traits (Mariotti et al., 2020; Kaya et al., 2019; Hernández et al., 2017; Atienza et al., 2014). These new tools could be highly useful for the development of molecular markers to assist breeders in the selection process by reducing the evaluation steps and the time and effort necessary.

1.4. Breeding for Verticillium wilt resistance in olive

Verticillium wilt in olive (VWO) is a vascular disease caused by the soil-borne fungal pathogen *Verticillium dahliae*. *V. dahliae* is a hemibiotrophic characterized by the production of melanized structures, which can remain viable in soils for many years, even in the absence of potential hosts (Klosterman et al., 2009). The disease cycle of this strictly asexual phytopathogen starts when infectious hyphae emerge from the microsclerotia. These hyphae penetrate plant roots and colonize the xylem vessels, disrupting water transport (Klimes et al., 2015). The development of symptoms is associated with the occlusion of plant water-conducting vessels by conidia and mycelium of *V. dahliae*, but also due to gums and tyloses produced by the plant as defense response (Keykhasaber et al., 2018; Klimes et al., 2015).

V. dahliae infects more than 400 different plant species, including other economically important annual and perennial crops (Klosterman et al., 2009). VWO was identified for first time in Italy 70 years ago. Thirty years later, the disease was detected in olive orchard in Córdoba (Spain) and soon thereafter in the main olive growing areas of Spain, partly due to the substitution of cotton fields by olive plantations and the pathogen spread by the irrigation systems and other agronomic practices (Montes-Osuna and Mercado-Blanco, 2020; Jiménez-Díaz et al., 2012; López-Escudero, 2010). The severity of symptoms depends on the pathotype virulence. The defoliating pathotype, which is the most virulent and abundant in olive growing areas of Spain (López-Escudero, 2010; Navas-Cortés et al., 2008),

cause defoliation in olive, whereas non-defoliating isolates produced canopy wilting. Additionally, two different syndromes could be observed in olive depending on the season. A quick wilting outbreak with rolling leaves occurs at the end of winter to early spring, named 'apoplexy'. A 'slow decline' occurs from spring to early summer, consisting in the defoliation of green leaves, chlorotic leaves and mummification of inflorescences (Jiménez-Díaz et al., 2012; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011). On the other hand, the severity of symptoms and physiological response also could vary among olive cultivars depending on the resistance level (Gharbi et al., 2017). In this sense, several genes have been described involving the disease response in olive (Cabanás et al., 2015), which are differentialy expressed among cultivars and even among plant tissues (Ramírez-Tejero et al., 2020; Leyva-Pérez et al., 2018; Jiménez-Ruiz et al., 2017).

Currently, attacks of *V. dahliae* in olive have been reported in many olive-growing areas, constituting a major constraint for olive productivity (Keykhasaber et al., 2018; Jiménez-Díaz et al., 2012; Tsror, 2011) and oil quality (Landa et al., 2019). Once *V. dahliae* infects a field, the control efforts must be focused on the reduction of microsclerotia in soil and incidence on the crop. The three factors hampering the control of verticillium wilt disease are the inaccessibility to the pathogen during infection, its long-term persistence in soil and the broad host range. In this sense, an integrated control management has been the most recommended strategy including the use of resistant cultivars as the most economical, environmental and efficient measure (Ostos et al., 2020; López-Escudero and Mercado-Blanco, 2011; Tsror, 2011; Klosterman et al., 2009). The evaluation of defoliating pathotype resistance have been carried out for a large set of olive cultivars by artificial inoculation under different conditions in growth chamber (López-Escudero et al., 2004; Martos-Moreno et al., 2006, Markakis et al., 2009; Erten and Yildiz, 2011), greenhouse (García-Ruiz et al., 2014, 2015; Sanei and Razavi, 2017) and fields (Trapero et al., 2013). As results of the evaluations carried out in Spain, only 'Frantoio', 'Empeltre' and 'Changlot Real' cultivars have shown a consistent resistant response to the defoliating pathotype, but unfortunately, these cultivars exhibit other agronomic disadvantages, affecting their adaptability to different growing system or productivity (Martos-Moreno et al., 2006; López-Escudero et al., 2004). Simultaneously, resistance to VWO have been evaluated in wild olives and some of them have shown high resistance levels (Arias-Calderón et al., 2015b; Colella et al., 2008), suggesting its potential use as resistant rootstock. It should be noted however that the effectiveness of using resistant rootstock have been recently proven not to be fully effective for controlling the disease symptoms in susceptible scions in the long term (Valverde et al., 2020).

According to these results, the olive breeding program of IFAPA started a new line of work in 2007 aiming at obtaining new cultivars combining both high resistance to VWO and improved agronomic characteristics. Progenies were obtained from crosses involving cultivars (or wild olives) of known resistance and the new generated genotypes were subsequently submitted to a selection process in several steps (León et al., 2015). The initial seedlings were firstly screened according to early bearing (short juvenile period) and high oil content and oil quality (De la Rosa et al., 2016; Arias-Calderón et al., 2014). Afterwards, the selected genotypes were evaluated for resistance to *V. dahliae* infection by artificial inoculations dipping bare roots in a conidial suspension and allowing symptom to develop

under controlled condition in a growth chamber (Arias-Calderón et al., 2015a; 2015b). The resistant breeding selections were then included in a final step in comparative trials under natural conditions, in which disease resistance and agronomical traits are re-evaluated. In this PhD Thesis, an intermediate evaluation under semi-controlled conditions in microplots with artificially infested soils has been also tested to check the usefulness and accuracy of different evaluation procedures (Chapter 1 of this Thesis).

1.5. Breeding for oil quality in olive

Extra virgin olive oil (EVOO) is the juice of olive fruit, extracted by mechanical and physical processes. EVOO is composed of two fractions, saponifiable (98-99%) and unsaponifiable (1-2%) (Capurso et al., 2018). The saponifiable fraction is characterized by high content of unsaturated fatty acids among which oleic acid is the most abundant (55-83%), whereas the saturated acids are minority. The unsaponifiable fraction differentiated EVOO from the rest of vegetable oils, containing many minor compounds (phenols, pigment, hydrocarbons, sterols, tocopherols, terpenes, aliphatic and aromatic alcohols, volatile compounds, among others). These minor compounds significantly contribute to the healthy (antioxidant, anti-inflamatory, cardioprotective and anti-tumoral activities) and organoleptic properties of EVOO (Jiménez-López et al., 2020; Capurso et al., 2018; Ros et al., 2014). Thus, these bioactive compounds are also responsible for the increasing demand of EVOO by the worldwide consumers.

Since most of the olive production is intended for oil extraction and EVOO is highly appreciated for its composition, breeding programs consider the oil composition as the essential characteristic to improve in new cultivars. The evaluation of large progenies from breeding crosses revealed a huge genetic variability among genotypes for oil composition including minor compounds content (Pérez et al., 2019, 2016; El Riachy et al., 2012a). Specific compounds revealed higher heritability depending on the genitors, as high oil content from 'Zard' and 'Roghani' (Zeinanloo et al., 2009) or 'Barnea', 'Kalamata' and 'Manzanillo' (Lavee and Avidana, 2011) and high oleic acid content from 'Sigoise' comparing with other Tunisian cultivars (Dabbou et al., 2010). Wild parents have been described as interesting genitors enhancing tocopherols content (León et al., 2018).

The most extended criteria for selection in olive breeding programs focused on oil quality are based on the fatty acid composition, specifically a high content of oleic acid (Dıraman et al., 2020; Medina et al., 2015; De la Rosa et al., 2013; Dabbou et al., 2012; León et al., 2008). Among minor compounds, phenolic compounds including those with tocopherol structure (pro-vitamin E) mainly contribute to EVOO's antioxidants properties (Capurso et al., 2018). Thus, improving the content of these bioactive compounds have been also considered in the selection process (Pérez et al., 2019; Medina et al., 2015; Riachy et al., 2012a, 2012b; León et al., 2011), in combination with phytosterols (Manai-Djebali et al., 2018). Additionally, phenols and volatiles compounds contribute to the organoleptic properties of EVOO, whose characterization is also important for new cultivars (García-González et al., 2010) in order to promote market differentiation and consumer acceptance (Jiménez-Guerrero et al., 2012).

Due to the importance of all these traits in breeding programs, the identification of genes or DNA variations affecting specific properties could be a powerful tool to accelerate the

selection process in progenies. In this sense, several candidate genes involved in terpenoids, phenols and fatty acid synthesis as well as quantitative trait loci associated with fatty acid composition, oil content or fruit traits have been identified (Mariotti et al., 2020; Hernández et al., 2017; Atienza et al., 2014). However, a thorough knowledge of these genes is still necessary to develop functional markers for genomic-assisted breeding in olive.

1.6. Genotype by Environment

Traditional olive cultivars were probably locally selected by the first farmers in original olive growing areas according to the adaptability to specific environmental and agronomical conditions (Diez et al., 2015). Nowadays, a limited number of these traditional cultivars have been introduced into intensive orchards in the new olive growing areas, which is an important drawback because of the lack of knowledge about the effect of climatic and growing conditions on the vegetative cycle of olive, productivity or oil quality (Rallo et al., 2018; Torres et al., 2017; Fernández-Escobar et al., 2013).

The genetic effects have been widely studied for different traits of the crop by several works demonstrating variability in the response to biotic and abiotic stresses among cultivars, as well as differences in productivity and oil quality (Pérez et al., 2019, 2016; Martos-Moreno et al., 2006; López-Escudero et al., 2004). The environmental effect on productivity and oil composition have also been described in works analyzing EVOOs from olives under different crop management practices or growing areas (Caporaso, 2016). However, the combined effect of genotype by environment interaction on different traits has been scarcely studied due to the requirement of experimentation in comparative field trials including factorial designs. Understanding the G x E interaction is crucial in olive breeding programs aiming to obtain new bred cultivars with stable phenotypes and suitable for different growing conditions.

Several cultivars have been studied in multi-environment trials in Andalusia (Spain) and Italy (Navas-López et al., 2019a, 2019b; Ripa et al., 2008), even at large scale comparing germplasm collections of Spain, Italy, Morocco, Lebanon and Argentina (Mousavi et al., 2019). These works determined olive traits reliant on the genetic, environmental factors and G x E interaction. From the phenological point of view, flowering is a highly disturbed process by G x E interaction, in which flowering timing is more regulated by the environment and flower quality is mainly under genetic control (Navas-López et al., 2019a). Fruit development is another unstable process because of G x E interaction affecting fruit ripening and oil accumulation. Thus, the final oil content in dry fruit weight is highly influenced by G x E interaction (Mousavi et al., 2019; Navas-López et al., 2019b, 2020). Fruit parameters such as fruit fresh weight is a character genetically conditioned, whereas fruit moisture is conditioned by the environment as well as the date of maximum oil content and fruit ripening index at that date (Mousavi et al., 2019; Navas-López et al., 2019b). From the oil quality point of view, the EVOO's composition is also highly variable regarding the effect of genotype, environment and G x E interaction on both fatty acids and minor compounds (Mousavi et al., 2019; Navas-López et al., 2020; Ripa et al., 2008; Pérez et al., 2018). In fact, some contradictory results have been reported so far. Thus, the genetic effect has been suggested as the main factor responsible for the variability in some quality components in

some works, but strong influence of the environment has been also reported. From the breeding point of view, low G x E interaction allowed similar ranking of genotypes on the different environments, which simplify the selection procedure in breeding programs.

Regarding the environment, high temperature has been suggested as one of the major constraints for olive growing and oil production in new growing areas as well as for potential future climate change impact (Torres et al., 2017, Gabaldón-Leal et al., 2017). Extreme temperatures have negative influence on oil accumulation and oleic acid content (Mousavi et al., 2019; Navas-López et al., 2019b) as well as mild temperatures throughout the year result in a lack of chilling hours for flowering (Torres et al., 2017, Gabaldón-Leal et al., 2017).

In short, these studies have shown that the genetic effect is very relevant for both agronomic traits and oil quality. Therefore, the new bred cultivars would provide variability regardless of the plantation area. The environmental effect on some traits support the differentiated production according to the growing area. Finally, the significant effect of the G x E interaction described for some parameters confirms the need to carry out field experimentation prior to introduce new cultivars or new growing areas. In general, further experimentation is needed to clarify some of the effects reported so far.

1.7. Objectives

The works addressed for this thesis were carried out in the framework of the olive breeding program for VWO resistance developed at IFAPA 'Alameda del Obispo'. Specifically, this thesis includes the works carried out in intermediate stages and final comparative trials of the selection process. For that purpose, experiments started with a set of preselections based on the results of artificial inoculations in growth chamber and preliminary agronomic evaluations of seedlings.

Two main objectives of this thesis were: the field experimentation to confirm the resistance response of breeding selections against the infection of the most virulent pathotype of *V. dahliae* in Andalusia and the agronomic characterization of the breeding selections; and the study of candidate resistant genes focusing on the development of useful tools to simplify selection processes.

To cover both aspects, different specific objectives have been established, each of which constitutes a chapter of this thesis:

- The evaluation of resistance response against the infection of the defoliant pathotype of *V. dahliae* in artificially infested microplots under natural climate conditions (Chapter 2).
- Characterization of the oil quality of the resistant selections growing in comparative trials under natural growing conditions (Chapter 3).
- Characterization of the volatile composition of the EVOO's from the resistant selections growing in comparative field trials (Chapter 4).

- Identification of genetic variations in candidate genes for resistance to VWO considering a wide germplasm collection representative of the genetic diversity of *O. europaea* specie and the disease response as well (Chapter 5).

1.8. References

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.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., De la Rosa, R., León, L., 2015a. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica 206, 619–629.

Arias-Calderón, Rocío, Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., Belaj, A., 2015b. Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. Crop Protection 75, 25–33.

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.

Atienza, S.G., De la Rosa, R., León, L., Martín, A., Belaj, A., 2014. Identification of QTL for agronomic traits of importance for olive breeding. Mol Breeding 34, 725–737.

Belaj, A., Muñoz-Diez, C., Baldoni, L., Porceddu, A., Barranco, D., Satovic, Z., 2007. Genetic Diversity and Population Structure of Wild Olives from the North-western Mediterranean Assessed by SSR Markers. Ann Bot 100, 449–458.

Belaj, A., Muñoz-Diez, C., Baldoni, L., Satovic, Z., Barranco, D., 2010. Genetic diversity and relationships of wild and cultivated olives at regional level in Spain. Scientia Horticulturae 124, 323–330.

Belaj, A., Veral, M., Sikaoui, H., Moukhli, A., Khadari, B., Mariotti, R., Baldoni, L., 2016. Olive Genetic Resources.

Besnard, G., Dupuy, J., Larter, M., Cuneo, P., Cooke, D., Chikhi, L., 2014. History of the invasive African olive tree in Australia and Hawaii: evidence for sequential bottlenecks and hybridization with the Mediterranean olive. Evolutionary Applications 7, 195–211.

Besnard, G., El Bakkali, A., Haouane, H., Baali-Cherif, D., Moukhli, A., Khadari, B., 2013. Population genetics of Mediterranean and Saharan olives: geographic patterns of differentiation and evidence for early generations of admixture. Ann Bot 112, 1293–1302.

Besnard, G., García-Verdugo, C., Rubio De Casas, R., Treier, U.A., Galland, N., Vargas, P., 2008. Polyploidy in the Olive Complex (*Olea europaea*): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses. Ann Bot 101, 25–30.

Besnard, G., Terral, J.-F., Cornille, A., 2018. On the origins and domestication of the olive: a review and perspectives. Ann Bot 121, 385–403.

Breton, C., Terral, J.-F., Pinatel, C., Médail, F., Bonhomme, F., Bervillé, A., 2009. The origins of the domestication of the olive tree. Comptes Rendus Biologies 332, 1059–1064.

Cabanás, C.G.-L., Schiliro`, E., Valverde-Corredor, A., Mercado-Blanco, J., 2015. Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. Front. Microbiol. 6.

Caporaso, N., 2016. Virgin Olive Oils: Environmental Conditions, Agronomical Factors and Processing Technology Affecting the Chemistry of Flavor Profile. J Food Chem Nanotechnol 2.

Capurso, A., Crepaldi, G., Capurso, C., 2018. Extra-Virgin Olive Oil (EVOO): History and Chemical Composition, in: Capurso, A., Crepaldi, G., Capurso, C. (Eds.), Benefits of the Mediterranean Diet in the Elderly Patient, Practical Issues in Geriatrics. Springer International Publishing, Cham, pp. 11–21.

Colella, C., Miacola, C., Amenduni, M., D'Amico, M., Bubici, G., Cirulli, M., 2008. Sources of verticillium wilt resistance in wild olive germplasm from the Mediterranean region. Plant Pathology 57, 533–539.

Cruz, F., Julca, I., Gómez-Garrido, J., Loska, D., Marcet-Houben, M., Cano, E., Galán, B., Frias, L., Ribeca, P., Derdak, S., Gut, M., Sánchez-Fernández, M., García, J.L., Gut, I.G., Vargas, P., Alioto, T.S., Gabaldón, T., 2016. Genome sequence of the olive tree, Olea europaea. Gigascience 5.

Dabbou, S., Chaieb, I., Rjiba, I., Issaoui, M., Echbili, A., Nakbi, A., Gazzah, N., Hammami, M., 2012. Multivariate Data Analysis of Fatty Acid Content in the Classification of Olive Oils Developed Through Controlled Crossbreeding. Journal of the American Oil Chemists' Society 89, 667–674.

Dabbou, S., Rjiba, I., Echbili, A., Gazzah, N., Mechri, B., Hammami, M., 2010. Effect of Controlled Crossing on the Triglyceride and Fatty Acid Composition of Virgin Olive Oils. Chemistry & Biodiversity 7, 1801–1813.

De la Rosa, R., Arias-Calderón, R., Velasco, L., León, L., 2016. Early selection for oil quality components in olive breeding progenies. European Journal of Lipid Science and Technology 118, 1160–1167.

De la Rosa, R., Talhaoui, N., Rouis, H., Velasco, L., León, L., 2013. Fruit characteristics and fatty acid composition in advanced olive breeding selections along the ripening period. Food Research International 54, 1890–1896.

Diez, C.M., Trujillo, I., Martínez-Urdiroz, N., Barranco, D., Rallo, L., Marfil, P., Gaut, B.S., 2015. Olive domestication and diversification in the Mediterranean Basin. New Phytologist 206, 436–447.

Dıraman, H., Karaman, H.T., Sefer, F., Ersoy, N., Arsel, A.H., 2020. Using Nutritional Lipid and Sensorial Profiles for the Characterization of Turkish Olive (Memecik X Gemlik cv) Hybrids obtained from Controlled Crossbreeding. Journal of the American Oil Chemists' Society 97, 943–954.

Duarte, F., Jones, N., Fleskens, L., 2008. Traditional olive orchards on sloping land: Sustainability or abandonment? Journal of Environmental Management, A Sustainable Future for Olive Production on Sloping Land 89, 86–98.

El Bakkali, A., Essalouh, L., Tollon, C., Rivallan, R., Mournet, P., Moukhli, A., Zaher, H., Mekkaoui, A., Hadidou, A., Sikaoui, L., Khadari, B., 2019. Characterization of Worldwide Olive Germplasm Banks of Marrakech (Morocco) and Córdoba (Spain): Towards management and use of olive germplasm in breeding programs. PLoS ONE 14, e0223716.

Erten L, Yıldız M. 2011. Screening for resistance of Turkish olive cultivars and clonal rootstocks to Verticillium wilt. Phytoparasitica 39, 83-92.

EU, European Commission. 2020. https://ec.europa.eu/info/food-farming-fisheries/plants-and-plant-products/plant-products/olive-oil_en

FAO, Food and Agriculture Organization of the United Nations (2018). http://www.fao.org/faostat/en/

Fernández Escobar R., De la Rosa R., León L., Gómez J.A., Testi F., Orgaz M., Gil-Ribes J.A., Quesada-Moraga E., Trapero A. Evolution and sustainability of the olive production systems. In : Arcas N. (ed.), Arroyo López F.N. (ed.), Caballero J. (ed.), D'Andria R. (ed.), Fernández M. (ed.), Fernández Escobar R. (ed.), Garrido A. (ed.), López-Miranda J. (ed.), Msallem M. (ed.), Parras M. (ed.), Rallo L. (ed.), Zanoli R. (ed.). Present and future of the Mediterranean olive sector. Zaragoza: CIHEAM/IOC, 2013. p. 11-42 (Options Méditerranéennes : Série A. Séminaires Méditerranéens; n. 106).

Gabaldón-Leal, C., Ruiz-Ramos, M., de la Rosa, R., León, L., Belaj, A., Rodríguez, A., Santos, C., Lorite, I.J., 2017. Impact of changes in mean and extreme temperatures caused by climate change on olive flowering in southern Spain. Int. J. Climatol. 37, 940–957.

García-González, D.L., Tena, N., Aparicio, R., 2010. Quality Characterization of the New Virgin Olive Oil Var. Sikitita by Phenols and Volatile Compounds. J. Agric. Food Chem. 58, 8357–8364.

Garcia-Ruiz, G.M., Trapero, C., Del Rio, C., López-Escudero, F.J., 2014. Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. Phytoparasitica 42, 205–212.

García-Ruiz, G.M., Trapero, C., Varo-Suárez, Á., Trapero, A., López-Escudero, F.J., 2015. Identifying resistance to Verticillium wilt in local Spanish olive cultivars. Phytopathologia Mediterranea 54.

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., Triki, M.A., 2017. Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the

hemibiotrophic pathogen *Verticillium dahliae.* Physiological and Molecular Plant Pathology 97, 30–39.

Gros-Balthazard, M., Besnard, G., Sarah, G., Holtz, Y., Leclercq, J., Santoni, S., Wegmann, D., Glémin, S., Khadari, B., 2019. Evolutionary transcriptomics reveals the origins of olives and the genomic changes associated with their domestication. The Plant Journal 100, 143–157.

Hernández, M.L., Belaj, A., Sicardo, M.D., León, L., De la Rosa, R., Martín, A., Martínez-Rivas, J.M., Atienza, S.G., 2017. Mapping quantitative trait loci controlling fatty acid composition in olive. Euphytica 213, 7.

IOC, International Olive Council. 2020, https://www.internationaloliveoil.org /wp-content/uploads/ 2020/10/ Merged-154-Newsletter.pdf

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., Del Mar Jiménez-Gasco, M., Antoniou, P.P., Tjamos, E.C., 2012. Verticillium Wilt, A Major Threat to Olive Production: Current Status and Future Prospects for its Management. Plant Dis 96, 304–329.

Jiménez-Guerrero, J.F., Gázquez-Abad, J.C., Mondéjar-Jiménez, J.A., Huertas-García, R., 2012. Consumer Preferences for Olive-Oil Attributes: A Review of the Empirical Literature Using a Conjoint Approach. Chapter 12 of Olive Oil - Constituents, Quality, Health Properties and Bioconversions. Editor: Boskou, D. ISBN: 978-953-307-921-9.

Jiménez-López, C., Carpena, M., Lourenço-Lopes, C., Gallardo-Gómez, M., Lorenzo, J.M., Barba, F.J., Prieto, M.A., Simal-Gandara, J., 2020. Bioactive Compounds and Quality of Extra Virgin Olive Oil. Foods 9, 1014.

Jiménez-Ruiz, J., Leyva-Pérez, M. de la O., Schilirò, E., Barroso, J.B., Bombarely, A., Mueller, L., Mercado-Blanco, J., Luque, F., 2017. Transcriptomic Analysis of Olea europaea L. Roots during the *Verticillium dahliae* Early Infection Process. The Plant Genome 10, 2016.07.0060.

Jiménez-Ruiz, J., Ramírez-Tejero, J.A., Fernández-Pozo, N., Leyva-Pérez, M. de la O., Yan, H., De la Rosa, R. de la, Belaj, A., Montes, E., Rodríguez-Ariza, M.O., Navarro, F., Barroso, J.B., Beuzón, C.R., Valpuesta, V., Bombarely, A., Luque, F., 2020. Transposon activation is a major driver in the genome evolution of cultivated olive trees (*Olea europaea* L.). The Plant Genome 13, e20010.

Julca, I., Marcet-Houben, M., Cruz, F., Gómez-Garrido, J., Gaut, B.S., Díez, C.M., Gut, I.G., Alioto, T.S., Vargas, P., Gabaldón, T., 2020. Genomic evidence for recurrent genetic admixture during the domestication of Mediterranean olive trees (*Olea europaea* L.). BMC Biology 18, 148.

Kaya, H.B., Akdemir, D., Lozano, R., Cetin, O., Sozer Kaya, H., Sahin, M., Smith, J.L., Tanyolac, B., Jannink, J.-L., 2019. Genome wide association study of 5 agronomic traits in olive (*Olea europaea* L.). Sci Rep 9.

Keykhasaber, M., Thomma, B.P.H.J., Hiemstra, J.A., 2018. Verticillium wilt caused by *Verticillium dahliae* in woody plants with emphasis on olive and shade trees. Eur J Plant Pathol 150, 21–37.

Khadari, B., El Bakkali, A., 2018. Primary Selection and Secondary Diversification: Two Key Processes in the History of Olive Domestication. International Journal of Agronomy. vol. 2018, Article ID 5607903, 9 pages.

Klimes, A., Dobinson, K.F., Thomma, B.P.H.J., Klosterman, S.J., 2015. Genomics Spurs Rapid Advances in Our Understanding of the Biology of Vascular Wilt Pathogens in the Genus Verticillium. Annual Review of Phytopathology 53, 181–198.

Klosterman, S.J., Atallah, Z.K., Vallad, G.E., Subbarao, K.V., 2009. Diversity, Pathogenicity, and Management of Verticillium Species. Annual Review of Phytopathology 47, 39–62.

Landa, B.B., Pérez, A.G., Luaces, P., Montes-Borrego, M., Navas-Cortés, J.A., Sanz, C., 2019. Insights Into the Effect of *Verticillium dahliae* Defoliating-Pathotype Infection on the Content of Phenolic and Volatile Compounds Related to the Sensory Properties of Virgin Olive Oil. Front. Plant Sci. 10, 232.

Lavee, S., Avidan, B., Ben-Ari, G., 2014. Trends in Breeding New Olive Varieties in Israel for Quality and Economic Management. Agricultural Sciences 2014.

Lavee, S., Avidana, B., 2011. Heredity diversity in populations of free-, self-, and specific cross-pollinated progenies of some olive (*Olea europaea* L.) cultivars. Israel Journal of Plant Sciences 59, 29–37.

León, L., Beltrán, G., Aguilera, M.P., Rallo, L., Barranco, D., De la Rosa, R., 2011. Oil composition of advanced selections from an olive breeding program. Eur. J. Lipid Sci. Technol. 113, 870–875.

León, L., De la Rosa, R., Gracia, A., Barranco, D., Rallo, L., 2008. Fatty acid composition of advanced olive selections obtained by crossbreeding. J. Sci. Food Agric. 88, 1921–1926.

León, L., De la Rosa, R., Velasco, L., Belaj, A., 2018. Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. Front. Plant Sci. 9.

León, L., De la Rosa, R. de la, Barranco, D., Rallo, L., 2007. Breeding for Early Bearing in Olive. HortScience 42, 499–502.

León, L., Velasco, L., De la Rosa, R., 2015. Initial selection steps in olive breeding programs. Euphytica 201, 453–462.

Leyva-Pérez, M. de la O., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J.B., Luque, F., Mercado-Blanco, J., 2018. Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. New Phytol. 217, 671–686.

López-Escudero, F.J., del Río, C., Caballero, J.M., Blanco-López, M.A., 2004. Evaluation of Olive Cultivars for Resistance to *Verticillium dahliae*. European Journal of Plant Pathology 110, 79–85.

López-Escudero, F.J., Mercado-Blanco, J., 2011. Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344, 1–50.

López-Escudero, F.J., Rio, C. del, Caballero, J.M., Blanco-López, M.A., 2004. Evaluation of olive cultivars for resistance to *Verticillium dahliae*. European Journal of Plant Pathology 110, 79-85.

López-Escudero, J., 2010. Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. Phytopathologia Mediterranea 49, 11.

Manai-Djebali, H., Oueslati, I., Martínez-Cañas, M.A., Zarrouk, M., Sánchez-Casas, J., 2018. Improvement of the Sterol and Triacylglycerol Compositions of Chemlali Virgin Olive Oils through Controlled Crossing with Mediterranean Cultivars. J Oleo Sci 67, 379–388.

MAPA, Ministry of Agriculture, Fisheries and Food. 2017. https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura /encuestas-plantaciones-arboles-frutales/

Mariotti, R., Belaj, A., De La Rosa, R., León, L., Brizioli, F., Baldoni, L., Mousavi, S., 2020. EST–SNP Study of *Olea europaea* L. Uncovers Functional Polymorphisms between Cultivated and Wild Olives. Genes 11, 916.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Epaminondas, J., 2009. Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. Eur. J. Plant. Pathol. 124, 603.

Martos-Moreno, C., López-Escudero, F.J., Blanco-López, M.Á., 2006. Resistance of Olive Cultivars to the Defoliating Pathotype of *Verticillium dahliae*. HortScience 41, 1313–1316.

Medina, V.S. de, Priego-Capote, F., Castro, M.D.L. de, 2015. The effect of genotype and ripening index on the phenolic profile and fatty acids composition of virgin olive oils from olive breeding programs. European Journal of Lipid Science and Technology 117, 954–966.

Montes-Osuna, N., Mercado-Blanco, J., 2020. Verticillium Wilt of Olive and its Control: What Did We Learn during the Last Decade? Plants 9, 735.

Mousavi, S., De la Rosa, R., Moukhli, A., El Riachy, M., Mariotti, R., Torres, M., Pierantozzi, P., Stanzione, V., Mastio, V., Zaher, H., El Antari, A., Ayoub, S., Dandachi, F., Youssef, H., Aggelou, N., Contreras, C., Maestri, D., Belaj, A., Bufacchi, M., Baldoni, L., Leon, L., 2019. Plasticity of fruit and oil traits in olive among different environments. Scientific Reports 9, 16968.

Navas-Cortés, J.A., Landa, B.B., Mercado-Blanco, J., Trapero-Casas, J.L., Rodríguez-Jurado, D., Jiménez-Díaz, R.M., 2008. Spatiotemporal Analysis of Spread of Infections by *Verticillium dahliae* Pathotypes Within a High Tree Density Olive Orchard in Southern Spain. Phytopathology 98, 167–180.

Navas-López, J.F., Cano, J., De la Rosa, R., Velasco, L., León, L., 2020. Genotype by environment interaction for oil quality components in olive tree. European Journal of Agronomy 119, 126115.

Navas-López, J. F., León, L., Rapoport, H.F., Moreno-Alías, I., Lorite, I.J., De la Rosa, R., 2019a. Genotype, environment and their interaction effects on olive tree flowering phenology and flower quality. Euphytica 215, 184.

Navas-López, J.F., León, L., Trentacoste, E.R., De la Rosa, R., 2019b. Multi-environment evaluation of oil accumulation pattern parameters in olive. Plant Physiology and Biochemistry 139, 485–494.

Ostos, E., Garcia-López, M.T., Porras, R., López-Escudero, F.J., Trapero-Casas, A., Michailides, T.J., Moral, J., 2020. Effect of Cultivar Resistance and Soil Management on Spatial-Temporal Development of Verticillium Wilt of Olive: A Long-Term Study. Front. Plant. Sci. 11, 584496.

Pagliuca, M.M., Scarpato, D., 2014. The Olive Oil Sector: A Comparison between Consumers and "experts" Choices by the Sensory Analysis. Procedia Economics and Finance 17, 221–230.

Pérez, A.G., De la Rosa, R., Pascual, M., Sánchez-Ortiz, A., Romero-Segura, C., León, L., Sanz, C., 2016. Assessment of volatile compound profiles and the deduced sensory significance of virgin olive oils from the progeny of Picual×Arbequina cultivars. Journal of Chromatography A 1428, 305–315.

Pérez, A.G., León, L., Pascual, M., De la Rosa, R., Belaj, A., Sanz, C., 2019. Analysis of Olive (*Olea Europaea* L.) Genetic Resources in Relation to the Content of Vitamin E in Virgin Olive Oil. Antioxidants 8, 242.

Pérez, A.G., León, L., Sanz, C., De la Rosa, R., 2018. Fruit Phenolic Profiling: A New Selection Criterion in Olive Breeding Programs. Front. Plant. Sci. 9, 241.

Perujo-Villanueva, M., Colombo, S., 2017. Cost analysis of parcel fragmentation in agriculture: The case of traditional olive cultivation. Biosystems Engineering 164, 135–146.

Rallo, L., Barranco, D., Castro-García, S., Connor, D.J., Campo, M.G. del, Rallo, P., 2013. High-Density Olive Plantations, in: Horticultural Reviews Volume 41. John Wiley & Sons, Ltd, pp. 303–384.

Rallo, L., Barranco, D., Díez, C.M., Rallo, P., Suárez, M.P., Trapero, C., Pliego-Alfaro, F., 2018. Strategies for Olive (*Olea europaea* L.) Breeding: Cultivated Genetic Resources and Crossbreeding, in: Al-Khayri, J.M., Jain, S.M., Johnson, D.V. (Eds.), Advances in Plant Breeding Strategies: Fruits: Volume 3. Springer International Publishing, Cham, pp. 535–600.

Rallo, L., Barranco, D., De la Rosa, R. de la, León, L., 2008. 'Chiquitita' Olive. HortScience 43, 529–531.

Ramírez-Tejero, J.A., Jiménez-Ruiz, J., Leyva-Pérez, M. de la O., Barroso, J.B., Luque, F., 2020. Gene Expression Pattern in Olive Tree Organs (*Olea europaea* L.). Genes 11, 544.

Riachy, M.E., Priego-Capote, F., León, L., Castro, M.D.L. de, Rallo, L., 2012a. Virgin olive oil phenolic profile and variability in progenies from olive crosses. Journal of the Science of Food and Agriculture 92, 2524–2533.

Riachy, M.E., Priego-Capote, F., Rallo, L., Castro, M.D.L., León, L., 2012b. Phenolic composition of virgin olive oils from cross breeding segregating populations. European Journal of Lipid Science and Technology 114, 542–551.

Ripa, V., Rose, F.D., Caravita, M.A., Parise, M.R., Perri, E., Rosati, A., Pandolfi, S., Paoletti, A., Pannelli, G., Padula, G., Giordani, E., Bellini, E., Buccoliero, A., Mennone, C., 2008. Qualitative evaluation of olive oils from new olive selections and effects of genotype and environment on oil quality. Advances in Horticultural Science 22, 95–103.

Rodríguez-Cohard, J.C., Sánchez-Martínez, J.D., Garrido-Almonacid, A., 2020. Strategic responses of the European olive-growing territories to the challenge of globalization. European Planning Studies 28, 2261–2283.

Ros, E., Martínez-González, M.A., Estruch, R., Salas-Salvadó, J., Fitó, M., Martínez, J.A., Corella, D., 2014. Mediterranean Diet and Cardiovascular Health: Teachings of the PREDIMED Study. Adv Nutr 5, 330S-336S.

Sacchi, R., Paduano, A., Savarese, M., Vitaglione, P., Fogliano, V., 2014. Extra Virgin Olive Oil: From Composition to "Molecular Gastronomy," in: Zappia, V., Panico, S., Russo, G.L., Budillon, A., Della Ragione, F. (Eds.), Advances in Nutrition and Cancer, Cancer Treatment and Research. Springer, Berlin, Heidelberg, pp. 325–338.

Sanei, S.J., Razavi, S.E. 2017. Resistance and vegetative growth analysis of some olive cultivars in response to a defoliating pathotype of Verticillium dahliae Kleb. Int. J. Hortic. Sci. 4, 239–250.

Sion, S., Taranto, F., Montemurro, C., Mangini, G., Camposeo, S., Falco, V., Gallo, A., Mita, G., Debbabi, O.S., Amar, F.B., Pavan, S., Roseti, V., Miazzi, M.M., 2019. Genetic Characterization of Apulian Olive Germplasm as Potential Source in New Breeding Programs. Plants 8, 268.

Torres, M., Pierantozzi, P., Searles, P., Rousseaux, M.C., García-Inza, G., Miserere, A., Bodoira, R., Contreras, C., Maestri, D., 2017. Olive Cultivation in the Southern Hemisphere: Flowering, Water Requirements and Oil Quality Responses to New Crop Environments. Front. Plant Sci. 8, 1830.

Trapero, C., Díez, C.M., Rallo, L., Barranco, D., López-Escudero, F.J., 2013. Effective inoculation methods to screen for resistance to Verticillium wilt in olive. Scientia Horticulturae 162, 252–259.

Trapero, C., Rallo, L., López-Escudero, F.J., Barranco, D., Díez, C.M., 2015. Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the Olea genus. Plant Pathology 64, 890–900.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., López-Escudero, F.J., 2013. Field Resistance to Verticillium Wilt in Selected Olive Cultivars Grown in Two Naturally Infested Soils. Plant Disease 97, 668–674.

Tsror, L., 2011. Epidemiology and control of Verticillium wilt on olive. Israel Journal of Plant Sciences 59, 59–69.

Unver, T., Wu, Z., Sterck, L., Turktas, M., Lohaus, R., Li, Z., Yang, M., He, L., Deng, T., Escalante, F.J., Llorens, C., Roig, F.J., Parmaksiz, I., Dundar, E., Xie, F., Zhang, B., Ipek, A., Uranbey, S., Erayman, M., Ilhan, E., Badad, O., Ghazal, H., Lightfoot, D.A., Kasarla, P., Colantonio, V., Tombuloglu, H., Hernández, P., Mete, N., Cetin, O., Van Montagu, M., Yang, H., Gao, Q., Dorado, G., Van de Peer, Y., 2017. Genome of wild olive and the evolution of oil biosynthesis. Proc Natl Acad Sci U S A 114, E9413–E9422.

Valverde, P., Trapero, C., Arquero, O., Serrano, N., Barranco, D., Díez, C.M., López-Escudero, F.J., 2020. Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. Plant Pathology n/a.

Vilar, J. and Pereira, J.E. (2018). International olive growing: Worldwide analysis and summary. Ed.: Fundación Caja Rural de Jaén. ISBN: 978-84-946394-9-4.

Zeinanloo, A., Shahsavari, A., Mohammadi, A., Naghavi, M.R., 2009. Variance component and heritability of some fruit characters in olive (*Olea europaea* L.). Scientia Horticulturae 123, 68–72.

2. Verticillium Wilt Evaluation of Olive Breeding Selections Under Semi-Controlled Conditions

This chapter has been published in the journal 'Plant Disease'.
Serrano, A., Rodríguez-Jurado, D., Román, B., Bejarano-Alcázar, J., De la Rosa, R., León, L. (2020).

2.1. Abstract

Genetic resistance is the most recommended measure to control verticillium wilt in olive (VWO), a vascular disease caused by the soil-borne fungus *Verticillium dahliae*, which has promoted in recent years the development of olive breeding programs aiming at obtaining new resistant and highly yielding cultivars. Screening has been commonly performed under controlled conditions in grow chamber after artificial inoculation at the early stage of breeding programs, but additional evaluation is necessary to confirm previous results as well as testing for additional agronomic traits. In this work, 20 breeding selections initially classified as resistant to the disease have been re-evaluated in artificially infested soils under natural environmental conditions. The maximum disease incidence (52.6%) was reached at 26 months after planting and the disease intensity index reached the maximum value of 38.5% at 29 months after plant. Nine breeding selections consistently confirm previous results of resistance to *V. dahliae* infection but contradictory results, compared to previous evaluation under controlled condition in grow chamber, were obtained for the rest of selections tested, which underlines the need of long-term experimentation under natural environmental conditions. Additional positive agronomic traits such as early bearing was also observed for some of the resistant selections, while variability for plant vigor was found. Some of them seems highly promising for releasing as new cultivars once characterization for other important agronomic traits is completed in future works.

2.2. Keywords

Defoliating pathotype, environmental conditions, genetic resistance, *Olea europaea* L., plant-pathogen interaction, screening, *Verticillium dahliae* Kleb.

2.3. Introduction

Olive (*Olea europaea* subsp. *europaea* var. *europaea* L.) is one of the most ancient fruit trees traditionally cultivated throughout the Mediterranean basin. In the last years, olive growing has been continuously expanding, both in traditional and non-traditional cultivation areas, boosted by technological improvements in growing systems including irrigation, mechanization, and higher planting density (Fernández-Escobar et al. 2013).

During the expanding process, new olive groves have been planted in areas traditionally cultivated with different crops facing, consequently, new challenges derived from these new conditions. Thus, in some areas olive orchards were established in infested soils by *Verticillium dahliae*, a soil-borne fungus causing wilting diseases in many crops and the causal agent of Verticillium Wilt in olive (VWO) (López-Escudero and Mercado-Blanco 2011; Jiménez-Díaz et al. 2011; Navas-Cortés et al. 2008). This pathogen becomes a major limitation for olive productivity in some new producing areas (Montes-Osuna and Mercado-Blanco 2020). Disease incidence and symptoms severity mainly depend on the virulence of the *V. dahliae* pathotype, whereas inoculum density in soil resulted not directly related with the disease parameters in both natural infested fields and artificially infested microplots (López-Escudero and Blanco-López 2007; Roca et al. 2016). In this sense, defoliating pathotype has been described more virulent than non-defoliating (López-Escudero et al.

2004) causing most of the infections in Andalusian olive orchards (López-Escudero et al. 2010).

The use of cultivars resistant to VWO is the most recommended prevention measure to be considered before planting in potentially infested soils. Nevertheless, most of the evaluated traditional cultivars resulted susceptible, at least to the defoliating (D) *V. dahliae* pathotypes, after many evaluation processes under different conditions in growth chamber (López-Escudero et al. 2004, 2007; Martos-Moreno et al. 2006), greenhouse (García-Ruiz et al. 2014, 2015), and field (Trapero et al. 2013a), in spite of differences linked to the inoculation process (Cirulli et al. 2008; Trapero et al. 2013b). In all cases, a wide variability on disease development was observed among cultivars, ranging from highly resistant to extremely susceptible (López-Escudero et al. 2004). However, some of the cultivars identified as resistant in these works have showed negative agronomic traits that could limit their commercial use. This includes late bearing, excessive vigor, frost susceptibility or low oil content. Additionally, some experiments revealed that VWO impose a limitation for plant growth (Birem et al. 2016; Sanei and Razavi 2017; Santos-Rufo et al. 2017) and yield (Gómez-Gálvez et al. 2020; Levin et al. 2003), and even affects oil quality (Landa et al. 2019). Even so, the negative impact of VWO showed variability among cultivars, with lower effect on resistant ones, which recommended their use as a genitor in future breeding programs.

Thus, after the evidence of genetic variability for VWO response of traditional cultivars, some breeding programs were initiated from crosses between resistant genitors aiming at obtaining new cultivars combining both high resistance to VWO and improved agronomical traits (Arias-Calderón et al. 2014; Trapero et al. 2015). Different evaluation procedures have been tested for screening purposes, but an evaluation step of resistance under natural environmental conditions is recommendable before finally selecting new resistant genotypes. This is necessary in order to determine variability in the effect of natural environment conditions on the olive genotype reaction to *V. dahliae*. Under field conditions, the disease is subjected to several factors of the natural environment, including virulence variation and pathotype prevailing as well as the distribution of fungus inoculum in the soil. The D pathotype causes higher rates of disease incidence than the ND pathotype and more severe epidemics (López-Escudero and Blanco-López 2007; Navas-Cortés et al. 2008). The number of patches in the field could affect to the disease incidence and severity as has been found in other perennial hosts (Goud et al. 2011). Differences in optimum soil temperatures have been also reported for specific pathotype infections (Calderón et al. 2014). Temperatures are also involved in the seasonal cycle of symptoms expression (Levin et al. 2003; Trapero et al. 2013a) and could contribute to the natural recovery of symptoms showed in different cultivars (López-Escudero and Blanco-López 2005).

In light of the above, this work aims to evaluate the VWO resistance of olive breeding selections under natural environmental conditions in a long-term experiment carried out in microplots infested with a known defoliating isolate of *V. dahliae*. Disease response as well as other important agronomic traits such as plant growth and earliness of bearing were evaluated to select the most promising selections to be recommended for olive growing in *V. dahliae* infested areas.

2.4. Materials and methods

2.4.1. Plant material

One-year-old plants of 20 breeding selections were obtained by vegetative propagation of semi-hardwood stem cutting (self-rooted plants). These selections were obtained in the olive breeding program of IFAPA from open pollination of different cultivars ('Arbequina', 'Empeltre', 'Frantoio', 'Koroneiki ', 'Picual', 'Picudo' and 'Manzanilla de Sevilla') or direct crosses ('Changlot Real' x 'Dolce Agogia', 'Frantoio' x 'Arbosana', 'Frantoio' x 'Dolce Agogia', 'Koroneiki' x 'Empeltre'). They were selected from the initial progenies on the basis of their early crop (short juvenile period) and high oil content (Arias-Calderón et al. 2014) and for having high level of resistance to *V. dahliae* when tested by root-dip inoculation under controlled conditions in growth chamber (Arias-Calderón et al. 2015a, b). Plants of four cultivars were also included in the experiment as controls: 'Changlot Real' and 'Frantoio' as resistant, 'Picual' as susceptible, and 'Arbequina' for which a variable disease response has been described depending on the evaluation conditions, i.e., susceptible in grow chamber experiment, but resistant in field conditions (Trapero et al. 2013a).

2.4.2. Inoculum production and soil infestation

A monoconidial isolate of *V. dahliae* defoliating-pathotype (Rodríguez-Jurado et al., 2008) belonging to the culture collection of the Sustainable Crop Protection Area of IFAPA 'Alameda del Obispo' (Córdoba, Spain) was used in this experiment. The inoculum multiplication was performed in 1L Erlenmeyer flasks containing 0.5 kg of a cornmeal-sand substrate consisting of a sterilized mixture of sand, cornmeal, and distilled water in a weight proportion of 9:1:2, respectively. Afterward, each flask was inoculated by adding 25 disks of 5mm diameter extracted from PDA (250 g peeled potato, 20 g agar and 20 g glucose per liter) plates with pathogen mycelia and was incubated for 8 weeks at 24±1℃ in dark, as previously described (Santos-Rufo et al. 2017). Flasks with PDA disks without fungus were included.

The experiment was conducted on 6×6 m experimental microplots delimited by a 2 mm thick high-density polyethylene sheet, located in open field (37°51'23.8"N 4°48'02.7"W) at the experimental field of IFAPA 'Alameda del Obispo', Córdoba, Spain. A total of 40 microplots were used, 20 of which were infested with the prepared inoculum and other 20 were used as controls in an inoculum-free soil. The 20 microplots of each treatment (infested/non infested) were divided in 10 elementary plots of 2 microplots each. In each elementary plot, one tree of each of the 24 genotypes tested (20 breeding selections and 4 control cultivars) was randomly planted. Therefore, 12 trees were planted in each microplot. Infested microplots were separated from control microplots by a perimeter mesh to avoid contaminations.

The microplots were infested one month prior to the planting date on March of 2016. The inoculation consisted on spreading 7.5 kg of the fungal substrate on each microplot. This was homogeneously distributed in the microplot using a motor hoe, deeping 15 cm into the soil. The same procedure was applied for the control microplots but using a non-infested

substrate. The soil in microplots was characterized by a loam texture (clay 19.5%, sand 41.5% and silt 39.0%) and basic pH (8.45) with 1.11% of organic matter.

2.4.3.Assessment of plant growth, pathogen evolution and environment conditions

Trunk diameter at 50 cm height was measured after planting and subsequently annually to follow-up plant growth. In addition, the number of bearing trees was recorded in November 2017 and 2018.

The *V. dahliae* inoculum density was estimated 0, 3, 7, 12, 19, 24 and 31 months after planting using the wet sieving technique (Huisman and Ashworth 1974). For each sampling date, six subsamples of 200 g were collected in each microplot with an Edelman sampler at a depth of 0 to 15 cm between the tree rows. The six soil subsamples were mixed to make a single sample per microplot, then processed by hand-crumbling and left to air dry for 4 weeks at room conditions. Afterward, each sample was sieved to remove organic debris and particles bigger than 0.8 mm. Duplicate samples containing 25 g of sieved soil from each microplot were suspended in 100 ml of sterile distilled water, shaken for 15 min at 250 rpm, and filtered through nested 250 and 20 µm sieves aiming to retain the most of persistent propagules of *V. dahliae* (Santos-Rufo et al. 2017 and Gómez-Gálvez et al 2019). The soil residues retained on the 20 µm sieve were recovered in 100 ml of agar solution (1 g of agar diluted on 1 L of sterile distilled water). Subsequently, 1 ml aliquots were plating onto sodium polypectate agar medium (Butterfield and DeVay 1977), using 10 plates for each replicate of 25 g of the soil sample. Plates were incubated for 14 days at 24±2 ℃ in the dark. After incubation, the soil particles onto the plates surface was washed with tap water and *V. dahliae* colonies were counted under a stereoscopic microscope. The number of colonies was expressed as propagules per gram of air-dried soil (ppg), considering that each colony was originated from a single propagule of the fungus.

Microplot soil temperature was hourly registered during one year of the total experiment period by probes and data loggers located in four different infested microplots at 15 cm of depth. Air temperatures were recorded by the meteorological station of the IFAPA 'Alameda del Obispo' (Córdoba, Spain) located near to the experimental trial.

2.4.4.Disease assessment

Olive trees were visually evaluated every month for 33 months from March 2016 until December of 2018. Symptoms severity was assessed based on the percentage of aerial part affected according to a scale from 0 to 4 (0=healthy tree; 1= 1-33% symptomatic; 2= 34-66% symptomatic; 3= 67-100% symptomatic; 4= completely dead) (Rodríguez-Jurado et al., 1993). During the evaluation period, the olive trees showing symptoms such as green defoliating or wilting leaves, chlorosis, curling leaves, or/and necrosis of shoots or branches were analyzed to confirm the infection by *V. dahliae*. For this assay, two stems were sampled from the symptomatic branches, washed in running tap water and disinfected in 10 % sodium hypochlorite for 1 min. Stem pieces of 3-5 mm were plated on chlortetracycline water agar medium and incubated at 24 ℃ in dark. Approximately, 9 days after plates were observed under microscope to identify *V. dahliae* conidiophores and/or microsclerotia.

Disease incidence (DI) was calculated as the percentage of symptomatic olive trees. Disease severity scores were used to calculate several disease parameters comprising the relative susceptibility index (RSI), which was calculated according to Arias-Calderón et al. (2015b).

$$RSI=[0.3\times RAUDPC+0.2\times FDII+0.3\times FDPI+0.05\times FDI+0.15\times(100-RDPF)]\times\frac{100}{SP}$$

where the relative area under the disease progress curve (RAUDPC) were calculated according to the following formula:

$$RAUDPC=\frac{100}{(s_{max}\times t_T)}\times\sum_{i=1}^{n}\frac{(s_i+s_{i+1})}{2}\times\Delta t$$

In RAUDPC: s_{max}= maximum value of severity (4); t_T= total evaluation period (33 months); s_i= severity value of the experimental unit in the observation i; n= number of observations; Δt= time between observations.

Disease intensity index (DII) was calculated for each observation time using the following formula:

$$DII=\sum_{x=1}^{n}\frac{(s_x\times n_x)}{(4\times n_T)}\times 100$$

In which s_x= disease severity value for individual olive tree; n_x= number of olive tree with s_x value; n_T= total number of olive trees for experimental unit.

Final disease incidence (FDI), final disease intensity index (FDII) and final dead plant incidence (FDPI) were estimated as the values for those parameters. Relative disease-free period (RDFP) corresponds to the percentage of the time before the appearance symptoms with reference to the total experiment period. SP represented the average of the susceptibility index obtained for the susceptible reference cultivar (‘Picual’).

Olive tree recovery and death rates were also calculated as a percentage of the total symptomatic trees evaluated for each genotype. Recovery from the disease was considered when an infected olive tree continued growing or re-sprouted diminishing the percentage of symptomatic aerial part until completely overcame the disease symptoms reaching a severity value of 0.

2.4.5.Statistical analysis

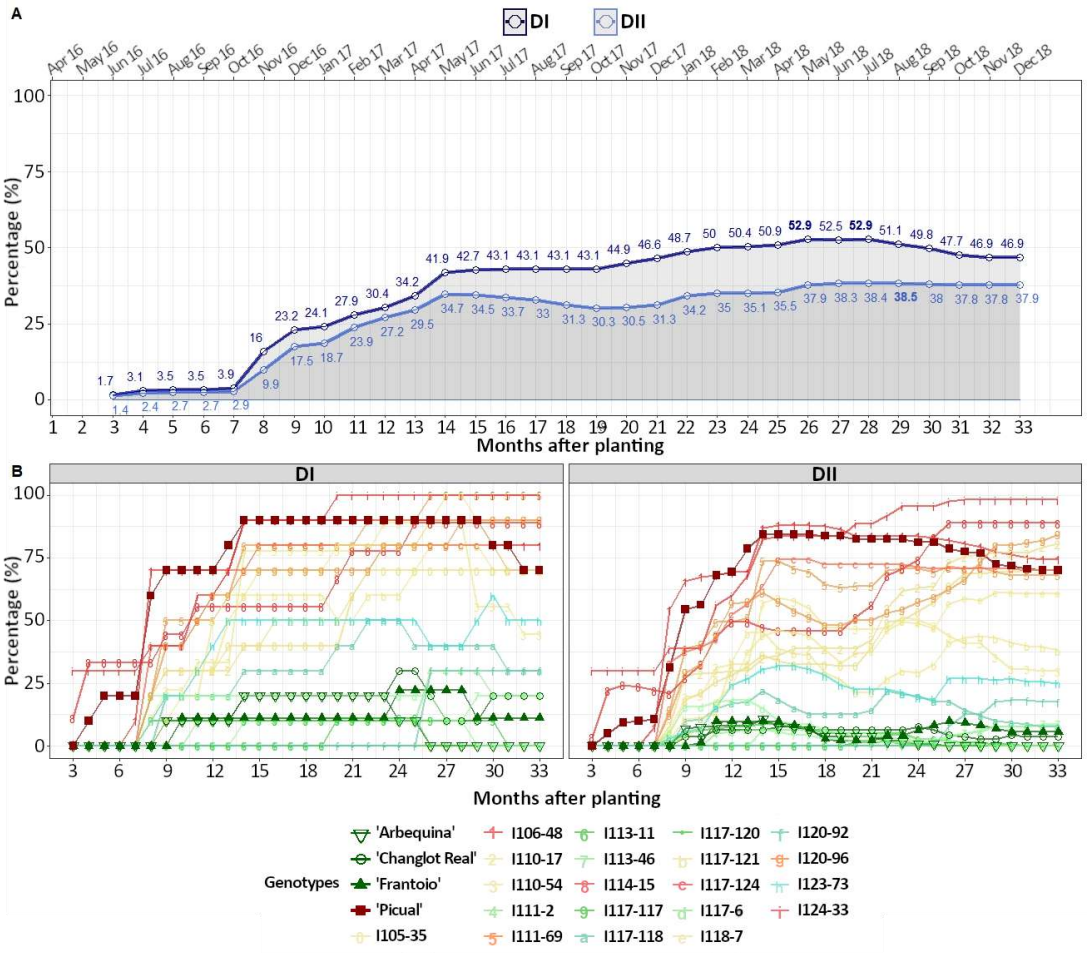
Statistical analysis was carried out using R version 3.6.2. Homogeneity of variances was tested applying Levene test of *car* package. Disease parameters, for which the homogeneity of variances was not met, were analyzed by Kruskal-Wallis test using *agricolae* package, followed by the post-hoc Dunn´s pairwise multiple comparison test of *dunn.test* package. Spearman rank correlation were performed using *corrplot* package, to evaluate relationships between RAUDPC and RSI at different evaluation times to decide the optimum evaluation period. Significant differences were considered for all analyses at P <0.05.

2.5. Results

2.5.1. Verticillium wilt development

Visual symptoms of disease were not observed on any of the olive trees from the control non-infested microplots throughout the experimental period, while, for olive trees in infested microplots, disease symptoms were first observed three months after planting (MAP) (Figure 2.1). Defoliation of green leaves was the most frequent symptom, although slow decline and apoplexy with leaf curling and wilting were observed in several cases but not linked to any specific genotype or observation time. In all the symptomatic cases, the *V. dahliae* infection was confirmed by fungal isolation in the laboratory.

Figure 2.1 Disease progression in 24 genotypes evaluated in V. dahliae-infested microplots for 33 months. A) Average disease incidence (DI) and Disease intensity index (DII) for the total of infested trees of the experiment. B) DI and DII for individual genotypes.



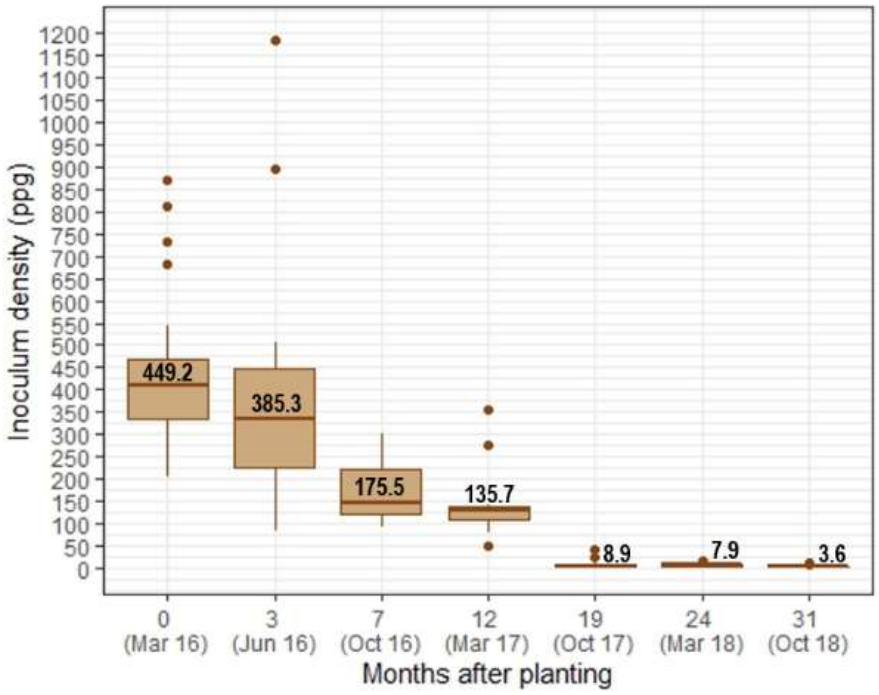
Symptoms increased irregularly in olive tree throughout the evaluation period. The first death plants were observed 3 months after planting, corresponding with the first plants showing symptoms. However, the disease development was variable among trees. Sometimes *V. dahliae* infection was lethal to the olive tree, whereas in other cases, symptoms affected only certain shoots or branches, while other healthy branches continued growing. Remission of symptoms was observed in several affected olive trees and, eventually, some of them were considered completely recovered from the disease symptoms.

Average disease incidence (DI) increased steadily up to 26 MAP, Spring 2018, when the percentage of total infected trees reached its maximum value of 52,9% (Figure 2.1A). From that moment an observed decline in average DI value was associated to symptoms recovery of some olive trees, which was more evident at the end of the experiment. Recovered olive trees were observed in the four control cultivars ('Picual', 'Arbequina', 'Changlot Real' and 'Frantoio') and 10 of the evaluated breeding selections (I106-48, I110-54, I111-2, I113-11, I117-117, I117-118, I117-121, I117-124, I117-6 and I123-73), as is shown by the decrease in the individual DI values of those genotypes (Figure 2.1B). It is worth noting that, the susceptible control 'Picual', reached its maximum incidence at 14 MAP (May 2017), similarly to I106-48, I117-117 and I117-6, whereas the incidence continued raising for the rest of genotypes. The genotypes showing initial symptoms later were registered at 30 MAP corresponding to 'Changlot Real' and I123-73.

Disease intensity index (DII) curve first increased and then presented fluctuations until the end of evaluation period (Figure 2.1). Although the first symptoms were observed 3 months after planting, it was not until the following autumn that the average DII start to increase significantly (from 7 MAP). One year after planting and after springtime (13-15 MAP), average DII reached values of 34.7-34.5%, quite close to the average value maximum (Figure 2.1A). A remission of severity symptom was observed during 16 to 19 MAP period (summertime and early fall), producing a decrease of average DII values. Afterwards, the severity of symptoms increased again until 29 MAP, when DII reached the maximum value of 38.5% (Figure 2.1A) just after another disease outbreak in the second spring of evaluation period (25-27 MAP). Declines on DII values from 29 to 33 MAP were due to lower severity of symptoms and total recovery of some symptomatic olive trees, which also reduced total DI values. However, several breeding selections reached the maximum individual DII value in the last months, such as I105-35, I110-17, I118-7 and I120-96 (Figure 2.1B). The fluctuations of DII values for single genotypes across time was due to the onset and remission of symptoms in some olive trees, which eventually turned out in the total or partial recovery or death of the tree.

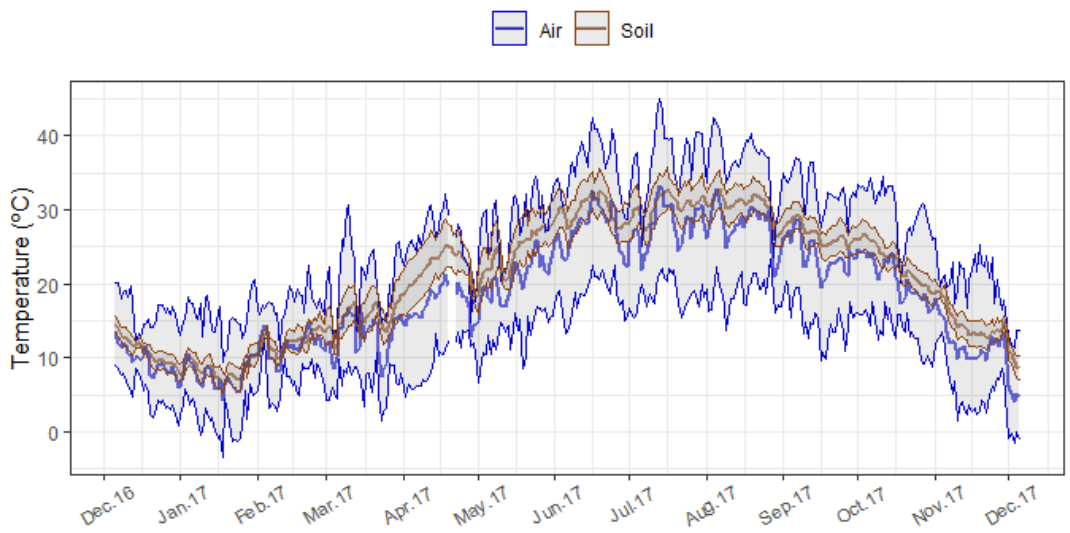
Quarterly analysis of soil inoculum revealed that the fungus remained viable along the evaluation period. Mean inoculum density of the total treatment continuously decreased over time, from 449.2 ppg at planting time to 385.3 ppg at first symptoms (3 MAP) and 3.6 ppg at 31 MAP, although wide variability was measured for the different microplots at each sampling time (Figure 2.2).

Figure 2.2 Variation of the V. dahliae inoculum density over time.



Environment (air and soil) temperature, considered an important factor in the disease progress and fungus survival, was monitored one year from December 2016 to December 2017 corresponding to the period 9 to 21 MAP (Figure 2.3). The overall fluctuation of temperatures was higher in air (-3.4 to 44.9℃) than in soil (5.1 to 35.8℃). Intraday variability in temperatures was also higher in air than soil due to higher minimum and lower maximum temperatures in the soil. The average daily mean temperature in soil was generally slightly higher than average daily air temperatures, ranging from 6.4 to 32.7℃ in soil and from 4.1 to 33.1℃ air for the total registered period.

Figure 2.3 Daily air and soil temperatures (average, maximum and minimum) in the experimental field from December 2016 (9 MAP) to December 2017 (21 MAP).



During this monitored period, a peak on incidence of the disease was observed at 14 MAP (corresponding to May 2017). Mean air temperature of 20.7°C and mean soil temperature of 24.4°C were registered at this time, with ranges of variation from 6.6°C to 34.5°C and 16.1°C to 31.2°C in air and soil, respectively.

2.5.2. Classification of genotypes

Significant differences among genotypes were observed for all disease parameters and two categories were clearly obtained for all of them, with none of the genotypes showing an intermediate disease response (Table 2.1). For RAUDPC, values above 24.3% corresponded to susceptible genotypes, while resistant genotypes showed values below 17.7%. It is noteworthy that I117-120 was the only genotype without symptomatic trees during the evaluation period. I113-11, I117-117, 'Arbequina' and I117-6 also showed null values of FDII and FDI, but disease symptoms were observed in some olive trees at some point in the experimental period which later were totally recovered. On the other hand, 100% of olive trees belonging to I124-33 and I111-69 were symptomatic (FDI = 100) at the end of the assessment period.

In general, susceptible genotypes began to develop symptoms earlier than resistant ones, therefore the latter ones showed higher RDFP values (Table 2.1). Similarly, susceptible genotypes reached higher values of FDPI, corresponding to higher death rates than the resistant ones (Figure 2.4). Among resistant genotypes, only I123-73 showed a FDPI value different from 0; additionally, this genotype displayed a 50% of FDI, which statistically differed from 'Arbequina' (Table 2.1). Therefore, although it was categorized as resistant based on RSI value, it could be reconsidered as susceptible, owing to the value of the other disease parameters. In this sense, 70% of I123-73 trees showed symptoms at any time of the assessment period, although registered a recovery rate of 29% (Figure 2.4). Likewise, some

susceptible genotypes also underwent higher incidence than those shown at the end of the experiment (FDI), such as 'Picual', I117-124, I118-7, I110-54 and I117-121, which had certain capacity to recover from the disease symptoms. In contrast, *V. dahliae* infection was especially lethal for symptomatic trees of I114-15 and I105-35 for which death rate reached 100% (Figure 2.4). In general, symptoms in susceptible genotypes were more severe, reaching higher death rate values, whereas resistant genotypes showed mainly mild or medium symptoms with high recovery rates and null death rates.

Table 2.1 Disease parameters measured 33 months after planting and resistance classification of the 24 genotypes evaluated (20 breeding selections and 4 control cultivars).

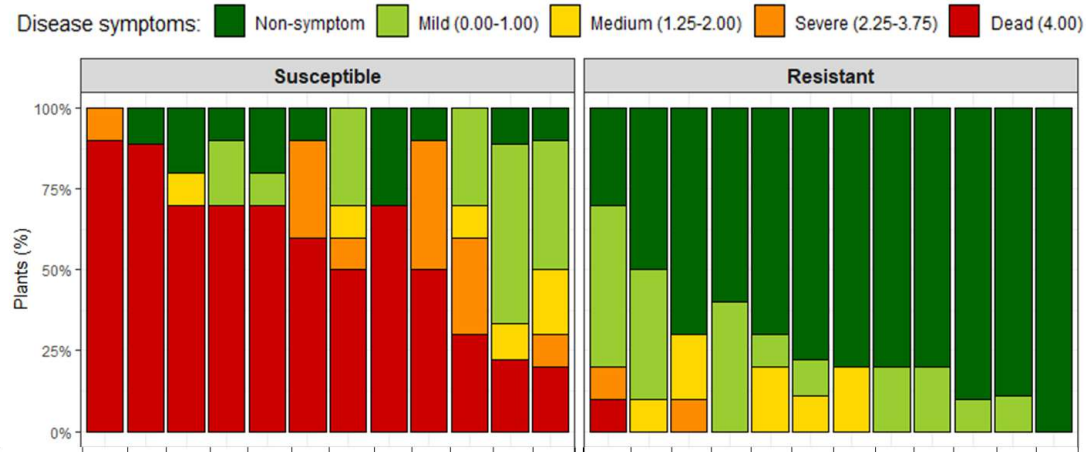
Genotype	Disease parameters[i]						Resistance Category[ii]
	RAUDPC	FDII	FDI	RDFP	FDPI	RSI	
I124-33	68.9 ACF	98.1 ACF	100.0 AF	30.3 ACF	90.0 ACF	125.1 ACF	S
I114-15	50.6 ACF	88.9 ACF	88.9 ACF	43.8 ACF	88.9 ACF	109.6 ACF	S
I106-48	60.9 ACF	74.4 ACF	80.0 ACF	35.2 ACF	70.0 ACF	102.6 ACF	S
'Picual'	58.9 ACF	70.0 ACF	70.0 ACF	33.3 ACF	70.0 ACF	100.0 ACF	S
I117-124	50.1 ACF	70.0 ACF	70.0 ACF	45.2 ACF	70.0 ACF	93.3 ACF	S
I120-96	43.8 ACF	84.4 ACF	90.0 ACF	47.0 ACF	60.0 ACF	91.4 ACF	S
I111-69	48.1 ACF	68.1 ACF	100.0 ACF	40.0 ACF	50.0 ACF	86.2 ACF	S
I105-35	34.1 ACF	70.0 ACF	70.0 ACF	60.9	70.0 ACF	82.5 ACF	S
I110-17	38.8 ACF	80.6 ACF	90.0 ACF	54.5 AF	50.0 ACF	81.7 ACF	S
I118-7	31.9 ACF	60.6 ACF	70.0 ACF	47.9 ACF	30.0	63.5 ACF	S
I110-54	31.0 ACF	29.9	44.4	47.1 ACF	22.2 P	48.5 ACF	S
I117-121	24.3 ACF	38.1 ACF	70.0 ACF	52.1 ACF	20.0 P	47.7 ACF	S
I123-73	17.7 P	25.0	50.0 A	63.9 P	10.0 P	32.1 P	R
I117-118	10.7 P	7.5 P	30.0	72.4 P	0.0 P	15.6 P	R
I120-92	3.4 P	17.5 P	30.0	93.6 P	0.0 P	10.6 P	R
'Changlot Real'	4.0 P	3.8 P	20.0 P	83.3 P	0.0 P	8.2 P	R
I111-2	3.6 P	8.1 P	20.0 P	89.1 P	0.0 P	8.1 P	R
'Frantoio'	4.6 P	5.6 P	11.1 P	89.2 P	0.0 P	7.0 P	R
I113-46	2.6 P	8.8 P	20.0 P	93.0 P	0.0 P	6.9 P	R
I117-6	5.2 P	0.0 P	0.0 P	84.8 P	0.0 P	5.8 P	R
'Arbequina'	2.7 P	0.0 P	0.0 P	87.0 P	0.0 P	4.2 P	R
I117-117	3.3 P	0.0 P	0.0 P	92.4 P	0.0 P	3.2 P	R
I113-11	0.2 P	0.0 P	0.0 P	95.6 P	0.0 P	1.1 P	R
I117-120	0.0 P	0.0 P	0.0 P	100.0 P	0.0 P	0.0 P	R

Disease parameters: Relative area under disease progress curve (RAUDPC), Final disease intensity index (FDII), Final disease incidence (FDI), Relative disease-free period (RDFP), Final dead plants incidence (FDPI), Relative susceptibility index (RSI). Superscript letters mean significative differences with reference cultivars ('Picual' (P), 'Arbequina' (A), 'Changlot Real' (C) and 'Frantoio' (F)) according to Dunn´s pairwise multiple comparison test at $p \leq 0.05$.[ii] Resistance category based on RSI value. Resistant (R), significantly different from 'Picual' and susceptible (S), significantly different from 'Frantoio'.

Relative susceptibility index (RSI) was finally used to categorize for verticillium wilt response of the different genotypes with two groups, resistant and susceptible, showing significant

differences between them. As expected, disease parameters for 'Picual' cultivar showed susceptible response to *V. dahliae*. In contrast, 'Arbequina' revealed values for disease parameters similar to traditional resistant controls 'Changlot Real' and 'Frantoio'. In total, 11 out of 20 breeding selections resulted susceptible, among which I124-33, I114-15 and I106-48 exhibited higher RSI values than the susceptible reference 'Picual'. Similarly, three resistant selections (I117-120, I113-11 and I117-117) showed lower RSI values than the best resistant control cultivar.

Figure 2.4 Frequency of trees with different classes of severity symptoms, and death and symptoms recovery rates for the 24 genotypes evaluated.



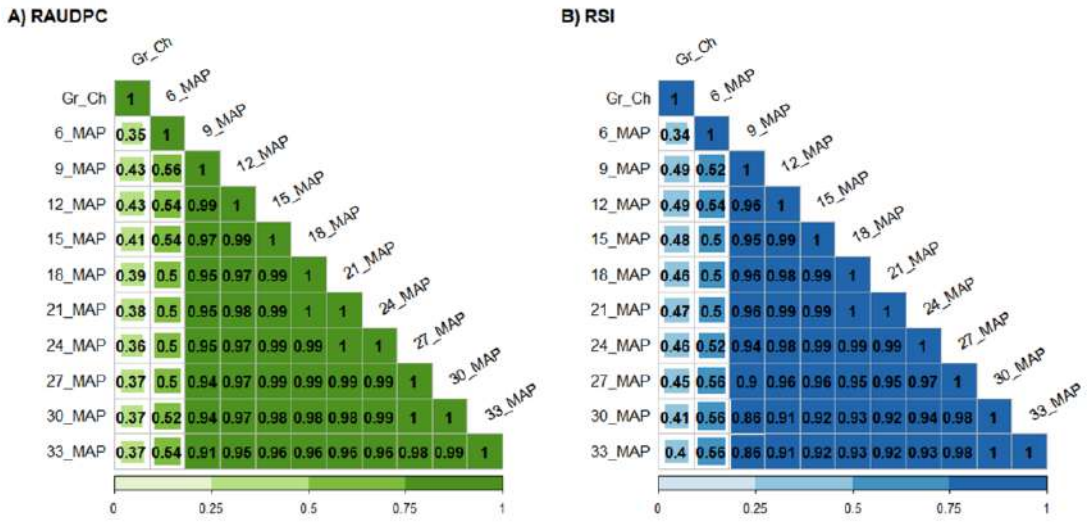
Genotype	I124-33	I114-15	I106-48	'Picual'	I117-124	I120-96	I111-69	I105-35	I110-17	I118-7	I110-54	I117-121	I123-73	I117-118	I120-92	'Changlot Real'	I111-2	'Frantoio'	I113-46	I117-6	'Arbequina'	I117-117	I113-11	I117-120
Death rate (%)	90	100	88	78	88	67	50	100	56	30	25	22	14	0	0	0	0	0	0	0	0	0	0	0
Recovery rate (%)	0	0	0	22	13	0	0	0	0	30	50	22	29	40	0	50	33	50	0	100	100	100	100	0

Disease symptoms severity was classified according to a scale from 0 to 4 in five classes as following: without symptoms, mild symptoms (0-1, including recovered trees), medium symptoms (1.25-2.00), severe symptoms (2.25-3.75) and dead trees (4). Death and recovery rates were calculated as a percentage of the total symptomatic trees.

Comparing the results obtained in this work with the previously obtained for the same genotypes under root-dip artificial inoculation and evaluation in growth chamber showed low correlation for RAUDPC and RSI parameters at the end of the experimental period for both methodologies, with Spearman r values of 0.37 and 0.4, respectively (Figure 2.5). However, a high correlation between RAUDPC and RSI was revealed for microplot results obtained at different timeframes. Correlation coefficients were high for evaluations made from 9 to 33 MAP, having an r value close to 1 those made from 28 to 33 MAP.

Figure 2.5 Spearman correlation analysis for average values per genotype for RAUDPC and RSI disease parameters among previous evaluation made in growth chamber (Arias-Calderón et al., 2015a, b) and

microplots evaluation from 6 to 33 MAP. A) Relative area under disease progress curve (RAUDPC). B) Relative susceptibility index (RSI).

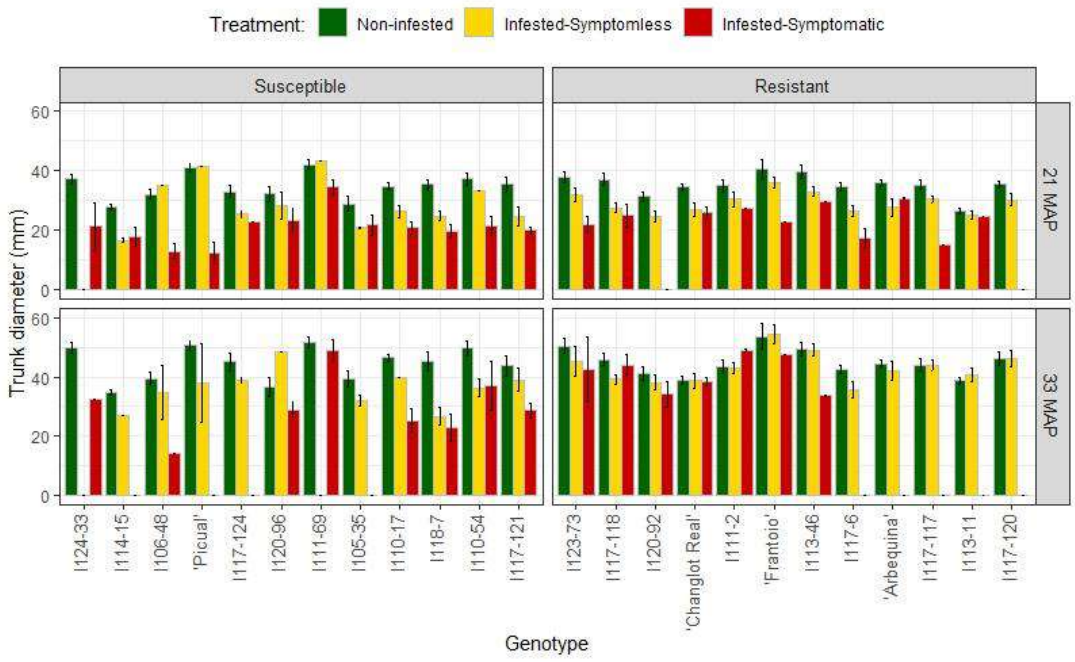


Growth chamber results (Gr_Ch), Microplot results at different months after planting (MAP).

2.5.3. Effect of Verticillium wilt on tree vigor and fruit bearing

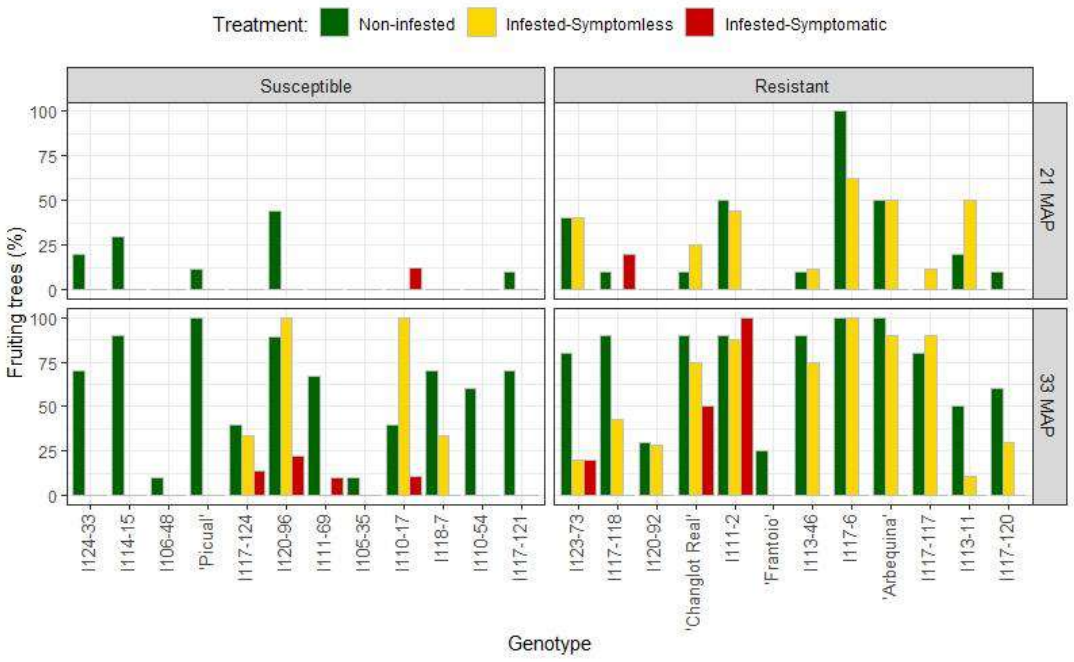
Tree growth assessed through measurements of trunk diameter was, in general, lower in infested than non-infested plants (Figure 2.6). The differences were more evident in trees expressing disease symptoms and more remarkable for susceptible genotypes, whereas light variability on trunk diameters was observed for resistant ones, particularly at the end of the experimental period. 'Frantoio' was the most vigorous among resistant cultivars, whereas 'Picual' and I111-69 were the most vigorous among susceptible genotypes (Figure 2.6). Resistant breeding selections showed wide variability in trunk diameter measurements, among them similar trunk diameters were registered for I117-117, I113-11, I117-120 and 'Arbequina'.

Figure 2.6 Trunk diameter in trees from non-infested control and infested treatment plots (symptomless and symptomatic trees) according to resistance category and genotype.



The effect of verticillium wilt on olive tree development was also observed for earliness of bearing. Notable differences in the proportion of fruiting trees were registered according to the inoculation treatment (non-infested vs infested) and resistance category of the genotypes (resistant vs susceptible) in both harvest seasons (Figure 2.7). At the first harvest season (21 MAP), early-bearing genotypes were mainly observed among resistant genotypes. 'Frantoio', in spite of its resistant response, showed a long unproductive period, similar to I106-48 and I105-35 susceptible breeding selections. On the contrary, I117-6 was the genotype with shorter unproductive period and higher productive potential, reaching both years the maximum number of fruiting trees. Remarkable results in terms of earliness of bearing were also observed for I111-2, which showed high percentage of fruiting trees regardless the treatment and symptomatology stage.

Figure 2.7 Percentage of fruiting trees from non-infested control and infested treatment plots (symptomless and symptomatic trees) according to resistance category and genotype.



2.6. Discussion

Testing breeding selections in *V. dahliae* microplots allowed the evaluation of tree growth and development of VWO epidemics in natural environment conditions in soil infested with one single pathotype. The defoliating pathotype of *V. dahliae* caused symptoms as defoliation of green leaves peaking from late fall through late spring as that occurring in natural infested soils (Navas-Cortés et al. 2008; Jiménez-Díaz et al. 2011). In this semi-controlled conditions, disease external symptoms started at three months after planting, whereas it could be detected 7 days after inoculation by root dip method in a grow chamber under favorable conditions (Arias-Calderón et al. 2015a). Soil inoculum could require longer time to establish in the soil and reach the roots. These results suggest that earliness of symptoms development depends on inoculation methods as previously described comparing evaluation procedures under controlled conditions in growth chamber, affecting also other disease parameters as FDI or FDII (López-Escudero et al. 2007; Jiménez-Fernández et al. 2016). The same happened when comparing different inoculation methods under controlled conditions in greenhouse (Cirulli et al. 2008). Different disease response was also observed for 'Picual' cultivar comparing root dip versus soil inoculation with cornmeal-sand medium (Varo et al. 2016a).

DI and DII showed a general tendence to increase during the disease evaluation period, although decreasing values for these disease variables were also observed in some genotypes at certain time points. This might be due to both the symptom remission and the recovery of some trees as previously observed in long-term assays (Santos-Rufo et al. 2017).

This behavior is usual in olive trees in naturally infested olive orchards (Navas-Cortés et al. 2008; Trapero et al. 2013a) where symptom has been observed from May to August (Bubici and Cirulli 2014), similar to our microplots observations. Natural symptoms recovery has been associated to resistant olive cultivars (López-Escudero and Blanco-López 2005), which is in line with our results in microplots, where genotypes such as I117-6, I117-117, I113-11, 'Arbequina', 'Frantoio' and 'Changlot Real', categorized as R, showed higher symptoms recovery rates. In this sense, tree recovery could be considered a resistant mechanism consisting on the ability to restrict the fungus invasion, which has also been associated to structural and compositional differences of vascular tissues (Keykhasaber et al. 2018). Lower values of the disease severity index and higher symptoms recovery rates observed in resistant genotypes could support the hypothesis of resistant trees having the capacity to activate mechanisms for the compartmentalization of infected xylem vessels after fungal infection (Keykhasaber et al. 2018).

In addition, symptom remission could be promoted by environmental conditions adverse to fungal development, with high air temperatures during summer causing the declines in DI and DII observed from 14 to 18 MAP in microplots. This effect is characteristic of VWO epidemics (López-Escudero et al. 2007; Navas-Cortés et al. 2008). Similarly, soil temperatures could determine the degree of VWO development among genotypes. In this sense, different optimum temperatures were estimated inducing *V. dahliae* infection depending on the olive cultivar which could range from 16 to 24℃ in 'Picual' and 'Arbequina' for defoliating pathotype, whereas temperatures up to 32℃ reduced disease progress in both cultivars (Calderón et al. 2014). In our work, average daily air and soil temperatures ranged from 4.3 to 25.8℃ and 6.4 to 28.4℃, respectively, between December 2016 and May 2017 (9-14 MAP), concomitant with an increase of DI and DII values i.e. in the susceptible 'Picual'. Furthermore, average soil temperatures over 30℃ were observed from June to September (14-18 MAP), together with a remission of DI and symptoms severity, and even with a significant decrease of inoculum density.

During the adverse conditions for VWO development described from June to September, olive trees continued growing and produced new shoots. This new growth remained healthy in the case of fully recovered trees such as 'Arbequina' and I111-2 or showed symptoms again when the environmental conditions became favorable for the progress of the disease as observed for I123-73. The reasons for this VWO relapses are not fully understood (López-Escudero and Blanco-López 2005; López-Escudero and Mercado-Blanco 2011; Bubici and Cirulli 2014). It could be attributable to fungus remaining viable inside the tree plant or due to new root infections produced by soil inoculum that remained viable over the whole assessment period. It should be noted that even though soil inoculum suffered the consequence of adverse high temperatures, registering a sharp decline after summer (at 7 and 19 MAP), as in other studies with similar type of inoculum (Santos-Rufo et al. 2017; Gómez-Gálvez et al. 2020), the remaining propagules amount was enough to cause epidemic or re-infect the roots (López-Escudero and Blanco-López 2007). In some cases, light increases or variability on inoculum densities were registered for specific microplots which could be also due to the measure technique or the presence of fungus aggregates into the soil samples as observed in another study in microplots (López-Escudero and Blanco-López 2007). Despite the different inoculum densities measured among microplots, there were no

differences on disease incidence values among experimental blocks. In this sense, similar inoculum densities could be found in soils of commercial olive orchard (Roca et al. 2016; Habib et al. 2017), but disease incidence has not been directly related to the microsclerotia density in soils (López-Escudero and Blanco-López 2007; Roca et al. 2016; Santos-Rufo et al. 2017).

According to RSI classification method, only two resistance categories resulted after assessment in microplot trial, compared with the three categories (susceptible, moderately susceptible and resistant) previously described for test in growth chamber (Arias-Calderón et al. 2015a, b). Differences in DI and symptoms severity was found between these works for the susceptible control 'Picual' used for RSI standardization. It was probably due to different experimental condition of inoculum and/or environment. In this work, the susceptible response of 'Picual' was consistent with previous evaluations, as well as, 'Frantoio' and 'Changlot Real' responded as resistant cultivars (López-Escudero et al. 2004; Martos-Moreno et al. 2006; Trapero et al. 2013a; Arias-Calderón et al. 2015a, b). Meanwhile, 'Arbequina' response have been described very variable, from extremely susceptible by root dip in growth chamber (López-Escudero et al. 2004; Martos-Moreno et al. 2006) to moderate susceptible in greenhouse (Trapero et al. 2015) or even resistant in fields (Trapero et al. 2013a) and microplots. These differences could be explained also by divergence experimental or by the symptom recovery typical of long-term experiments.

Eleven out of the 20 tested genotypes reached a DI higher than 70% and were characterized as susceptible, which was observed in olive cultivars evaluated in naturally infested soils with a high inoculum density (Trapero et al. 2013a). This represents contrasting results, since all of the genotypes evaluated in this work were previously selected as potentially resistant when inoculated by root-dip and evaluated in a growth chamber (Arias-Calderón et al. 2015a, b). Thus, the results in microplot with infested soil showed a very low correlation values with the previous evaluations under growth chamber conditions, despite the fact that the same defoliating isolate of *V. dahliae* was used (Arias-Calderón et al. 2015a, 2015b). The different category assigned to the genotypes could be due to experimental escapes in the previous inoculation by root-dip method or also because of the non-limited root growth in microplot, which could increase the contact probability between roots and *V. dahliae* propagules (Navas-Cortés et al. 2008). Disease response could be affected also in different ways for using different fungal structures as inoculum (conidia or microsclerotia) (Gómez-Gálvez et al. 2019) or different media for fungal culture in the laboratory (Varo et al. 2016a, b).

Despite the differences found between the two testing methods, root dip is considered an effective first screening test in early stages of the breeding program showing results in shorter time and requiring less space (Trapero et al. 2013b; Arias-Calderón et al. 2015a, b). While, soil inoculation could be useful for a long-term re-evaluation (Cirulli et al. 2008) during which the reaction to VWO could be studied in the first months after field planting when the disease impact used to be more damaging (Jiménez-Díaz et al. 2011; Trapero et al. 2013b). Results in this work support the possibility of shortening the assessment period in infested microplots conditions to only around 12-15 MAP. The results obtained for RAUDPC and RSI values showed a strong correlation from that point of the experiment and, therefore, similar classification of genotypes was obtained since then.

The trial results revealed also important differences in tree growth and earliness of bearing. Several reports showed the reduction of vegetative growth of olive after artificial inoculations with *V. dahliae*, describing the delay or cessation of stem growth as a typical symptom of VWO (Martos-Moreno et al. 2006) even in symptomless trees (García-Ruiz et al. 2015). The inhibition of olive growth caused by the infection of the defoliating pathotype of *V. dahliae* have been analyzed through the fresh and dry weight of olive trees (Birem et al. 2016; Sanei and Razavi 2017; Santos-Rufo et al. 2018; Gómez-Gálvez et al. 2020). Shoot length, stem diameter, number of leaves per tree, average leaf area, canopy volume and relative below-ground biomass were significantly reduced by 17-38.5 % in 'Picual' grown in infested soil by defoliating pathotype in natural environmental conditions (Santos-Rufo et al. 2018; Gómez-Gálvez et al. 2020). Differential behavior was described among olive cultivars for reduction of root and canopy growth (Sanei and Razavi 2017). In this work, olive tree growth in microplots was evaluated through trunk diameter measurements, which is considered a highly representative measure for the total tree vigor (León et al. 2007). Smaller trunk diameters were measured in olive trees in infested plots than in non-infested ones, being even smaller for symptomatic trees of both susceptible and resistant genotypes. Among the studied cultivars, 'Frantoio' registered the highest trunk diameter values, confirming the high vigor of this cultivar (León et al. 2007).

A negative effect of the disease on earliness of bearing was also observed, particularly for susceptible genotypes. Therefore, the cultivation of susceptible genotypes could imply a substantial decrease of fruit production even when the plants are asymptomatic, because of both longer unproductive period and higher disease severity, in contrast to resistant genotypes. This loss of productivity caused for VWO has been previously described for many olive cultivars all around the world (Jiménez-Díaz et al. 2011). For 'Picual', the infection caused by the defoliating pathotype reduced by 88,7% the number of fruits per tree in a study with infested soil (Gómez-Gálvez et al. 2020). Among resistant cultivars, 'Frantoio' confirmed the longest unproductive period compared to 'Arbequina' or 'Picual', as previously reported (León et al. 2007). Among the breeding selections, some resistant genotypes such as I111-2 and I117-6 showed shorter unproductive period and high percentage of fruiting trees, even under inoculation conditions, which represents an agronomic trait of paramount importance.

The usefulness of the evaluation of breeding selections for VWO resistance by artificial infested soils with propagules of *V. dahliae* defoliating pathotype was demonstrated under natural environmental conditions. Disease and tree growth followed a similar progress that those in commercial olive orchards. The evaluation process resulted more restrictive in identifying resistant genotypes than a previous evaluation in growth chamber. The lack of correlations among disease parameters registered from disease assessment under controlled and natural environment conditions revealed the important role of the environment in the olive genotype-*V. dahliae* interaction. Future works will be necessary to determine the effect of environmental conditions on the resistance stability in olive across different environmental conditions. A comprehensive agronomic characterization of the resistant promising selections for characters such as oil content and quality and suitability for different olive growing systems will be also necessary before releasing them as new cultivars.

2.7. Acknowledgements

This research was financially supported by IFAPA project AVA2019.027, partially funded by European Regional Development Fund (ERDF). Alicia Serrano is grateful for the funding received from the Researcher Training Program in INIA-Autonomous Communities Research Centers (FPI-INIA).

2.8. References

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., De la Rosa, R., and Rodríguez-Jurado, D. 2015a. Resistance to Verticillium wilt in olive progenies from open-pollination. Scientia Horticulturae. 185:34–42.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., De la Rosa, R., and León, L. 2015b. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica. 206:619–629.

Arias-Calderón, R., Rouiss, H., Rodríguez-Jurado, D., De la Rosa, R., and León, L. 2014. Variability and heritability of fruit characters in olive progenies from open-pollination. Scientia Horticulturae. 169:94–98.

Birem, F., Alcántara-Vara, E., and López-Escudero, F. J. 2016. Water consumption and vegetative growth progress in resistant and susceptible olive cultivars infected by *Verticillium dahliae*. AS. 07:230–238.

Bubici, G., and Cirulli, M. 2014. Natural recovery from Verticillium wilt in olive: can it be exploited in a control strategy? Plant Soil. 381:85–94.

Butterfield, E. J., and DeVay, J. E. 1977. Reassessment of soil assays for *Verticillium dahliae*. Phytopathology 67:1073-1078.

Calderón, R., Lucena, C., Trapero-Casas, J. L., Zarco-Tejada, P. J., and Navas-Cortés, J. A. 2014. Soil temperature determines the reaction of olive cultivars to *Verticillium dahliae* pathotypes. PLoS ONE. 9:e110664.

Cirulli, M., Colella, C., D'Amico, M., Amenduni, M., and Bubici, G. 2008. Comparison of screening methods for the evaluation of olive resistance to *Verticillium dahliae* Kleb. Journal of Plant Pathology. 90:7–14.

Fernández-Escobar R., De la Rosa R., León L., Gómez J.A., Testi F., Orgaz M., Gil-Ribes J.A., Quesada-Moraga E., Trapero A., Msallem M. 2013. Evolution and sustainability of the olive production systems. In : Arcas N. (ed.), Arroyo López F.N. (ed.), Caballero J. (ed.), D'Andria R. (ed.), Fernández M. (ed.), Fernández-Escobar R. (ed.), Garrido A. (ed.), López-Miranda J. (ed.), Msallem M. (ed.), Parras M. (ed.), Rallo L.(ed.), Zanoli R. (ed.). Present and future of the Mediterranean olive sector. Zaragoza: CIHEAM / IOC, p. 11-42 (Options Méditerranéennes: Série A. Séminaires Méditerranéens; n. 106).

García-Ruiz, G. M., Trapero, C., Del Rio, C., and López-Escudero, F. J. 2014. Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. Phytoparasitica. 42:205–212.

García-Ruiz, G. M., Trapero, C., Varo-Suárez, Á., Trapero, A., and López-Escudero, F. J. 2015. Identifying resistance to Verticillium wilt in local Spanish olive cultivars. Phytopathologia Mediterranea. 54:453-460.

Gómez-Gálvez, F. J., Hidalgo-Moya, J. C., Vega-Macías, V., Hidalgo-Moya, J. J., and Rodríguez-Jurado, D. 2019. Reduced introduction of *Verticillium dahliae* through irrigation systems and accumulation in soil by injection of peroxygen-based disinfectants. Plant Pathology. 68:116–126.

Gómez-Gálvez, F. J., Vega-Macías, V., Hidalgo-Moya, J. C., Hidalgo-Moya, J. J., and Rodríguez-Jurado, D. 2020. Application to soil of disinfectants through irrigation reduces *Verticillium dahliae* in the soil and verticillium wilt of olive. Plant Pathology. 69:272–283.

Goud, J.-K. C., Termorshuizen, A. J., and van Bruggen, A. H. C. 2011. Verticillium wilt in nursery trees: damage thresholds, spatial and temporal aspects. Eur J Plant Pathol. 131:451–465.

Habib, W., Choueiri, E., Baroudy, F., Tabet, D., Gerges, E., Saab, C., et al. 2017. Soil inoculum density of *Verticillium dahliae* and Verticillium wilt of olive in Lebanon. Annals of Applied Biology. 170:150–159.

Huisman, O. C., and Ashworth, L. J. 1974. Quantitative assessment of *Verticillium albo-atrum* in field soils: procedural and substrate improvements. Phytopathology 64:1043-1044.

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., del Mar Jiménez-Gasco, M., Antoniou, P. P., and Tjamos, E. C. 2011. Verticillium wilt, a major threat to olive production: current status and future prospects for its management. Plant Disease. 96:304–329.

Jiménez-Fernández, D., Trapero-Casas, J. L., Landa, B. B., Navas-Cortés, J. A., Bubici, G., Cirulli, M., et al. 2016. Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. Plant Pathol. 65:1279–1291.

Keykhasaber, M., Thomma, B. P. H. J., and Hiemstra, J. A. 2018. Verticillium wilt caused by *Verticillium dahliae* in woody plants with emphasis on olive and shade trees. Eur J Plant Pathol. 150:21–37.

Landa, B. B., Pérez, A. G., Luaces, P., Montes-Borrego, M., Navas-Cortés, J. A., and Sanz, C. 2019. Insights into the effect of *Verticillium dahliae* defoliating-pathotype infection on the content of phenolic and volatile compounds related to the sensory properties of virgin olive oil. Front. Plant Sci. 10:232.

León, L., De la Rosa, R. de la, Barranco, D., and Rallo, L. 2007. Breeding for early bearing in olive. HortScience. 42:499–502.

Levin, A. G., Lavee, S., and Tsror (Lahkim), L. 2003. Epidemiology of *Verticillium dahliae* on olive (cv. Picual) and its effect on yield under saline conditions. Plant Pathology. 52:212–218.

López-Escudero, F. J., and Blanco-López, M. A. 2005. Recovery of young olive trees from *Verticillium dahliae*. Eur J Plant Pathol. 113:367–375.

López-Escudero, F. J., and Blanco-López, M. A. 2007. Relationship between the inoculum density of *Verticillium dahliae* and the progress of Verticillium wilt of Olive. Plant Disease. 91:1372–1378.

López-Escudero, F. J., Blanco-López, M. Á., Rincón, C. D. R., and Reig, J. M. C. 2007. Response of olive cultivars to stem puncture inoculation with a defoliating pathotype of *Verticillium dahliae*. HortScience. 42:294–298.

López-Escudero, F. J., and Mercado-Blanco, J. 2011. Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. Plant Soil. 344:1–50.

López-Escudero, F. J., Mercado-Blanco, J., Roca, J. M., Valverde-Corredor, A., and Blanco-López, A. M. 2010. Verticillium wilt of olive in the Guadalquivir Valley (Southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. Phytopathologia Mediterranea. 49:370–380.

López-Escudero, F. J., del Río, C., Caballero, J. M., and Blanco-López, M. A. 2004. Evaluation of olive cultivars for resistance to *Verticillium dahlia*e. European Journal of Plant Pathology. 110:79–85.

Martos-Moreno, C., López-Escudero, F. J., and Blanco-López, M. A. 2006. Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae.* HortScience. 41:1313–1316.

Montes-Osuna, N., and Mercado-Blanco, J. 2020. Verticillium wilt of olive and its control: What did we learn during the last decade? Plants. 9:735.

Navas-Cortés, J. A., Landa, B. B., Mercado-Blanco, J., Trapero-Casas, J. L., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. 2008. Spatiotemporal analysis of spread of infections by *Verticillium dahliae* pathotypes within a high tree density olive orchard in southern Spain. Phytopathology. 98:167–180.

Roca, L. F., Moral, J., Trapero, C., Blanco-López, M. Á., and López-Escudero, F. J. 2016. Effect of inoculum density on Verticillium wilt incidence in commercial olive orchards. Journal of Phytopathology. 164:61–64.

Rodríguez-Jurado, D. 1993. Interacciones huésped-parásito en la marchitez del olivo (*Olea europaea* L.) inducida por *Verticillium dahliae* Kleb. PhD Thesis, University of Córdoba, Spain (in Spanish).

Rodríguez-Jurado, D., Moraño-Moreno, R., Bejarano-Alcázar, J. 2008. Dispersion of defoliating and non-defoliating pathotype of *Verticillium dahliae* in host crops by irrigation water in southern Spain. European Journal of Plant Pathology. 90:419–420.

Sanei, S. J., and Razavi, S. E. 2017. Resistance and vegetative growth analysis of some olive cultivars in response to a defoliating pathotype of *Verticillium dahliae* Kleb. International Journal of Horticultural Science and Technology. 4:239–250.

Santos-Rufo, A., Hidalgo, J. J., Hidalgo, J. C., Vega, V., and Rodríguez-Jurado, D. 2018. Morphophysiological response of young olive trees to Verticillium wilt under different surface drip irrigation regimes. Plant Pathology. 67:848–859.

Santos-Rufo, A., Vega, V., Hidalgo, J. J., Hidalgo, J. C., and Rodríguez-Jurado, D. 2017. Assessment of the effect of surface drip irrigation on *Verticillium dahliae* propagules differing in persistence in soil and on verticillium wilt of olive. Plant Pathology. 66:1117–1127.

Trapero, C., Díez, C. M., Rallo, L., Barranco, D., and López-Escudero, F. J. 2013b. Effective inoculation methods to screen for resistance to Verticillium wilt in olive. Scientia Horticulturae. 162:252–259.

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. 2015. Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. Plant Pathology. 64:890–900.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., and López-Escudero, F. J. 2013a. Field resistance to Verticillium wilt in selected olive cultivars grown in two naturally infested soils. Plant Disease. 97:668–674.

Varo, A, Moral, J., Lozano-Tóvar, M. D., and Trapero, A. 2016a. Development and validation of an inoculation method to assess the efficacy of biological treatments against Verticillium wilt in olive trees. BioControl. 61:283–292.

Varo, A., Raya-Ortega, M. C., and Trapero, A. 2016b. Enhanced production of microsclerotia in recalcitrant *Verticillium dahliae* isolates and its use for inoculation of olive plants. Journal of Applied Microbiology. 121:473–484.

3. Chemical Components Influencing Oxidative Stability and Sensorial Properties of Extra Virgin Olive Oil and Effect of Genotype and Environment on Their Expression

This chapter has been published in the journal 'LWT-Food science and technology'. Serrano, A., De la Rosa, R., Sánchez-Ortiz, A., Cano, J., Pérez, A.G., Sanz, C., Arias-Calderón, R., Velasco, L., León, L. (2021).

3.1. Abstract

Extra virgin olive oil (EVOO) chemical composition is characterized by high content of monounsaturated fatty acids and minor compounds including phenols, sterols, tocopherols, squalene and volatile compounds. These components are related to EVOO quality in terms of healthy properties, shelf life alteration due to susceptibility to oxidative degeneration and sensory properties. In this work, the variability of 66 different chemical compounds, oxidative stability and sensory attributes of EVOO was analyzed in order to study the relationships among them and the effect of cultivar, growing location and their interaction on their expression. Partial least squares (PLS) regression models allowed accurate prediction for EVOO stability on the basis of the chemical composition of the oils, with marked positive influence of oleic acid and 3,4-DHPEA-EA phenol content on stability values, while poor prediction results were obtained for sensory attributes. Cultivar and location showed limited effect on the sensory properties of EVOO, even though the same factors provide significant effect for the rest of chemical compounds and stability. These results should be taken into account in breeding programs aimed to obtain new cultivars with improved EVOO characteristics and to determine the best cultivar to be planted in each environment.

3.2. Keywords

Fatty acid composition; minor components; oil quality; *Olea europaea*; olive breeding.

3.3. Introduction

Extra virgin olive oil (EVOO) contributes to the healthy and nutritional properties of the Mediterranean diet inscribed in 2013 on the Representative List of the Intangible Cultural Heritage of Humanity by UNESCO (Radd-Vagenas et al., 2017). Its fatty acid composition, mainly composed of monounsaturated fatty acids, as well as a myriad of minor components including phenols, sterols, tocopherols and squalene, are the main responsible for the healthy properties of EVOO, particularly regarding cardiovascular diseases, inflammation, cancer and a general increase in life expectancy (Francisco et al., 2019; Gouvinhas et al., 2017). These components are also responsible for EVOO quality in terms of shelf life, mainly related to alteration due to susceptibility to oxidative degeneration (Aparicio et al., 1999; Mateos et al., 2003). The EVOO sensory profile is the result of a combination of taste, odor and chemical responses produced by different compounds. Among these sensorial properties, three main positive attributes (fruity, bitter and pungent) are used for classification of EVOO (IOC, 2018).

Several associations between individual components or groups of components and oxidative stability have been attempted (Aparicio et al., 1999; Mateos et al., 2003). Similarly, correlations among several phenolic compounds and EVOO sensorial attributes bitterness and pungency as well as several volatile compounds and fruity sensorial attribute have been reported (Andrewes et al., 2003; Campestre et al., 2017; Cerretani et al., 2008; Mateos et al., 2004). However, comprehensive studies including proper experimental design able to identify the main factors affecting the chemical composition of EVOO have not been carried

out. Also, the potential effects of these factors on the association between chemical composition and oxidative stability and sensorial properties are poorly understood.

Recent works indicate that the genetic effect is the main source of variation for most EVOO chemical components and a high variability for oil composition has been reported in different olive plant materials (Cerretani et al., 2008; de la Rosa et al., 2016; García-Vico et al., 2017; León et al., 2018). This genetic influence is also claimed regarding both oxidative stability and sensorial properties. In fact, the peculiarity of certain local cultivars is considered one of the main singularities for EVOO Protected Denomination of Origin declarations. Moreover, environmental influence on chemical components, oxidative stability and sensorial properties of EVOO has also been reported, particularly from studies of single cultivars grown in different locations (Ben Mansour et al., 2017; Issaoui et al., 2010).

However, genotype by location studies on EVOO quality are very scarce and necessaries, as recent works indicate a differential performance of cultivars under different environments for olive fruit traits (Navas-López et al., 2019). Particularly, as far as we know, the combined effect of genotype and location on the potential associations among chemical components, oxidative stability and sensorial properties of EVOO is completely unknown. Therefore, the present work aims to determine the genetic and location effects and their interaction on the variability of 66 chemical components of EVOO, and in its stability and sensory profile. For that, four different cultivars were evaluated in this work. 'Picual' is the most widely grown cultivar in Spain and (Barranco et al., 2000). It shows many favorable agronomic characteristics, such as early bearing, high productivity and easy mechanical harvesting, and also produces highly appreciated EVOO characterized by high oleic acid content and stability. However, its high susceptibility to Verticillium wilt caused by the soil fungus *Verticillium dahliae* hindered its cultivation in some areas, which promotes the development of breeding programs for Verticillium wilt resistance (Arias-Calderón et al., 2015). EVOO from three advanced selections of this breeding program were also evaluated in this work. Data gathered were also used to investigate how the variability of EVOO chemical composition is influencing both its stability and sensory profile.

3.4. Materials and methods

3.4.1. Plant materials

Three advanced selections of the breeding program for Verticillium wilt resistance developed at IFAPA were evaluated, together with 'Picual' as a reference cultivar. One of the selections (Sel1) comes from open pollination of 'Koroneiki' and the other two (Sel2 and Sel3) from crosses between 'Frantoio' and 'Arbosana'. All four genotypes were planted in comparative trials in spring of 2015 in three locations in Jaén province, Arjona, Begíjar and Úbeda, hereafter named as Loc1, Loc2 and Loc4 respectively. In 2016, the four genotypes were also planted in experimental microplots at IFAPA research Centre, Córdoba (Loc3). In all these comparative trials, the genotypes were distributed in three randomized blocks with 4 to 6 plants per elementary plot. Olive fruit samples of 4 kg were randomly picked by hand from each elementary plot in November 2018. An almost complete set of samples from 4 genotypes x 4 locations x 3 replicates was collected, with only one missing sample of Sel3 in

Loc4. After harvesting, olive fruit samples were immediately transported to the laboratory and stored at 4°C until olive oil extraction within 24h.

3.4.2. EVOO extraction

Only healthy fruits, without visible damage, were processed. EVOO was extracted using the Abencor system (Comercial Abengoa, S.A., Seville, Spain), which is a laboratory set for olive extraction composed by stainless hammer mill, thermo-mixer and centrifugal machine, reproducing the industrial process of mechanical extraction. Firstly, olive fruits were milled at 3000 rpm with a 5 mm sieve. 2.5 g/100 g of talc was added to the resulting olive paste that then was malaxed at 28°C for 30 min, adding 100 ml of water at room temperature for the last 10 minutes of malaxation. Then, the olive paste was centrifuged for 1 min at 1372 g relative centrifugal force. The EVOO obtained was decanted, filtered through paper, transferred into dark glass bottles and stored in the dark at 4°C until analysis. As expected from healthy fruit samples without damage, all the extracted oils were classified as EVOO, meeting the regulatory values established for quality criteria. For instance, only two samples showed free acidity values higher than 0.4, and all of them lower than the 0.8 value regulated for classification as EVOO (data not shown).

3.4.3. Chemical composition

A total of 66 chemical compounds of different groups were quantified (Table 3.1).

Table 3.1 Traits evaluated in EVOO samples

Group	Compound	Abbreviation	Units
Oxidative Stabililty		IT	h
Sensory properties		Fruity	(0-10)
		Bitter	(0-10)
		Pungent	(0-10)
Fatty acids	Palmitic	C160	%
	Palmitoleic	C161	%
	Stearic	C180	%
	Oleic	C181	%
	Linoleic	C182	%
	Linolenic	C183	%
	Arachidic	C200	%
	Eicosenoic	C201	%
	Behenic	C220	%
Tocopherols	Total	Tocopherols	mg/kg
	α-Tocopherol	aToc	%
	β-Tocopherol	BToc	%
	γ-Tocopherol	γToc	%
Squalene		Squalene	mg/kg
Phenols	Total	Phenols	mg/kg
	Hydroxytyrosol	HTyr	%
	Tyrosol	Tyr	%
	Vanillic acid	Van	%
	Vanillin	Vani	%
	pCumaric acid	pCum	%
	Hydroxytyrosol acetat	AcHTyr	%
	Oleacein	3,4-DHPEA-EDA	%
	Oleocanthal	p-HPEA-EDA	%
	Pinoresinol	Pino	%
	Cinnamic acid	Cin	%
	Acetoxypinoresinol	AcPino	%
	Oleuropein aglycone	3,4-DHPEA-EA	%
	Ligstroside aglycone	p-HPEA-EA	%
	Ferulic acid	Fer	%
	Luteolin	Lut	%
	Apigenin	Api	%

Group	Compound	Abbreviation	Units
Sterols	Total	Sterols	mg/kg
	Campesterol	Camp	%
	Stigmasterol	Stig	%
	Δ7-Campesterol	Δ7Camp	%
	Clerosterol	Clero	%
	β-sitosterol	Sito	%
	Δ5-avenasterol	Δ5Av	%
	Δ5-24-stigmastadienol	Δ524Stig	%
	Δ7-stigmastenol	Δ7Stig	%
	Δ7-avenasterol	Δ7Av	%
Volatiles	Total	Volatiles	µg/kg
	(E)+ (Z)-hex-3-enal	V01	%
	(Z)-hex-2-enal	V02	%
	(E)-hex-2-enal	V03	%
	(Z)-hex-3-enol	V04	%
	(E)-hex-2-enol	V05	%
	Hexanal	V06	%
	Hexan-1-ol	V07	%
	(Z)-pent-2-enal	V08	%
	(E)-pent-2-enal	V09	%
	Pent-1-en-3-ol	V10	%
	(Z)-pent-2-en-1-ol	V11	%
	(E)-pent-2-en-1-ol	V12	%
	Penten dimer-1	V13	%
	Penten dimer-2	V14	%
	Penten dimer-3	V15	%
	Penten dimer-4	V16	%
	Penten dimer-5+6	V17	%
	Penten dimer-7	V18	%
	Pentan-3-one	V19	%
	Pentanal	V20	%
	Hexyl acetate	V21	%
	(Z)-hex-3-en-1-yl acetate	V22	%
	Limonene	V23	%
	Ocimene	V24	%

- *Fatty acid composition*

Fatty acid composition was analyzed by gas chromatography (GC) on a Perkin Elmer Clarus 600 GC (Perkin Elmer Inc, Waltham, MA, USA) equipped with a BPX70 30 m x 0.25 mm internal diameter x 0.25 µm film thickness capillary column (SGE Analytical Science Pty Ltd, Ringwood, Australia). Hydrogen was used as carrier gas at a constant flow of 0.8 ml/min. A split injector and flame ionization detector were maintained at 300 °C. The initial oven temperature was 140 °C maintained for 2 min, followed by a rate increase of 20 °C / min up to 250 °C, maintained for 2 min.

- *Analysis of Tocopherols*

Tocopherol extraction, separation by high-performance liquid chromatography (HPLC) and quantification was done on around 100 mg of EVOO using a fluorescence detector (Waters 474) at 295-nm excitation and 330-nm emission and iso-octane/tert-butylmethylether (94:6)

as eluent at an isocratic flow rate of 0.8 ml/min (Velasco et al., 2019). Chromatographic separation of the tocopherols was performed on a LiChrospher 100 diol column (250 mm 9 2 mm I.D.) with 5-lm spherical particles, connected to a silica guard column (LiChrospher Si 60, 5 mm 9 4 mm I.D.). Quantitative determination of tocopherols was done by using external calibration curves obtained for each of the tocopherol homologs α-, β-, γ-, and δ-tocopherol using tocopherol standards (Calbiochem Tocopherol Set, catalog no. 613424, Merck KGaA, Darmstadt, Germany). Total tocopherol content was calculated as the sum of α-, β-, γ-, and δ-tocopherol contents.

- *Analysis of Phytosterols and Squalene*

Sterols and squalene contents in EVOO were analyzed by GLC of the unsaponifiable fraction following silylation, without preliminary thin-layer chromatography (TLC) fractionation. Alkaline hydrolysis was performed by adding 2 g/100 mL of a solution of potassiumhydroxide dissolved in ethanol at a concentration of 2%. After vortexing, the tubes were left in a water bath at 80 °C for 15 min. The unsaponifiable was extracted by vortexing with 1 mL hexane and 1.5 mL water. The upper hexane layer was transferred to 2-mL glass vials that were maintained in an oven at 37.5 °C overnight. Fifty microliter hexane and 50 µL silylating mixture composed of pyridine:hexamethyldisilazane:trimethylchlorosilane 9:3:1 by vol (Cat. No. 355650.0922, Panreac Química, Barcelona, Spain) were added and the vials were left at room temperature for 15 min. The solution was transferred to 2-mL vials containing 200 µL inserts and centrifuged at 4,000 rpm for 10 min. The vials were capped and conserved at −20 °C until analysis, usually within 24 h of preparation. GC analyses were performed on a Perkin Elmer Clarus 600 GC (Perkin Elmer Inc, Waltham, MA, USA) equipped with a ZB-5 capillary column (id = 0.25 mm, length = 30 m, film thickness = 0.10 µm; Phenomenex, Torrance, CA, USA) using hydrogen as carrier gas at a pressure of 125 KPa. Split injector and flame ionization detector were maintained at 320 °C. The oven thermal regime was the following: initial temperature of 240 °C was increased at 5 °C / min to final temperature of 265 °C and held for 10 min. Total analytical time was 15 min. Total phytosterol content was calculated as the sum of individual phytosterols and expressed as mg/kg. Sterol peaks were identified by comparison with a sample analysed at the reference laboratory of the Instituto de la Grasa (CSIC) at Sevilla, Spain. Squalene was identified using a commercial standard (Cat. No. S3626, Sigma-Aldrich). 5α-cholestan-3β-ol (Cat. No. D6128, Sigma- Aldrich, St. Louis, MO, USA) and squalene (Cat. No. S3626, Sigma- Aldrich) were used as internal standard.

- *Analysis of volatile compounds*

Volatile compounds were extracted and analyzed by means of HS-SPME/GC-MS-FID. EVOO samples (1 g) were prepared in duplicate vials of 10 mL and placed in a vial heater at 40 °C for a 10 min equilibration time. Volatile compounds from the headspace were adsorbed onto SPME fiber DVB/Carboxen/PDMS 50/30 µm (Supelco Co., Bellefonte, PA, USA). The sampling time was 50 min at 40 °C, and the desorption of volatile compounds was performed directly into the GC injector. Volatile compounds were identified on a Bruker model Scion 456-GC-TQ MS system (Bruker, Massachusetts, USA) equipped with a Supelcowax 10 capillary column (30 m × 0.25 mm i.d.; thickness, 0.25 µm; Sigma-Aldrich Co. LLC) working under the following conditions: helium (carrier gas) flow rate of 1mL/min; injection by splitless method at 250 °C; 5 min of column holding time at 50 °C and then ramped up at 4 °C/min to 200 °C;

the mass detector operated in electronic impact mode at 70 eV, with the temperature source set at 250 °C and the mass spectra were scanned at 7 scans/s in the m/z 30–250 mass-to-charge ratio range. Volatile compounds were matched to the Wiley/NBS and NIST libraries and by with GC retention time in comparison with standards. For the quantification of volatile compounds, calibration curves were obtained for each one by adding known amounts of the pure standards to deodorized olive oil at six level (Acesur, Seville, Spain). The absence of target volatile compounds in the matrix was checked and this olive oil was used to build calibration curves. As control of the extraction and analysis, samples containing a mixture of volatile standards and blank samples (no oil) were run at the beginning and during sample analysis.

- *Analysis of phenolic compounds*

EVOO phenolics were isolated by solid phase extraction (SPE) according to a previously published methodology (Mateos et al., 2001). 0.5 ml of a methanol solution containing two internal standards, p-hydroxyphenyl-acetic and o-coumaric acids (p-HPA and o-com) was added to each oil sample (2.5 g) before the extraction. The solvent was evaporated in a rotary evaporator at 40 °C under vacuum, and the residue was dissolved in 6 mL of hexane. This oil solution was applied to a diol-bonded phase cartridge (Supelco, Bellefonte, PA) previously conditioned. The column was washed twice with hexane (3 ml) and once with 4 mL of hexane/ethyl acetate (90:10, v/v). Finally, the column was eluted with 10 mL of methanol, later evaporated until dryness in a rotary evaporator at room temperature and under vacuum. The residue was extracted with 500 µL of methanol/water (1:1, v/v) at 40 °C. Phenolic extracts were analyzed by HPLC on a Beckman Coulter liquid chromatography system equipped with a System Gold 168 detector, a solvent module 126, an autosampler module 508 and a Waters column heater module. A Superspher RP 18 column (4.6 mm i.d. × 250 mm, particle size 4 µm: Dr Maisch GmbH, Germany). Elution was performed at a flow rate of 1.0 mL/min, using water/phosphoric acid (99.5:0.5) (solvent A) and methanol/acetonitrile (50:50) (solvent B) as the mobile phases and the following elution program: (A) 0–25 min, 5–30% solvent B; (B) 25–35 min, 30–38% solvent B; 35–40 min, 38% solvent B; 40–45 min, 38–100% solvent B. The quantification of phenolic components was done at 280 nm. The identification of compounds was confirmed by HPLC/ESI-qTOF-HRMS. The liquid chromatograph system was Dionex Ultimate 3000 RS UHPLC liquid chromatograph system (Thermo Fisher Scientific, Waltham, MA, USA) equipped with a similar Superspher RP 18 column but with formic acid (1%) instead of phosphoric acid (0.5%) in solvent A. A split post-column of 0.4 mL/min was introduced directly on the mass spectrometer electrospray ion source. The HPLC/ESI-qTOF operated for mass analysis using a micrOTOF-QII High Resolution Time-of- Flight mass spectrometer (UHRTOF) with qQ-TOF geometry (Bruker Daltonics, Bremen, Germany) equipped with an electrospray ionization (ESI) interface. Mass spectra were acquired in MS fullscan mode and data were processed using TargetAnalysis 1.2 software (Bruker Daltonics, Bremen, Germany).

3.4.4. Oxidative stability

Induction period was determined by Rancimat method. Oil samples (3.0 g) were heated at 120 °C in a Rancimat equipment (Metrohm AG, Herisau, Switzerland), with a continuous air flow of 20 L/h passing through the samples. Induction time (IT) was calculated as the time

needed (hours) for the appearance of a sudden water conductivity rise caused by the adsorption of volatiles derived from oil oxidation.

3.4.5. Sensory analysis

Sensory analysis was carried out by the EVOO sensory panel of PDO Priego de Córdoba, Andalucía, Spain, established in 1995. The panel was formed by 8 judges trained in the method for the organoleptic assessment of EVOO according to the official method of the IOC (2018). Positive attributes considered in the official methodology were: fruity (set of olfactory sensations perceived directly and/or through the back of the nose), bitter (characteristic primary taste of oil perceived in the circumvallate papillae of the tongue) and pungent (biting tactile sensation perceived throughout the whole of the mouth cavity, particularly in the throat). Sensory analysis was carried out in 41 out of the 47 EVOO samples due to lack of enough amount for some of them, well balanced among cultivars and locations and including all the combinations cultivar x location tested.

3.4.6. Statistical analysis

EVOO samples were obtained from three randomized blocks replicates for each cultivar x location combination and all the chemical analyses were performed in duplicate. Principal components analysis (PCA) was used to investigate the relationships among traits and the variability between and within the different groups of samples evaluated (by cultivar and location). Partial least squares (PLS) regression was used to study the associations of chemical components with oxidative stability and sensorial properties of EVOO. Full cross-validation (i.e. leave-one-out) was used for determining the performance of the models. Correlation between actual and predicted values (r), standard error of cross validation (RMSECV) and residual predictive deviation (RPD), defined as the ratio of the standard deviation for any given constituent to the standard error of cross validation or prediction for the same constituent, were determined to indicate the relative accuracy of each model, as previously described in PLS applications (Nicolaï et al., 2007). Analysis of variance was performed for the most important constituents to test differences between sources of variation (cultivar, location and interaction) and separation of means was carried out accordingly. Unscrambler (CAMO A/S, Trondheim, Norway) and Statistix (Analytical Software, Tallahassee, FL, United States) software were used for the statistical analysis.

3.5. Results

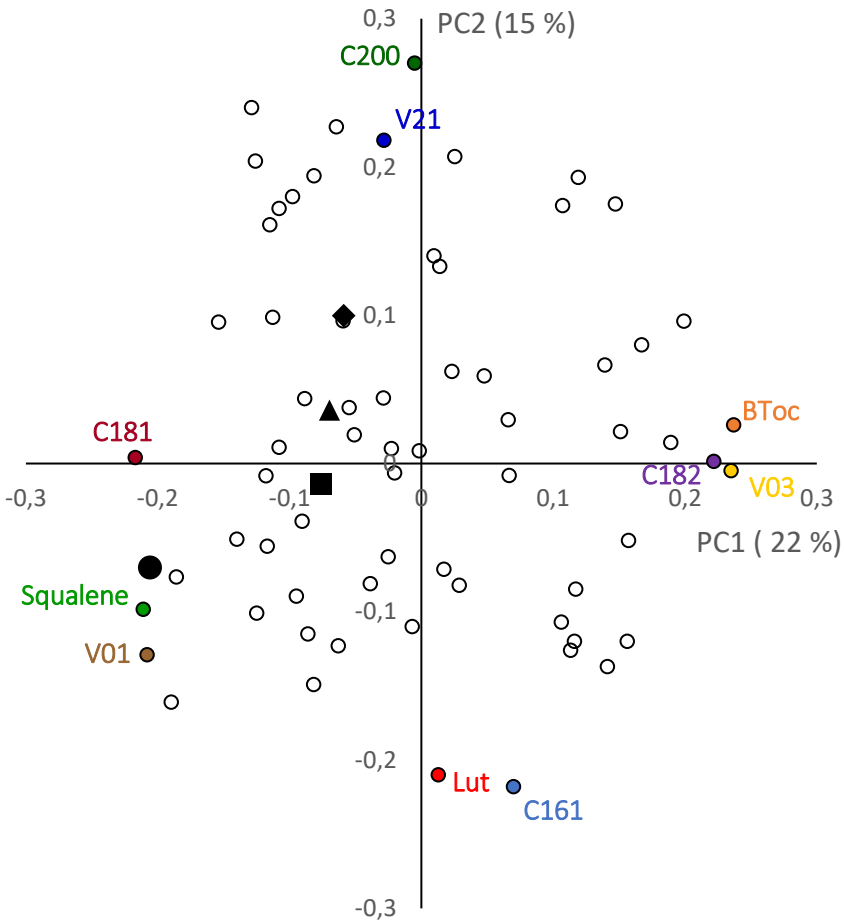
Descriptive statistics of the full data set showed wider variability for induction time compared to sensorial properties, with coefficient of variation (CV) of 43.98 % for IT vs. 15.86-19.94 % for fruity, bitter and pungent sensorial traits (Table 3.2). Among the evaluated sensorial traits, fruity showed the highest range of variability (3.00-6.10) and pungent the lowest (2.00-3.50). As expected for EVOO, negative attributes were not detected in any of the evaluated samples. Regarding the main fatty acid (C18:1) and total amount of minor components, C18:1 showed the lowest CV, while Phenol, Volatile and Squalene contents showed much higher variability with CV 64.046, 43.30 and 38.90 and range of variation 1236.80-11882.00, 195.50-1079.30 and 11656-63797, respectively, much higher than C18:1 and tocopherol content.

Table 3.2 Descriptive statistics for stability (induction time, IT), sensorial traits and main chemical compounds of EVOO samples (n=47).

Trait/compound	Mean	SD	CV	Min.	Max.
IT (h)	14.79	6.50	43.98	4.75	27.03
Fruity (0-10)	4.89	0.83	17.00	3.00	6.10
Bitter (0-10)	2.75	0.55	19.94	1.90	4.00
Pungent (0-10)	2.70	0.43	15.86	2.00	3.50
C18:1 (%)	75.43	5.49	7.28	65.58	82.53
Tocopherol (mg/kg)	248.14	45.16	18.20	155.32	382.95
Squalene (mg/kg)	5,245.00	3,380.70	64.46	1,236.80	11,882.00
Sterols (mg/kg)	1,674.10	313.58	18.73	1,025.90	2,179.10
Phenol (mg/kg)	465.77	201.68	43.30	195.50	1,079.30
Volatile (μg/kg)	28,732.00	11,176.00	38.90	11,656.00	63,797.00

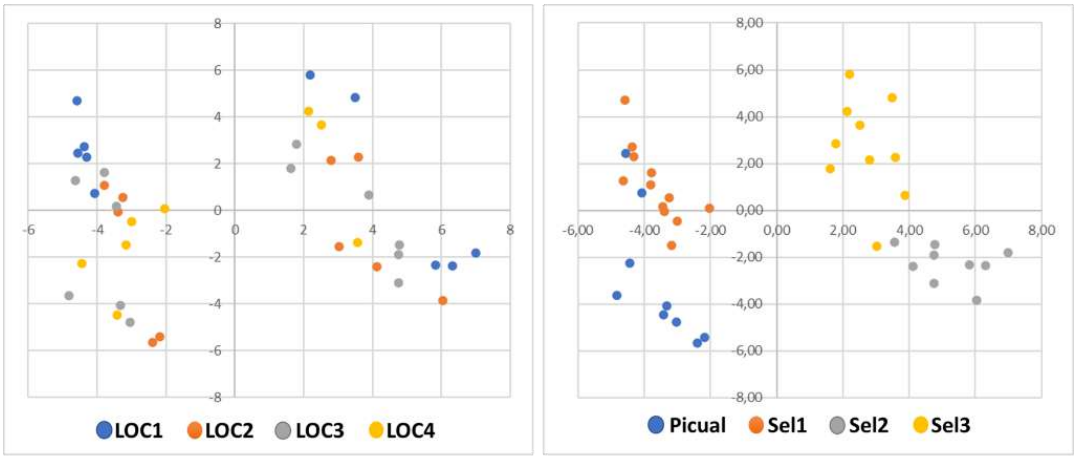
Exploratory analysis by PCA showed a wide variability for both samples scores and variables loadings in the model. The first two components of PCA carried out from the whole dataset including 66 chemical components plus oxidative stability and sensorial data evaluated in 47 EVOO samples, explained 22 and 15% of the total variability, respectively (Figure 3.1). PC1 was positively correlated mainly with linoleic acid (C18:2), (E)-hex-2-enal (V03) and β-Tocopherol (BToc) and negatively with stability (IT) and chemical compounds such as oleic acid (C18:1), squalene and (E)+(Z)-hex-3-enal (V01). PC2 was associated positively with volatiles such as hexyl acetate (V21) and arachidic acid (C20:0) and negatively with palmitoleic acid (C16:1) and luteolin phenolic compound (Lut).

Figure 3.1 Loading plot of PCA model developed from 66 chemical compounds (white circles), stability (black circle) and sensorial traits including fruity (black diamond), bitter (black square) and pungent (black triangle) evaluated in EVOO samples. Compounds abbreviations are given in Table 3.1.



The position of oxidative stability on the loading biplot, located nearby chemical compounds such as oleic acid (C18:1), Squalene and (E)+(Z)-hex-3-enal (V01), suggest a positive correlation among them. Fruity, bitter and pungent sensorial traits were on the contrary located closer to the loading plot center, which indicate low weight for these components on the general variability of the dataset. Besides, these results suggest no correlation among stability and sensorial data. The score biplot showed clear separation of EVOO samples according to cultivars, while no grouping could be observed regarding location of the trials (Figure 3.2). Main separation between cultivars was obtained through PC1, with Sel2 and 3 occupying the right (positive) side and the opposite for 'Picual' and Sel1. Therefore, higher values for stability and C18:1/C18:2 ratio can be expected for 'Picual' and Sel1 compared with Sel2 and Sel3.

Figure 3.2 Scores plot of PCA model developed from 66 chemical compounds, stability and sensorial traits evaluated in EVOO samples. (A) Distribution by cultivar; (B) Distribution by location



PLS models developed from 66 chemical compounds for stability (IT) showed high correlation and RPD values, while the opposite was obtained for the three sensorial traits (Table 3.3, Figure 3.3). In all cases, only one or two components were included in the models. Scores plot of PLS model developed for stability reflects the same grouping by cultivar and location previously described for PCA model (data not shown). Regression coefficients of this PLS model showed the highest positive values for C18:1 and 3,4-DHPEA-EA, while negative for C18:2 and 3,4-DHPEA-EDA (Figure 3.4). Total phenolic and squalene content (positive) and sterols content (negative) play also important role in the model.

Table 3.3 Cross-validation results for PLS models developed for stability (induction time, IT) and sensorial traits of EVOO samples (n=47).

	nPLS	r	RMSECV	RPD
IT (h)	2	0.88	3.44	1.89
Fruity (0-10)	1	0.21	0.85	0.98
Bitter (0-10)	1	0.29	0.54	1.02
Pungent (0-10)	1	0.11	0.45	0.96

[1]Number of latent variables (nPLS), Correlation between actual and predicted constituent values (r), Standard error of cross validation (RMSECV), Residual predictive deviation (RPD), Range Error Ratio (RER).

Figure 3.3 Predicted vs. reference values from PLS models developed for stability and sensorial traits based on values of 66 chemical compounds evaluated in EVOO.

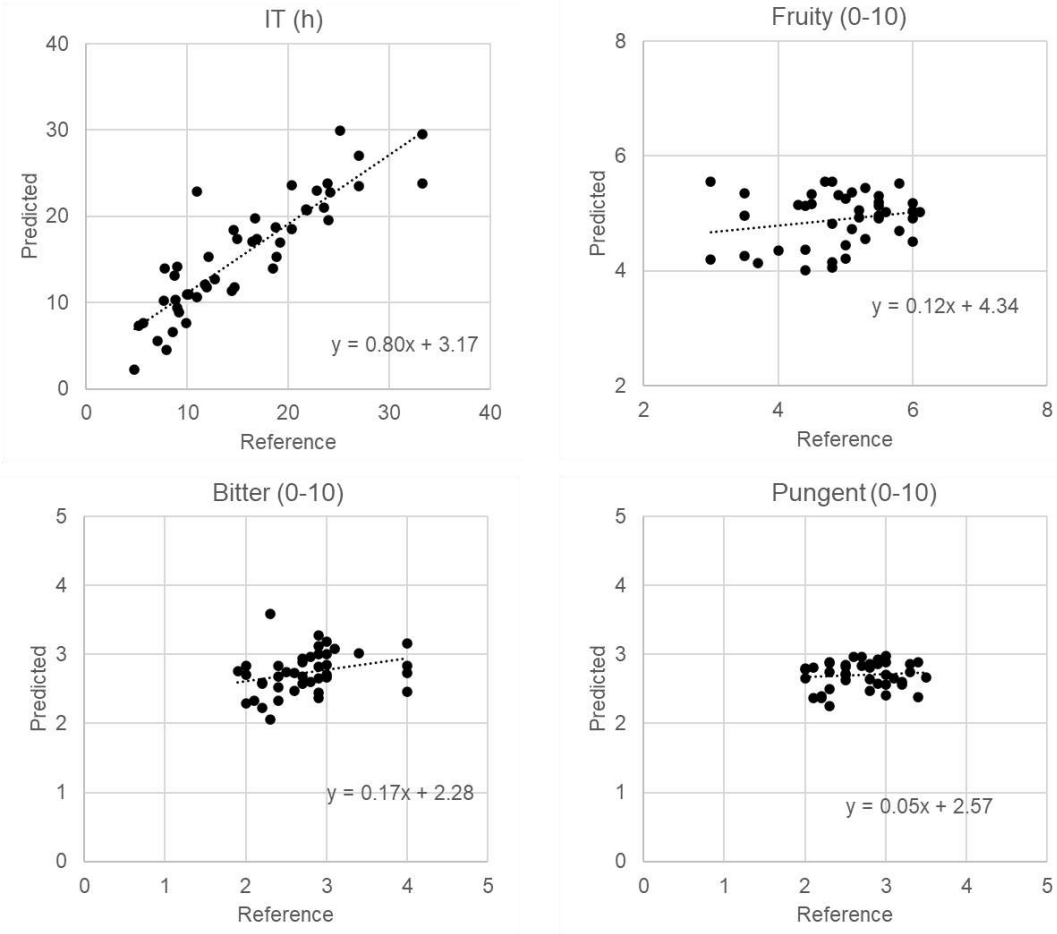
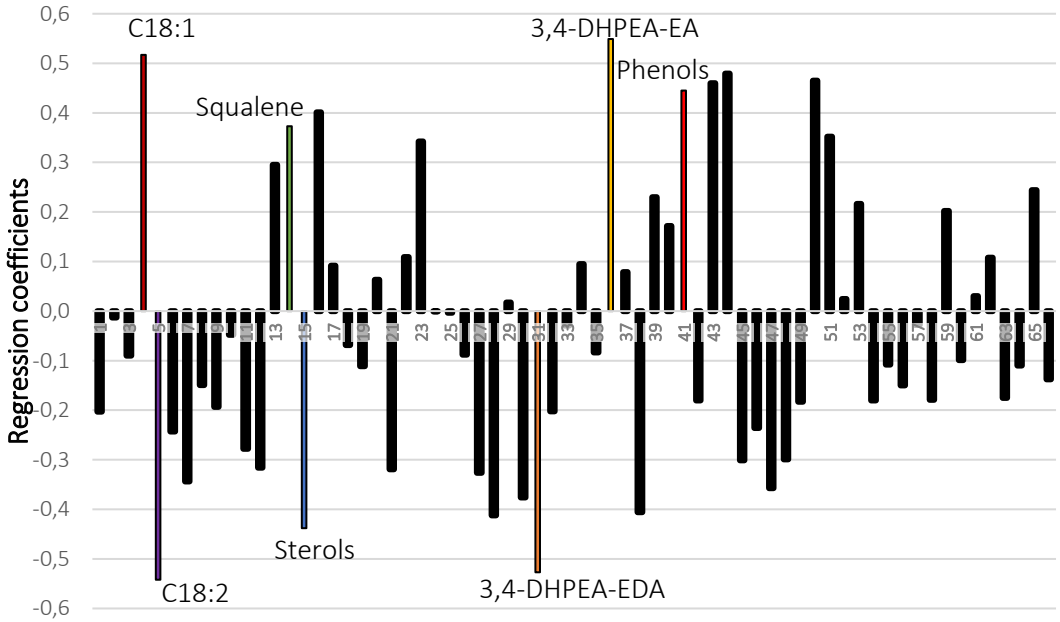


Figure 3.4 Regression coefficients of PLS model developed for stability (induction time, IT) from 66 chemical compounds evaluated in EVOO. Main components in the model are indicated.



Analysis of variance showed significant differences by cultivar and location for all the main chemical compounds of EVOO except location for oleic acid (C18:1). In all cases, non-significant differences were obtained for cultivar x location interaction. Cultivar effect was the main contributor of sums of squares for C18:1, total tocopherol, squalene and sterols content, while location was higher for total phenols and volatile contents (Table 3.4). Comparison of means showed similar chemical composition in 'Picual' and Sel1 on the one hand (high C18:1 and squalene content and low sterols content) and Sel2 and Sel3 (both coming from crosses between 'Frantoio' and 'Arbosana') on the other hand. Comparison of means among locations showed different trends for the different evaluated traits. For example, Loc1 differed from other locations in the lowest amount of squalene, the highest phenols content was quantified in EVOO samples from Loc3 and higher volatile contents were observed for Loc2 and Loc4.

Table 3.4 Percentage of sums of squares for each source of variation and comparison of means by Cultivar and Location for main chemical compounds of EVOO.

Source	df	C18:1 (%)	Tocopherol (mg/kg)	Squalene (mg/kg)	Sterols (mg/kg)	Phenols (mg/kg)	Volatiles (µg/kg)
Cultivar	3	91.6	42.6	83.4	66.3	11.8	18.4
Location	3	1.2^{NS} (1)	15.7	7.8	11.9	40.5	60.0
C x L	9	3.1	11.8	5.5	8.4	11.9	7.7
Error	31	4.1	29.9	3.3	13.3	35.8	14.0
'Picual'		81.0 a (2)	257.7 a	7,765.6 b	1,426.6 b	460.7 ab	24,009 b
Sel1		78.5 b	256.3 a	8,669.4 a	1,428.2 b	491.3 ab	36,634 a
Sel2		67.3 d	276.3 a	1,969.2 c	1,934.4 a	362.0 b	27,116 b
Sel3		74.9 c	198.1 b	2,333.2 c	1,928.5 a	556.7 a	27,027 b
Loc1		74.7	266.0 a	3,658.1 b	1,808.8 a	425.2 b	24,796 b
Loc2		75.9	262.8 ab	6,117.4 a	1,754.9 ab	391.5 b	34,578 a
Loc3		75.0	231.6 bc	5,445.7 a	1,610.9 bc	681.8 a	17,100 c
Loc4		76.1	230.8 c	5,805.6 a	1,508.0 c	355.5 b	39,337 a

(1)NS: non-significant differences at P<0.05. (2)Different letter by Cultivar or Location indicates significant differences at P<0.05.

Regarding stability (IT) and sensorial traits of EVOO, analysis of variance showed significant differences by cultivar only for IT and bitter, and location effect for IT. No significant differences for fruity and pungent sensorial traits were found neither by genotype nor by location. Like for chemical compounds, non-significant differences were obtained for Cultivar x Location interaction. Cultivar effect was the main contributor of sums of squares only for IT, while error sums of squares was predominant for sensorial traits (Table 3.5). Again, comparison of means suggests a general higher similitude between 'Picual' and Sel1 compared to Sel2 and Sel3, mainly due to higher oxidative stability.

Table 3.5 Percentage of sums of squares for each source of variation and comparison of means by Cultivar and Location for stability (induction time, IT) and sensorial traits of EVOO.

Source	df	IT (h)	Fruity (0-10)	Bitter (0-10)	Pungent (0-10)
Cultivar	3	69.9	24.0	16.8	3.9
Location	3	10.5	0.5	15.9	5.3
C x L	9	5.2	6.1	25.8	20.7
Error	31	14.4	69.3	41.6	70.1
'Picual'		21.83 a	4.69	3.10 a	2.81
Sel1		18.11 b	5.24	2.71 ab	2.75
Sel2		7.73 d	4.18	2.40 b	2.48
Sel3		12.45 c	5.25	2.79 ab	2.75
Loc1		12.81 b	4.82	2.61	2.54
Loc2		15.22 ab	4.97	2.61	2.82
Loc3		17.80 a	4.93	3.09	2.78
Loc4		13.32 b	4.84	2.60	2.66

Different letter by Cultivar or Location indicates significant differences at P<0.05.

3.6. Discussion

A wide variability has been observed for stability (induction time, IT), sensorial traits and main chemical compounds of the set of EVOO samples. The average values observed for 'Picual' are in general comparable to previous references. Thus, EVOO from 'Picual' have been traditionally characterized by a high C18:1, phenol content and oil stability, being its EVOO chemical composition one of the main reason for its widespread use as a genitor in breeding programs (León et al., 2011). However, it should be noted that wide variability for some of these components such as total phenol content has also been reported in some works, as high as from 133–1295 mg/kg (Beltrán et al., 2007). High bitterness is also characteristic of 'Picual' (Mateos et al., 2004). Similar values for total volatiles content have been reported for 'Picual' in previous works (Pérez et al., 2016), although much lower values, around 8,000-9,000 mg/kg have been also observed (Sánchez-Ortiz et al 2007). The average values obtained in 'Picual' for others minor components such as squalene, phytosterol and tocopherols are also similar than previously reported for this cultivar (Aparicio et al., 1999; Velasco et al., 2015). No significant correlation was observed among the different stability and sensorial traits evaluated. On the contrary, significant correlations among fruity, bitter and pungent sensorial traits from 0.60 to 0.77 were obtained in a previous work from a set of 100 samples from an annual competition (Pedan et al., 2019). It is unknown to what extent the origin of samples could have affected these results.

3.6.1. Chemical components influencing oxidative stability and sensorial properties

Accurate predictive PLS model was obtained only for EVOO stability (induction time) using the data of 66 chemical components analyzed, with high correlation between actual and predicted values (around 0.9). This value could be considered accurate enough for ranking and selection of genotypes and discrimination into high, medium and low values. Similarly,

RPD values near 2 indicates that coarse quantitative predictions are possible, although values around 3 are recommended for excellent prediction accuracy (Nicolaï et al., 2007). However, poor prediction results were obtained in the models developed for positive sensorial properties (fruity, bitter and pungent).

The effect of various compounds on EVOO stability measured by Rancimat has been reported in previous studies. A good correlation (R2=0.91) has been previously found, using stepwise linear regression analysis, between stability and both the oleic/linoleic ratio and the contents of phenols and tocopherols (Aparicio et al., 1999). However, that study was performed with only two cultivars with contrasting behaviors in terms of stability ('Picual' and 'Hojiblanca') in a single environment. Grouping of samples can be inferred also in correlations reported from other works (Bendini, 2007). In the present work, the PLS model developed for stability showed highly significant correlation with only two latent variables. The model showed high and positive regression coefficients values for C18:1 and 3,4-DHPEA-EA and negative for C18:2 and 3,4-DHPEA-EDA. It is well established the negative correlation between oleic and linoleic fatty acids in all vegetable oils including EVOO. Regarding secoiridoid derivatives, all of them are produced by β-glucosidase hydrolysis of olive fruits glycosides during crushing and malaxation (Bendini, 2007). Similar relationships among individual phenols and IT measured by Rancimat were also reported from the analysis of EVOOs obtained from a wide variability of malaxation conditions, suggesting the use of the ratio (3,4-DHPEA-EA + *p*-HPEA-EA)/(3,4-DHPEA-EDA + *p*-HPEA-EDA) as a good estimator of EVOO stability (Miho et al., 2020). Comparison of the antioxidant capacity of isolated individual phenolic compounds using a similar accelerated oxidation test showed high antioxidant activity for deacetoxy oleuropein aglycon and oleuropein aglycon, while pro-oxidant effect was found for ligstroside aglycon (Carrasco-Pancorbo et al., 2005). Stability was therefore related to the amount and composition of individual phenols rather than to the total phenolic content.

Up to one hundred and eighty different volatile compounds belonging to several chemical groups (carbonyl, ester, alcohol, hydrocarbon) have been found in EVOO aromas (Angerosa, 2002). Among them, those produced enzymatically from the lipoxygenase (LOX) pathway have been generally considered the main responsible in the formation of EVOO positive aroma attributes, while many others responsible for negative attributes (defects), such as rancid, winey-vinegary, fusty, muddy sediment, musty, are not present in EVOO (Angerosa, 2002; Campestre et al., 2017). EVOO fruitiness has been previously correlated positively with the content of individual volatiles such as Z-2-penten-1-ol; 3,5-dimethyl-1,6-heptadiene; and sum of aldehydes C6, and negatively with 3-methyl-1-butanol; 2-methyl-1-butanol; 2,4-dimethylheptane; hexyl acetate; nonanal; decanal; Z-2-decenal, although the extent of these correlations was not reported (Cerretani et al., 2008). However, associations between individual volatile concentration and specific EVOO aromas such as fruity could be hindered by different odor thresholds, the complex interactions between volatiles and receptors responsible of EVOO smell, the existence of multiple volatiles responsible for a flavor sensation, and the combinations of volatiles yielding flavors different to those expected from individual compounds (Campestre et al., 2017; Chambers & Koppel, 2013; Genovese et al., 2019). PLS results obtained in this work confirm these difficulties as the model developed was not able to accurately predict the level of fruitiness, that is the main positive odor

attribute of EVOO. Using a similar PLS approach, good predictions were previously achieved for some negative attributes such as vinegar, not detected in our work as we were working only with EVOO samples, but satisfactory cross-validation was not obtained for prediction of other sensory attributes (Servili et al., 1995). Similarly, PLS models based on volatile fingerprint have been reported to be able to discriminate between olive oil categories, i.e. extra virgin vs. non-extra virgin samples; virgin vs. lampante categories with 97% correct classification in cross-validation (Quintanilla-Casas et al., 2020).

The secoiridoid derivatives resulting from the enzymatic hydrolysis of oleuropein, ligstroside and demethyloleuropein, identified as the dialdehydic forms of decarboxymethyloleuropein and decarboxymethylligstroside aglycones (3,4-DHPEA-EDA and *p*-HPEA-EDA, respectively) and the aldehydic forms of oleuropein and ligstroside aglycones (3,4-DHPEA-EA and *p*-HPEA-EA, respectively) are the most abundant phenolic components found in EVOO. These compounds have been suggested to underlay the bitter and pungent sensory attributes of EVOO. In fact, the absorbance of the phenolic extract obtained from EVOO measured at 225 nm was proposed as a simple method for bitterness evaluation, although comparison of samples from cultivars with very different phenolic profiles was considered non accurate (Gutiérrez Rosales et al., 1992; Mateos et al., 2004). Total phenol content, measured as the absorbance at 726 nm after reaction with the Folin-Ciocalteau reagent, was also suggested as an easy tool for bitterness assessment without sensory evaluation (Beltrán et al., 2007). More specifically, *p*-HPEA-EDA (oleocanthal) was described as the main phenolic responsible for the EVOO pungency (Andrewes et al., 2003), while 3,4-DHPEA-EA was suggested as the main responsible for bitterness attribute (Mateos et al., 2004), even though the magnitude of these relationships is discussed (Campestre et al., 2017; Cerretani et al., 2008; Pedan et al., 2019). Literature reviews show different results, relating bitterness intensity to the presence of oleuropein derivatives, to both oleuropein and ligstroside aglycons, or only to ligstroside derivatives (Campestre et al., 2017). Our results indicate that prediction of positive sensorial properties (fruity, bitter and pungent) was not possible from chemical constituents.

It should be noted that, unlike previous studies, our work was conducted using a wide EVOO sample set with combined effects of genotype and location, and cross-validation was carried out for testing the results. Generalization of results obtained from simple pair comparison of highly different EVOO could have occurred in previous works (Bendini, 2007; Lukić et al., 2018). The use of commercial EVOO samples without controlling the potential effects of other factors such as harvest time or extraction system could also difficult the analysis of results (Beltrán et al., 2007; Gutiérrez Rosales et al., 1992; Mateos et al., 2004). Finally, it cannot be excluded some differences in determination and identification of the different phenolic compounds among works, as a wide variability of methodologies are used for these analyses.

3.6.2. Cultivar and location effects

Significant differences among cultivars and locations have been obtained in this work for the main chemical components of EVOO. These differences among chemical components led to subsequent differences regarding EVOO stability. This was expected based on the relationships between oil composition and stability discussed above. A stronger effect of

cultivar, compared to some environmental factors such as year of harvest and ripening stage, has been previously reported for some compositional and antioxidant properties of EVOO (Borges et al., 2019). In our work, Loc3 showed the highest stability, probably due to its higher phenol content. The geographical area of origin has been also found to play a role in the qualitative and quantitative characteristics of EVOO in previous works (Ben Mansour et al., 2017).

On the contrary, significant differences among cultivars and locations for chemical components of EVOO were not translated into significant differences in sensory attributes. Previous studies indicate the importance of the genetic effect on the volatile composition of EVOO. Comparison of contrasting cultivars such as 'Arbequina' and 'Picual' showed clear genotypic effect for both the availability of non-esterified polyunsaturated fatty acids, especially linolenic acid, and the enzymatic activity of the LOX system responsible of the biosynthesis of VOO aroma compounds and therefore its sensorial characterization (Sánchez-Ortiz et al., 2007). Consequently, the effects of cultivar on the sensorial properties of EVOO has been underlined, linking these sensorial differences to the activities of the different enzymes involved in the different pathways (Campestre et al., 2017; Sánchez-Ortiz et al., 2007). However, similar to our work, no significant differences in sensory parameters were observed between Italian and Spanish EVOOs in relation to their area origin and olive cultivar and, therefore, the inclusion of additional positive sensory notes was recommended for regulations of some PDO-EVOOs (Genovese et al., 2019). Fruitiness was also found to be poor inter-cultivar but potent intra-cultivar typicity discriminator for Istrian cultivars, even though significant differences for many volatile compounds were observed (Lukić et al., 2018).

3.7. Conclusions

Our results suggest that other parameters, apart from cultivar and location, provide significant variation for sensory properties of EVOO together with the inherent difficulties associated to sensory evaluation. A deeper knowledge of these additional factors could open up the possibilities of modulating sensory attributes regardless cultivar and location of origin. The implication of these results regarding current PDOs regulation should be further studied in future works. On the other hand, the lack of accuracy in the models developed for prediction of sensory attributes underline the need for maintaining sensory evaluation panel test as a tool of paramount importance for evaluating EVOO sensory quality. On the contrary, EVOO stability seems to be easy to predict based on the chemical composition. For this trait, the influence of genotype and location conditions could be quantified. This is of paramount importance in breeding programs aimed to obtain new cultivars with improved EVOO characteristics and to determine the best cultivar to be planted in each growing area.

3.8. Acknowledgements

This work was supported by projects of Andalusian Institute of Agricultural Research and Training (AVA201601.2, AVA2019.027), partly funded by European Regional Development Fund (ERDF). AS acknowledges Spanish National Institute of Agricultural Research for financial support. Field and lab staff assistance is highly acknowledged. Thank also to the

members of the Tasting Panel from the Protected Denomination of Origin "Priego de Córdoba" (Priego de Córdoba, Córdoba, Spain) who carried out the sensory analysis of EVOO samples.

3.9. References

Andrewes, P., Busch, J. L. H. C., de Joode, T., Groenewegen, A., & Alexandre, H. (2003). Sensory Properties of Virgin Olive Oil Polyphenols: Identification of Deacetoxy-ligstroside Aglycon as a Key Contributor to Pungency. Journal of Agricultural and Food Chemistry, 51(5), 1415–1420. https://doi.org/10.1021/jf026042j

Angerosa, F. (2002). Influence of volatile compounds on virgin olive oil quality evaluated by analytical approaches and sensor panels. European Journal of Lipid Science and Technology, 104, 639–660. https://doi.org/10.1002/1438-9312(200210)104:9/10<639::AID-JLT639>3.0.CO;2-U

Aparicio, R., Roda, L., Albi, M. A., & Gutiérrez, F. (1999). Effect of Various Compounds on Virgin Olive Oil Stability Measured by Rancimat. Journal of Agricultural and Food Chemistry, 47(10), 4150–4155. https://doi.org/10.1021/jf9812230

Barranco, D., Rallo, L. (2000). Olive Cultivars in Spain. Horttechnology, 10(1), 107–110. https://doi.org/10.21273/horttech.10.1.107

Beltrán, G., Ruano, M. T., Jiménez, A., Uceda, M., & Aguilera, M. P. (2007). Evaluation of virgin olive oil bitterness by total phenol content analysis. European Journal of Lipid Science and Technology, 109(3), 193–197. https://doi.org/10.1002/ejlt.200600231

Ben Mansour, A., Chtourou, F., Khbou, W., Flamini, G., & Bouaziz, M. (2017). Phenolic and volatile compounds of Neb Jmel olive oil cultivar according to their geographical origin using chemometrics. European Food Research and Technology, 243(3), 403–418. https://doi.org/10.1007/s00217-016-2754-5

Bendini, A., Cerretani, L., Carrasco-Pancorbo, A., Gómez-Caravaca, A.M., Segura-Carretero, A., Fernández-Gutiérrez, A., & Lercker, G. Phenolic molecules in virgin olive oils: a survey of their sensory properties, health effects, antioxidant activity and analytical methods. An overview of the last decade. Molecules, 12(8), 1679-1719. doi: 10.3390/12081679.

Borges, T. H., Serna, A., López, L. C., Lara, L., Nieto, R., & Seiquer, I. (2019). Composition and Antioxidant Properties of Spanish Extra Virgin Olive Oil Regarding Cultivar, Harvest Year and Crop Stage. Antioxidants, 8(7), 217. https://doi.org/10.3390/antiox8070217

Campestre, C., Angelini, G., Gasbarri, C., & Angerosa, F. (2017). The Compounds Responsible for the Sensory Profile in Monovarietal Virgin Olive Oils. Molecules, 22(11), 1833. https://doi.org/10.3390/molecules22111833

Carrasco-Pancorbo, A., Cerretani, L., Bendini, A., Segura-Carretero, A., Del Carlo, M., Gallina-Toschi, T., Lercker, G., Compagnone, D., & Fernández-Gutiérrez, A. (2005). Evaluation of the Antioxidant Capacity of Individual Phenolic Compounds in Virgin Olive Oil. Journal of Agricultural and Food Chemistry, 53(23), 8918–8925. https://doi.org/10.1021/jf0515680

Cerretani, L., Salvador, M. D., Bendini, A., & Fregapane, G. (2008). Relationship Between Sensory Evaluation Performed by Italian and Spanish Official Panels and Volatile and Phenolic Profiles of Virgin Olive Oils. Chemosensory Perception, 1(4), 258–267. https://doi.org/10.1007/s12078-008-9031-3

Chambers, E., & Koppel, K. (2013). Associations of Volatile Compounds with Sensory Aroma and Flavor: The Complex Nature of Flavor. Molecules, 18(5), 4887–4905. https://doi.org/10.3390/molecules18054887

de la Rosa, R., Arias-Calderón, R., Velasco, L., & León, L. (2016). Early selection for oil quality components in olive breeding progenies: Olive breeding for oil quality components. European Journal of Lipid Science and Technology, 118(8), 1160–1167. https://doi.org/10.1002/ejlt.201500425

Francisco, V., Ruiz-Fernández, C., Lahera, V., Lago, F., Pino, J., Skaltsounis, L., González-Gay, M. A., Mobasheri, A., Gómez, R., Scotece, M., & Gualillo, O. (2019). Natural Molecules for Healthy Lifestyles: Oleocanthal from Extra Virgin Olive Oil. Journal of Agricultural and Food Chemistry, 67(14), 3845–3853. https://doi.org/10.1021/acs.jafc.8b06723

García-Vico, L., Belaj, A., Sánchez-Ortiz, A., Martínez-Rivas, J., Pérez, A., & Sanz, C. (2017). Volatile Compound Profiling by HS-SPME/GC-MS-FID of a Core Olive Cultivar Collection as a Tool for Aroma Improvement of Virgin Olive Oil. Molecules, 22(1), 141. https://doi.org/10.3390/molecules22010141

Genovese, A., Caporaso, N., Leone, T., Paduano, A., Mena, C., Perez-Jimenez, M. A., & Sacchi, R. (2019). Use of odorant series for extra virgin olive oil aroma characterisation: Aroma of extra virgin olive oil by odorant series. Journal of the Science of Food and Agriculture, 99(3), 1215–1224. https://doi.org/10.1002/jsfa.9293

Gouvinhas, I., Machado, N., Sobreira, C., Domínguez-Perles, R., Gomes, S., Rosa, E., & Barros, A. (2017). Critical Review on the Significance of Olive Phytochemicals in Plant Physiology and Human Health. Molecules, 22(11), 1986. https://doi.org/10.3390/molecules22111986

Gutiérrez Rosales, F., Perdiguero, S., Gutiérrez, R., & Olias, J. M. (1992). Evaluation of the bitter taste in virgin olive oil. Journal of the American Oil Chemists' Society, 69(4), 394–395. https://doi.org/10.1007/BF02636076

Issaoui, M., Flamini, G., Brahmi, F., Dabbou, S., Hassine, K. B., Taamali, A., Chehab, H., Ellouz, M., Zarrouk, M., & Hammami, M. (2010). Effect of the growing area conditions on differentiation between Chemlali and Chétoui olive oils. Food Chemistry, 119(1), 220–225. https://doi.org/10.1016/j.foodchem.2009.06.012

León, L., de la Rosa, R., Velasco, L., & Belaj, A. (2018). Using Wild Olives in Breeding Programs: Implications on Oil Quality Composition. Frontiers in Plant Science, 9, 232. https://doi.org/10.3389/fpls.2018.00232

Lukić, I., Horvat, I., Godena, S., Krapac, M., Lukić, M., Vrhovsek, U., & Brkić Bubola, K. (2018). Towards understanding the varietal typicity of virgin olive oil by correlating sensory and

compositional analysis data: a case study. Food Research International, 112, 78–89. https://doi.org/10.1016/j.foodres.2018.06.022

Mateos, R., Cert, A., Pérez-Camino, M. C., & García, J. M. (2004). Evaluation of virgin olive oil bitterness by quantification of secoiridoid derivatives. Journal of the American Oil Chemists' Society, 81(1), 71–75. https://doi.org/10.1007/s11746-004-0859-x

Mateos, R., Domínguez, M. M., Espartero, J. L., & Cert, A. (2003). Antioxidant Effect of Phenolic Compounds, α-Tocopherol, and Other Minor Components in Virgin Olive Oil. Journal of Agricultural and Food Chemistry, 51(24), 7170–7175. https://doi.org/10.1021/jf034415q

Mateos, R., Espartero, J. L., Trujillo, M., Rios, J. J., León Camacho, M., Alcudia, F., & Cert, A. (2001). Determination of phenols, flavones, and lignans in virgin olive oils by solid-phase extraction and high-performance liquid chromatography with diode array ultraviolet detection. Journal of Agricultural and Food Chemistry, 49 (5), 2185–2192. doi: 10.1021/jf0013205

Miho, H., Moral, J., López-González, M. A., Díez, C. M., & Priego-Capote, F. (2020). The phenolic profile of virgin olive oil is influenced by malaxation conditions and determines the oxidative stability. Food Chemistry, 314, 126183. https://doi.org/10.1016/j.foodchem.2020.126183

Navas-Lopez, J. F., León, L., Trentacoste, E. R., & de la Rosa, R. (2019). Multi-environment evaluation of oil accumulation pattern parameters in olive. Plant Physiology and Biochemistry, 139, 485–494. https://doi.org/10.1016/j.plaphy.2019.04.016

Nicolaï, B. M., Beullens, K., Bobelyn, E., Peirs, A., Saeys, W., Theron, K. I., & Lammertyn, J. (2007). Nondestructive measurement of fruit and vegetable quality by means of NIR spectroscopy: A review. Postharvest Biology and Technology, 46(2), 99–118. https://doi.org/10.1016/j.postharvbio.2007.06.024

Pedan, V., Popp, M., Rohn, S., Nyfeler, M., & Bongartz, A. (2019). Characterization of Phenolic Compounds and Their Contribution to Sensory Properties of Olive Oil. Molecules, 24(11), 2041. https://doi.org/10.3390/molecules24112041

Pérez, A. G., León, L., Pascual, M., Romero-Segura, C., Sánchez-Ortiz, A., de la Rosa, R., & Sanz, C. (2014). Variability of Virgin Olive Oil Phenolic Compounds in a Segregating Progeny from a Single Cross in Olea europaea L. and Sensory and Nutritional Quality Implications. PLOS ONE, 9(3), e92898. https://doi.org/10.1371/journal.pone.0092898

Quintanilla-Casas, B., Bustamante, J., Guardiola, F., García-González, D. L., Barbieri, S., Bendini, A., Toschi, T. G., Vichi, S., & Tres, A. (2020). Virgin olive oil volatile fingerprint and chemometrics: Towards an instrumental screening tool to grade the sensory quality. LWT – Food Science and Technology, 121, 108936. https://doi.org/10.1016/j.lwt.2019.108936

Radd-Vagenas, S., Kouris-Blazos, A., Singh, M.F., Flood, V.M. (2017). Evolution of Mediterranean diets and cuisine: concepts and definitions. Asia Pacific Journal of Clinical Nutrition, 26 (5), 749-763. https://doi.org/10.6133/apjcn.082016.06

Sánchez-Ortiz, A., Pérez, A. G., & Sanz, C. (2007). Cultivar Differences on Nonesterified Polyunsaturated Fatty Acid as a Limiting Factor for the Biogenesis of Virgin Olive Oil Aroma. Journal of Agricultural and Food Chemistry, 55(19), 7869–7873. https://doi.org/10.1021/jf071202i

Servili, M., Conner, J. M., Piggott, J. R., Withers, S. J., & Paterson, A. (1995). Sensory characterisation of virgin olive oil and relationship with headspace composition. Journal of the Science of Food and Agriculture, 67(1), 61–70. https://doi.org/10.1002/jsfa.2740670111.

Velasco, L., Fernandez-Cuesta, A., De la Rosa, R., Victoria Ruiz-Mendez, M., & Leon, L. (2014). Selection for Some Olive Oil Quality Components Through the Analysis of Fruit Flesh. Journal of the American Oil Chemists Society, 91(10), 1731-1736. doi:10.1007/s11746-014-2523-1

4. GENETIC AND ENVIRONMENTAL EFFECT ON VOLATILE COMPOSITION OF EXTRA VIRGIN OLIVE OIL

This chapter has been published in the journal 'European Journal of Lipid Science and Technology'.
Serrano, A., De la Rosa, R., Sánchez-Ortiz, A., León, L. (2020).

4.1. Abstract

Several factors affect the amount and composition of the volatile fraction of extra virgin olive oil (EVOO), key component responsible for the aroma of this product. In this work, advanced selections of the olive breeding program developed in the Institute of Agricultural and Fishery Research and Training (IFAPA) were evaluated in comparative fields established in different climatic conditions, to test and quantify the effect of genetic (G) and environmental (E) factors. Significant differences between genotypes and environments were found for total content and proportions of volatile compounds, except for C5/LA and terpenes. Genetic factor was responsible for the volatile profile variation, representing the variance component attributable to genotype up to 46% for C6/LnA compounds, while environmental factor represented the 62% of the variation in total volatile content. On the contrary, significant G x E interaction was only found for the percentage of C5/LA and esters. EVOOs obtained from three breeding selections were quantitatively and qualitatively differentiated from 'Picual' cultivar in the four experimental fields tested. In qualitative terms, EVOOs from 'Picual' and 'IFAPA111-2' clearly differentiated from those of 'IFAPA117-117' and 'IFAPA117-120' genotypes due to their volatile profiles. In quantitative terms, EVOO from 'IFAPA111-2' genotype showed the highest total content of volatile compounds.

4.2. Practical applications

The results revealed a qualitative and quantitative differentiation among olive genotypes regarding their EVOOs' volatile fractions, demonstrating that breeding selections could be a suitable alternative for cultivar diversification in olive orchards and extend the commercial offer of EVOOs regarding to aroma characteristics.

4.3. Keywords

Olive oil quality, Olea europaea L., multi-environment, breeding selections, multivariate analysis.

4.4. Introduction

Extra virgin olive oil (EVOO) is the juice obtained exclusively from olive fruits by mechanical and physical processes. EVOOs are essentially composed of triacylglycerols (about 98%) among which the most abundant fatty acid is the monounsaturated oleic acid, followed by smaller amounts of other fatty acids such as linoleic, palmitic, linolenic, palmitoleic and stearic. Nevertheless, EVOO composition highly differs from other vegetable oils mainly due to the presence of minor components (around 2%). Among them, phenols and volatile compounds are the main responsible for EVOOs singularity providing organoleptic and health-promoting properties. Specifically, volatiles have been closely linked with EVOO aroma including positive and negative attributes [1–4]. Therefore, volatile composition plays an important role for EVOOs classification into commercial categories through the sensory analysis [3,5], which could be complemented by instrumental methods [6,7]. In this sense, volatiles compounds could also contribute to the consumer acceptance [4,8].

Volatile fraction includes a large number of compounds [2,3], among which the most important are hydrocarbons, alcohols, aldehydes, ketones, esters and furans, whose synthesis begins in the milling step of the oil extraction process by the activation of several enzymes [9,10]. EVOOs volatiles are mainly enzymatically produced from linoleic (LA) and linolenic (LnA) acids through the lipoxygenase (LOX) pathway. During the first step, 9- and 13-hydroperoxides are generated [11]. Subsequently, specific hydroperoxide lyases (HPL) catalyze the production of C6 aldehydes [12], which are later reduced by alcohol dehydrogenase arising C6 alcohols [13]. Finally, the mediation of alcohol acetyl transferases (AAT) produce different kind of esters [14]. Moreover, C5 compounds are produced through an additional branch of the LOX pathway from a cleavage reaction of 13-hydroperoxide of linoleic acid involving its alkoxyl radical [2]. Volatile compounds originated by the lipoxygenase cascade from polyunsaturated fatty acid (linoleic and linolenic acids) are responsible for pleasant flavors. Among them, C5 and C6 compounds are the most representative in EVOOs [3,15]. Whereas, unpleasant flavors come from other chemical compounds, such as aliphatic carbonyls, alcohols or acids, produced by fatty acid oxidation process, amino acids biochemical transformations and other enzymatic activities or fermentations [2,15,16].

Final volatile composition of EVOOs is determined by these biochemical processes, which are in turn influenced by agronomic and technological aspects [2,15]. Specifically, environmental, and genetic factors have an important influence on the volatile fraction of EVOOs [17–20]. Thus, volatile profiles have been even proposed as useful characteristic for frauds detection in commercial EVOOs, regarding to the cultivar or geographical origin authenticity [21–24].

Cultivar genetic origin has been considered one the most important factor influencing volatile composition of EVOOs because of the enzyme's activity and substrates (LA and LnA) availability are genetically determined [9,11,13,25]. The variability in volatile composition due to genetic influence has been characterized in a wide number of cultivars including local cultivar grown in specific regions [21,26] or representative cultivars from all over the world growing in cultivar collections [27–29]. Moreover, a high variability, even transgressing the genitors, has been found among progenies of breeding programs [30], which showed the potential to develop new cultivars with different EVOOs composition. Therefore, olive breeding programs include minor compounds among its breeding objectives together with many other important agronomic traits such as high productivity, disease resistance and suitability to different growing systems. Furthermore, the stability of improved traits across environments should be tested since olive orchards are constantly expanding through cultivation areas with diverse climatic conditions [31]. However, information on the combined effect of genotype and environment for volatile composition of EVOO is scarce. Besides to the best of our knowledge, specifically designed comparative fields in different environments have not been used to properly quantify the importance of these factors (genotype, environment, and interaction). In the olive breeding program of IFAPA, three genotypes were selected based on their high level of resistance to Verticillium wilt and high productivity and oil content [32–34]. These three breeding selections together with 'Picual' cultivar were evaluated in four comparative fields established in different locations of Jaén and Córdoba provinces in Andalusia, Southern Spain. In the present work, the variability of volatile composition was tested in EVOOs from these genotypes

considering the influence of the different environmental conditions and the effect of G x E interaction.

4.5. Materials and methods

4.5.1. Plant material and olive fruit sampling

Volatile compounds were analyzed in EVOOs from three advanced selections of the breeding program for Verticillium wilt resistance developed at IFAPA 'Alameda del Obispo'. These three genotypes were previously selected due to their high level of resistance to Verticillium wilt and potential high productivity and oil content [32–34]. Two of them come from crosses between 'Frantoio' and 'Arbosana' cultivars ('IFAPA117-117' and 'IFAPA117-120') and the other one from open pollination of the cultivar 'Koroneiki' ('IFAPA111-2'). 'Picual' was also evaluated as a reference cultivar because it is the most grown cultivar in Andalusia with highly appreciated EVOO. One-year-old plants of the four genotypes were planted in three comparative fields in spring of 2015 in three different locations in Jaén province (Arjona, Begíjar and Úbeda) and in the experimental field of IFAPA (Córdoba) in spring of 2016. A randomized complete block design was used including 3 blocks and from four (Córdoba, Arjona and Úbeda) to six (Begíjar) plants/genotype/block. Characteristics of each experimental field are described in Figure 4.1. Córdoba is differentiated from the other locations by lower altitude and higher temperatures and, exceptionally high annual rainfall was recorded that harvest season. Úbeda, located at the highest altitude, was the coldest environment, while Arjona and Begíjar fields were characterized by similar environmental conditions.

Olive fruit samples of 4 kg were picked by hand from each elementary plot in November 2018. This was the second harvest season for all the experimental fields, corresponding with two years after planting in Córdoba field and the third year after planting in Arjona, Begíjar and Úbeda environments. A total of 48 samples (4 genotypes x 4 fields x 3 blocks) were transported to the laboratory immediately after collection and stored at 4°C until olive oil extraction within 24h.

Figure 4.1 Location and characteristics of experimental fields

Environment	1 Microplots (Córdoba, Andalusia)	2 Arjona (Jaén, Andalusia)	3 Begíjar (Jaén, Andalusia)	4 Úbeda (Jaén, Andalusia)
Latitude:	37°51'22" N	37°55'06" N	37°57'24" N	37°53'23" N
Longitude:	4°48'02" W	3°58'16" W	3°37'00" W	3°15'05" W
Altitude (m):	91	269	278	470
Rainfall (mm):	802.4	516.4	536.6	536.2
T° mean (°C):	17.2	16.6	16.4	14.4
T° min. (°C):	-3.4	-6.3	-3.9	-5
T° max.(°C):	44.5	43.1	42.5	42.8

*Climatologycal data referred to 2018

4.5.2. Olive oil extraction

Only healthy fruits, without visible damage, were processed. EVOO was extracted using the Abencor system (Commercial Abengoa, S.A., Seville, Spain). Firstly, olive fruits were milled at 3000 rpm with a 5 mm sieve. 2.5% of talc was added to the resulting olive paste that then was malaxed at 28ºC for 30 min, adding 100 ml of water for the last 10 minutes of malaxation, and centrifuged for 1 min at 3500 rpm. The EVOO obtained was decanted, filtered through paper,

transferred into dark glass bottles, and stored in the dark at 4°C until the moment of analysis (less than one week). As expected, obtained from healthy fruit samples without damage, all the extracted oils were classified as EVOO, meeting the regulatory values established for quality criteria. For instance, all samples showed free acidity values lower than the 0.8 value regulated for classification as EVOO, even more, only two samples showed free acidity values higher than 0.4 (data not shown).

4.5.3.Analysis of volatile compounds

Volatile compounds were extracted and analyzed by means of HS-SPME/GC-MS [12]. EVOO samples (1g) were prepared in duplicate vials of 10mL and placed in a vial heater at 40°C for a 10 min equilibration time. Volatile compounds from the headspace were adsorbed onto SPME fiber DVB/Carboxen/PDMS 50/30 µm (Supelco Co., Bellefonte, PA, USA). The sampling time was 50 min at 40°C, and the desorption of volatile compounds was performed directly into the GC injector. Volatile compounds were identified on a Bruker model Scion 456-GC-TQ MS system (Bruker, Massachusetts, USA) equipped with a Supelcowax 10 capillary column (30 m × 0.25 mm i.d.; thickness, 0.25 µm; Sigma-Aldrich Co. LLC) working under the following conditions: helium (carrier gas) flow rate of 1mL/min; injection by splitless method at 250°C; 5 min of column holding time at 50°C and then ramped up at 4°C/min to 200°C; the mass detector operated in electronic impact mode at 70 eV, with the temperature source set at 250°C and the mass spectra were scanned at 7 scans/s in the 30–250 m/z ratio range. Volatile compounds were matched to NIST 17 (version 2.3) library and by with GC retention time in comparison with standards.

For the quantification of volatile compounds, calibration curves were obtained for each one by adding known amounts of the pure standards to deodorized olive oil at six level (Acesur, Seville, Spain).The absence of target volatile compounds in the matrix was checked and this olive oil was used to build calibration curves. As control of the extraction and analysis, samples containing a mixture of volatile standards and blank samples (no oil) were run at the beginning and during sample analysis.

4.5.4.Statistical analysis

Volatile compounds were clustered into groups based on the fatty acid of origin (LA or LnA) and the number of carbons (C6 and C5 compounds), as well as esters formed through the LOX pathway and terpenes (Table 4.1). In order to understand the contribution of each volatile compound to the EVOO's aroma, odor thresholds were considered [30], as well as the odor activity value (OAV) [29].

Analysis of variance was carried out for total volatile compounds and percentage of the groups to test the effect of genotype, environment, and their interaction. The distribution of individual volatile compounds within groups by genotype and environment was tested by Chi-Square Test ($\chi 2$). Principal components analysis (PCA) was performed to test the relations among the different volatile compounds as well as their grouping according to genotype and environment. Statistix (Analytical Software, Tallahassee, FL, USA) and Unscrambler (CAMO A/S, Trondheim, Norway) were used for the statistical analysis. Both individual volatile compounds and groups of compounds, as well as the total amount of volatiles, were included

in PCA analysis, for a total of 31 variables defined in Table 4.1. Data were expresses as percentage and standardized before analysis.

4.6. Results and discussion

EVOOs were produced from healthy fruits, hand-picked and following a proper extraction process, so most of the detected compounds produce pleasant flavors [3] and no compounds related to negative attributes were detected.

Among the volatile compounds analyzed in the volatile fraction of EVOO, those enzymatically synthetized from polyunsaturated fatty acids through the LOX pathway have been described as the most important contributors to EVOO's aroma, both qualitatively and quantitatively; therefore, they were selected as target analytes being the major component of the volatile fraction of EVOOs analyzed [2,3,26,29].

The table 4.1 shows the results of volatile analysis considering the total of samples. The quantification was expressed by grouping volatile compounds according to the number of carbons (C6 and C5) and the fatty acid of origin (LnA and LA), esters and terpenes. Each group was codified to simplify the following references. Average of total amount of target volatile compounds in EVOOs from the genotypes and environments tested was 28,732 µg/kg. Both C6 and C5 compounds were found in similar mean quantities of 14,392 and 13,939 µg/kg, respectively (Table 4.1). The most abundant compounds were those derived from linolenic acid (LnA), such as C6/LnA and C5/LnA (14,118 and 13,909 µg/kg, respectively), which was observed in previous studies carried out on large collections of olive cultivars [27–29]. However, among all compounds detected, only few of them were found in quantities above its odor threshold (OAV>1), so a low number of compounds could be considered to have a highlighted contribution to EVOOs' aroma.

In this sense, most of C6 compounds were detected in high enough quantities to exceed the odor threshold in several samples (Table 4.1). (E)-hex-2-enal was the most abundant volatile compound, with a concentration ranging from 1,351 to 21,730 µg/kg and showed OAV greater than 1 for all the samples analyzed (Table 4.1). Similarly, (E)-hex-2-enal was the main compound contributing to the aroma of EVOOs from different cultivars representing a wide genetic diversity of the Word Olive Germplasm Bank of IFAPA (Córdoba) [27–29]. Volatile compounds in sets of 36, 39 and 61 olive cultivars were previously studied, and most of the cultivars were characterized by high (E)-hex-2-enal content reaching the odor threshold in 100% of the EVOO samples [27–29]. Moreover, similar results regarding the importance and variability of this compound have been previously described in EVOO [27,30,29]. (E)+(Z)-hex-3-enal, were the second most important contributors to EVOO's aroma of C6/LnA, also having an AOV>1 in 74.5% of the samples analyzed.

C6/LA compounds (group G2), represented by hexanal and hexan-1-ol, had a lower contribution to the EVOOs' aroma than C6/LnA, with a concentration ranging from 35 to 709 µg/kg (Table 4.1).

C5/LnA group of compounds (G3) reached a similar content than C6/LnA. In this group, the proportion of pentene dimers was higher than C5 aldehydes, alcohols, and ketone (Table 4.1).

Nonetheless, C5/LnA has a low influence on the EVOOs odor as most of them exhibited an AOV<1, except for (Z)-pent-2-en-1-ol, which could provide a perceptible pleasant flavor in 14.9% of the samples. Similar contents of C5/LnA compounds were previously described for different olive cultivars and progenies [27,28,30].

C5/LA compounds (group G4) were identified, such as pentan-3-one and pentanal, but none of them was detected over its odor threshold (Table 4.1). Pentan-3-one was described in similar quantities in different EVOOs from several cultivars [27,35]. Whereas pentanal may not be present in EVOO, for example, a previous study about four Tunisian cultivars, it was only detected in 'Dhokar Douirat' cultivar [36].

Table 4.1 Average content and variability of volatile compounds detected in the EVOO samples analyzed (µg/kg oil) classified by chemical.

Group	Subgroup	Volatile compound	Code*	Mean (µg/kg oil)	Min. (µg/kg oil)	Max. (µg/kg oil)	C.V.**	Samples OAV>1 (%)	Odor threshold***
C6/LnA	*Total*		*G1*	14,118	4,724	31,373	40		
	C6/LnA aldehydes	(E)+(Z)-hex-3-enal	*G1-V01*	4,375	n.d.	18,215	117	74.5	1.7
		(Z)-hex-2-enal	*G1-V02*	302	48	861	77	21.3	424
		(E)-hex-2-enal	*G1-V03*	8,860	1,351	21,730	67	100	424
	C6/LnA alcohols	(Z)-hex-3-enol	*G1-V04*	445	30	4,417	187	8.5	1,100
		(E)-hex-2-enol	*G1-V05*	137	n.d.	1,314	168	0	5,000
C6/LA	*Total*		*G2*	274	35	709	69		
	C6/LA aldehyde	Hexanal	*G2-V06*	203	27	626	74	17	300
	C6/LA alchool	Hexan-1-ol	*G2-V07*	71	7	433	122	2.1	400
C5/LnA	*Total*		*G3*	13,909	3,283	30,719	47		
	C5/LnA aldehydes	(Z)-pent-2-enal	*G3-V08*	26	10	52	36	0	300
		(E)-pent-2-enal	*G3-V09*	28	10	64	44	0	300
	C5/LnA alcohols	Pent-1-en-3-ol	*G3-V10*	101	34	195	41	0	400
		(Z)-pent-2-en-1-ol	*G3-V11*	185	93	365	34	14.9	250
		(E)-pent-2-en-1-ol	*G3-V12*	22	7	39	39	0	250
	Pentene dimers	Penten dimer-1	*G3-V13*	577	107	1,312	55	0	13,500
		Penten dimer-2	*G3-V14*	505	94	1,135	54	0	13,500
		Penten dimer-3	*G3-V15*	3,901	791	8,978	52	0	13,500
		Penten dimer-4	*G3-V16*	3,690	791	8,208	49	0	13,500
		Penten dimer-5+6	*G3-V17*	1,434	466	3,008	42	0	13,500
		Penten dimer-7	*G3-V18*	3,440	835	7,665	45	0	13,500

C5/LA	*Total*		*G4*	30	4	195	113		
	C5/LA ketone	Pentan-3-one	*G4-V19*	13	1	171	214	0	7,000
	C5/LA aldehyde	Pentanal	*G4-V20*	17	2	88	90	0	240
Esters	*Total*		*G5*	317	16	1,795	134		
	LOX esters	Hexyl acetate	*G5-V21*	72	4	391	111	0	1,040
		(Z)-hex-3-en-1-yl acetate	*G5-V22*	245	8	1,405	147	34	200
Terpenes	*Total*		*G6*	85	n.d.	423	130		
		Limonene	*G6-V23*	15	n.d.	98	120	0	250
		Ocimene	*G6-V24*	70	n.d.	421	162	8.5	250
TOTAL				28,732	11,656	63,797	39		

*Code: Volatile compounds were classified by chemical groups and codified to reference in the text. **CV: Coefficient of Variation. ***Odor threshold [30]. LA= Linoleic acid; LnA= Linolenic acid; OAV= Odor activity value; n.d.= below the detection limit.

To a lesser extent, esters and terpenes also contributed to the aroma of EVOOs. (Z)-hex-3-en-1-yl acetate and hexyl acetate constituted esters group (G5). These compounds originated through the LOX pathway could be detected in different concentrations depending on the AAT activity, which also varies among cultivars [14,24]. Higher content of (Z)-hex-3-en-1-yl acetate in combination with C5 and C6 compounds could enhance the EVOOs' pleasant flavors [15]. Limonene and ocimene were the two terpene compounds found in the EVOOs samples, although only ocimene reach its odor threshold in 8.5% of samples. Similarly, these two terpenes were described in previous studies comparing Spanish and French EVOOs, in which ocimene resulted more abundant than limonene, mainly for 'Blanquetier' cultivar [24]. However, limonene was described in higher proportion than ocimene in Tunisian cultivars ('Jemri', 'Touffehi' and 'Fakhari') in which, limonene was the main terpene contributing to the EVOO's aroma [37].

According to the results, significant differences were found among genotypes and environments (fields) for total content and proportions of groups of volatile compounds, except for C5/LA and terpenes (Table 4.2). Variance attributable to represented 62% of the total variance in total volatile content. Regarding the proportions of the different groups of volatile compounds, the genetic factor represented up to 46% of the total variance for C6/LnA. These results are in line with previous works describing differences on volatile compounds for single cultivars like the Tunisian cultivars 'Oueslati' and 'Chétoui' grown in different geographical areas [18,19], i.e. variability associated to the environmental effect. Additionally, genetic differences have been described for several cultivars growing in the same area in Tunisia [37], Algeria [38], Italy [26], and even large sets of cultivars from the World Olive Germplasm Bank of IFAPA (Córdoba, Spain) [27,28]. Several works have also observed the effect of both factors (genetic and environmental) studying the volatile composition of different olive cultivars in different regions in Italy (6 cultivars and 4 regions) [39], Turkey (2 cultivar and 2 regions) [40] and Chile (3 cultivars and 2 regions) [35]. However, complete factorial design was not available in these works, and a proper G x E interaction could not be evaluated. Only in a previous work G x E interaction was suggested as an important source of variation for volatile composition from the characterization of three Italian cultivars ('Casaliva', 'Frantoio' and 'Leccino') in three grown regions (Abruzzo, Lombardy and Tuscany) [41]. Therefore, it is noteworthy that we found a quantitative small G x E effect compared with individual effects of these two factors. Variance for G x E interaction represents 15.8% and 23.8%, of the total variance respectively, for C6/LA and esters concentrations, showing significant effect ($p<0.05$) for both groups of compounds.

Table 4.2 Variability of volatile compound content depending on genotype, environment and their interaction.

		df	Total (µg/kg oil)	G1 (%)	G2 (%)	G3 (%)	G4 (%)	G5 (%)	G6 (%)
FACTORS	E	3	62 ***	23.7 ***	20.5 ***	18.1 ***	0.0 NS	16.8 ***	1.1 NS
	G	3	16 ***	46.0 ***	30.3 ***	45.2 ***	5.2 NS	24.7 ***	4.5 NS
	G x E	9	5 NS	7.1 NS	15.8 *	9.0 NS	8.8 NS	23.8 *	4.4 NS
	Error	31	17	23.2	33.4	27.7	85.9	34.7	90.0
GENOTYPE	Picual		24009 b	55.2 a	0.7 b	42.0 c	0.1	1.5 ab	0.5
	UCI1112		36634 a	39.5 c	0.7 b	57.3 a	0.1	1.9 a	0.5
	UCI117117		27116 b	57.2 a	1.4 a	41.0 c	0.1	0.1 c	0.2
	UCI117120		27027 b	49.3 b	0.8 b	49.0 b	0.1	0.7 bc	0.1
ENVIRONMENT	Arjona		24796 b	42.3 c	0.7 b	54.3 a	0.1	2.0 a	0.5
	Begíjar		34578 a	54.1 ab	1.1 a	44.2 b	0.1	0.5 b	0.2
	Córdoba		17100 c	55.3 a	0.7 b	43.0 b	0.1	0.6 b	0.3
	úbeda		39337 a	49.4 b	1.2 a	47.6 ab	0.1	1.3 ab	0.4

*Genotype factor (G), environment growing condition (E), genotype and environment interaction (G x E). Volatile compound groups: G1 = C6/LnA; G2 = C6/LA; G3 = C5/LnA; G4 C5/LA; G5 = Esters; G6 = Terpenes. Variance analysis results: NS = No significative; *** p<0,05; * p<0,1.*

Differences among genotypes for the total content of volatile compounds, were due to the high value for 'IFAPA111-2' (36,634µg/kg), mainly owing to a high content of C5/LnA in this cultivar (Table 4.2). On the other hand, EVOO from 'IFAPA117-117' and 'Picual' was differentiated by a greater C6/LnA percentage than C5/LnA in comparison with the other genotypes. These differences on volatile fractions among genotypes were frequently found in olive cultivars growing under the same environmental conditions [26–28,38] or also in different regions [42,43]. These results support the general idea of the volatile fraction like a character highly genetically regulated.

Among environments, higher volatile content was observed in Úbeda and Begíjar fields, which were quantitative and qualitatively similar between them (39,337 and 34,578 µg/kg, respectively). In contrast the lowest volatile content (17,100 µg/kg) (Table 4.2) was observed in Córdoba, the environment with higher temperature and lower altitude (Figure 4.1), which is in line with the study contrasting cultivars in south and center of Tunisia [44,45]. Quantitative and qualitative differences on volatile content of EVOOs were previously found in studies under different environmental conditions of Morocco [46], Turkey [40,47], Tunisia [19,45], Italy [20] and Chile [35]. The differences found in those studies were attributed to the influence of the environmental growth conditions, as altitude, latitude, temperatures, or rainfall, on the enzyme activities [3,18]. In this sense, specific compounds were associated with specific

geographical origins. For instance, 1-Hexanol and (E)-2-hexenal content could differentiate EVOOs from north and south of Tunisia [45], terpenes content varied among Croatian regions [22] and several compounds were revealed useful to distinguish the growing region of 'Picholine Marocaine' in Morocco [46]. According to our results, in qualitative terms, Arjona environment stood out because of having the lowest percentage of C6/LnA compounds together with the highest content of C5/LnA ones.

Therefore, in general terms, the results obtained for the first time from a complete factorial G x E experimental design, showed that a wide volatile fractions variability could be attributed to differences among cultivars and growing environments, while G x E had a low impact (Table 4.2). The low influence of G x E interaction found in volatile composition was in line with the difficulties in distinguishing EVOOs from single cultivars growing in different regions of Andalusia (Spain) [48].

To find out in detail volatile compounds responsible of the variability between genotypes, a statistical analysis was carried out for individual compounds within each group (Table 4.3). In general, compounds of each group were significatively involved in the genotype differentiation, except for C5/LnA. C6/LnA and esters compounds were useful to distinguish between 'Picual' and 'IFAPA111-2' from 'IFAPA117-117' and 'IFAPA117-120'. Thus, volatile compounds within C6/LnA showed a characteristic larger proportion of (E)-hex-2-enal in EVOOs from 'IFAPA117-117' and 'IFAPA117-120' genotypes, whereas 'Picual' and 'IFAPA111-2' showed a high percentage of (E)+(Z)-hex-3-enal and its alcohol (Z)-hex-3-enol (Table 4.3). The preponderant proportion of one of these two volatile compounds was also observed in EVOOs of Tunisian cultivars [36]. Higher amount of (E)-hex-2-enal, could also be due to a low activity of alcohol dehydrogenase (ADH), which was previously observed in some cultivars such as 'Coratina' and described as a genetically dependent characteristic [49]. Hence, genotypes with lower alcohol content could be caused by a less ADH enzyme activity, which was also observed in 'IFAPA117-117' for hexan-1-ol (Table 4.3). 'IFAPA117-117' and 'IFAPA117-120' EVOOs showed also higher percentage of hexyl acetate, which is an esters from LA, whereas (Z)-hex-3-en-1-yl acetate from LnA was more representative of 'Picual' and 'IFAPA111-2' EVOOs (Table 4.3). (Z)-hex-3-en-1-yl acetate was also described as an important volatile compound for olive cultivars as 'Arbequina', 'Chorruo', 'Koroneiki', 'Manzanilla' and 'Picholine Marocaine' [29]. Differences in esters content could indicate a different availability of the LnA or LA fatty acids or because of the AAT activity, both reasons were previously described as genetically dependent [9,17,50]. Similarly, differences in terpenes composition (Table 4.3) have been proposed as a characteristic highly determined by genetic and environmental factors [17,45,50].

Table 4.3 Differences in volatile composition of EVOOs depending on the genetic factor.

Volatile compound		'Picual'	IFAPA 111-2	IFAPA 117-117	IFAPA 117-120	χ^2
C6/LnA	(E)+(Z)-hex-3-enal	63.19	58.58	1.89	0.21	202.85***
	(Z)-hex-2-enal	3.57	3.15	0.92	0.85	
	(E)-hex-2-enal	29.96	31.16	95.67	96.62	
	(Z)-hex-3-enol	3.12	5.97	0.50	0.94	
	(E)-hex-2-enol	0.16	1.14	1.03	1.37	
C6/LA	Hexanal	74.76	73.69	85.91	62.41	14.00**
	Hexan-1-ol	25.24	26.31	14.09	37.59	
C5/LnA	(Z)-pent-2-enal	0.37	0.13	0.21	0.16	2.34^{NS}
	(E)-pent-2-enal	0.40	0.14	0.22	0.17	
	Pent-1-en-3-ol	0.83	0.56	0.81	0.94	
	(Z)-pent-2-en-1-ol	1.78	0.99	1.51	1.66	
	(E)-pent-2-en-1-ol	0.18	0.10	0.25	0.17	
	Penten dimer-1	3.93	4.27	3.81	3.75	
	Penten dimer-2	3.37	3.69	3.41	3.41	
	Penten dimer-3	27.94	29.11	24.80	27.41	
	Penten dimer-4	25.84	25.63	28.29	25.15	
	Penten dimer-5+6	11.14	10.98	9.79	11.56	
	Penten dimer 7	24.22	24.40	26.90	25.62	
C5/LA	Pentan-3-one	33.33	26.70	21.91	39.81	8.97*
	Pentanal	66.67	73.30	78.09	60.19	
Esters	Hexyl acetate	15.26	16.78	56.62	53.35	67.44***
	(Z)-hex-3-en-1-yl acetate	84.74	83.22	43.38	46.65	
Terpenes	Limonene	35.83	24.24	24.60	60.72	37.86***
	Ocimene	64.17	75.76	75.40	39.28	

Statistical significance of Chi-Square test ($\chi 2$): NS = Non-Significant, * p<0.05, ** p<0.01, *** p<0.001

Similarities on volatile profiles between 'IFAPA117-117' and 'IFAPA117-120' could be partly attributable to the fact that both were obtained from the same cross combination ('Frantoio' x 'Arbosana'), although proper analysis of the parents as well as extended progenies of the same cross should be carried out in future works before generalization.

Comparison among experimental fields revealed slighter differences on volatile fraction by the environmental influence in comparison with the genetic effect (Table 4.4), which is consistent with the results of previous works [22,35]. No significant differences between environment were found for C6/LnA, C5/LnA and esters volatile compounds (Table 4.4), in spite of variability of (E)-hex-2-enal content being previously described as a characteristically influenced by the environment [47,45]. The lack of differences for hexyl acetate content among environments has been previously reported in other studies and justified by a great stability of AAT enzyme under different climatic conditions [20].

On the other hand, EVOOs showed significant differences on C6/LA volatile components among environments (Table 4.4). Although, as previously reported in other studies involving a single cultivar under different regional conditions [19,51], a general higher proportion of C6 aldehydes than C6 alcohols was found in all environments. EVOOs from Begíjar had a

distinctive lower percentage of hexan-1-ol, which was previously proposed as a marker for geographical origin differentiation of Tunisian EVOOs [44]. In addition, variable proportion of C5/LA volatile compounds were detected among environments (Table 4.4), which is in accordance with the composition of EVOOs from areas around north Italy [20]. EVOOs from Úbeda field showed the highest content of pentan-3-one.

The proportion of individual terpenes compounds was also significantly different among environments (Table 4.4), which suggest the potential usefulness of these compounds as markers of origin in agreement with previous works [17,48,50].

Table 4.4 Differences in volatile composition of EVOOs depending on the environmental factor.

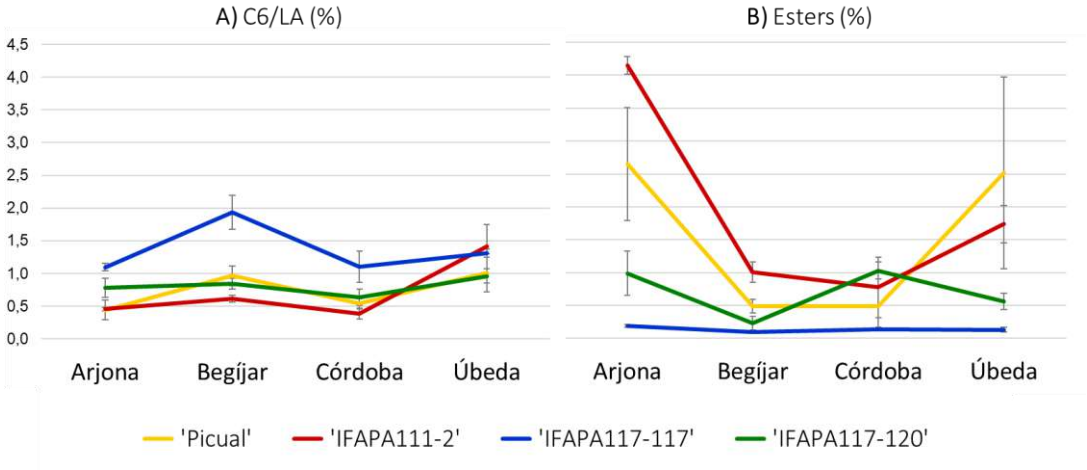
Volatile coumpound		Arjona	Begíjar	Córdoba	Úbeda	χ2
C6/LnA	(E)+(Z)-hex-3-enal	33.94	34.80	33.73	23.33	8.93^{NS}
	(Z)-hex-2-enal	2.23	2.34	2.09	1.91	
	(E)-hex-2-enal	61.15	59.48	61.95	68.49	
	(Z)-hex-3-enol	2.03	2.91	1.40	4.48	
	(E)-hex-2-enol	0.65	0.46	0.83	1.79	
C6/LA	Hexanal	72.66	85.87	70.29	68.45	9.40*
	Hexan-1-ol	27.34	14.13	29.71	31.55	
C5/LnA	(Z)-pent-2-enal	0.22	0.17	0.30	0.20	3.65^{NS}
	(E)-pent-2-enal	0.22	0.20	0.26	0.25	
	Pent-1-en-3-ol	0.78	0.76	0.87	0.71	
	(Z)-pent-2-en-1-ol	1.40	1.37	1.81	1.33	
	(E)-pent-2-en-1-ol	0.20	0.17	0.19	0.16	
	Penten dimer-1	3.83	4.23	3.24	4.53	
	Penten dimer-2	3.42	3.68	2.92	3.90	
	Penten dimer-3	27.69	28.53	24.70	28.41	
	Penten dimer-4	26.00	26.73	24.39	28.04	
	Penten dimer-5+6	10.46	9.77	14.05	8.97	
	Penten dimer 7	25.79	24.39	27.28	23.50	
C5/LA	Pentan-3-one	23.11	28.10	28.05	42.74	9.56*
	Pentanal	76.89	71.90	71.95	57.26	
Esters	Hexyl acetate	35.95	34.45	33.07	37.19	0.39^{NS}
	(Z)-hex-3-en-1-yl acetate	64.05	65.55	66.93	62.81	
Terpenes	Limonene	1.91	54.92	57.94	27.89	91.55***
	Ocimene	98.09	45.08	42.06	72.11	

Statistical significance of chi-Square test (χ2): NS = Non-Significant, * p<0.05, ** p<0.01, *** p<0.001

The significant effect of G x E interaction in C6/LA compounds was due to the higher content of them in EVOOs from 'IFAPA117-117' and 'IFAPA111-2' in Begíjar and Úbeda, respectively (Figure 4.2A). Additionally, significant effect of G x E interaction revealed a higher proportion of esters in EVOO samples of 'Picual' and 'IFAPA111-2' from Arjona and Úbeda, along with

the variability of esters content detected in EVOO from 'IFAPA117-120' in Begíjar and Úbeda (Figure 4.2B). These results suggested the influence of G x E interaction on the potential content of esters and C6/LA compounds of EVOO, as previously observed for the flavor profile of Italian EVOOs [41]. This fact could explain the differentiation of EVOOs from the same cultivar grown into nearby areas.

Figure 4.2 G x E interaction for individual volatile compounds percentage belonging to C6/LA (A) and esters (B) groups.

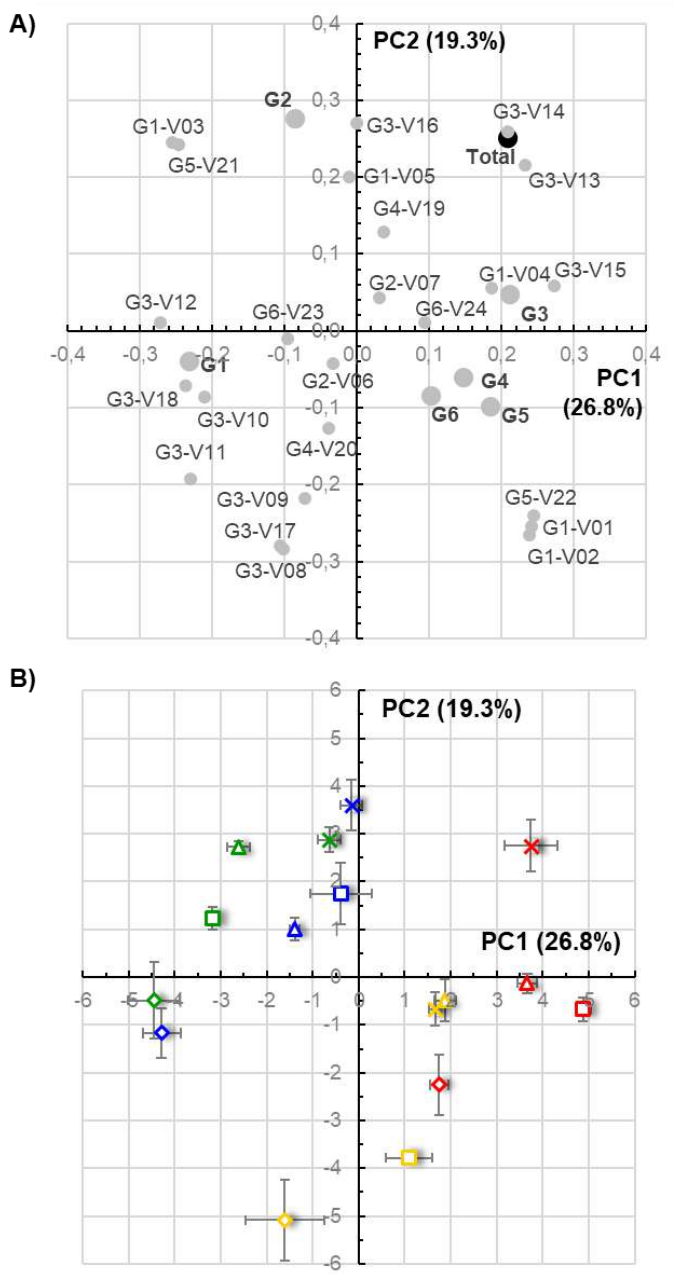


PCA analysis was also performed to assess the influence of E and G factors considering total amounts and percentage of volatile compounds (Figure 4.3). Two main principal components explained 46.1% of the total variance for volatile composition in the EVOOs studied. The loading plot of variables showed the main correlations between volatile compounds above mentioned, such as the negative correlation between C6/LnA and C5/LnA characterizing Arjona environment, and between (E)-hex-2-enal (G1-V03) and (E)+(Z)-hex-3-enal (G1-V01) useful compounds to distinguish between 'Picual' and 'IFAPA111-2' from 'IFAPA117-117' and 'IFAPA117-120' (Figure 4.3B).

No clear groupings were observed by environments, while two clusters were clearly formed by genotypes (Figure 4.3B), supporting the importance of genetic factor also observed in Table 4.3. 'IFAPA117-117' and 'IFAPA117-120' genotypes appeared together in second and third quadrants, where (E)-2-hex-enal (G1-V03), hexyl acetate (G5-V21) and limonene (G6-V23) were the most important compounds, among others. As mentioned above, these two genotypes shared a common parentage ('Frantoio' x 'Arbosana'), although their common genetic origin cannot be considered the only reason to explain their similar volatile fractions. For instance, variability on volatile composition was described among EVOOs extracted from progenies of 'Picual' x 'Arbequina' [30]. EVOOs from 'Picual' and 'IFAPA111-2' were mainly clustered around the fourth quadrant and characterized by (E)+(Z)-hex-3-enal, (Z)-hex-2-enal, and (Z)-hex-3-en-1-yl acetate volatile compounds (G1-V01, G1-V02 and G5-V22, respectively).

Figure 4.3 PCA loadings (A) and scores (B) biplots for volatile compounds. Average scores values of EVOOs samples are coded according to the genotype (yellow= 'Picual'; red= 'IFAPA111-2'; green=

'IFAPA117-120'; blue= 'IFAPA117-117') and environment (□ = Arjona; Δ = Begíjar; x = Úbeda; ◊ = Córdoba). Abbreviations are given in Table 4.1.



Similar odors properties can be expected for genotypes grouped according to their volatile composition in the PCA analysis as reported in previous works [2,21,28]. Thus, aromas of 'IFAPA117-117' and 'IFAPA117-120' could be differentiated from 'Picual' cultivar, whereas a more similar aromatic profile can be expected between 'IFAPA111-2' and 'Picual'. This characteristic mainly genetically determined was also quite constant under different

environments. Future works should investigate how the total content may affect the intensity of the sensory scores.

4.7. Conclusions

In this study, EVOOs' volatile fractions were mainly composed of C5 and C6 aldehydes and alcohols, from LA and LnA fatty acids. LOX esters and terpenes are also important components. C5 and C6 compounds from LnA were more abundant than those from LA and the content of aldehydes compounds was higher than alcohols in most of the EVOOs under study. The results showed significant variability on volatile composition among of EVOOs due to both the genotype and environmental factors. However, except for C6/LA and esters compounds, the different genotypes respond to environmental variation in the same way, i.e. no significant G x E interaction was found.

The overall results of this work indicated that volatile composition of EVOOs from breeding selections ('IFAPA111-2', 'IFAPA117-117' and 'IFAPA117-120') were quantitatively or qualitatively different to 'Picual' cultivar, when all of them grown in the same Andalusian fields and were harvested at the same time. So, the genotypes studied could be a suitable alternative for cultivar diversification in olive orchards and extend the commercial offer of EVOOs regarding to its aroma. Future works will be planned over several harvest seasons to decipher the contribution of location and year on the environmental variability for volatile composition of EVOO.

4.8. Acknowledgement

This research was financially supported by IFAPA project AVA201601.2, partially funded by European Regional Development Fund (ERDF). Alicia Serrano is grateful for the funding received from the Researcher Training Program in INIA-Autonomous Communities Research Centers (FPI-INIA). Thanks are also due to Angjelina Belaj for critical review of the manuscript.

4.9. References

[1] G. Procida, A. Cichelli, C. Lagazio, L.S. Conte, J. Sci. Food Agric. 2016, 96, 311-318.

[2] F. Angerosa, M. Servili, R. Selvaggini, A. Taticchi, S. Esposto, G. Montedoro, J. Chromatogr. A. 2004, 1054, 17-31.

[3] C.M. Kalua, M.S. Allen, D.R. Bedgood, A.G. Bishop, P.D. Prenzler, K. Robards, Food Chem. 2007, 100, 273-286.

[4] C. Oliver-Pozo, R. Aparicio-Ruiz, I. Romero, D.L. García-González, J. Agric. Food Chem. 2015, 63, 48, 10477-10483.

[5] C. Sales, T. Portolés, L.G. Johnsen, M. Danielsen, J. Beltran, Food Chem. 2019, 271, 488-496.

[6] B. Quintanilla-Casas, J. Bustamante, F. Guardiola, D.L. García-González, S. Barbieri, A. Bendini, T.G. Toschi, S. Vichi, A. Tres, LWT-Food Sci. and Technol. 2020, 121, 108936.

[7] E. Valli, F. Panni, E. Casadei, S. Barbieri, C. Cevoli, A. Bendini, D.L. García-González, T. Gallina Toschi, Foods. 2020, 9, 657.

[8] G. Caporale, S. Policastro, A. Carlucci, E. Monteleone, Food Qual. Prefer. 2006, 17, 116-125.

[9] A. Sánchez-Ortiz, A.G. Pérez, C. Sanz, Food Res. Int. 2013, 54, 1972-1978.

[10] A. Sánchez-Ortiz, C. Romero, A.G. Pérez, C. Sanz, J. Agric. Food Chem. 2008, 56, 4681-4685.

[11] A. Sánchez-Ortiz, C. Romero-Segura, C. Sanz, A.G. Pérez, J. Agric. Food Chem. 2012, 60, 812-822.

[12] A. Sánchez-Ortiz, M.A. Bejaoui, A. Quintero-Flores, A. Jiménez, G. Beltrán, Food Res. Int. 2018, 111, 220-228.

[13] A. Sánchez-Ortiz, C. Romero-Segura, V.E. Gazda, I.A. Graham, C. Sanz, A.G. Pérez, J. Agric. Food Chem. 2012, 60, 1300-1307.

[14] J.J. Salas, J. Agric. Food Chem. 2004, 52, 3155-3158.

[15] C. Campestre, G. Angelini, C. Gasbarri, F. Angerosa, Molecules. 2017, 22, 1833.

[16] L. Cecchi, M. Migliorini, E. Giambanelli, A. Rossetti, A. Cane, N. Mulinacci, J. Agric. Food Chem. 2019, 67, 13150-13163.

[17] T. Cecchi, B. Alfei, Food Chem. 2013, 141,2025-2035.

[18] O. Youssef, F. Guido, I. Manel, N.B. Youssef, C.P. Luigi, H. Mohamed, D. Daoud, Z. Mokhtar, Food Chem. 2011, 124, 1770-1776.

[19] S.B. Temime, E. Campeol, P.L. Cioni, D. Daoud, M. Zarrouk, Food Chem. 2006, 99, 315-325.

[20] S. Vichi, L. Pizzale, L.S. Conte, S. Buxaderas, E. López-Tamames, J. Agric. Food Chem. 2003, 51,6572-6577.

[21] F. Blasi, L. Pollini, L. Cossignani, Foods. 2019, 8, 58.

[22] I. Lukić, S. Carlin, I. Horvat, U. Vrhovsek, Food Chem. 2019, 270, 403-414.

[23] D. Melucci, A. Bendini, F. Tesini, S. Barbieri, A. Zappi, S. Vichi, L. Conte, T. Gallina Toschi, Food Chem. 2016, 204, 263-273.

[24] J.F. Cavalli, X. Fernandez, L. Lizzani-Cuvelier, A.-M. Loiseau, Food Chem. 2004, 88, 151-157.

[25] A. Sánchez-Ortiz, A.G. Pérez, C. Sanz, J. Agric. Food Chem. 2007, 55, 7869-7873.

[26] D. Tura, O. Failla, D. Bassi, S. Pedò, A. Serraiocco, Sci. Hortic. 2008, 118, 139-148.

[27] C. Sanz, A. Belaj, A. Sánchez-Ortiz, A. Pérez, Separations. 2018, 5, 24.

[28] L. García-Vico, A. Belaj, A. Sánchez-Ortiz, J. Martínez-Rivas, A. Pérez, C. Sanz, Molecules. 2017, 22, 141.

[29] G. Luna, M.T. Morales, R. Aparicio, Food Chem. 2006, 98, 243-252.

[30] A.G. Pérez, R. de la Rosa, M. Pascual, A. Sánchez-Ortiz, C. Romero-Segura, L. León, C. Sanz, J. Chromatogr. A. 2016, 1428, 305-315.

[31] J.F. Navas-López, L. León, E.R. Trentacoste, R. de la Rosa, Plant Physiol. Biochem. 2019, 139, 485-494.

[32] R. Arias-Calderón, L. León, J. Bejarano-Alcázar, A. Belaj, R. de la Rosa, D. Rodríguez-Jurado, Sci. Hortic. 2015, 185, 34-42.

[33] R. Arias-Calderón, D. Rodríguez-Jurado, J. Bejarano-Alcázar, A. Belaj, R. de la Rosa, L. León, Euphytica. 2015, 206, 619-629.

[34] R. de la Rosa, R. Arias-Calderón, L. Velasco, L. León, Eur. J. Lipid Sci. Technol. 2016, 118, 1160-1167.

[35] N. Romero, J. Saavedra, F. Tapia, B. Sepúlveda, R. Aparicio, J. Sci. Food Agric. 2016, 96, 583-592.

[36] I. Oueslati, F.M. Haddada, H. Manaï, W. Zarrouk, W. Taamalli, X. Fernández, L. Lizzani-Cuvelier, M. Zarrouk, J. Agric. Food Chem. 2008, 56, 7992-7998.

[37] S. Ben Brahim, A. Amanpour, F. Chtourou, H. Kelebek, S. Selli, M. Bouaziz, J. Agric. Food Chem. 2018, 66, 2851-2861.

[38] M. Cherfaoui, T. Cecchi, S. Keciri, L. Boudriche, Int. J. Food Prop. 2018, 21, 36-49.

[39] M. Servili, S. Esposto, R. Selvaggini, A. Taticchi, S. Urbani, G.F. Montedoro, I. Ricco. Acta Hortic. 2008, 791, 729-734.

[40] S. Kesen, H. Kelebek, S. Selli, J. Agric. Food Chem. 2014, 62, 391-401.

[41] D. Tura, O. Failla, D. Bassi, C. Attilio, A. Serraiocco, Eur. J. Lipid Sci. Technol. 2013, 115, 196-210.

[42] O. Baccouri, L. Cerretani, A. Bendini, M.F. Caboni, M. Zarrouk, L. Pirrone, D.D.B. Miled, Eur. J. Lipid Sci. Technol. 2007, 109, 1208-1217.

[43] F.M. Haddada, H. Manai, D. Daoud, X. Fernandez, L. Lizzani-Cuvelier, M. Zarrouk, Food Chem. 2007, 103, 467-476.

[44] A.B. Mansour, B. Gargouri, G. Flamini, M. Bouaziz, J. Oleo Sci. 2015, 64, 381-392.

[45] M. Issaoui, G. Flamini, F. Brahmi, S. Dabbou, K.B. Hassine, A. Taamali, H. Chehab, M. Ellouz, M. Zarrouk, M. Hammami, Food Chem. 2010, 119, 220-225.

[46] A. Bajoub, A. Sánchez-Ortiz, E.A. Ajal, N. Ouazzani, A. Fernández-Gutiérrez, G. Beltrán, A. Carrasco-Pancorbo, Food Res. Int. 2015 76, 410-417.

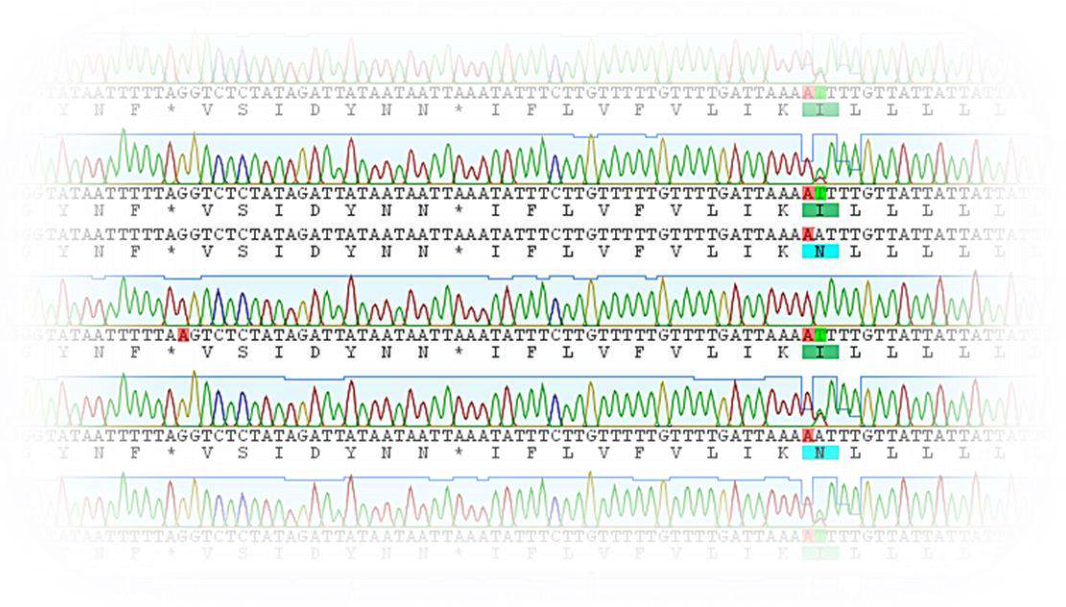
[47] M. Kiralan, G. Ozkan, F. Koyluoglu, H.A. Ugurlu, A. Bayrak, A. Kiritsakis, Eur. J. Lipid Sci. Technol. 2012, 114, 552-557.

[48] R. Aparicio-Ruiz, D.L. García-González, A. Lobo-Prieto, R. Aparicio, Eur. J. Lipid Sci. Technol. 2019, 121, 1800133.

[49] A. Runcio, L. Sorgonà, A. Mincione, S. Santacaterina, M. Poiana, Food Chem. 2008, 106, 735-740.

[50] S. Vichi, J.M. Guadayol, J. Caixach, E. López-Tamames, S. Buxaderas, J. Chromatogr. A. 2006, 1125, 117-123.

[51] Ş. Şişik Oğraş, G. Kaban, M. Kaya, Int. J. Food Prop. 2018, 21, 1833-1843.



5. Nucleotide Diversity Analysis of Candidate Genes for Verticillium Wilt Resistance in Olive

This chapter has been published in the journal 'Scientia Horticulturae':
Serrano, A., León, L., Belaj, A., Román, B. (2020).

5.1. Abstract

Analyzing the molecular variability of genes potentially related to disease resistance in olive (*Olea europaea*, L.) could be useful to develop marked assisted strategies for breeding as well as for general management of genetic resources. This work studied nucleotide diversity of 77 different genotypes from cultivated and wild subsp. *europaea*, subsp. *guanchica* and subsp. *cerasiformis* in coding regions of 7 disease response genes involved in different resistant mechanism against the infection of the fungal phytopathogen *Verticillium dahliae*. A total of 92 functional SNPs and 2 InDels were revealed unevenly distributed among each gen with frequencies from 0.0019 to 0.0614. Haplotype analysis were carried out to identify relationships between SNPs and disease resistance phenotypes or subspecies. Although a clear correlation attending to resistance evaluation could not be observed, phylogenetic and structure analysis showed some interesting association regarding the origin of plant materials for some genes. These are the first functional SNPs associated to Verticillium wilt resistance genes in olive and can contribute to stablish of a set of valuable markers for the management of germplasm collections and selection process in breeding programs.

5.2. Keywords

Olea europaea L., *Verticillium dahliae* Kleb, resistance genes, SNPs, marker-assisted breeding.

5.3. Introduction

The cultivated olive (*Olea europaea* subsp. *europaea* var. *europaea*) is one of the most important and ancient crop species within the Mediterranean Basin. Many studies demonstrated that the cultivated olive comes mainly from the domestication of wild Mediterrean olive (*O. europaea* subsp. *europaea* var. *sylvestris*) (Diez et al., 2015; Belaj et al., 2007; Baldoni et al., 2006). Besides, there are five more wild olive subspecies (*cuspidata*, *laparrinei*, *marocanna*, *guanchica* and *cerasiformis*) endemic from specific non-Mediterranean areas, which also contributed to the local olive diversification (Besnard et al., 2018, 2008; Brito et al., 2008).

The study of the existing variability and the identification of olive cultivars was traditionally based on morphological and agronomical traits (Blazakis et al., 2017; Barranco Navero et al., 2000). Then, molecular methods complemented these descriptors mainly focusing on the study of DNA variations (Belaj et al., 2012, 2007; Trujillo et al., 2014). Currently, SNP markers have been more efficient for molecular diversity analysis in olive compared to other kind of DNA markers, and special attention is being paid to their development and use (Belaj et al., 2018; D'Agostino et al., 2018; Kaya et al., 2013). So far, SNPs discovery has been generated by Sanger sequencing and Next Generation Sequencing (NGS) techniques (Ganal et al., 2009; Kaya et al., 2013), which have allowed to reveal an important number of SNPs very useful for genetic diversity studies (Belaj et al., 2018). Genotype-by-sequencing (GBS) technologies was applied to identify SNPs among a F1 progeny and construct a high-density genetic linkage map (İpek et al., 2016). This approach was also applied for cultivar identification and genetic relationships studies (Biton et al., 2015; D'Agostino et al., 2018).

SNPs discovery could be important also for early selection assistance in breeding programs (Belaj et al., 2018; Mammadov et al., 2012). Olive breeding programs are currently being developed to obtain new cultivars with improved agronomic characteristics including disease resistance (Arias-Calderón et al., 2015; Arias-Calderón et al., 2015a). However, classical breeding in woody plants requires long time due to the long juvenile period of the species and the need for developing accurate selection techniques (León et al., 2016; Santos-Antunes et al., 2005). In breeding for disease resistance, additional difficulties can be associated with the evaluation methods, like heterogeneity among infected plants, differences in pathogen colonization, unspecific symptoms and lack of high throughput screening techniques (Arias-Calderón et al., 2015a; Mercado-Blanco et al., 2003).

The development of marker assisted selection strategies requires knowledge and understanding the molecular basis of olive characteristics interesting to improve, which could be provided through various research approaches. Thus, contrasting transcriptome sequencing was applied to identify changes in gene expression and reveal SNPs in coding regions associated with different biological process (Kaya et al., 2013). Similarly, several SNPs potentially involved in domestication process were found contrasting gene sequences and transcriptomes between wild and cultivated olives (Cultrera et al., 2019; Gros-Balthazard et al., 2019; Unver et al., 2017). Moreover, differentially-expressed gene (DEGs) analysis has been widely used to reveal genes associated to important agronomic traits including disease response, particularly to Verticillium wilt caused by the fungus *V. dahliae* (Cabanás et al., 2015; Jiménez-Ruiz et al., 2017; Leyva-Pérez et al., 2018). Advances in NGS and improvement in olive genome availability enabled genome wide association studies to detect association between olive leaf or fruit characteristics and specific SNPs (Kaya et al., 2019a) and even a wide characterization of resistance genes using cultivated and wild olives genomes (Bettaieb and Bouktila, 2020). Transcriptome studies have also revealed a high number of candidate resistance genes for vascular diseases such as Verticillium wilt, which underlined the complexity of plant responses (Yadeta and J. Thomma, 2013; Wally and Punja, 2010).

For that reasons, this work aims to study polymorphisms among coding region of 7 candidate genes involved in *V. dahliae* infection response, comparing 77 olive genotypes with different phenotype reaction to this disease and coming from different origins. These genes were previously described in olive or different host plants, concerning several defense mechanisms such as physical barrier to avoid pathogens penetration and spreading inside plant tissues as well as biochemical reaction that inhibits the pathogen growth. The usefulness of the polymorphisms identified are discussed in terms of potential value for development of molecular markers for both management of germplasm collections and marked assisted breeding.

5.4. Materials and Methods

5.4.1. Plant material and DNA extraction

A set of 77 olive genotypes were considered for this study including cultivars, wild genotypes and breeding selections from IFAPA collections in Córdoba (Spain) (Table 5.1). Thirty-eight cultivars, representing different olive growing areas, were selected from the World Olive

Germplasm Bank (WOGBC). In addition, eleven wild olive genotypes preserved in the Wild Olive Tree Collection (WOTC) were also included: 7 genotypes belonging to *O. europaea* subsp. *europaea* var. *sylvestris* from different areas of the Iberian Peninsula and South Morocco, 2 genotypes of *O. europaea* subsp. *cerasiformis* representative from Madeira Islands and 2 genotypes of *O. europaea* subsp. *guanchica* from Canary Islands (Belaj et al., 2010). Finally, 28 genotypes were selected representing advanced selections obtained by the Olive Breeding Program, coming from different crosses and open pollination progenies.

The selection criterion of the germplasm set was to collect a wide diversity considering both differences in the origin and variability in response to Verticillium wilt. Thus, most of them had been previously phenotyped for verticillium wilt resistance by artificial inoculation in different experiments (Arias-Calderón et al., 2015a; Arias-Calderón et al., 2015; Arias-Calderón et al., 2015b; Martos-Moreno et al., 2006; López-Escudero et al., 2004) following the same technique based on dipping root system in a conidia suspension (Rodríguez-Jurado, 1993) and were classified in the following categories: highly resistant (HR), resistant (R), moderately resistant (MR), susceptible (S), extremely susceptible (ES) (López-Escudero et al., 2004). The genotype identification and register number, germplasm source, *O. europaea* subspecie or origin and resistant category of each genotype are given in Table 5.1.

Table 5.1 Olive germplasm set

Cultivar name or identification code	Register number/Code	Germplasm source[1]	*O. europaea* subspecie or crossbreed[2]	Category Resistance[3]
'Arbequina'	231	WOGB	subsp. *europaea* var. *europaea*	ES
'Arbosana'	666	WOGB	subsp. *europaea* var. *europaea*	MR
'Ayvalik'	97	WOGB	subsp. *europaea* var. *europaea*	S
'Azapa'	726	WOGB	subsp. *europaea* var. *europaea*	MR
'Bodoquera'	631	WOGB	subsp. *europaea* var. *europaea*	UN
'Bouteillan'	63	WOGB	subsp. *europaea* var. *europaea*	S
'Caninese'	77	WOGB	subsp. *europaea* var. *europaea*	UN
'Changlot Real'	15	WOGB	subsp. *europaea* var. *europaea*	R
'Chemlal de Kabilye'	118	WOGB	subsp. *europaea* var. *europaea*	S
'Cobrancosa'	124	WOGB	subsp. *europaea* var. *europaea*	S
'Coratina'	79	WOGB	subsp. *europaea* var. *europaea*	UN
'Cornicabra'	10	WOGB	subsp. *europaea* var. *europaea*	ES
'De Sal'	420	WOGB	subsp. *europaea* var. *europaea*	S
'Dokkar'	539	WOGB	subsp. *europaea* var. *europaea*	S
'Dolce Agogia'	1163	WOGB	subsp. *europaea* var. *europaea*	R
'Empeltre'	13	WOGB	subsp. *europaea* var. *europaea*	R
'Escarabajuelo de	353	WOGB	subsp. *europaea* var. *europaea*	UN
'Frantoio'	80	WOGB	subsp. *europaea* var. *europaea*	MR
'Hojiblanca'	2	WOGB	subsp. *europaea* var. *europaea*	S
'Jabaluna'	392	WOGB	subsp. *europaea* var. *europaea*	UN
'Kalamon'	105	WOGB	subsp. *europaea* var. *europaea*	UN
'Kato Drys'	848	WOGB	subsp. *europaea* var. *europaea*	R
'Koroneiki'	218	WOGB	subsp. *europaea* var. *europaea*	MR
'Leccino'	82	WOGB	subsp. *europaea* var. *europaea*	MR
'Manzanilla de Sevilla'	21	WOGB	subsp. *europaea* var. *europaea*	ES
'Manzanilla Picua'	377	WOGB	subsp. *europaea* var. *europaea*	R
'Maurino'	84	WOGB	subsp. *europaea* var. *europaea*	UN
'Lianolia Kerkyras'	108	WOGB	subsp. *europaea* var. *europaea*	S
'Menya'	669	WOGB	subsp. *europaea* var. *europaea*	R
'Meski'	115	WOGB	subsp. *europaea* var. *europaea*	S
'Mission Moojeski'	708	WOGB	subsp. *europaea* var. *europaea*	UN
'Moraiolo'	85	WOGB	subsp. *europaea* var. *europaea*	UN
'Negrillo de Arjona'	27	WOGB	subsp. *europaea* var. *europaea*	UN
'Nevadillo Negro de	45	WOGB	subsp. *europaea* var. *europaea*	S
'Picual'	97	WOGB	subsp. *europaea* var. *europaea*	S
'Picudo'	3	WOGB	subsp. *europaea* var. *europaea*	S
'Royal de Cazorla'	390	WOGB	subsp. *europaea* var. *europaea*	UN
'Sevillenca'	227	WOGB	subsp. *europaea* var. *europaea*	MR

Jeac9	109	WOTC	subsp. *europaea* var. *sylvestris*	S
W01	234	WOTC	subsp. *europaea* var. *sylvestris*	R
W02	225	WOTC	subsp. *europaea* var. *sylvestris*	R
W03	44	WOTC	subsp. *europaea* var. *sylvestris*	R
W04	55	WOTC	subsp. *europaea* var. *sylvestris*	R
W05	69	WOTC	subsp. *europaea* var. *sylvestris*	HR
W06	52	WOTC	subsp. *europaea* var. *sylvestris*	HR
W07	163-45	WOTC	subsp. *cerasiformis*	R
W08	163-126	WOTC	subsp. *cerasiformis*	R
W09	33	WOTC	subsp. *guanchica*	R
W10	1048	WOTC	subsp. *guanchica*	HR
I95-41		OBP	'Picual' x 'Jeac9'	R
I96-41		OBP	'Picual' x 'Jeac9'	HR
I105-35		OBP	'Picual' o.p.	R
I106-48		OBP	'Picudo' o.p.	MR
I110-17		OBP	'Frantoio' o.p.	R
I110-54		OBP	'Empeltre' o.p.	R
I111-2		OBP	'Koroneiki' o.p.	R
I111-69		OBP	'Picudo' o.p.	R
I113-11		OBP	'Arbequina' o.p.	R
I113-46		OBP	'Koroneiki' o.p.	HR
I114-15		OBP	'Manzanilla de Sevilla' o.p.	R
117-113		OBP	'Frantoio' x 'Arbosana'	MR
I117-120		OBP	'Frantoio' x 'Arbosana'	HR
I117-121		OBP	'Frantoio' x 'Arbosana'	R
I117-125		OBP	'Frantoio' x 'Arbosana'	S
I117-6		OBP	'Frantoio' x 'Dolce Agogia'	R
I119-15		OBP	'Changlot Real' x 'Dolce Agogia'	S
I119-44		OBP	'Changlot Real' x 'Dolce Agogia'	ES
I120-92		OBP	'Changlot Real' x 'Dolce Agogia'	R
I120-96		OBP	'Changlot Real' x 'Dolce Agogia'	HR
I123-58		OBP	'Koroneiki' x 'Empeltre'	S
I123-73		OBP	'Koroneiki' x 'Empeltre'	HR
I124-33		OBP	'Koroneiki' x 'Empeltre'	R
I124-65		OBP	'Koroneiki' x 'Empeltre'	MR
'Sikitita'		OBP	'Picual' x 'Arbequina'	UN
UCI10-30		OBP	'Frantoio' x 'Picual'	UN
UCI2-68		OBP	'Picual' x 'Arbequina'	UN
UCI4-62		OBP	'Frantoio' x 'Picual'	UN

[1]

Germplasm source: WOGB = World Olive Germplasm Bank of IFAPA (Córdoba, Spain); WOTC = Wild Olive Tree Collection established at IFAPA (Córdoba, Spain); OBP = Breeding selections from the Olive Breeding Program for Verticillium wilt resistance developed at IFAPA (Córdoba, Spain).

[2]Genotype subspecies, and parental crossed. 'o.p.' means open pollination.

[3]Category resistance to Verticillium Wilt: Highly Resistant (HR), Resistant (R), Moderately Resistant (R), Susceptible (S), Extremely Susceptible (ES) according to previous evaluations (Arias-Calderón et al., 2015, 2015a, 2015b; López-Escudero et al., 2004; Martos-Moreno et al., 2006). Unknown (UN) for those genotypes that have not been previously evaluated.

Total genomic DNA was extracted from young leaves following the CTAB method (de la Rosa et al., 2003). Leaf tissue were powdered in liquid nitrogen, mixed with 1ml of CTAB buffer (100 mM Tris-HCl pH 8, 1.4 M NaCl, 20 mM EDTA, 2% CTAB, 1% PVP, 0.2% β-mercaptoethanol, 0.1 % NaHSO4) and incubated at 65°C for 30 minutes, then was added to each sample an equal volume of chloroform–isoamyl alcohol (24:1), and centrifuged at 13000 rpm for 15 minutes. The aqueous phase was recovered and mixed with 750µL of isopropanol and let DNA to precipitate for one hour in the freezer, subsequently, was washed with 1ml of 70% ethanol, dried, and resuspended in 50µl of TE buffer (10mM Tris-HCl pH 8, 0.1mM EDTA). The concentrations of extracted DNA were estimated with Thermo Scientific™ NanoDrop 2000 and adjusted to 40ng/µl for further PCR analyses.

5.4.2. Candidate gene selection and primers design

A total of 7 genes were considered in this study. Four of them were selected from two SSH libraries generated with 'Frantoio' cultivar infected with *V. dahliae* and whose expression pattern were validated by qRT-PCR (Cabanás et al., 2015). They were revealed in up or down regulation and represent different functional genes such as a disease resistance-responsive protein (DRR2), a transcription factor (GRAS1), an acetone-cyanohydrin lyase (ACL) and a caffeoyl-o-methyltransferase (CO-MT). The other three genes were chosen from different species based on available works in which the role of the gen inhibiting *V. dahliae* infections was analyzed in plants with induced mutations. That genes are: a cythocrome P450 gene (CYP77A2), from wild eggplant of *Solanum torvum* (Yang et al., 2015), a thaumatin-like protein (TLP1) from *Gossypium barbadense L.* (Munis et al., 2010) and a cotton profilin gene (PFN2) (Wang et al., 2017).

In the case of the genes selected from other host species, the homologous sequence in *Olea europaea L.* was obtained by BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and sequences of the other candidate genes were also searched on NCBI and OLEA EST databases.

Primer design considered homologue sequences was carried out using Primer3web (version 4.1.0) online software (http://primer3.ut.ee). A pair of primers were designed for each candidate gene, trying to amplify mainly coding regions. In case of CYP77A2 gene, two pair of primers were designed to amplify two different exons non overlapping. Data about the selected candidate genes and primers sequences are available in Table 5.2.

Table 5.2 Candidate genes analyzed and primer designed.

Candidate	Reference	Homologue sequence[1]	Primers 5' - 3'
DRR2	JZ823156.1	OLEEUCl007459:Contig2	Fw: ATCATGGGAAAGCTCAGCAC Rv: GGTCCTGATACAAATCCTAAGCA
GRAS1	JZ844283.1	XM_023022114.1	Fw: AGAAATGGCACCTAGCATGG Rv: CTCTCGTCTGGCATGTGGT
ACL	JZ823163.1	OLEEUCl004759:Contig1	Fw: CCGGTTACCGTGTCTCAAAT Rv: ACTAAAGGACACAGGGAAATGC
CO-MT	JZ844695.1	XM_023042716.1	Fw: AACCAGAGGCCATGAAAGA Rv: TTTCTCAATGGTGCATCAGG
PFN2	GU237487.1	XM_023026745.1	Fw: ACCAGGCGGTGTTACTGTG Rv: TCCATTAAAAACCACCGAAAA
CYP77A2	HM480300.1	XM_023001563.1	Fw: ATTTTCACAGCCTTCGCATT Rv: TGAAGCAGCAGTTGGATCAG Fw: ATGGAGGGACCGATACAACA Rv: CATCAAAGCACAATACAACCA
TLP1	DQ912960.1	XM_023024708.1	Fw: TTTCAGCCACAGTTTTCACG Rv: CAAATAATCCGAACCCGAAC

[1]Homologue sequences were found on NCBI and OLEA EST databases. Primer design were done using homologue sequences on Primer 3web (version 4.1.0) online software.

5.4.3.PCR amplification and sequencing of PCR products

Extracted DNA for single plants of each genotype were PCR amplified using the designed primers for each candidate gene and performed in a Bio-Rad MyCycler Thermal Cycler PCR. DNA amplification was carried out in triplicate in total volumes of 25µl containing 40ng of genomic DNA, 0.5µM of each primer, 2.5µl of 10x NH4 Reaction Buffer, 0.75µl of 50 mM MgCl2 solution, 0.2mM of dNTP mixture, 1.25 unit of Taq DNA polymerase (Bioline). Cycling conditions included initial denaturation for 8 min at 94°C, followed by 35 cycles of 35s at 94°C, 35s at 56°C and 1 min at 72°C, with a final extension step of 7 min at 72°C. Amplification of each sample was confirmed by loading 5 µl of PCR reaction onto 2% agarose gel stained with Safe-Red™ dye and visualized under UV light.

PCR products were purified by FavorPrep™ GEL/PCR Purification Kit (Favorgen Biotech), according to the manufacturer's instructions, quantified as described above for genomic DNA and used for direct DNA sequencing (sanger sequencing) using respective forward and reverse primers at the STABVIDA sequencing facilities (Caparica, Portugal). Thus, each DNA sample was sequenced in duplicate. Moreover, a cDNA sample of 'Frantoio' cultivar (available from a previous experiment) was sequenced for each gene to confirm coding regions by alignment with the genomic DNA sequence.

5.4.4.Sequence analysis and polymorphisms detection

Forward and Reverse sequences of each sample were aligned to generate consensus sequences using Geneious 7.0.6. software by geneious pairwaise alignment (Kearse et al.,

2012). Contig sequences were manually checked for possible sequencing errors. ClustalW algoritm (Thompson et al., 1994) was applied for consensus sequences alignment in Geneious to determine the correct reading frame and for the phylogenetic analysis in MEGA 6.06 (Tamura et al., 2013), which was performed using the Neighbor-Joining method (Saitou and Nei, 1987). Alignment of the consensus sequences was translated into protein in MEGA 6.06 for each candidate gene and exported to DnaSP 5.10 software (Librado and Rozas, 2009) for nucleotide polymorphism analysis including allelic haplotypes, haplotype diversity (H), nucleotide diversity (π) and neutrality tests of Tajima's D and Fu and Li´s D* and F* (Fu and Li, 1993; Tajima, 1989). However, ka/ks ratio was estimated by MEGA 6.06 based on Nei and Gojobori's equation (Nei and Gojobori, 1986). Polymorphism Information Content (PIC) was calculated for locus based on allelic frequencies among all 77 genotypes analyzed. Two different analysis of molecular variance (AMOVA) were performed to study differences among genotypes according to disease resistance category (HR, R, MR, S, ES and UN) and origin of the plant materials (breeding selections, subsp. *europaea* var. *europaea*, subsp. *europaea* var. *sylvestris*, subsp. *cerasiformis* and subsp. *guanchica*). Finally, consensus sequences alignments were converted into NEXUS format as needed for PopART program (Population Analysis with Reticulate Trees), which was used to generate haplotype networks by TCS algorithm (Clement et al., 2000). The structure of the genetic diversity was determined using STRUCTURE v2.3.4. (Pritchard et al., 2000). The number of genetically homogeneous clusters (K) were performed considering 7 genes together, for K between one and six clusters with 20 interaction for each K value. Bayesian analysis was run under admixture model for 100 generations after a brun-on period of 1,000. The most likely number of clusters was determined by ΔK value. AMOVA was performed using GenAlEx 6.5 for Excel 2016.

5.5. Results

5.5.1.Sequence diversity analysis of candidate genes

Five out of the 7 verticillium wilt resistance candidate genes studied (DRR2, GRAS1, COMT, PFN2 and TLP1) were successfully amplified and sequenced from the 77 genotypes. However, ACL gene was not amplified from one genotype of the subsp. *guanchica* (W09) and poor-quality sequence was obtained from one genotype of subsp. *cerasiformis* (W07) for CYP77A2 exon 2. Therefore, sequences from 76 genotypes were considered for these two candidate genes.

Firstly, the alignment of 'Frantoio' cDNA and DNA allowed to verify the coding regions of candidate genes (Figure S5.1). In the case of ACL, COMT and PFN2 genes, both coding and non-coding regions have been sequenced, including two introns in ACL and COMT and one intron for PFN2. However, the sequence analysis has been only applied to the coding regions and only high-quality reads have been selected. The fragments size ranged from 302 bp to 1269 bp considering that in the case of introns containing genes this size was reduced only to exons.

Forward and reverse sequences for each candidate gene were used to construct contigs that were aligned for sequence diversity analysis. Polymorphisms data on coding regions are summarized in Table 5.3.

Table 5.3 Sequence diversity among 77 olive genotypes for 7 candidate disease response genes.

Candidate gene	DRR2	GRAS1	ACL	COMT	PFN2	CYP77A2	TLP1
Exons lenght (bp)	618	525	684	357	302	1269	535
Number of exons	1	1	3	3	2	2	1
Number of InDels	0	0	0	0	1 (20bp)	0	1(1bp)
Number of SNPs	15	1	18	6	9	10	33
Transitions	8	1	9	4	3	8	20
Transversion	7	0	9	2	6	2	13
Ka/Ks ratio	0.4693	-	0.2155	0.0858	0.3140	1,0611	0.4064
SNPs frequency	0.0242	0.0019	0.0263	0.0168	0.0298	0.0079	0.0614
Nucleotide diversity	0.0056	0.0009	0.0012	0.0041	0.0031	0.0018	0.0061
Number of haplotypes	17	2	7	10	7	19	16
Haplotypes diversity	0.557	0.469	0.177	0.697	0.491	0.873	0.472
Neutrality test							
Tajima's D	0.382^{ns}	1.580^{ns}	−2.311**	0.439*	-1.349^{ns}	0.274^{ns}	-1.679^{ns}
Fu and Li's D*	0.047^{ns}	0.510^{ns}	−2.372*	0.219*	-0.104^{ns}	1.385^{ns}	-1.395^{ns}
Fu and Li's F*	0.198^{ns}	0.960^{ns}	−2.798*	0.342*	-0.622^{ns}	1.190^{ns}	-1.798^{ns}
PIC value	0,83	0,00	0,87	0,59	0,64	0,71	0,90

The results correspond to polymorphism analysis carried out on coding regions. Sequence diversity were analyzed using DNAsp 5.10, except ka/ks ratio, which was calculated using MEGA 6.06. ns = Non significative. *significative, p<0.05. **significative, p<0.01.

Allelic diversity among genotypes is mainly represented by different SNPs frequencies for each gene analyzed fragment. GRAS1 and CYP77A2 are characterized by a low SNPs frequency of 0.0019 and 0.0079, respectively. DRR2, ACL and PFN2 showed similar SNPs frequencies between 0.0242 and 0.0298. A slightly lower value was observed for COMT with 6 SNPs. The highest SNPs frequency was observed in TLP1 (0.0614) as well as the highest nucleotide diversity (0.0061) due to the identification of 33 SNPs and 1bp indel. An indel of 20 bp was also found into the second exon of PFN2 gene resulting in a nucleotide diversity of 0.0031.

Analyzing SNPs mutations, for most genes more transitions than transversions were observed and synonymous substitutions predominate over the non-synonyms (values of Ka/Ks ratio below 1), which means that genetic diversity generated during the evolution of these genes was predominantly under purifying selection. By contrast, CYP77A2 gene showed a Ka/Ks value of 1.0611, which indicates that new variants appeared during the evolution (diversifying selection). The lack of non-synonymous substitution in GRAS1 gene hindered the estimation of ka/ks ratio.

The number of haplotypes and their diversity also varied among the analyzed genes, ranging from 2 (GRAS1) to 19 (CYP77A2) and from 0.177 (ACL) to 0.873 (CYP77A2), respectively (Table 5.3).

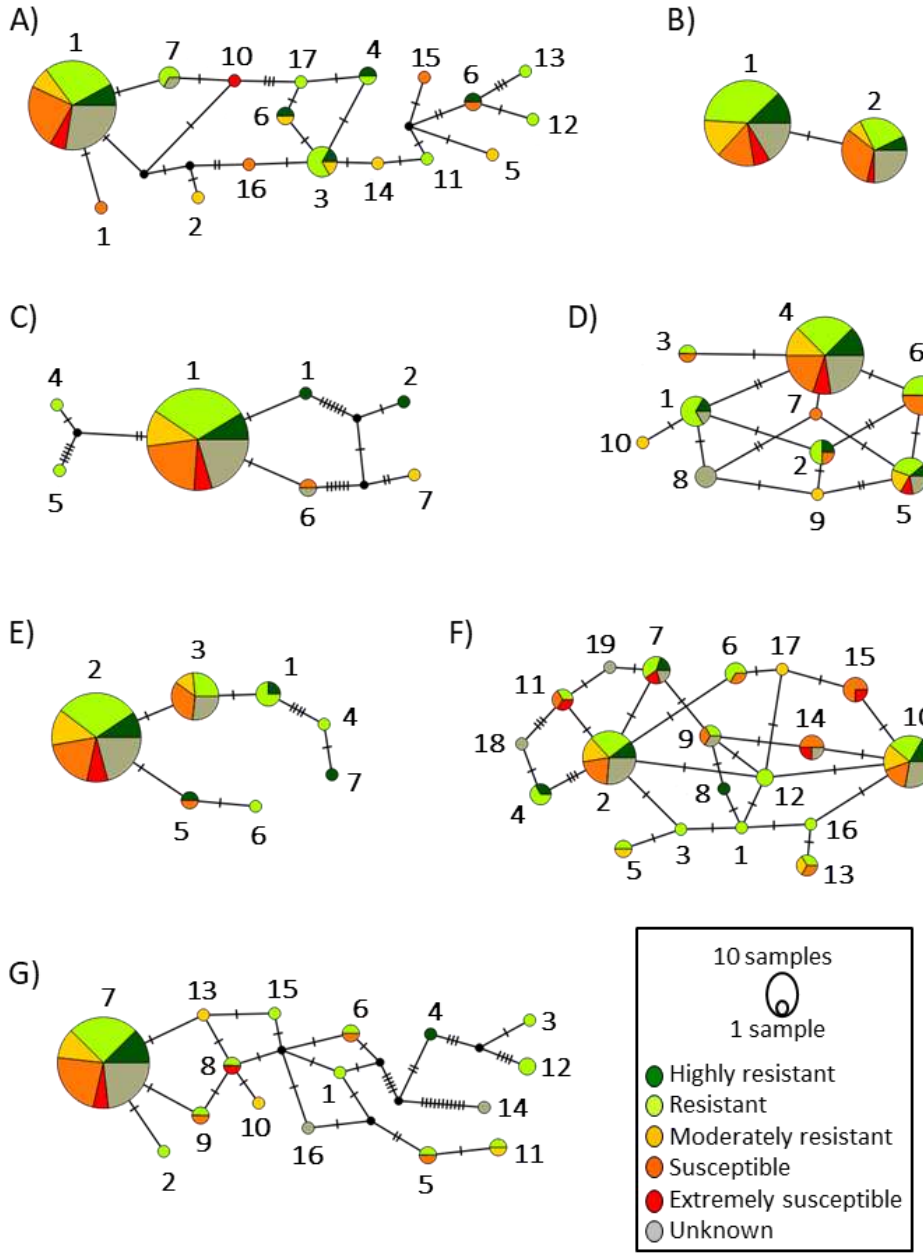
The three neutrality tests applied to analyze the polymorphisms showed both positive and negative scores. However, only ACL and COMT ratios showed significant values. In case of ACL, negative scores confirm a purifying selection, while positive scores for COMT are related to rare alleles or lack of singletons.

The reduced number of polymorphisms in the GRAS gene was also reflected in the null value of PIC parameter. For the other genes, the mean of PIC value was 0.76 ranged from 0.59 (COMT) to 0. 90 (PLT1) which means that all of them could be highly informative markers (PIC≥0.50).

5.5.2. Haplotype characterization for candidate genes

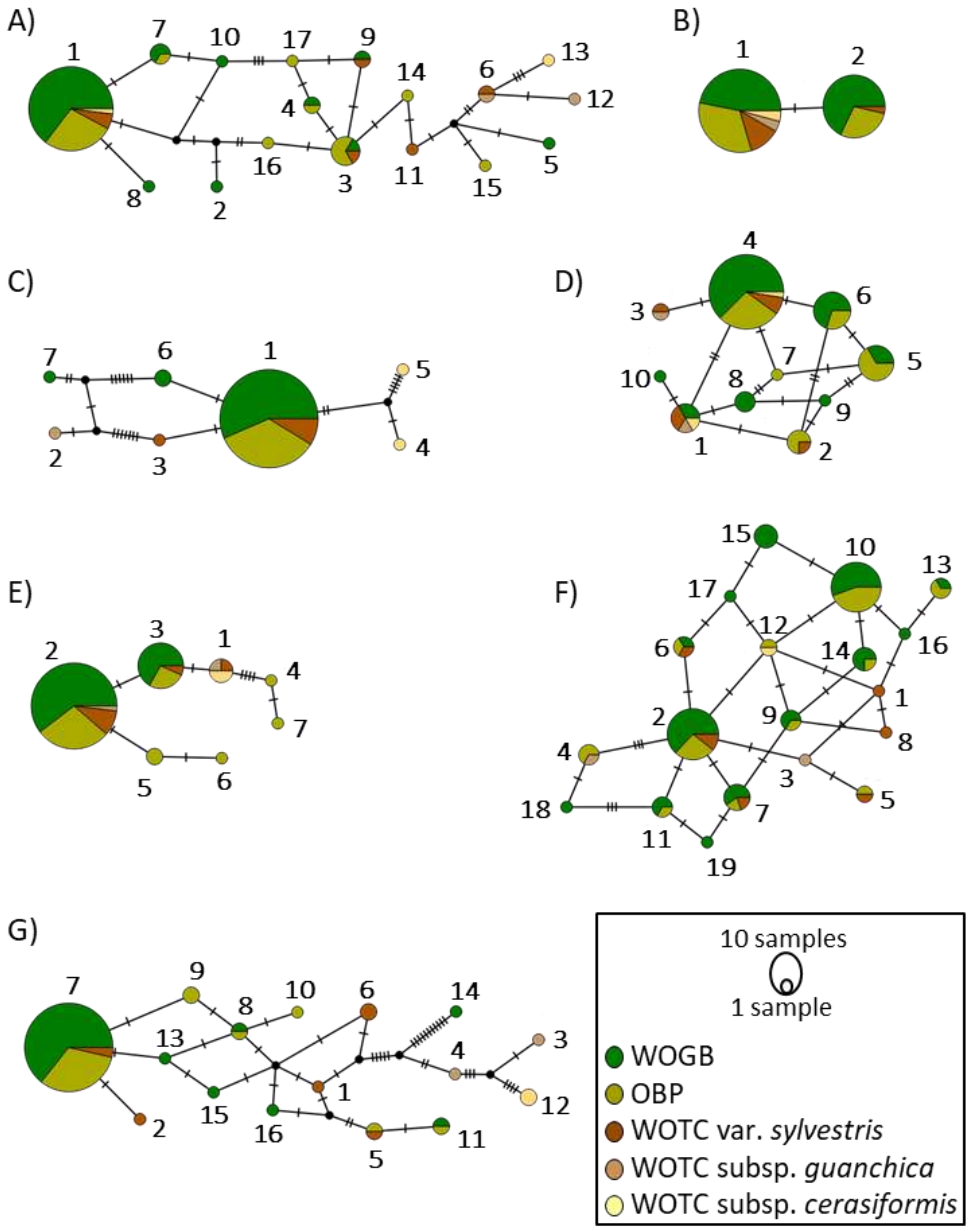
Haplotype networks are very variable between genes, with more complex networks correspond to genes with more haplotypes (Figures 5.1 and 5.2). COMT and CYP77A2 genes revealed a high number of interconnected haplotypes mainly by single mutations events because of a higher haplotype diversity with respect to the nucleotide diversity. On the other hand, haplotypes not connected to each other were observed in PFN2 gene while GRAS1 gene showed a network only composed by two haplotypes connected by a single mutation event. Lack of ancestral relations were observed in DRR2, ACL and TLP1 haplotypes networks, although several mutations events dividing some haplotypes were found in ACL and TLP1 genes. The polymorphism distribution within haplotypes of different genes is represented in Figure S5.2.

Figure 5.1 Haplotype network of different candidate gene based on the resistance response to V. dahliae infection of each genotype.



The haplotype network was constructed using the PopART program (Population Analysis with Reticulate Trees). A) DRR2 gene; B) GRAS1 gene; C) ACL gene; D) COMT gene; E) PFN2 gene; F) CYP77A2 gene; G) TLP1 gene. Each circle represents a haplotype and was labeled accordingly. Circle size is proportional to the number of sequences presenting that haplotype. The color represents the level of resistance to infection by *V. dahliae* pathogen. The black nodules represent the possible ancestral and black sticks correspond to mutational steps between haplotypes.

Figure 5.2 Haplotype network of different candidate gene based on the origin of each genotype.



The haplotype network was constructed using the PopART program (Population Analysis with Reticulate Trees). A) DRR2 gene; B) GRAS1 gene; C) ACL gene; D) COMT gene; E) PFN2 gene; F) CYP77A2 gene; G) TLP1 gene. Each circle represents a haplotype and was labeled accordingly. Circle size is proportional to the number of sequences presenting that haplotype. The color represents the genotypes origin: World Olive Germplasm Bank (WOGB); breeding selection from the Olive Breeding Program (OBP); Wild Olive Tree Collection (WOTC) *sylvestris*, *guanchica* and *cerasiformis* subspecies. The black nodules represent the possible ancestral and black sticks correspond to mutational steps between haplotypes.

- *DRR2*

Sequenced genotypes were divided into 17 haplotypes and among them 11 were represented by only one genotype; each of these haplotypes corresponded to a different disease response from resistant (Hap 11, 12, 13 and 17) to extremely susceptible (Hap 10) (Figure 5.1A). Single haplotype also represented different germplasm origins, including breeding selections (Hap 14-17), cultivars (Hap 2,5, 8 and 10), wilds (Hap 11) as well as the subsp. *guanchica* and *cerasiformis* (Hap 12 and 13, respectively) (Figure 5.2A). The haplotypes 3, 4 and 9 included cultivated and wild genotypes that display different response upon *V. dahliae* infection ranging from highly resistant to moderately resistant. The haplotype 7 also represented 2 resistant genotypes ('Dolce Agogia' cultivar and the breeding selection 'I117_6') as well as 'Caninese' cultivar, whose disease response is unknown. However, the haplotype 1 represented 51 genotypes, including all kind of disease responses (Figure 5.1A) and origins, except *guanchica* genotypes (Figure 5.2A).

- *GRAS1*

Allele diversity for GRAS1 gene originated a network composed by two haplotypes. The haplotype 1 represented 63.6% of the total. Both included genotypes with different disease response, from highly resistant to extremely susceptible (Figure 5.1B) and genotypes from different origin, although genotypes belonging to *cerasiformis* and *guanchica* subspecies joined into haplotype 1 (Figure 5.2B).

- *ACL*

Most of europaea subspecie genotypes (both cultivated and wild) are included in the haplotype 1 (Figure 5.2C) whose verticillium wilt disease response varies from highly resistant to extremely susceptible (Figure 5.1C). The genotypes belonging to subsp. *cerasiformis* are represented by the haplotypes 4 and 5, whereas haplotype 2 corresponds to *guanchica* genotype. These 3 haplotypes including resistant genotypes along with Hap 7 ('Sevillenca' cultivar) were differentiated by several mutation events, also with lack of ancestral relations.

- *COMT*

Haplotypes 4 and 5 represent 40 and 9 genotypes, respectively, including all levels of disease resistance. A total of 12 resistant and moderately resistant genotypes are represented by the haplotypes 3 and 6 (Figure 5.1D). Haplotype 8 represented cultivars from WOGB with unknown response to *V. dahliae* infection (Figures 5.1D and 5.2D). Haplotypes 7, 9 and 10 represent a single susceptible breeding selection (I123-58) or a single moderately resistant cultivar ('Frantoio' and 'Leccino'). *Cerasiformis* and *guanchica* genotypes are represented by haplotypes 1, 3 and 4 along with other cultivated and wild genotypes of *europaea* subspecie (Figure 5.2D).

- *PFN2*

The set of genotypes was divided into 7 different haplotypes for PFN2 gene. A 20bp delection were found in haplotypes 1, 4 and 7 (Figure 5.2S) which represent highly resistant and resistant genotypes (Figure 5.1E) from different subspecies. The dominant haplotype 2 represents 53

genotypes without disease response discrimination and including both *europaea* and *guanchica* subspecies. Haplotype 3 represented only *europaea* genotypes with different levels of disease resistance. (Figures 5.1E and 5.2E). Two breeding selection from OBP with different disease response were represented by haplotype 5, whereas haplotype 6 represented a single resistant breeding selection. These two haplotypes were differentiated from the greatest group by single mutation events.

- *CYP77A2*

CYP77A2 gene showed the largest number of haplotypes (19), with haplotypes 2 and 10 comprising the highest number of genotypes from *europaea* subspecies with variable response to *V. dahliae* infection (Figure 5.1F and 5.2F). Thus, extremely susceptible genotypes were included into haplotypes 7, 11, 14, 15 along with susceptible (Hap 11, 14 and 15) or even resistant (Hap 7,11) ones. Haplotypes 1 and 8 represented single wild genotypes, whereas haplotype 3 was exclusively for a single *guanchica* genotype. Other genotypes of these origins were included into different haplotypes joined to resistant selections. Interestingly, haplotypes 1, 3, 4, 8, 12 and 16 were associated to HR and R genotypes.

- *TLP1*

Allele diversity of TLP1 gene divided the set of olive germplasm into 16 haplotypes. Haplotype 7 represented the largest number of genotypes including cultivars from WOGBC, breeding selections and wild olives, all of them belonging to *europaea* subspecie (Figure 5.1G). However, they belonged to different resistance categories (Figure 5.1G). Several additional mutations originated the haplotypes 3, 4, 12 and 14 which corresponds to *guanchica* and *cerasiformis* subspecies and 'Coratina' cultivar. A deletion of 1pb was found in haplotypes 5 and 11 representing four genotypes of *europaea* subspecie (Figure 5.1G) but different disease resistance levels.

5.5.3. Genetic diversity structure and phylogenetic relationships

Analysis of molecular variance (AMOVA) show significant differences according to disease resistance category (HR, R, MR, S, ES and UN) but not according to origin of plant materials (breeding selections, subsp. *europaea* var. *europaea*, subsp. *europaea* var. *sylvestris*, subsp. *cerasiformis* and subsp. *guanchica*) (Table 5.4). However, in both cases most of the molecular variation was attributable to differences within groups rather than between groups.

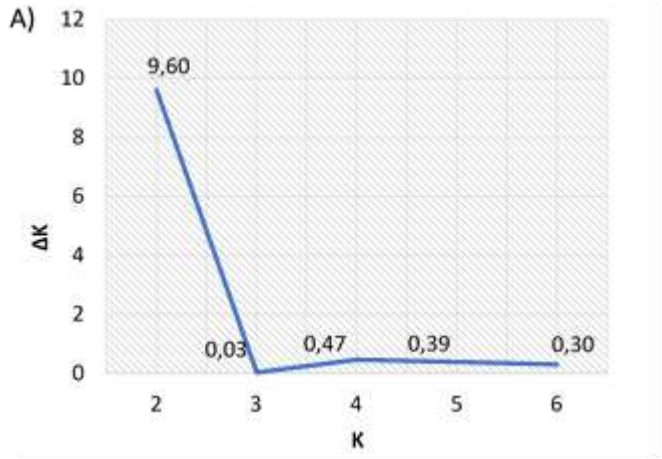
Table 5.4 AMOVA analysis for 77 genotypes and 94 polymorphisms (total for 7 genes) according to resistant category and origin of plant materials

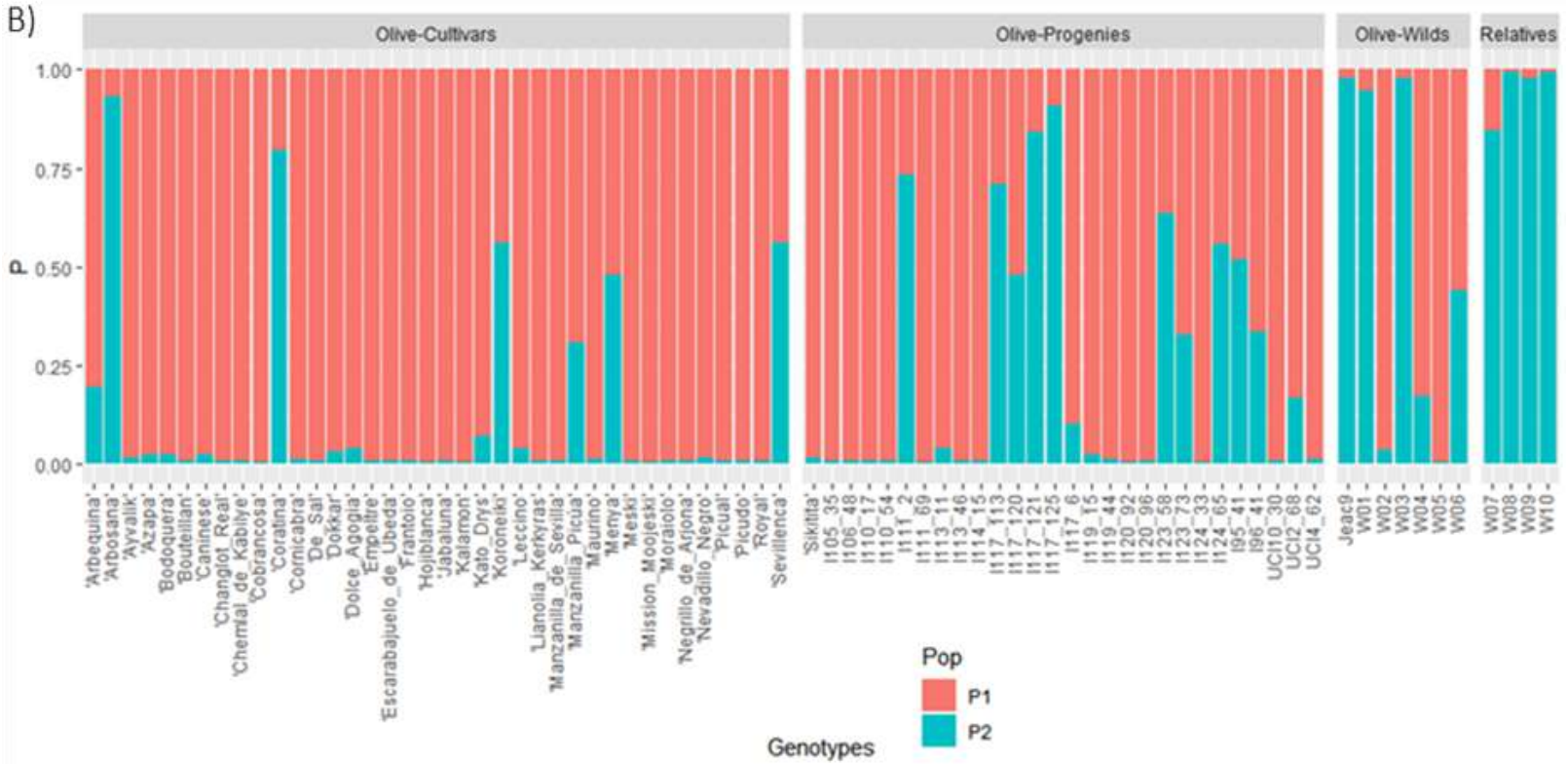
	Df	SS	MS	Variance component	Variance component (%)	P
For resistance categories						
Among groups	5	49.8	10.0	0.3	4.0	0.018
Within groups	71	461.8	6.5	6.5	96.0	
For plant material origin						
Among groups	4	34.3	8.6	0.2	2.0	0.166
Within groups	72	477.3	6.6	6.6	98.0	

d.f.: degree of freedom, SS: sum of squares, MS: mean squares, PhiPT: index for genetic differentiation among populations, p: level of significance.

A clustering study was implemented in STRUCTURE software considering admixed model. The Ln P(D) value was used to estimate the number of clusters of the 77 genotypes based on the genomic data of all locus analyzed. In order to determine the optimum k value, the number of cluster (K) was plotted against Δk (Figure 5.3A). The maximum value of Δk (9.60) was reached at k=2, declining sharply to keep steady for k>2. Genotypes were grouped into two inferred clusters whose thresholds resulted very different: 0.764 for gene pool 1 and 0.236 for gene pool 2. Most of the cultivated genotypes and breeding selections clustered in gene pool 1 (Figure 5.3B). The genotypes from relative subspecies (*cerasiformis* and *guanchica*) in conjunction with other wild olives (subsp. *europaea* var. *sylvestris*) were more characteristic of gene pool 2. However, some cultivars such as 'Arbosana and 'Coratina' also clustered in this gene pool. While the cultivars, 'Koroneiki' 'Menya' and 'Sevillenca' were almost equally assigned to the two genepools. Interestingly all the breeding selections obtained from crosses between 'Frantoio' and 'Arbosana' clustered mainly in the same gene pool related to wild olives and relative subspecies or showed high admixtures levels between the two gene pools (I117-120). The same was obtained for the breeding selection coming from 'Koroneiki' open pollination progeny (I111-2).

Figure 5.3 A) Δk values for different number of population (k) from 2 to 6. B) Population structure of 77 olive genotypes for k=2.

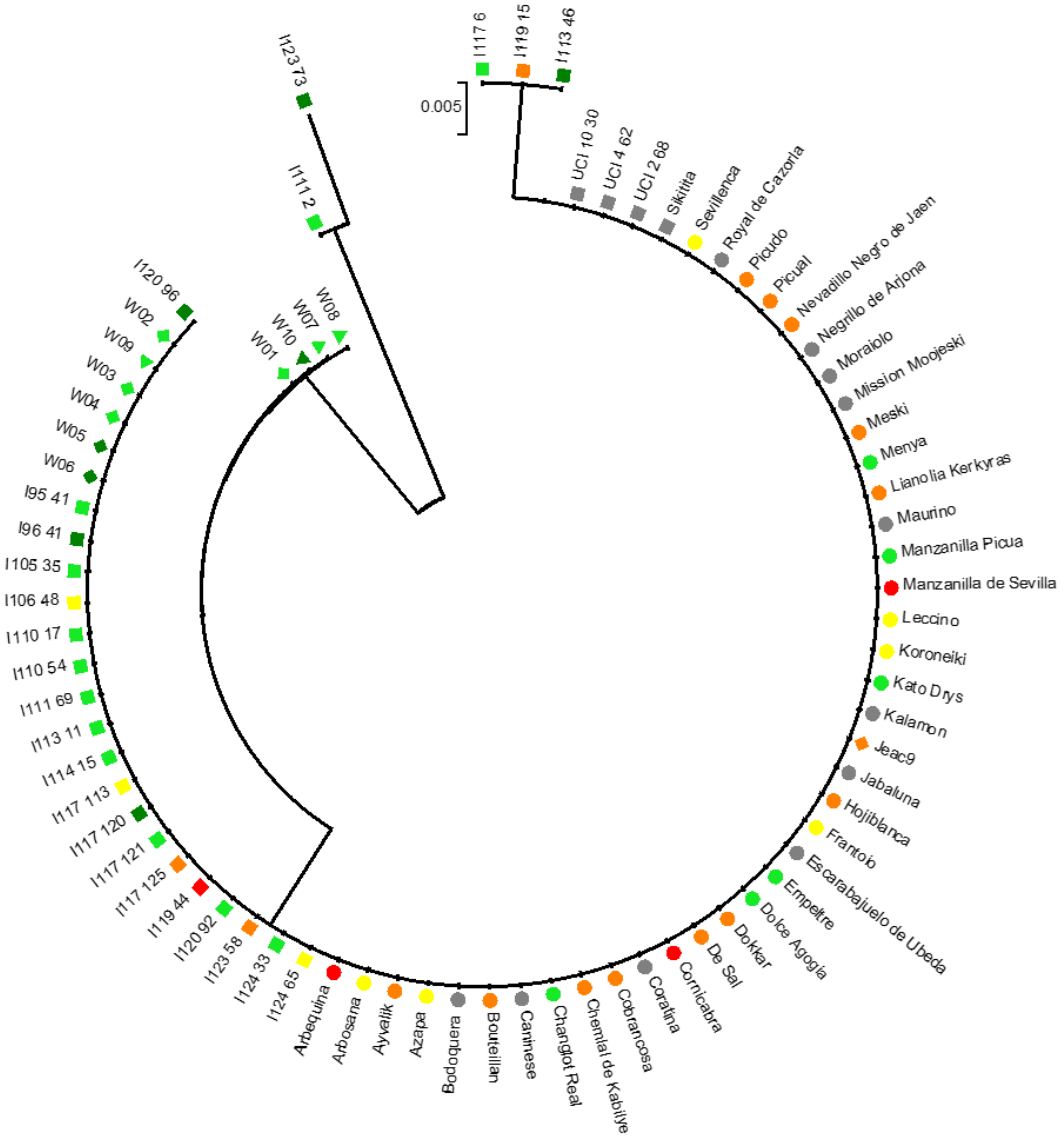




Genotypes were split in four groups based on cultivated olives (subsp. *europaea* var. *europaea*) breeding selections, wild olives (subsp. *europaea* var. *sylvestris*) and genotypes from relative subspecies (subsp. *cerasiformis* and *guanchica*). Each genotype is represented by a vertical column, which is partitioned into colored segments that represent the cultivar's estimated common fractions in the k clusters.

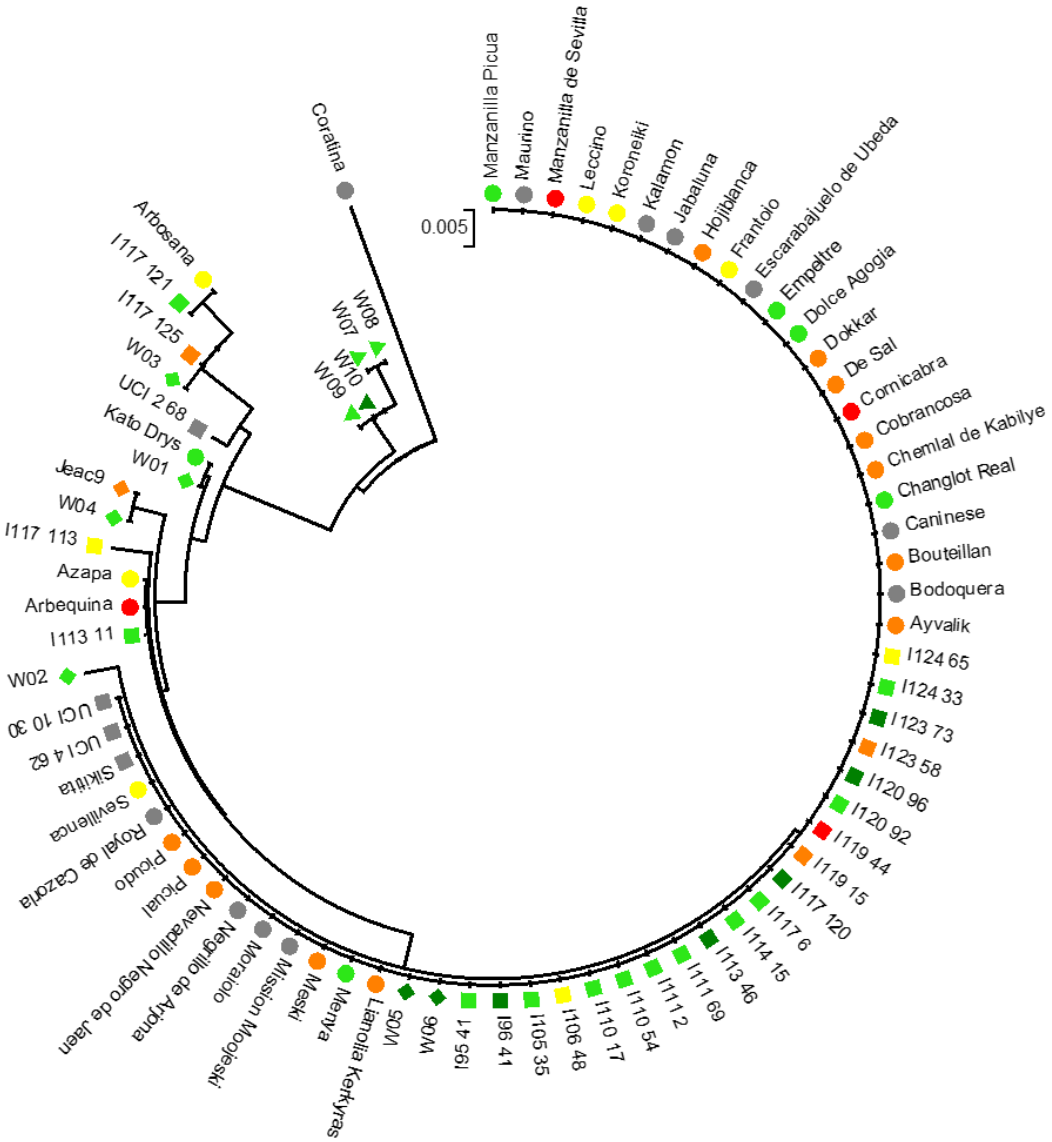
Phylogenetic analysis of each single gene revealed relationships among genotypes (Figures 5.4 and 5.5; and Supplemental Figures S5.3-S5.6) except GRASS gene which resulted very homogeneous among genotypes. PFN2 and TLP1 dendrograms showed some clustering for *O. europaea* subspecies (Figures 5.4 and 5.5). Thus, in PFN2, three genotypes of subsps. *cerasiformis* (W07 and W08) and *guanchica* (W10) clustered together with two advanced breeding selections (I111-2, I123-73) sharing a common 'Koroneiki' parentage, as well as with one wild genotype, being all of them resistant or highly resistant. These genotypes were differentiated due to the presence of a deletion of 20bp. The two above mentioned breeding selections as s well as other three ones (I117-6, I119-15 and I113-46) contained minor allele for other SNPs (Figure 5.4). TLP1 phylogenetic dendrogram showed the best separation among different subspecies genotypes with a group composed by the genotypes of *guanchica* subspecie, another very related group included *cerasiformis* genotypes and 'Coratina' cultivar from *europaea* subspecie. All of them, shared the minor allele of 5 SNPs. The presence of 11 SNPs only found in 'Coratina' may explain the clustering of this cultivar apart from the rest. While the wild genotype (W03), the cultivar 'Arbosana', and two breeding selection obtained from its cross with 'Frantoio' (I117-121 and I117-125) shared a 1pb deletion. Several divergent events separated the different subsp. *europaea* genotypes into different groups, although without phylogenetic differences among most cultivars and breeding selections (Figure 5.5). No clear clustering according to plant material origin or resistance category was observed for any of the other evaluated genes (Supplemental Figures S5.3-S5.6), regardless the number of divergent events. It should be noted that some breeding selections showed different alleles from parental ones and joined into different phylogenetic groups.

Figure 5.4 Phylogenetic tree for PFN2 gene fragment



Phylogenetic dendrogram based on PFN2 gene fragment of 77 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subsp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly resistant; Light green= resistant, Yellow = moderately resistant, Orange = susceptible; Red = extremely susceptible; Grey = unknown.

Figure 5.5 Phylogenetic tree for TLP1 gene fragment



Phylogenetic dendrogram based on TLP1 gene fragment of 77 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subsp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly resistant; Light green= resistant, Yellow = moderately resistant, Orange = susceptible; Red = extremely susceptible; Grey = unknown.

5.6. Discussion

Wide allelic diversity among genotypes was observed for the evaluated genes, with most of the polymorphisms based on SNPs variation in comparison with InDels. Among them, higher

frequencies of transitions than transversions were found, in agreement with previous studies considering sequence variation in olive (Besnard and El Bakkali, 2014; Cultrera et al., 2019; Zhu et al., 2019). Higher SNPs frequency for ACL gene was observed compare to previous results from the transcriptomic analysis of 'Frantoio' cultivar after *V. dahliae* infection (Cabanás et al., 2015), which suggests the existence of different genes belonging to the ACL family. In the case of GRAS candidate gene, our results are in agreement with the low levels of diversity previously described for this family of genes (Cenci and Rouard, 2017; Guo et al., 2017).

The nucleotide diversity values (π) observed in this work (from 0.0009 to 0.0061) are lower than the obtained in genes involved in fatty acid and triterpenes production or sugar transport throughout the plant (0.007 to 0.029) (Cultrera et al., 2019). And they are far below the average value (0.318) reported in genetic diversity analysis of different olive cultivars (Zhu et al., 2019), which could be higher due to the analysis of non-coding regions. However, nucleotide diversity analysis of 5 genes among different O. *europaea* subspecies showed similar values and confirmed that π is lower in coding regions than in introns (Besnard and El Bakkali, 2014). Regarding to ka/ks ratio most of the genes were under purifying selection which is in accordance with the results of a wide analysis of olive NBS resistance genes (Bettaieb and Bouktila, 2020). In accordance with previous findings in olive by means of both SSR and SNP markers (Belaj et al., 2018; Kaya et al., 2013; Zhu et al., 2019), high informativeness values were determined in this study for all the polymorphic fragments, except for GRASS, thus indicating that most of them could be useful for molecular markers development.

The association of SNPs into the same haplotype with resistance response to biotic stresses has been previously reported for traits such as *V. albo-atrum* infection in potato (Simko et al., 2004), bymovirus resistance in barley (Yang et al., 2017) or cyst nematode resistance in soybean (Yuan et al., 2016). In our case the aim of the haplotype-based analysis of polymorphisms was to detect association with Verticillium wilt evaluation since the analyzed genes were selected from differential expression studies in olive upon *V. dahliae* infection (DRR2, GRAS1, COMT, ACL) or functional validation in different host species where the genes resulted efficient against this pathogen (PFN2, TLP1 and CYP77A2). Moreover GRAS1, COMT and ACL were also induced in 'Arbequina' cultivar after infection of *Pseudomonas fluorescens* (Schilirò et al., 2012), even other genes belonging to the same family than CYP77A2, TLP1 and PFN2 were also found in transcriptomic analysis of 'Frantoio' cultivar under *V. dahliae* infection (Cabanás et al., 2015). DRR2 and COMT are involved in lignin production providing protection against the pathogen. The accumulation of lignin as defense mechanism against *V. dahliae* infection has been identified in olive (Gharbi et al., 2017) and deeply described in cotton (Xu et al., 2011). Similarly, TLP1 was selected due to its role in secondary cell wall thickening and rigidity (Munis et al., 2010) even enhancing resistance in roots (Zhang et al., 2013). TLP1 belongs to PR5 protein family related to stress resistance response that inhibits hyphal growth and sporulation of fungal pathogens. In olive, genes coding for PR5 were previously found highly expressed in resistant cultivar after *V. dahliae* infection (Gharbi et al., 2017). Another gene involved in biochemical pathways is CYP77A2, which belongs to cytochrome P450. Two genes of this family were identified as putative fatty acid oxidases and could produce antifungal compound through the epoxidation of linoleic

acid (Bak et al., 2011). PFN2 is related to constitutive defense mechanism such as filaments density and number of bundles in the actin cytoskeleton (Wang et al., 2017). The importance of actin cytoskeleton in plant as innate resistance mechanism has been previously described (Li and Staiger, 2018). In contrast to the above described genes, ACL gene is a salicylic acid-binding protein 2-like (SABP2) and GRAS1 is a transcription factor belonging to GRAS protein, both inducing systemic acquired resistance upon pathogen attack. GRAS gene was observed conferring resistance to Verticillium wilt in wild cotton (Cai et al., 2019). ACL acts as a lipase promoted by salicylic acid (SA) gene and induces the expression of other pathogenesis-related genes affecting the resistance response to *V. dahliae* in olive (Gharbi et al., 2017) and cotton (Gong et al., 2017).

Despite the functional evidence of the selected genes, most haplotypes observed for them in this work represented a wide variability of disease resistance categories, with only few exceptions associated with specific resistance response such as haplotype 4 of DRR2 and CYP77A2 genes and haplotype 1 of COMT and PFN2 genes. These results could be explained by different factors. Firstly, *V. dahliae* resistance is a multigenic response in olive and each genotype could be activating a different battery of genes to fight against the pathogen. Additional associated difficulties in olive are due to the lack of Verticillium wilt QTLs reported and validated across years and environments on mapping populations. Moreover, the absence of functional characterization of Verticillium wilt resistance genes has hampered this strategy. In this sense, only recently a functional validation of a NPR1 gene in olive transgenic plants has been reported (Narváez et al., 2020), in which different effectiveness was observed depended on the fungus pathotype or even the race. Secondly, resistance evaluation to *V. dahliae* is a difficult task and even when using equal inoculation protocol to evaluate the same cultivar, different measures could be observed, as we found for some cultivars of our germplasm set (Arias-Calderón et al., 2015, 2015a, 2015b; Martos-Moreno et al., 2006; López-Escudero et al., 2004). The phenotyping process based on visual observations could classify genotypes with similar molecular responses to different resistant categories and/or assign the same resistance class to genotypes with different molecular responses. In this sense, differences in symptoms expression and vascular tissue colonization observed when phenotyping the trait, could also suggest the activation of both tolerance and resistance mechanisms depending on the evaluated accession (Arias-Calderón et al., 2015a). Complementary methods such as the pathogen quantification in plant could be useful in future studies to better classify the disease response and highlight differences between resistant or tolerant host behavior (Mercado-Blanco et al., 2003). The exploration of more variability among cultivated, wild and related olive subspecies may shed more light about the diversity on *V. dahliae* resistance and possible associations between polymorphisms and phenotypes, as already described for other traits in olive (Cultrera et al., 2019; Kaya et al., 2019b; Zhu et al., 2019).

Haplotype analysis was also performed considering the origin of the evaluated germplasm set. Some specific polymorphisms were revealed for relative subspecies. Thus, five haplotypes belonging to 4 different genes were associated to *guanchica* subspecie and 4 haplotypes representing 3 genes identified *cerasiformis* subspecie. Whereas in previous works, SNP discovery has been carried out considering cultivated material that later would be validated on relative subspecies (Belaj et al., 2018). Nonetheless, the set of germplasm

under study was composed only by a reduce number of *guanchica* and *cerasiformis* genotypes and future studies should be recommended to increase the number of wild accessions considered. The phylogenetic analysis also sustained the clustering of subspecies mainly for DRR2, ACL, PFN2 and TLP1 genes. Therefore, the SNPs reported in this study could be useful to characterize *O. europaea* germplasm as already confirmed in previous studies (Belaj et al., 2018; Cultrera et al., 2019; Zhu et al., 2019).

The population structure was in accordance with phylogenetic and subspecies haplotype analysis. Thus, genotypes from *guanchica* and *cerasiformis* subspecies were differentiated along with some wild olives, breeding selections and some cultivars. Moreover, cultivated and wild olives of *europaea* subspecies grouped together in many haplotypes and remained closely related in phylogenetic analysis, which agrees with the theory of cultivated olives coming from the domestication of wild genotypes previously described in evolutionary studies (Baldoni et al., 2006; Belaj et al., 2011; Kassa et al., 2019). Wild olives and cultivars were also related due to the gene flow in some regions, where could be difficult to distinguish among real wild olives and feral forms (Baldoni et al., 2006; Belaj et al., 2010). Even transcriptomic analysis comparing wild and cultivated olives suggested that the domestication process mainly affected gene expression with moderate genomic consequences (Gros-Balthazard et al., 2019). Phylogenetic and domestication studies of *O. europaea* subspecies, including tetraploid *cerasiformis* endemic from Madeira Island and diploid *guanchica* from Canary Islands, suggested two different spread events separated by the Ocean. As expected, the genotypes of subsp. *cerasiformis* showed higher gene diversity than the ones of subsp. *guanchica* which has been described more related to the Mediterranean wild olives (Kassa et al., 2019). Phylogenetic analysis also demonstrated the relationships among cultivated and *cerasiformis* subspecie (Cultrera et al., 2019). According to these results, SNPs of DRR2, ACL, PFN2 and TLP1 genes could be useful for molecular-marker design with subspecies identification purposes. In this sense, TLP1 could be deeply evaluated for marker development to identify subspecies of *O. europaea*, since it was the only gene in which relative subspecies were completely differentiated from the rest of genotypes, even *cerasiformis* and *guanchica* remained in separated groups.

5.7. Conclusions

Genetic diversity study among subspecies of *O. europaea*, including *europaea* (cultivated and wild), *cerasiformis* and *guanchica* revealed SNPs in coding regions of putative resistance genes involving different disease defense mechanism. The identification of 92 functional SNPs and 2 InDels were identified, that could contribute to the development of a SNPs set that could be useful to characterize germplasm collections regarding Verticillium wilt resistance and thus allowing assistance in the selection process of breeding programs. The haplotype analysis revealed most of them represented genotypes with different disease resistant response and belonging to different origins. However, independent gene analysis allowed identification of SNPs on TLP1 gene useful to distinguish among subspecies, as well as a deletion on PFN2 fragment potentially related to resistance response. Further studies are needed to validate these SNPs and InDels in a wide number of genotypes showing differentiated disease response and coming from plant material origin.

5.8. Acknowledgement

This research was financially supported by INIA project RTA2013-00019 and IFAPA project AVA201601.2, both partially funded by European Regional Development Fund (ERDF). Alicia Serrano is grateful for the funding received from the Researcher Training Program in INIA-Autonomous Communities Research Centers (FPI-INIA).

5.9. References

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. https://doi.org/10.1016/j.scienta.2015.01.015

Arias-Calderón, Rocío, Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., León, L., 2015a. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica 206, 619–629. https://doi.org/10.1007/s10681-015-1463-7

Arias-Calderón, Rocío, Rodríguez-Jurado, D., León, L., Bejarano-Alcázar, J., De la Rosa, R., Belaj, A., 2015b. Pre-breeding for resistance to Verticillium wilt in olive: Fishing in the wild relative gene pool. Crop Protection 75, 25–33. https://doi.org/10.1016/j.cropro.2015.05.006

Bak, S., Beisson, F., Bishop, G., Hamberger, B., Höfer, R., Paquette, S., Werck-Reichhart, D., 2011. Cytochromes P450. Arabidopsis Book 9. https://doi.org/10.1199/tab.0144

Baldoni, L., Tosti, N., Ricciolini, C., Belaj, A., Arcioni, S., Pannelli, G., Germana, M.A., Mulas, M., Porceddu, A., 2006. Genetic Structure of Wild and Cultivated Olives in the Central Mediterranean Basin. Ann Bot 98, 935–942. https://doi.org/10.1093/aob/mcl178

Barranco Navero, D., International Olive Oil Council, 2000. World catalogue of olive varieties. International Olive Oil Council, Madrid, Spain.

Belaj, A., Domínguez-García, M. del C., Atienza, S.G., Martín Urdíroz, N., De la Rosa, R., Satovic, Z., Martín, A., Kilian, A., Trujillo, I., Valpuesta, V., Del Río, C., 2012. Developing a core collection of olive (*Olea europaea* L.) based on molecular markers (DArTs, SSRs, SNPs) and agronomic traits. Tree Genetics & Genomes 8, 365–378. https://doi.org/10.1007/s11295-011-0447-6

Belaj, A., León, L., Satovic, Z., de la Rosa, R., 2011. Variability of wild olives (*Olea europaea* subsp. *europaea* var. *sylvestris*) analyzed by agro-morphological traits and SSR markers. Scientia Horticulturae 129, 561–569. https://doi.org/10.1016/j.scienta.2011.04.025

Belaj, A., Muñoz-Díez, C., Baldoni, L., Porceddu, A., Barranco, D., Satovic, Z., 2007. Genetic Diversity and Population Structure of Wild Olives from the North-western Mediterranean Assessed by SSR Markers. Ann Bot 100, 449–458. https://doi.org/10.1093/aob/mcm132

Belaj, A., Muñoz-Díez, C., Baldoni, L., Satovic, Z., Barranco, D., 2010. Genetic diversity and relationships of wild and cultivated olives at regional level in Spain. Scientia Horticulturae 124, 323–330. https://doi.org/10.1016/j.scienta.2010.01.010

Belaj, A., Rosa, R. de la, Lorite, I.J., Mariotti, R., Cultrera, N.G.M., Beuzón, C.R., González-Plaza, J.J., Muñoz-Mérida, A., Trelles, O., Baldoni, L., 2018. Usefulness of a New Large Set of High Throughput EST-SNP Markers as a Tool for Olive Germplasm Collection Management, in: Front. Plant Sci. https://doi.org/10.3389/fpls.2018.01320

Besnard, G., El Bakkali, A., 2014. Sequence analysis of single-copy genes in two wild olive subspecies: nucleotide diversity and potential use for testing admixture. Genome 57, 145–153. https://doi.org/10.1139/gen-2014-0001

Besnard, G., García-Verdugo, C., Rubio De Casas, R., Treier, U.A., Galland, N., Vargas, P., 2008. Polyploidy in the Olive Complex (*Olea europaea*): Evidence from Flow Cytometry and Nuclear Microsatellite Analyses. Ann Bot 101, 25–30. https://doi.org/10.1093/aob/mcm275

Besnard, G., Terral, J.-F., Cornille, A., 2018. On the origins and domestication of the olive: a review and perspectives. Ann Bot 121, 385–403. https://doi.org/10.1093/aob/mcx145

Bettaieb, I., Bouktila, D., 2020. Genome-wide analysis of NBS-encoding resistance genes in the Mediterranean olive tree (*Olea europaea* subsp. *europaea* var. *europaea*): insights into their molecular diversity, evolution and function. Tree Genetics & Genomes 16, 23. https://doi.org/10.1007/s11295-020-1415-9

Biton, I., Doron-Faigenboim, A., Jamwal, M., Mani, Y., Eshed, R., Rosen, A., Sherman, A., Ophir, R., Lavee, S., Avidan, B., Ben-Ari, G., 2015. Development of a large set of SNP markers for assessing phylogenetic relationships between the olive cultivars composing the Israeli olive germplasm collection. Mol Breeding 35, 107. https://doi.org/10.1007/s11032-015-0304-7

Blazakis, K.N., Kosma, M., Kostelenos, G., Baldoni, L., Bufacchi, M., Kalaitzis, P., 2017. Description of olive morphological parameters by using open access software. Plant Methods 13. https://doi.org/10.1186/s13007-017-0261-8

Brito, G., Loureiro, J., Lopes, T., Rodriguez, E., Santos, C., 2008. Genetic characterisation of olive trees from Madeira Archipelago using flow cytometry and microsatellite markers. Genet Resour Crop Evol 55, 657–664. https://doi.org/10.1007/s10722-007-9268-8

Cabanás, C.G.-L., Schiliro`, E., Valverde-Corredor, A., Mercado-Blanco, J., 2015. Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. Front. Microbiol. 6. https://doi.org/10.3389/fmicb.2015.00928

Cai, Y., Cai, X., Wang, Q., Wang, P., Zhang, Yu, Cai, C., Xu, Y., Wang, K., Zhou, Z., Wang, C., Geng, S., Li, B., Dong, Q., Hou, Y., Wang, H., Ai, P., Liu, Z., Yi, F., Sun, M., An, G., Cheng, J., Zhang, Yuanyuan, Shi, Q., Xie, Y., Shi, X., Chang, Y., Huang, F., Chen, Y., Hong, S., Mi, L., Sun, Q., Zhang, L., Zhou, B., Peng, R., Zhang, X., Liu, F., n.d. Genome sequencing of the Australian wild diploid species *Gossypium australe* highlights disease resistance and delayed gland morphogenesis. Plant Biotechnology Journal n/a. https://doi.org/10.1111/pbi.13249

Cenci, A., Rouard, M., 2017. Evolutionary Analyses of GRAS Transcription Factors in Angiosperms. Front. Plant Sci. 8. https://doi.org/10.3389/fpls.2017.00273

Clement, M., Posada, D., Crandall, K.A., 2000. TCS: a computer program to estimate gene genealogies. Molecular Ecology 9, 1657–1659. https://doi.org/10.1046/j.1365-294x.2000.01020.x

Cultrera, N.G.M., Sarri, V., Lucentini, L., Ceccarelli, M., Alagna, F., Mariotti, R., Mousavi, S., Ruiz, C.G., Baldoni, L., 2019. High Levels of Variation Within Gene Sequences of *Olea europaea* L. Front. Plant Sci. 9. https://doi.org/10.3389/fpls.2018.01932

D'Agostino, N., Taranto, F., Camposeo, S., Mangini, G., Fanelli, V., Gadaleta, S., Miazzi, M.M., Pavan, S., di Rienzo, V., Sabetta, W., Lombardo, L., Zelasco, S., Perri, E., Lotti, C., Ciani, E., Montemurro, C., 2018. GBS-derived SNP catalogue unveiled wide genetic variability and geographical relationships of Italian olive cultivars. Scientific Reports 8, 1–13. https://doi.org/10.1038/s41598-018-34207-y

De la Rosa, R., Angiolillo, A., Guerrero, C., Pellegrini, M., Rallo, L., Besnard, G., Bervillé, A., Martin, A., Baldoni, L., 2003. A first linkage map of olive (*Olea europaea* L.) cultivars using RAPD, AFLP, RFLP and SSR markers. Theor Appl Genet 106, 1273–1282. https://doi.org/10.1007/s00122-002-1189-5

Diez, C.M., Trujillo, I., Martinez-Urdiroz, N., Barranco, D., Rallo, L., Marfil, P., Gaut, B.S., 2015. Olive domestication and diversification in the Mediterranean Basin. New Phytologist 206, 436–447. https://doi.org/10.1111/nph.13181

Fu, Y.X., Li, W.H., 1993. Statistical tests of neutrality of mutations. Genetics 133, 693–709.

Ganal, M.W., Altmann, T., Röder, M.S., 2009. SNP identification in crop plants. Curr. Opin. Plant Biol. 12, 211–217. https://doi.org/10.1016/j.pbi.2008.12.009

Gharbi, Y., Barkallah, M., Bouazizi, E., Gdoura, R., Triki, M.A., 2017. Differential biochemical and physiological responses of two olive cultivars differing by their susceptibility to the hemibiotrophic pathogen *Verticillium dahliae*. Physiological and Molecular Plant Pathology 97, 30–39. https://doi.org/10.1016/j.pmpp.2016.12.001

Gong, Q., Yang, Z., Wang, X., Butt, H.I., Chen, E., He, S., Zhang, C., Zhang, X., Li, F., 2017. Salicylic acid-related cotton (*Gossypium arboreum*) ribosomal protein GaRPL18 contributes to resistance to *Verticillium dahliae*. BMC Plant Biol. 17, 59. https://doi.org/10.1186/s12870-017-1007-5

Gros-Balthazard, M., Besnard, G., Sarah, G., Holtz, Y., Leclercq, J., Santoni, S., Wegmann, D., Glémin, S., Khadari, B., 2019. Evolutionary transcriptomics reveals the origins of olives and the genomic changes associated with their domestication. The Plant Journal 100, 143–157. https://doi.org/10.1111/tpj.14435

Guo, Y., Wu, H., Li, X., Li, Q., Zhao, X., Duan, X., An, Y., Lv, W., An, H., 2017. Identification and expression of GRAS family genes in maize (*Zea mays* L.). PLOS ONE 12, e0185418. https://doi.org/10.1371/journal.pone.0185418

İpek, A., Yılmaz, K., Sıkıcı, P., Tangu, N.A., Öz, A.T., Bayraktar, M., İpek, M., Gülen, H., 2016. SNP Discovery by GBS in Olive and the Construction of a High-Density Genetic Linkage Map. Biochem Genet 54, 313–325. https://doi.org/10.1007/s10528-016-9721-5

Jiménez-Ruiz, J., Leyva-Pérez, M. de la O., Schilirò, E., Barroso, J.B., Bombarely, A., Mueller, L., Mercado-Blanco, J., Luque, F., 2017. Transcriptomic Analysis of *Olea europaea* L. Roots during the *Verticillium dahliae* Early Infection Process. The Plant Genome 10, 0. https://doi.org/10.3835/plantgenome2016.07.0060

Kassa, A., Konrad, H., Geburek, T., 2019. Molecular diversity and gene flow within and among different subspecies of the wild olive (*Olea europaea* L.): A review. Flora 250, 18–26. https://doi.org/10.1016/j.flora.2018.11.014

Kaya, H.B., Akdemir, D., Lozano, R., Cetin, O., Sozer Kaya, H., Sahin, M., Smith, J.L., Tanyolac, B., Jannink, J.-L., 2019a. Genome wide association study of 5 agronomic traits in olive (*Olea europaea* L.). Sci Rep 9. https://doi.org/10.1038/s41598-019-55338-w

Kaya, H.B., Akdemir, D., Lozano, R., Cetin, O., Sozer Kaya, H., Sahin, M., Smith, J.L., Tanyolac, B., Jannink, J.-L., 2019b. Genome wide association study of 5 agronomic traits in olive (*Olea europaea* L.). Sci Rep 9, 18764. https://doi.org/10.1038/s41598-019-55338-w

Kaya, H.B., Cetin, O., Kaya, H., Sahin, M., Sefer, F., Kahraman, A., Tanyolac, B., 2013. SNP Discovery by Illumina-Based Transcriptome Sequencing of the Olive and the Genetic Characterization of Turkish Olive Genotypes Revealed by AFLP, SSR and SNP Markers. PLoS One 8. https://doi.org/10.1371/journal.pone.0073674

Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., Buxton, S., Cooper, A., Markowitz, S., Duran, C., Thierer, T., Ashton, B., Meintjes, P., Drummond, A., 2012. Geneious Basic: An integrated and extendable desktop software platform for the organization and analysis of sequence data. Bioinformatics 28, 1647–1649. https://doi.org/10.1093/bioinformatics/bts199

León, L., Arias-Calderón, R., de la Rosa, R., Khadari, B., Costes, E., 2016. Optimal spatial and temporal replications for reducing environmental variation for oil content components and fruit morphology traits in olive breeding. Euphytica 207, 675–684. https://doi.org/10.1007/s10681-015-1569-y

Leyva-Pérez, M. de la O., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J.B., Luque, F., Mercado-Blanco, J., 2018. Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. New Phytol 217, 671–686. https://doi.org/10.1111/nph.14833

Li, J., Staiger, C.J., 2018. Understanding Cytoskeletal Dynamics During the Plant Immune Response. Annu. Rev. Phytopathol. 56, 513–533. https://doi.org/10.1146/annurev-phyto-080516-035632

Librado, P., Rozas, J., 2009. DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. Bioinformatics 25, 1451–1452. https://doi.org/10.1093/bioinformatics/btp187

López-Escudero, F.J., Rio, C. del, Caballero, J.M., Blanco-López, M.A., 2004. Evaluation of olive cultivars for resistance to *Verticillium dahliae*. European journal of plant pathology 110, 79–85. https://doi.org/10.1023/B:EJPP.0000010150.08098.2d

Mammadov, J., Aggarwal, R., Buyyarapu, R., Kumpatla, S., 2012. SNP Markers and Their Impact on Plant Breeding. International Journal of Plant Genomics 2012, 1–11. https://doi.org/10.1155/2012/728398

Martos-Moreno, C., López-Escudero, F.J., Blanco-López, M.Á., 2006. Resistance of Olive Cultivars to the Defoliating Pathotype of *Verticillium dahliae*. HortScience 41, 1313–1316. https://doi.org/10.21273/HORTSCI.41.5.1313

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., Jiménez-Díaz, R.M., 2003. Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. https://doi.org/10.1016/j.pmpp.2003.10.001

Munis, M.F.H., Tu, L., Deng, F., Tan, J., Xu, L., Xu, S., Long, L., Zhang, X., 2010. A thaumatin-like protein gene involved in cotton fiber secondary cell wall development enhances resistance against *Verticillium dahliae* and other stresses in transgenic tobacco. Biochemical and Biophysical Research Communications 393, 38–44. https://doi.org/10.1016/j.bbrc.2010.01.069

Narváez, I., Pliego Prieto, C., Palomo-Ríos, E., Fresta, L., Jiménez-Díaz, R.M., Trapero-Casas, J.L., López-Herrera, C., Arjona-López, J.M., Mercado, J.A., Pliego-Alfaro, F., 2020. Heterologous Expression of the AtNPR1 Gene in Olive and Its Effects on Fungal Tolerance. Front. Plant Sci. 11. https://doi.org/10.3389/fpls.2020.00308

Nei, M., Gojobori, T., 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol Biol Evol 3, 418–426. https://doi.org/10.1093/oxfordjournals.molbev.a040410

Pritchard, J.K., Stephens, M., Donnelly, P., 2000. Inference of Population Structure Using Multilocus Genotype Data. Genetics 155, 945–959.

Rodríguez-Jurado, D.R., 1993. Interacciones huésped-parasito en la marchitez del olivo (*Olea europaea* L.) inducida por *Verticillium dahliae* kleb. PhD Thesis, University of Córdoba, Spain. 324 pp.

Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. 4, 406–425. https://doi.org/10.1093/oxfordjournals.molbev.a040454

Santos-Antunes, F., León, L., Rosa, R. de la, Alvarado, J., Mohedo, A., Trujillo, I., Rallo, L., 2005. The Length of the Juvenile Period in Olive as Influenced by Vigor of the Seedlings and the Precocity of the Parents. HortScience 40, 1213–1215. https://doi.org/10.21273/HORTSCI.40.5.1213

Schilirò, E., Ferrara, M., Nigro, F., Mercado-Blanco, J., 2012. Genetic Responses Induced in Olive Roots upon Colonization by the Biocontrol Endophytic Bacterium *Pseudomonas fluorescens* PICF7. PLOS ONE 7, e48646. https://doi.org/10.1371/journal.pone.0048646

Simko, I., Haynes, K.G., Ewing, E.E., Costanzo, S., Christ, B.J., Jones, R.W., 2004. Mapping genes for resistance to *Verticillium albo-atrum* in tetraploid and diploid potato populations using haplotype association tests and genetic linkage analysis. Mol Genet Genomics 271, 522–531. https://doi.org/10.1007/s00438-004-1010-z

Tajima, F., 1989. Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. Genetics 123, 585–595.

Tamura, K., Stecher, G., Peterson, D., Filipski, A., Kumar, S., 2013. MEGA6: Molecular Evolutionary Genetics Analysis Version 6.0. Mol Biol Evol 30, 2725–2729. https://doi.org/10.1093/molbev/mst197

Thompson, J.D., Higgins, D.G., Gibson, T.J., 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22, 4673–4680.

Trujillo, I., Ojeda, M.A., Urdiroz, N.M., Potter, D., Barranco, D., Rallo, L., Diez, C.M., 2014. Identification of the Worldwide Olive Germplasm Bank of Córdoba (Spain) using SSR and morphological markers. Tree Genetics & Genomes 10, 141–155. https://doi.org/10.1007/s11295-013-0671-3

Unver, T., Wu, Z., Sterck, L., Turktas, M., Lohaus, R., Li, Z., Yang, M., He, L., Deng, T., Escalante, F.J., Llorens, C., Roig, F.J., Parmaksiz, I., Dundar, E., Xie, F., Zhang, B., Ipek, A., Uranbey, S., Erayman, M., Ilhan, E., Badad, O., Ghazal, H., Lightfoot, D.A., Kasarla, P., Colantonio, V., Tombuloglu, H., Hernandez, P., Mete, N., Cetin, O., Van Montagu, M., Yang, H., Gao, Q., Dorado, G., Van de Peer, Y., 2017. Genome of wild olive and the evolution of oil biosynthesis. Proc Natl Acad Sci U S A 114, E9413–E9422. https://doi.org/10.1073/pnas.1708621114

Wally, O., Punja, Z.K., 2010. Genetic engineering for increasing fungal and bacterial disease resistance in crop plants. GM Crops 1, 199–206. https://doi.org/10.4161/gmcr.1.4.13225

Wang, W., Sun, Y., Han, L., Su, L., Xia, G., Wang, H., 2017. Overexpression of GhPFN2 enhances protection against *Verticillium dahliae* invasion in cotton. Sci. China Life Sci. 60, 861–867. https://doi.org/10.1007/s11427-017-9067-2

Xu, L., Zhu, L., Tu, L., Liu, L., Yuan, D., Jin, L., Long, L., Zhang, X., 2011. Lignin metabolism has a central role in the resistance of cotton to the wilt fungus *Verticillium dahliae* as revealed by RNA-Seq-dependent transcriptional analysis and histochemistry. J. Exp. Bot. 62, 5607–5621. https://doi.org/10.1093/jxb/err245

Yadeta, K.A., J. Thomma, B.P.H., 2013. The xylem as battleground for plant hosts and vascular wilt pathogens. Front Plant Sci 4. https://doi.org/10.3389/fpls.2013.00097

Yang, L., Shi, C., Mu, X., Liu, C., Shi, K., Zhu, W., Yang, Q., 2015. Cloning and expression of a wild eggplant cytochrome P450 gene, StoCYP77A2, involved in plant resistance to

Verticillium dahliae. Plant Biotechnol Rep 9, 167–177. https://doi.org/10.1007/s11816-015-0355-6

Yang, P., Habekuß, A., Hofinger, B.J., Kanyuka, K., Kilian, B., Graner, A., Ordon, F., Stein, N., 2017. Sequence diversification in recessive alleles of two host factor genes suggests adaptive selection for bymovirus resistance in cultivated barley from East Asia. Theor Appl Genet 130, 331–344. https://doi.org/10.1007/s00122-016-2814-z

Yuan, C.P., Wang, Y.J., Zhao, H.K., Zhang, L., Wang, Y.M., Liu, X.D., Zhong, X.F., Dong, Y.S., 2016. Genetic diversity of rhg1 and Rhg4 loci in wild soybeans resistant to soybean cyst nematode race 3. Genet. Mol. Res. 15. https://doi.org/10.4238/gmr.15027386

Zhang, Y., Wang, X.F., Ding, Z.G., Ma, Q., Zhang, G.R., Zhang, S.L., Li, Z.K., Wu, L.Q., Zhang, G.Y., Ma, Z.Y., 2013. Transcriptome profiling of *Gossypium barbadense* inoculated with *Verticillium dahliae* provides a resource for cotton improvement. BMC Genomics 14, 637. https://doi.org/10.1186/1471-2164-14-637

Zhu, S., Niu, E., Shi, A., Mou, B., 2019. Genetic Diversity Analysis of Olive Germplasm (*Olea europaea* L.) With Genotyping-by-Sequencing Technology. Front. Genet. 10. https://doi.org/10.3389/fgene.2019.00755

5.10. Supplementary material

Figure S 5.1 cDNA and DNA alignment of 'Frantoio' cultivar using Geneious 7.0.6. software for the identification of coding and non-coding

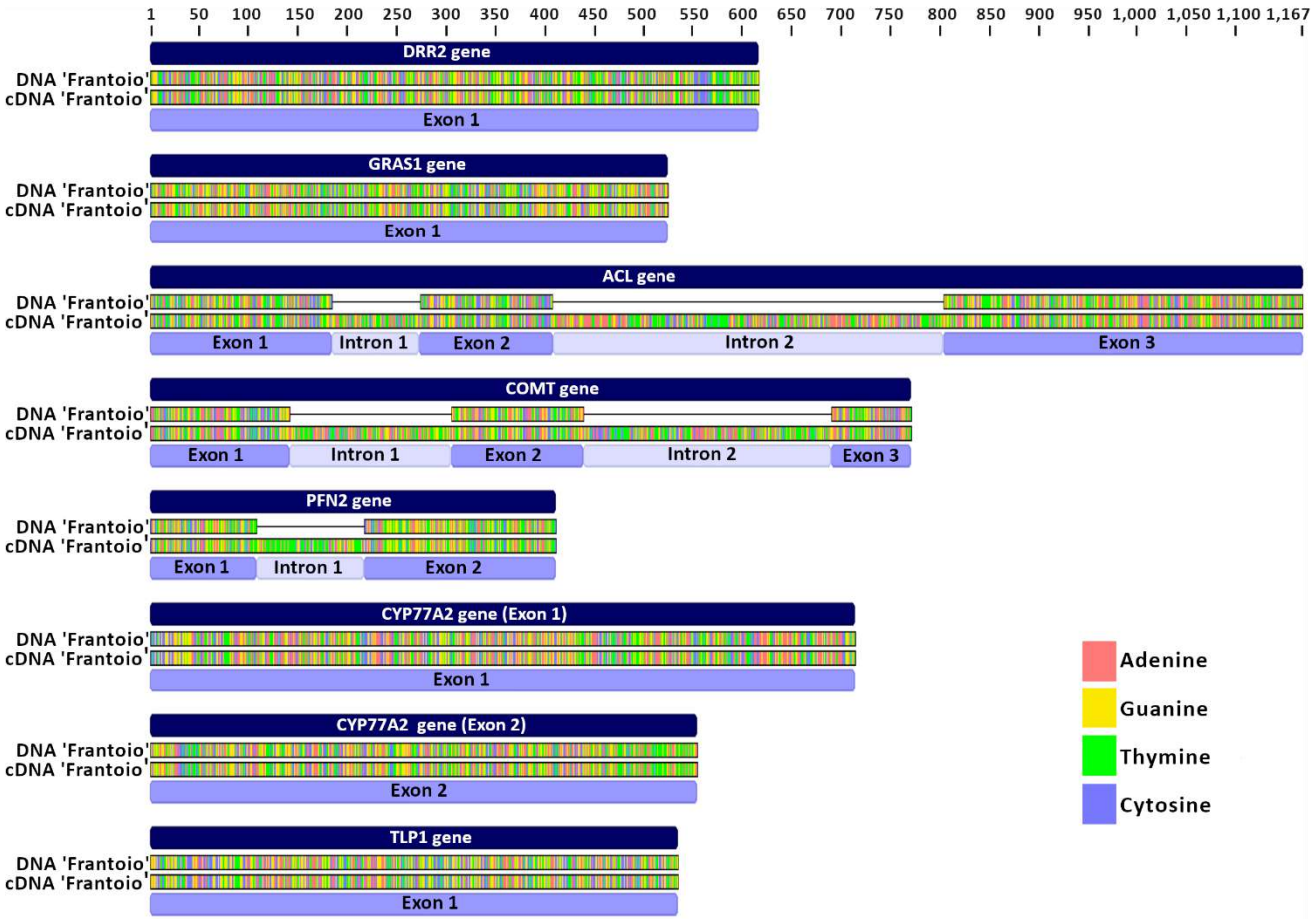
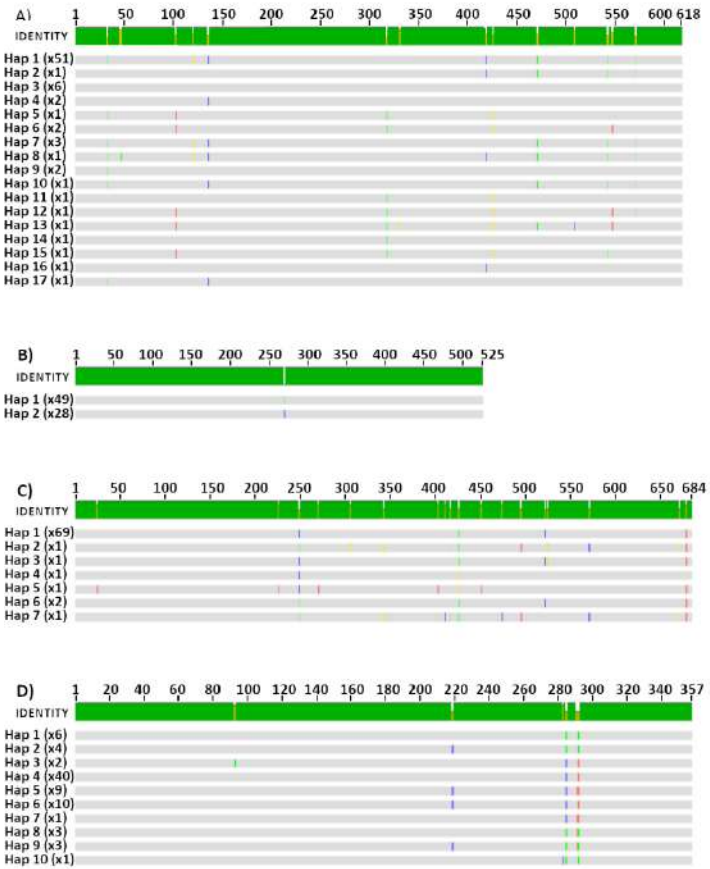
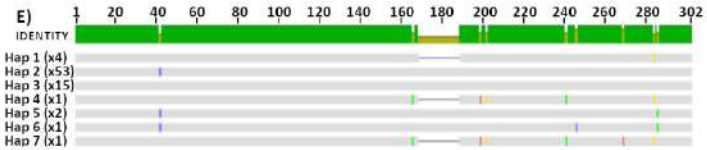
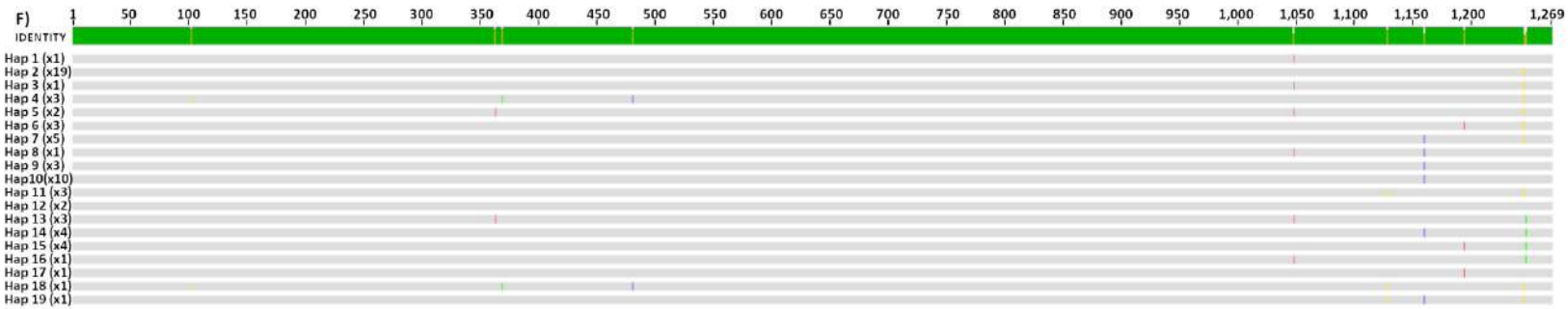
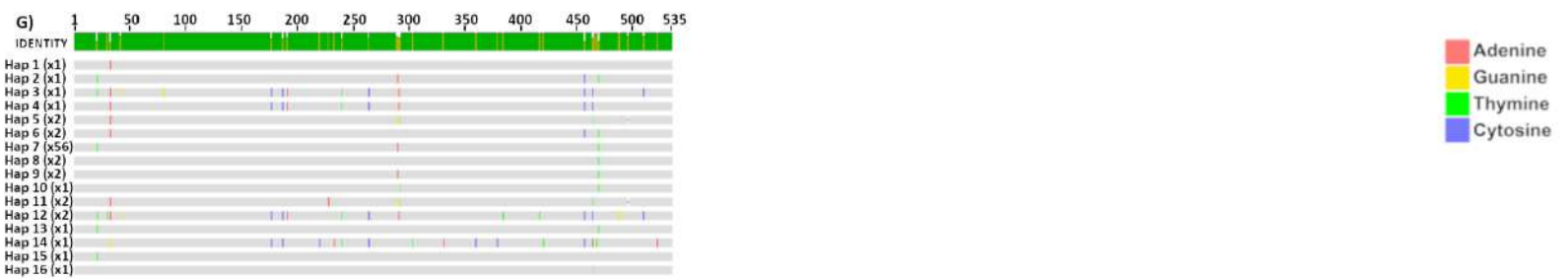


Figure S 5.2 Haplotype alignment of different candidate gene fragments developed based on polymorphism analysis.



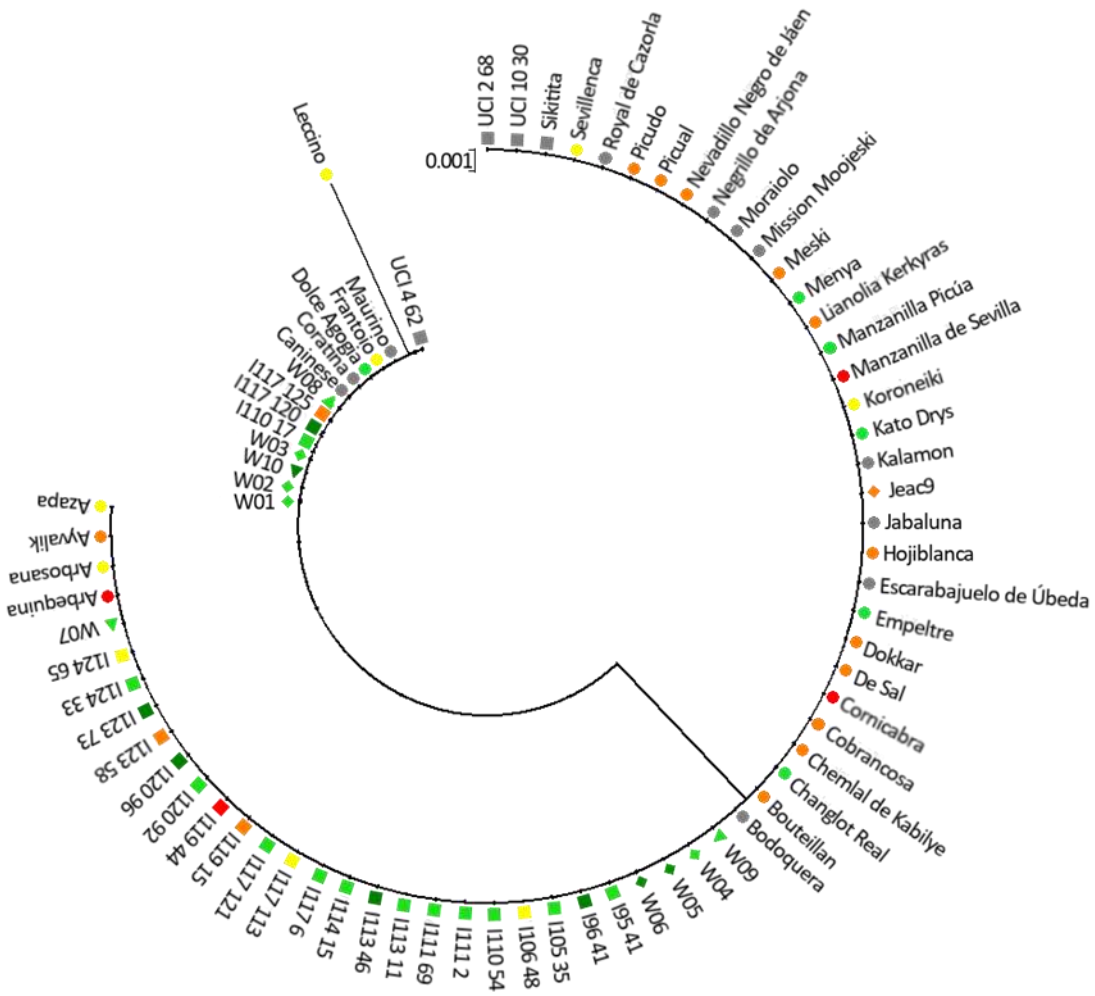






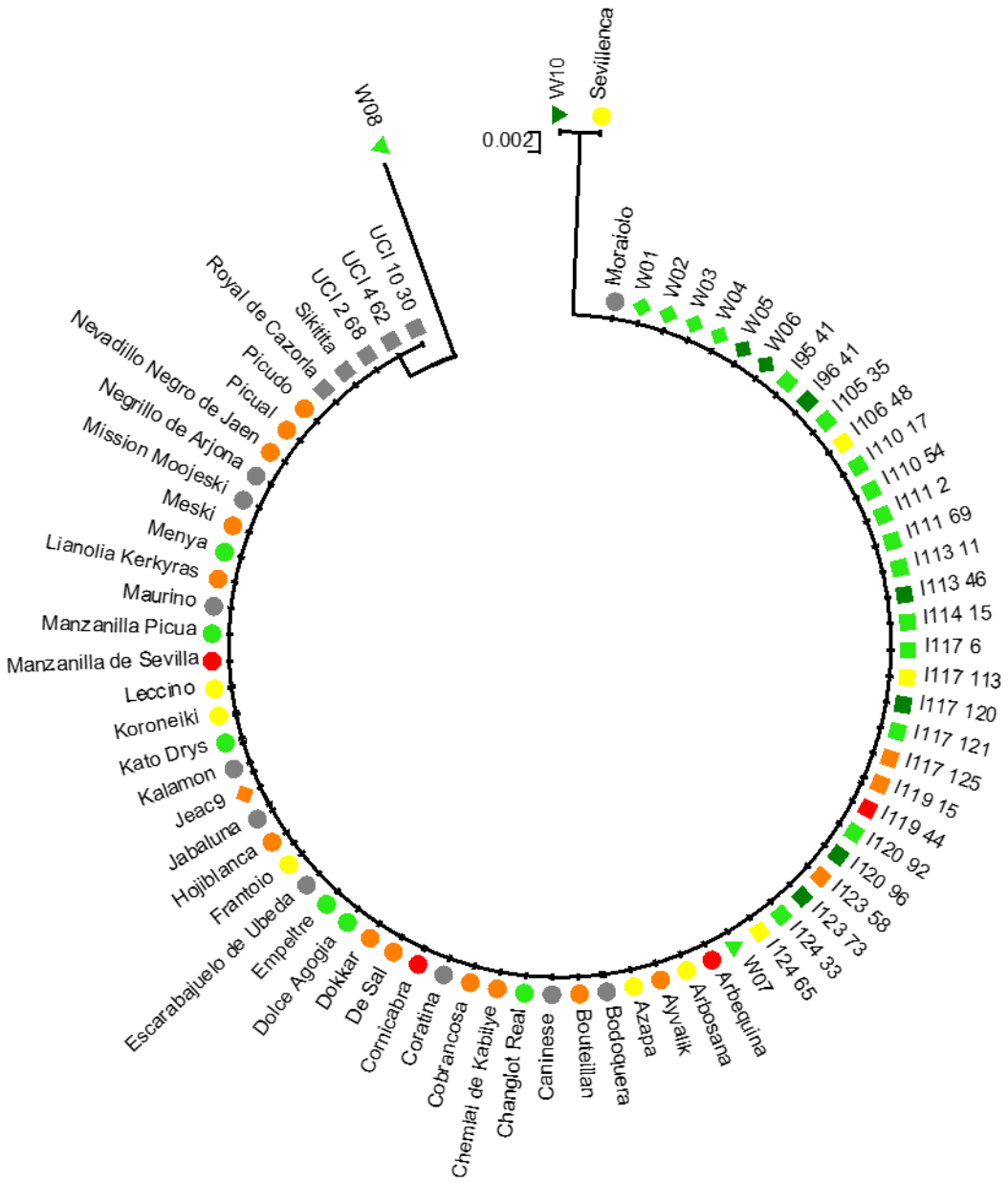
The haplotype alignment was carried out in Geneious 7.0.6 using ClustalW algoritm. A) DRR2 gene; B) GRAS1 gene; C) ACL gene; D) COMT gene; E) PFN2 gene; F) CYP77A2 gene; G) TLP1 gene. The number in parentheses indicates the number of genotypes included into each haplotype. The colored marks represent polymorphisms (SNPs and indels).

Figure S 5.3 Phylogenetic tree for COMT gene fragment



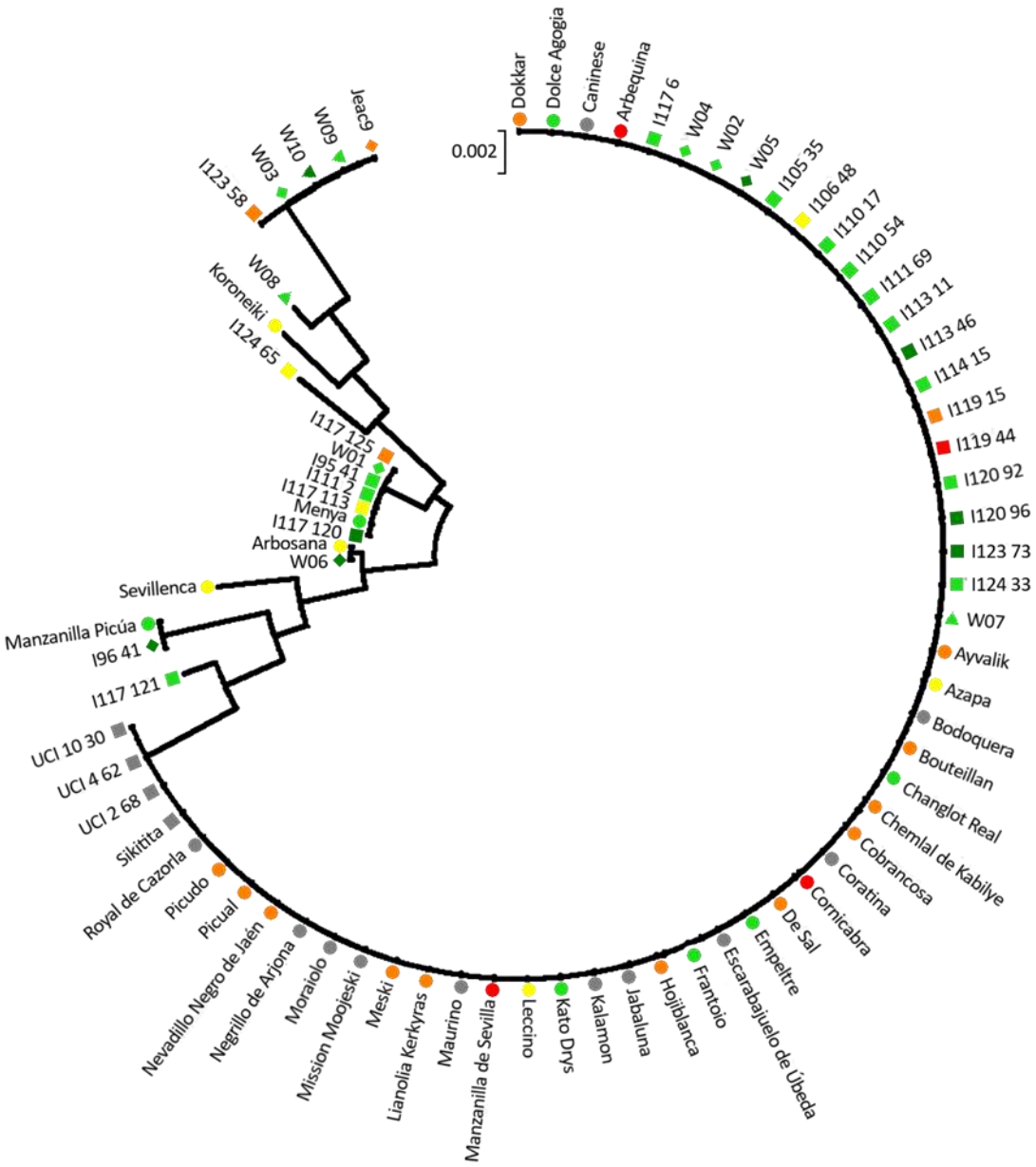
Phylogenetic dendrogram based on COMT gene fragment of 77 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly-resistant; Light green= resistant, Yellow = moderately-resistant, Orange = susceptible; Red = extremely-susceptible; Grey = unknown.

Figure S 5.4 Phylogenetic tree for ACL gene fragment.



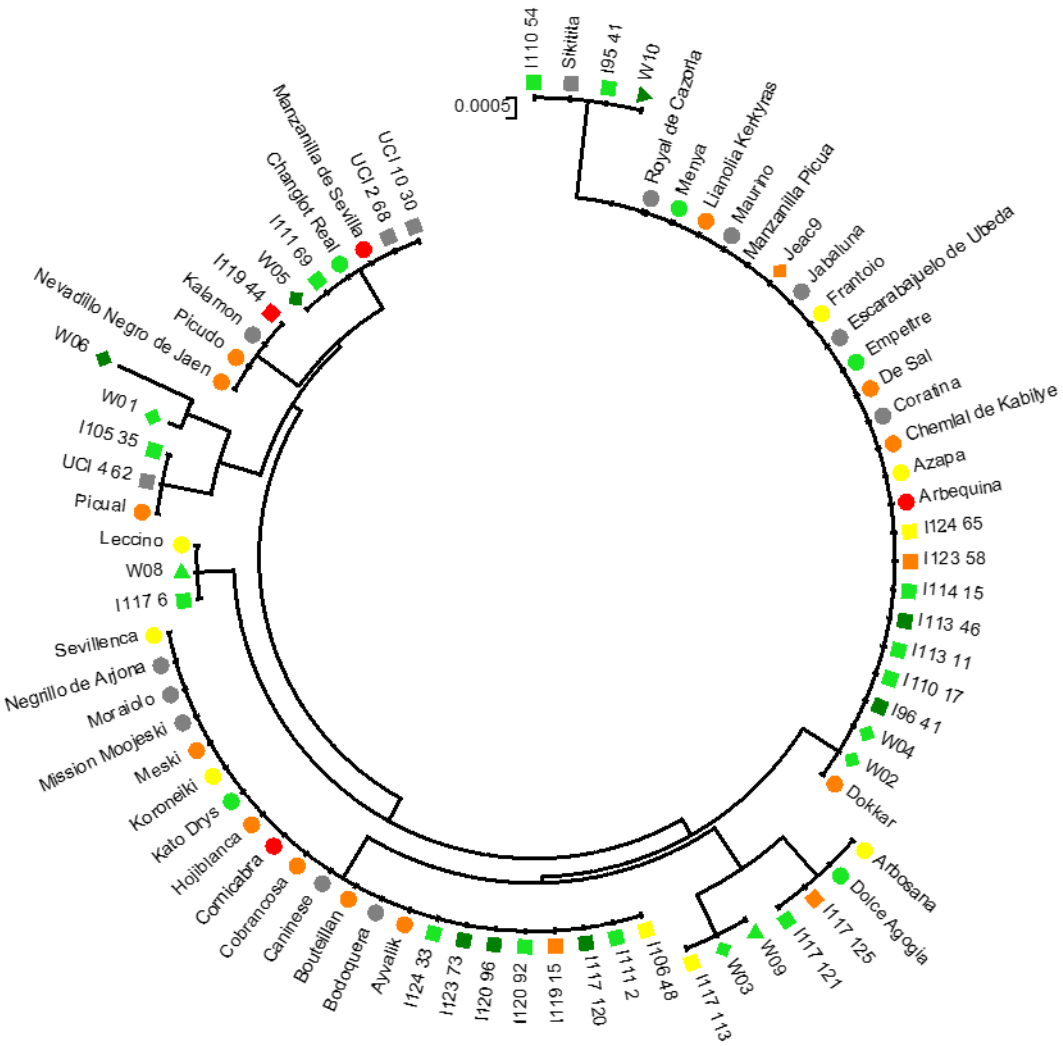
Phylogenetic dendrogram based on ACL gene fragment of 76 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly-resistant; Light green= resistant, Yellow = moderately-resistant, Orange = susceptible; Red = extremely-susceptible; Grey = unknown.

Figure S 5.5 Phylogenetic tree for DRR2 gene fragment.



Phylogenetic dendrogram based on DRR2 gene fragment of 77 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly-resistant; Light green= resistant, Yellow = moderately-resistant, Orange = susceptible; Red = extremely-susceptible; Grey = unknown.

Figure S 5.6 Phylogenetic tree for CYP77A2 gene fragment.



Phylogenetic dendrogram based on CYP77A2 gene fragment of 76 genotypes generated using MEGA 6.06 software by Neighbor-Joining method. Symbols indicate the origin of each genotype: ○ = Cultivar from the world olive germplasm bank; □ = Breeding selections; ◊ = *O. europaea* subp. *europaea* var. *sylvestris*; Δ = subsp. *cerasiformis*; ∇ = subspecie *guanchica*. Color indicate the response to *V. dahliae* infection: Dark green = Highly-resistant; Light green= resistant, Yellow = moderately-resistant, Orange = susceptible; Red = extremely-susceptible; Grey = unknown.

6. General Discussion

Breeding programs are underway in several olive producing countries based on intraspecific cross-breeding between cultivars of known merit aiming at combining the good qualities of the genitors in some of the genotypes of the progenies (Bellini et al., 2008). The interest in olive breeding is increasing from some year now, as up to 9 olive plant variety rights applications are granted or active at EU level by Community Plant Variety Office (CPVO, 2020). Even though, olive breeding is still far away from other fruit tree species, where the numbers of new-bred cultivars is higher, as peach (613) or apple (390).

Routine selection process in fruit tree breeding programs is usually arranged in several steps. In each one, the number of genotypes under evaluation is reduced and the number of replications per genotype and characters under evaluation increases, up to the final release of new promising cultivars (León et al., 2015). This process could be highly variable according to the specific dimension of the breeding program and the financial and human resources available to manage the trials, so that many different possibilities of allocation of resources have been reported for different crops (Badenes and Byrne 2011).

In olive, three main steps, i.e. the initial seedling population, an intermediate step of selection and a final set of comparative trials in different environments, are usually recommended to fulfill the whole cycle of selection in breeding programs (León et al., 2015). Due to the long juvenile phase and unproductive period of olive, this selection process can take up to 15-20 years. Several methodological advances carried out in the last years allowed a general simplification of some breeding steps, by shortening of the juvenile period, establishing some early selection criteria and developing easier and faster selection procedures for some traits, thus reducing the time and effort for advancing in the selection process (De la Rosa et al., 2016, 2006; Santos-Antunes et al., 2005).

Early bearing, high productivity, high oil content, oil quality, suitability for mechanical harvesting and resistance to main pest and diseases are mentioned among the main objectives of these breeding programs (Fernández-Escobar et al., 2013). Considering breeding for disease resistance, Verticillium Wilt in olive (VWO) represents a major limitation for olive productivity in many producing areas (Montes-Osuna and Mercado-Blanco 2020). Hence, olive breeding for VWO has been considered an important objective in several breeding works due to the difficulties eradicating its causal agent, *V. dahliae,* in infested soils and the scarce of traditional cultivars showing a significant level of resistant to this disease (Trapero et al., 2015; Arias-Calderón et al., 2014; Erten and Yıldız, 2011).

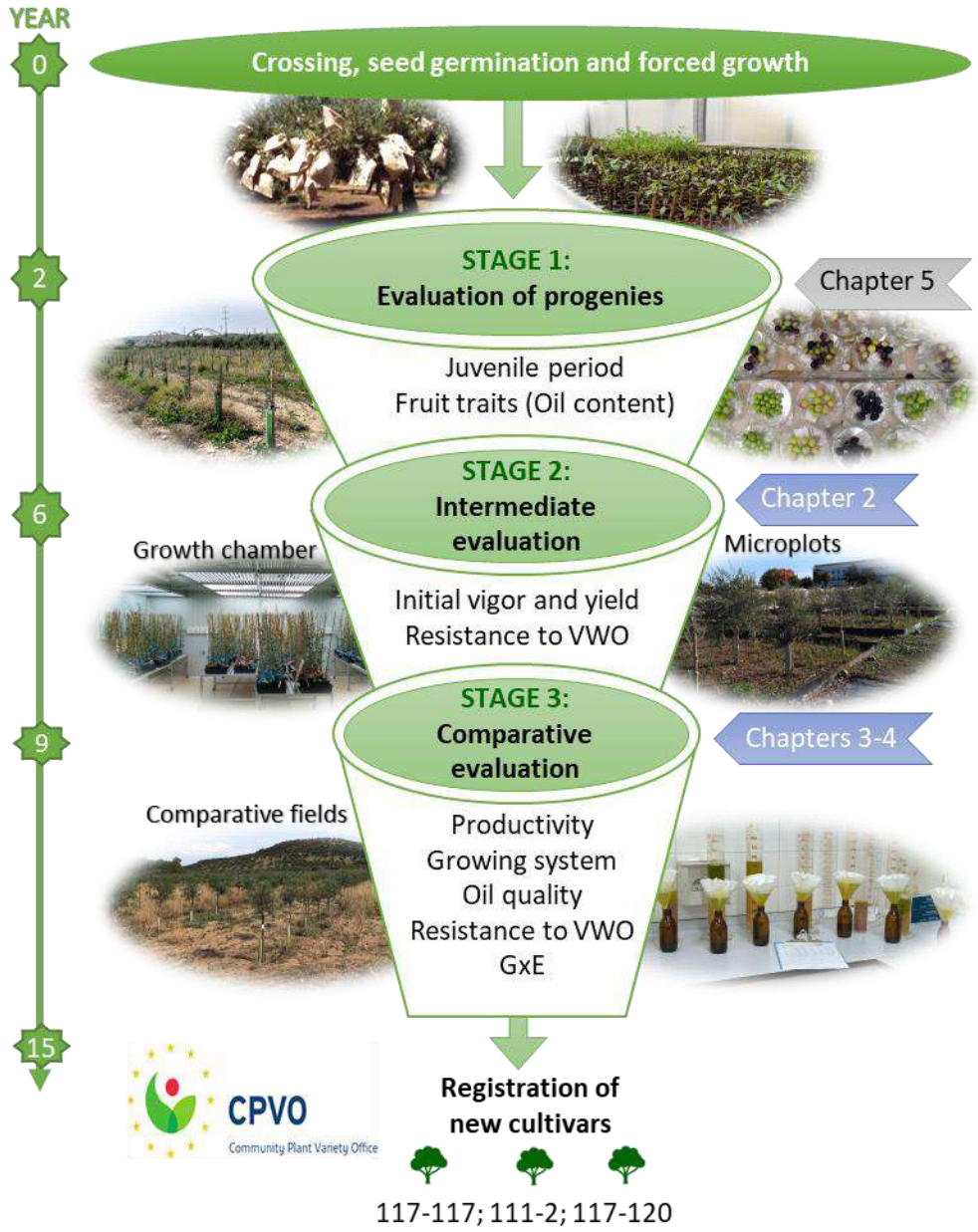
Several alternative schemes can be used in olive breeding programs for VWO resistance according to the step of selection chosen for evaluation. Screening for resistance in the first step of selection at the initial seedling stage could imply high risk of selection of genotypes that will probably show poor agronomic performance afterwards. In fact, 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). Alternatively, later screening at intermediate step increases the chances of final selection of genotypes combining both high disease resistance and appropriated agronomic characters. The step of selection chosen for resistance screening also influences the availability of plant materials and the inoculation method to be used. Screening at intermediate step allows the availability of vegetative propagated

replications of the genotypes under evaluation, instead of single seedling plants available at the initial step. In this way, appropriate experimental design can be used for testing, which guarantee more consistent evaluation of disease reaction. Different inoculation methods, inoculum type, environments, age and type of plants have been used to screen olive for resistance to VWO (Trapero et al. 2013a; Cirulli et al., 2008; López-Escudero et al., 2007). Among them, testing in microplots artificially inoculated with *V. dahliae* allows the evaluation of VWO epidemics under natural environment conditions in infested soil with a single pathotype, thus producing homogeneous results.

Taking all the above-mentioned into consideration, the olive breeding program for VWO developed at IFAPA is based on three steps of selection. For that purpose, several crosses, some of them involving genitors (cultivars) with high level of resistance to VWO were performed to produce a seedling population. This seedling population was evaluated, in a firs step, for productivity, oil content and fruit size. On the basis of this evaluation around 10% of the seedlings were selected for the second and then third steps of breeding selection. This thesis is part of the second and third stages of the olive breeding program, in which the genotypes selected in previous steps are characterized for disease resistant response and preliminary productivity data. In addition, the genetic studies have been focused on the development of molecular tools to assist olive breeders shortening the screening process (Figure 6.1).

In previous works, huge variability for resistance to *V. dahliae* infection was demonstrated in progenies from both open-pollination and breeding crosses (Arias-Calderón et al., 2014; 2015a; 2015b). Higher levels of resistance to VWO were observed in genotypes from resistant genitors such as 'Frantoio', 'Empeltre' or 'Changlot Real'. However, resistant genotypes were also selected from genitors with good agronomic behavior but susceptible to VWO such as 'Picual', 'Manzanilla de Sevilla', 'Picudo' and moderately resistant like 'Koroneiki', 'Arbosana' and 'Arbequina' (Arias-Calderón et al., 2015a, 2015b; Trapero et al., 2015). They could provide other convenient agronomic traits for olive oil production to the new VWO-resistant cultivars to be selected (Arias-Calderón et al., 2014, De la Rosa et al., 2016; Trapero et al., 2015). Wild olives have been also used as genitor in breeding programs for verticillium wilt resistance and resistant genotypes were detected among progenies. However, adequate agronomic performance could require additional backcross generations with cultivated genotypes with good agronomic behavior (Arias-Calderón et al., 2015c; Trapero et al., 2015). The usefulness of resistant wild olives and relative subspecies as rootstocks have also been studied to control VWO, but contradictory results have been described. In this sense, the susceptible cultivars grafted onto resistant 'Frantoio' or wild olives rootstocks avoided the disease development (Porras-Soriano et al., 2003, Bubici and Cirulli, 2012; Jiménez-Fernández et al., 2016); although long-term evaluation in infested field including different rootstock/scion combinations only revealed a delay in symptoms development (Valverde et al., 2021). So, most efforts have been focused on breeding from crosses among traditional cultivars and seedlings assessment.

Figure 6.1: Workflow of the olive breeding program for verticillium wilt resistance.



Plant materials evaluated in this work come from breeding progenies initially selected for short juvenile period and high oil content in a first step of the breeding selection scheme (Arias-Calderón et al., 2014). Initial evaluation for oil quality traits was also carried out at this stage for quality components that can be directly evaluated through the analysis of fruit flesh (Velasco et al., 2014; De la Rosa et al., 2016). According to these results, genotypes with higher oil content and promising oil composition were selected in this first step.

Subsequently, in the second step, resistance to VWO was firstly evaluated in a growth chamber by root-dipping inoculations in a conidia suspension of the defoliating pathotype of *V. dahliae*. This evaluation process has been described as the most effective method for early screening of resistant genotypes, enabling the artificial inoculation of a large number of genotypes in a short time and limited space (Trapero et al., 2013a). However, evaluations under field conditions are recommended to corroborate the disease response observed in growth chamber evaluations (Arias-Calderón et al., 2015a;2015b;2015c; Trapero et al., 2013).

In this sense, the olive breeding program of IFAPA included an evaluation under semi-controlled conditions as intermediate evaluation step to evaluate 20 genotypes potentially resistant after evaluation by root-dipping inoculation in growth chamber. This experiment, reported in the chapter 2 of the present Thesis, consisted on the evaluation under natural climatic conditions of 20 genotypes stablished in microplots with artificially infested soil with microsclerotia of the defoliating pathotype of *V. dahliae*.

Results of testing in artificially inoculated microplots carried out in this work seems to mimic that occurring in natural infested soils with symptoms as defoliation of green leaves peaking from late fall through late spring (Navas-Cortés et al., 2008; Jiménez-Díaz et al., 2011). The disease response differed from the symptoms described in growth chamber, which is in line with previous studies comparing evaluations under field and controlled conditions (Trapero et al., 2013b). In this step only 9 genotypes, out of the 20 tested, revealed a resistant reaction to VWO. However, only 3 were selected according to resistance and productivity criteria.

Symptom development was homogeneously observed among microplots showing an important outbreak from 3 to 14 months after planting and reaching the maximum disease severity incidence at 26-29 months after planting. According to these results, *V. dahliae*-olive interaction in microplots experiments under semi-controlled condition could provide similar results to those under natural conditions in olive orchards with advantages on experiment management and time-consuming.

In microplots, different effects were observed even on asymptomatic plants growing in infested soils as the reduction of plant growth and the delay on fruit production, which were more evident for susceptible genotypes than resistant ones. These results support the usefulness of resistant genotypes in infested soils as an efficient measure for VWO control, which have also been observed in a long-term study in commercial olive orchards (Ostos et al., 2020).

At the same time, resistant genotypes selected in growth chamber (the same genotypes evaluated in microplots), have been evaluated in final comparative trials stablished in naturally infested soils in commercial olive orchards. Four field trials were distributed in Jaén province (Arjona, Úbeda, Begíjar and Villatorres), the main producing area of olive oil in Andalusia (Spain) (MAPA, 2020). Similar quantities of *V. dahliae* propagules (microsclerotia per gram of soil) were detected in soils among field trials, quantifying lower inoculum densities to those applied in artificially infested soils of microplots. In this case, disease incidence was highly variable ranging from 2,7% to 23,9%, in Villatorres and Begijar fields, respectively (unpublished data). This variability on disease incidence was also depicted in

the unevenly development of symptoms in some genotypes among fields, which have been also observed in previous field evaluations of olive cultivars (Trapero et al., 2013b). In field conditions, the disease incidence could be affected by the unequal distribution of the pathogen in soil, crop management practices or other environmental factors, so these results support the compelling necessity of comparative trials. Additionally, the analysis of symptomatic plants in comparative trials revealed the genetic variability of *V. dahliae* isolates in Jaén olive orchards, where the defoliating pathotype caused most of the infections (unpublished data). These results are in line with previous studies about the distributions of *V. dahliae* in Andalusia (López-Escudero et al., 2010; Navas-Cortés et al., 2008). Despite the difficulties, the comparative field trials corroborated the resistant response of the genotypes selected in the microplots experiment.

Resistant selections must always fulfill a good performance for others important agronomic and quality traits in order to be considered good candidates to be released as new cultivars. Among then, in breeding programs aiming at obtaining new cultivars for olive oil production, the composition of the oil must be always considered of paramount importance. Extra virgin olive oil (EVOO) contributes to the healthy and nutritional properties of the Mediterranean diet, which was inscribed in 2010 on the Representative List of the Intangible Cultural Heritage of Humanity by UNESCO (Ros et al., 2014). EVOO fatty acid composition, mainly represented by monounsaturated fatty acids, as well as a myriad of minor components are the main responsible for the healthy properties of EVOO, particularly regarding cardiovascular diseases, inflammation, cancer and a general increase in life expectancy (Jiménez-López et al., 2020).

Evaluation for these compounds should be, therefore, always considered in routine evaluation and selection of breeding works. A high genetic influence on EVOO composition has been reported in different olive plant materials and a wide genetic variability has been observed in olive progenies, even form a single cross, similar or even wider than the variability observed from traditional cultivar sample sets (León et al., 2004; De la Rosa et al., 2016; Pérez et al., 2014). Thus, evaluation and selection for EVOO quality components in breeding programs can be initiated at early stages of the breeding process, even at the initial seedling population.

However, due to the relative contribution of environmental variance on these traits (Navas-López et al., 2020), selection for EVOO composition in usually carried out later in the breeding process, at the intermediate step or, more frequently, at the final step of comparative trials in different environments. Following this approach, EVOO composition of the most promising resistant selections identified in our work, together with 'Picual' as a reference cultivar, was evaluated in comparative trials established both in experimental microplots and under field conditions in different locations.

Moreover, information currently available on the genetic and environmental interaction for EVOO composition is still quite limited, even though this information would be of paramount importance for accurate selection of new cultivars with improved composition. Thus, our experimental approach was also used to gain deeper knowledge on the effect of genotype, environment and their interaction (G x E) on chemical components, oxidative stability and sensorial properties of EVOO. These studies are described in Chapters 3 and 4 of this Thesis.

In chapter 3, the analysis of 66 different chemical compounds, oxidative stability and sensory attributes of fruity, bitter and pungent were carried out in order to determine the effect of cultivar, environment and G x E interaction on their quantitative and qualitive expression. The results showed a strong influence of the genotype on oleic acid content, tocopherols, squalene and sterols, while the environmental effect was predominant for phenols and volatile compounds. Similar results have been previously described for traditional olive cultivars studied in five different growing areas of Andalusia (Spain), and for breeding selections evaluated in three Italian regions as well (Navas-López et al., 2020; Ripa et al., 2008). However, contradictory results have been reported for another work considering a set of olive cultivars from germplasm collections located in different growing countries, in which the environmental factor resulted the main influence for oleic acid content (Mousavi et al., 2019).

Genetic effect was also predominant for oxidative stability (induction time) and fruity, while G x E interaction was important for bitter and pungent properties. The effect of G x E interaction was significative on EVOO compounds supporting the requirement of experimentation under different field conditions, which could be relevant for both oil characteristics and other traits as flowering and oil accumulation (Navas-López et al., 2019a; 2019b; 2020).

In addition to the genetic and environmental factors, EVOO compounds influence the oxidative stability and sensorial properties of EVOOs. Among them, oleic acid and 3,4-DHPEA-EA were positively correlated with stability, in contrast to 3,4-DHPEA-EDA, linoleic acid or sterols. Previous works also concluded that fatty acid and phenols profiles are the most influential factors for EVOO oxidative stability describing opposite effect for oleic and linoleic acids and suggesting (3,4-DHPEA-EA + p-HPEA-EA)/(3,4-DHPEA-EDA + p-HPEA-EDA) as a reliable estimator for oxidative stability (Miho et al., 2020; Montaño et al., 2016).

Regarding the sensory properties, fruity, bitter and pungent were not correlated with EVOO chemical compounds, in spite of several works have previously described the relationship among volatiles and phenols with sensorial attributes (Pedan et al., 2019; Campestre et al., 2017; Cecchi et al., 2013). The lack of correlation between EVOO compounds and sensory properties analyzed in our work could be due to EVOO samples showed similar flavors intensities and the specific aromas were not considered in the sensorial analysis.

Nonetheless, the breeding selections were diferentiated according to EVOO composition, distinguishing ‘Picual’ and ‘IFAPA111-2’ from ‘IFAPA117-117’ and ‘IFAPA117-120’. This differentiation among genotypes was observed considering only volatile compounds as well. In this sense, particular attention was paid to volatile composition in Chapter 4, in which the paramount effect of the genetic factor was ratified for volatile composition of EVOO. These results revealed that the genetic factor mainly influences the volatile profile, and the environmental factor affects the total content, while G x E interaction was significant for C5/LA compounds and esters. The variability on volatile fraction among olive cultivars has been studied in previous works for large sets of traditional cultivars due this trait has been described as an important character for genitors in breeding programs (García-Vico et al., 2017; Luna et al., 2006).

In general, according to our results, the genetic effect prevailed over environment for most of the EVOO compounds. This is an important finding for olive breeding programs aiming to obtain new bred selections with improved EVOO characteristics. However, the scarce knowledge on the genetic control of the different agronomic traits still limits the progress of olive breeding programs compared to other fruit trees species (Lavee, 2013). Several attempts to implement marker-assisted selection (MAS) in olive have been based on the development of genetic linkage maps and localization of quantitative trait loci (QTLs) associated to agronomical traits of interest, as previously widely reported in other fruit tree species. This represents a promising strategy for improving the efficiency of breeding programs, allowing selection of interesting genotypes from the initial seedling populations (Collard et al., 2005). Thus, attempts for QTLs mapping related to flowering, fruiting, vigor, fruit and oil traits (fatty acid composition) have been reported (Ben Sadok et al. 2013; Atienza et al., 2014). However, those works were based in single cross progenies planted in single locations. Therefore, they are not considering the wide genetic variability harbored in the cultivated germplasm nor its interaction with the environment. Similar approaches have not yet been attempted for VWO. The difficulties for extensive and accurate phenotyping for this trait make quite complex to develop these studies.

Transcriptomic approaches have been also widely used in the last years to reveal genes associated to important agronomic traits including cold acclimation (Leyva-Pérez et al., 2015), fruit abscission (Gil-Amado and Gómez-Jiménez, 2013), response to pest such as olive fruit fly *Bactrocera oleae* (Grasso et al., 2017), plant architecture (González-Plaza et al., 2016), early plant development and juvenile period (Jiménez-Ruiz et al., 2018) and oil quality components (Alagna et al., 2012). These works allowed the identification of several transcripts putatively involved in the regulation of these traits, but unscrambling the whole picture involved in these processes is still far from be accomplished. This approach has also been applied to study the olive response to *V. dahliae* infection VWO (Jiménez-Ruiz et al., 2019; 2017; Leyva-Pérez et al., 2018; Cabanás et al., 2015), revealing a large number of differentially expressed genes comparing resistant and susceptible cultivars. These transcriptomic differences were observed in both conditions before and after infection, revealing a complex defense mechanism against VWO in olive.

'Frantoio' and 'Picual' are the cultivars used for these transcriptomic studies, which are considered references for resistance and susceptible responses to VWO, respectively. Additional cultivars have been rarely included in these works, with a few exceptions such as validation of some genes in 'Changlot Real' as resistant cultivar (Cabanás et al., 2015). In this sense, the Chapter 5 of this Thesis comprises the analysis of 7 resistance candidate genes in 77 different genotypes including cultivars, breeding selections, wild olives and related subspecies (*guanchica* and *cerasiformis*). Nucleotide diversity was analyzed in coding regions of 7 genes involved in different resistant mechanism selected from previous works. Four of them were selected from transcriptomic analysis of infected olives by the defoliating pathotype of *V. dahliae* and three genes were selected from different host species in which their effects had been validated through transgenic plants. This analysis revealed 94 coding region polymorphisms in putative resistance genes, which could contribute to develop valuable markers along with SNPs detected in previous works (Cultrera et al., 2019; Zhu et al., 2019; Belaj et al., 2018). In spite of this work did not provide a clear correlation attending

to resistance evaluation, some interesting associations regarding the origin of plant materials were observed for some genes, which could be useful for germplasm collection management and selection process assistance in breeding programs. Many different genes have been identified in the disease response, which could trigger different defense mechanism among genotypes leading to resistant or tolerant responses as previously described in different works (Arias-Calderón et al., 2015a; Robb, 2007). So, better characterization of the disease response and deeper understanding of *V.dahlie*-olive interaction in a large set of genotypes are needed for future genetic works. Advances in next generation sequencing technologies would also enable genome studies revealing important resistance/susceptibility genes.

Recently, the genome of the *Olea europaea* cultivar 'Picual' has been sequenced revealing an assembly size of 1.68 Gb with up to 79667 gene models (Jiménez-Ruiz et al., 2020). Using 'Picual' as the reference, new whole genome sequences could be expected to be soon available, which paves the way towards the development of new genomic tools. Among these tools, genome-wide association studies (GWAS) represent a very promising strategy with increased potential compared to QTL mapping, by using more diverse populations with lower levels of linkage disequilibrium and higher marker density, which narrows the genomic regions to search for potential candidate genes and causal polymorphisms (Korte and Farlow, 2013). In this sense, the first GWAS in olive have provided 19 candidate genes for agronomic traits (Kaya et al., 2019). Currently, we are testing this approach with highly promising results for Verticillium wilt resistance from the evaluation of a core collection (work in progress). The analysis of several disease parameters arose some discrepancies arose in resistance categorization comparing with the traditional classification method based on the RAUDPC values (López-Escudero et al., 2004). The slight differences in symptom development among genotypes could be related to differences in genes expression or defense mechanisms. So, a detailed description of phenotype could provide a better understanding of studies at genome level contributing to detect resistance genes for the development of molecular markers, which could represent a convenient strategy to enhance olive breeding for VWO in the near future.

In summary, the selection process carried out by the olive breeding program of IFAPA have so far enabled the selection of 3 highly resistant genotypes with differentiated oil quality (IFAPA111-2, IFAPA117-117 and IFAPA117-120), which means that the procedure has been effective in obtaining new varieties.

The workflow of the olive breeding program for verticillium wilt resistance could be applied to other olive diseases, such as the olive quick decline syndrome, which is a vascular disease caused by *X. fastidiosa* subsp. *pauca* strain CoDiRO that emerged in 2013 in Southern Italy (Saponari et al., 2014). This disease shares some characteristics with VWO, such a huge host species, genetic variability of the pathogen, inefficient chemical control and reduce resistant olive cultivars (Saponari et al., 2019; Marcelletti and Scortichini, 2016).

In this sense, two predoctoral research stays have been accomplished by the internationalization of this Thesis, focused to learn new techniques for the evaluation of olive resistance against CoDiRO. The first step consisted on resistance evaluation of traditional cultivars using different methods including both artificial inoculation in

greenhouse and natural infestation in fields by insects or rootstocks transmission. In any case, the disease development requires longer period than VWO, which makes difficult the evaluation process.

Currently, field observations since the emergence of the disease in Apulia (South-eastern Italy) identified resistant cultivars prevailing in infected olive orchards in this area. So, 'Fs-17' and 'Leccino' cultivars showed tolerance to CoDiRO, while 'Ogliarola Salentina' and 'Cellina di Nardò' resulted highly susceptible. According to these results 'Fs-17' and 'Leccino' could be used as genitors in a new work line of the IFAPA olive breeding program for resistance to *X. fastidiosa* subsp. *pauca*. Nonetheless, the lack of knowledge about the reaction of a large set of cultivars limit the possibilities in breeding program, as well as the unknown reaction against different isolates o subspecies of *X. fastidiosa*.

At the same time CoDiRO-olive interaction have been studied at the transcriptomic level revealing differences in gene expression between 'Ogliarola Salentina' and 'Leccino' assotiated with a limitation of the bacterial multiplication in tolerant cultivars (Giampetruzzi et al., 2016). Validation of these putative genes in 'Cellina di Nardò' and 'Leccino' was carried out during a predoctoral stay at The Institute for Sustainable Plant Protection (Bari, Italy) (unpublished results). The validation of genes involved in the olive reaction to *X. fastidiosa* infection could be very useful for the development of molecular markers, as previously proposed for VWO.

The complex control of vascular diseases requires also knowledge about the pathogen. For that reason, the second research stay in the Department of Microbiology and Plant Pathology at the University of California (Riverside, California) was focused on the study of the virulence mechanism of *X. fastidiosa* strain Temecula, causal agent of Pierce's Disease in grapes. The major virulence factors of *X. fastidiosa* is the twitching motility mediated by type IV pili (Rapicavoli et al., 2018). This collaboration consisted on development of *X. fastidiosa* mutants knocking three different genes involved in type IV pili development (*pilA1*, *pilA2* and *pilB*) (Kandel et al., 2018; Meng et al., 2005). The purpose of this study was the functional validation of *pil* genes in grapes after artificial inoculations in greenhouse (work in progress), whose results could further elucidate the bacterium-host interaction.

In summary, the development of new bred cultivars for resistance to olive disease requires a broad understanding about vascular diseases, focusing on the host plant, but considering the importance of the pathogen and environment. Moreover, olive breeding could contribute to increase the EVOO productivity and diversity obtaining selections adapted to the new conditions of olive growing.

6.1. References

Alagna, F., Mariotti, R., Panara, F., Caporali, S., Urbani, S., Veneziani, G., Esposto, S., Taticchi, A., Rosati, A., Rao, R., Perrotta, G., Servili, M., Baldoni, L., 2012. Olive phenolic compounds: metabolic and transcriptional profiling during fruit development. BMC Plant Biology 12, 162.

Arias-Calderón, R., León, L., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., Rodríguez-Jurado, D., 2015a. Resistance to Verticillium wilt in olive progenies from open-pollination. Scientia Horticulturae 185, 34–42.

Arias-Calderón, R., Rodríguez-Jurado, D., Bejarano-Alcázar, J., Belaj, A., de la Rosa, R., León, L., 2015b. Evaluation of Verticillium wilt resistance in selections from olive breeding crosses. Euphytica 206, 619–629.

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.

Atienza, S.G., de la Rosa, R., León, L., Martín, A., Belaj, A., 2014. Identification of QTL for agronomic traits of importance for olive breeding. Mol Breeding 34, 725–737.

Badenes, M.L., Byrne, D.H. (Eds.), 2012. Fruit Breeding, Handbook of Plant Breeding. Springer-Verlag, New York.

Bellini, E., Giordani, E., Rosati, A., 2008. Genetic improvement of olive from clonal selection to cross-breeding programs. Advances in Horticultural Science 22, 73–86.

Ben Sadok, I., Celton, J.-M., Essalouh, L., El Aabidine, A.Z., Garcia, G., Martinez, S., Grati-Kamoun, N., Rebai, A., Costes, E., Khadari, B., 2013. QTL mapping of flowering and fruiting traits in olive. PLoS One 8, e62831.

Bubici, G., Cirulli, M., 2012. Control of Verticillium wilt of olive by resistant rootstocks. Plant Soil 352, 363–376.

Cabanás, C.G.-L., Schiliro`, E., Valverde-Corredor, A., Mercado-Blanco, J., 2015. Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root colonization by the vascular pathogen *Verticillium dahliae*. Front. Microbiol. 6.

Campestre, C., Angelini, G., Gasbarri, C., Angerosa, F., 2017. The Compounds Responsible for the Sensory Profile in Monovarietal Virgin Olive Oils. Molecules 22, 1833.

Cecchi, T., Alfei, B., 2013. Volatile profiles of Italian monovarietal extra virgin olive oils via HS-SPME–GC–MS: Newly identified compounds, flavors molecular markers, and terpenic profile. Food Chemistry 141, 2025–2035.

Cirulli, M., Colella, C., D'Amico, M., Amenduni, M., Bubici, G., 2008. Comparison of screening methods for the evaluation of olive resistance to *Verticillium dahliae* kleb. Journal of Plant Pathology 90, 7–14.

Collard, B.C.Y., Jahufer, M.Z.Z., Brouwer, J.B., Pang, E.C.K., 2005. An introduction to markers, quantitative trait loci (QTL) mapping and marker-assisted selection for crop improvement: The basic concepts. Euphytica 142, 169–196.

CVPO, Community Plant Variety Office. 2020, https://cpvo.europa.eu/en/applications-and-examinations/cpvo-varieties-database.

Erten, L., Yıldız, M., 2011. Screening for resistance of Turkish olive cultivars and clonal rootstocks to Verticillium wilt. Phytoparasitica 39, 83–92.

Fernández-Escobar, R., Gil-Ribes, J.A., Quesada-Moraga, E., Trapero, A., Msallem, M., 2013. Evolution and sustainability of the olive production systems 32.

García-Vico, L., Belaj, A., Sánchez-Ortiz, A., Martínez-Rivas, J., Pérez, A., Sanz, C., 2017. Volatile Compound Profiling by HS-SPME/GC-MS-FID of a Core Olive Cultivar Collection as a Tool for Aroma Improvement of Virgin Olive Oil. Molecules 22, 141.

Giampetruzzi, A., Morelli, M., Saponari, M., Loconsole, G., Chiumenti, M., Boscia, D., Savino, V.N., Martelli, G.P., Saldarelli, P., 2016. Transcriptome profiling of two olive cultivars in response to infection by the CoDiRO strain of *Xylella fastidiosa* subsp. *pauca*. BMC Genomics 17, 475.

Gil-Amado, J.A., Gomez-Jimenez, M.C., 2013. Transcriptome Analysis of Mature Fruit Abscission Control in Olive. Plant and Cell Physiology 54, 244–269.

González-Plaza, J.J., Ortiz-Martín, I., Muñoz-Mérida, A., García-López, C., Sánchez-Sevilla, J.F., Luque, F., Trelles, O., Bejarano, E.R., De La Rosa, R., Valpuesta, V., Beuzón, C.R., 2016. Transcriptomic Analysis Using Olive Varieties and Breeding Progenies Identifies Candidate Genes Involved in Plant Architecture. Front. Plant Sci. 7.

Grasso, F., Coppola, M., Carbone, F., Baldoni, L., Alagna, F., Perrotta, G., Pérez-Pulido, A.J., Garonna, A., Facella, P., Daddiego, L., López, L., Vitiello, A., Rao, R., Corrado, G., 2017. The transcriptional response to the olive fruit fly (*Bactrocera oleae*) reveals extended differences between tolerant and susceptible olive (*Olea europaea* L.) varieties. PLOS ONE 12, e0183050.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., del Mar Jiménez-Gasco, M., Antoniou, P.P., Tjamos, E.C., 2011. Verticillium Wilt, A Major Threat to Olive Production: Current Status and Future Prospects for its Management. Plant Disease 96, 304–329.

Jiménez-Fernández, D., Trapero-Casas, J.L., Landa, B.B., Navas-Cortés, J.A., Bubici, G., Cirulli, M., Jiménez-Díaz, R.M., 2016. Characterization of resistance against the olive-defoliating *Verticillium dahliae* pathotype in selected clones of wild olive. Plant Pathol 65, 1279–1291.

Jiménez-López, C., Carpena, M., Lourenço-Lopes, C., Gallardo-Gómez, M., Lorenzo, J.M., Barba, F.J., Prieto, M.A., Simal-Gandara, J., 2020. Bioactive Compounds and Quality of Extra Virgin Olive Oil. Foods 9, 1014.

Jiménez-Ruiz, J., de la O Leyva-Pérez, M., Vidoy-Mercado, I., Barceló, A., Luque, F., 2018. Transcriptomic time-series analysis of early development in olive from germinated embryos to juvenile tree. BMC Genomics 19.

Jiménez-Ruiz, J., Leyva-Pérez, M. de la O., Gómez-Lama Cabanás, C., Barroso, J.B., Luque, F., Mercado-Blanco, J., 2019. The Transcriptome of *Verticillium dahliae* Responds Differentially Depending on the Disease Susceptibility Level of the Olive (*Olea europaea* L.) Cultivar. Genes (Basel) 10.

Jiménez-Ruiz, J., Leyva-Pérez, M. de la O., Schilirò, E., Barroso, J.B., Bombarely, A., Mueller, L., Mercado-Blanco, J., Luque, F., 2017. Transcriptomic Analysis of *Olea europaea* L. Roots during the *Verticillium dahliae* Early Infection Process. The Plant Genome 10, 0.

Kandel, P.P., Chen, H., Fuente, L.D.L., 2018. A Short Protocol for Gene Knockout and Complementation in *Xylella fastidiosa* Shows that One of the Type IV Pilin Paralogs (PD1926) Is Needed for Twitching while Another (PD1924) Affects Pilus Number and Location. Appl. Environ. Microbiol. 84.

Kaya, H.B., Akdemir, D., Lozano, R., Cetin, O., Sozer Kaya, H., Sahin, M., Smith, J.L., Tanyolac, B., Jannink, J.-L., 2019. Genome wide association study of 5 agronomic traits in olive (*Olea europaea* L.). Sci Rep 9, 18764.

Korte, A., Farlow, A., 2013. The advantages and limitations of trait analysis with GWAS: a review. Plant Methods 9, 29.

Lavee, S., 2013. Evaluation of the need and present potential of olive breeding indicating the nature of the available genetic resources involved. Scientia Horticulturae 161, 333–339.

León, L., Martín, L.M., Rallo, L., 2004. Repeatability and Minimum Selection Time for Fatty Acid Composition in Olive Progenies. HortScience 39, 477–480.

León, L., Velasco, L., de la Rosa, R., 2015. Initial selection steps in olive breeding programs. Euphytica 201, 453–462.

Leyva-Pérez, M. de la O., Jiménez-Ruiz, J., Gómez-Lama Cabanás, C., Valverde-Corredor, A., Barroso, J.B., Luque, F., Mercado-Blanco, J., 2018. Tolerance of olive (*Olea europaea*) cv Frantoio to *Verticillium dahliae* relies on both basal and pathogen-induced differential transcriptomic responses. New Phytol. 217, 671–686.

Leyva-Pérez, M. de la O., Valverde-Corredor, A., Valderrama, R., Jiménez-Ruiz, J., Muñoz-Merida, A., Trelles, O., Barroso, J.B., Mercado-Blanco, J., Luque, F., 2015. Early and delayed long-term transcriptional changes and short-term transient responses during cold acclimation in olive leaves. DNA Res 22, 1–11.

López-Escudero, F.J., Blanco-López, M.Á., Rincón, C.D.R., Reig, J.M.C., 2007. Response of Olive Cultivars to Stem Puncture Inoculation with a Defoliating Pathotype of *Verticillium dahliae*. HortScience 42, 294–298.

López-Escudero, F.J., del Río, C., Caballero, J.M., Blanco-López, M.A., 2004. Evaluation of Olive Cultivars for Resistance to Verticillium dahliae. European Journal of Plant Pathology 110, 79–85.

López-Escudero, J., 2010. Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of Verticillium dahliae. Phytopathologia Mediterranea 49, 11.

Luna, G., Morales, M.T., Aparicio, R., 2006. Characterisation of 39 varietal virgin olive oils by their volatile compositions. Food Chemistry 98, 243–252.

MAPA, Ministry of Agriculture, Fisheries and Food. 2020. https://www.mapa.gob.es/es/estadistica/temas/estadisticasagrarias/agricultura/superficies-producciones-anuales-cultivos/

Marcelletti, S., Scortichini, M., 2016. Xylella fastidiosa CoDiRO strain associated with the olive quick decline syndrome in southern Italy belongs to a clonal complex of the subspecies pauca that evolved in Central America. Microbiology, 162, 2087–2098.

Meng, Y., Li, Y., Galvani, C.D., Hao, G., Turner, J.N., Burr, T.J., Hoch, H.C., 2005. Upstream Migration of Xylella fastidiosa via Pilus-Driven Twitching Motility. Journal of Bacteriology 187, 5560–5567.

Montaño, A., Hernández, M., Garrido, I., Llerena, J.L., Espinosa, F., 2016. Fatty Acid and Phenolic Compound Concentrations in Eight Different Monovarietal Virgin Olive Oils from Extremadura and the Relationship with Oxidative Stability. International Journal of Molecular Sciences 17, 1960.

Montes-Osuna, N., Mercado-Blanco, J., 2020. Verticillium Wilt of Olive and its Control: What Did We Learn during the Last Decade? Plants 9, 735.

Mousavi, S., de la Rosa, R., Moukhli, A., El Riachy, M., Mariotti, R., Torres, M., Pierantozzi, P., Stanzione, V., Mastio, V., Zaher, H., El Antari, A., Ayoub, S., Dandachi, F., Youssef, H., Aggelou, N., Contreras, C., Maestri, D., Belaj, A., Bufacchi, M., Baldoni, L., Leon, L., 2019. Plasticity of fruit and oil traits in olive among different environments. Scientific Reports 9, 16968. https://doi.org/10.1038/s41598-019-53169-3

Navas-Cortés, J.A., Landa, B.B., Mercado-Blanco, J., Trapero-Casas, J.L., Rodríguez-Jurado, D., Jiménez-Díaz, R.M., 2008. Spatiotemporal Analysis of Spread of Infections by Verticillium dahliae Pathotypes Within a High Tree Density Olive Orchard in Southern Spain. Phytopathology® 98, 167–180.

Navas-López, J.F., Cano, J., de la Rosa, R., Velasco, L., León, L., 2020. Genotype by environment interaction for oil quality components in olive tree. European Journal of Agronomy 119, 126115.

Navas-López, J. F., León, L., Rapoport, H.F., Moreno-Alías, I., Lorite, I.J., de la Rosa, R., 2019a. Genotype, environment and their interaction effects on olive tree flowering phenology and flower quality. Euphytica 215, 184.

Navas-López, J.F., León, L., Trentacoste, E.R., de la Rosa, R., 2019b. Multi-environment evaluation of oil accumulation pattern parameters in olive. Plant Physiology and Biochemistry 139, 485–494.

Ostos, E., García-López, M.T., Porras, R., López-Escudero, F.J., Trapero-Casas, A., Michailides, T.J., Moral, J., 2020. Effect of Cultivar Resistance and Soil Management on Spatial-Temporal Development of Verticillium Wilt of Olive: A Long-Term Study. Front Plant Sci 11, 584496.

Pedan, V., Popp, M., Rohn, S., Nyfeler, M., Bongartz, A., 2019. Characterization of Phenolic Compounds and Their Contribution to Sensory Properties of Olive Oil. Molecules 24, 2041.

Pérez, A.G., León, L., Pascual, M., Romero-Segura, C., Sánchez-Ortiz, A., Rosa, R. de la, Sanz, C., 2014. Variability of Virgin Olive Oil Phenolic Compounds in a Segregating Progeny from a Single Cross in Olea europaea L. and Sensory and Nutritional Quality Implications. PLOS ONE 9, e92898.

Porras Soriano, A., Soriano Martín, M.L., Porras Piedra, A., 2003. Grafting olive cv. Cornicabra on rootstocks tolerant to Verticillium dahliae reduces their susceptibility. Crop Protection 22, 369–374.

Rallo, L., Barranco, D., Díez, C.M., Rallo, P., Suárez, M.P., Trapero, C., Pliego-Alfaro, F., 2018. Strategies for Olive (Olea europaea L.) Breeding: Cultivated Genetic Resources and Crossbreeding, in: Al-Khayri, J.M., Jain, S.M., Johnson, D.V. (Eds.), Advances in Plant Breeding Strategies: Fruits: Volume 3. Springer International Publishing, Cham, pp. 535–600.

Rapicavoli, J., Ingel, B., Blanco-Ulate, B., Cantu, D., Roper, C., 2018. Xylella fastidiosa: an examination of a re-emerging plant pathogen. Mol Plant Pathol 19, 786–800.

Ripa, V., Rose, F.D., Caravita, M.A., Parise, M.R., Perri, E., Rosati, A., Pandolfi, S., Paoletti, A., Pannelli, G., Padula, G., Giordani, E., Bellini, E., Buccoliero, A., Mennone, C., 2008. Qualitative evaluation of olive oils from new olive selections and effects of genotype and environment on oil quality. Advances in Horticultural Science 22, 95–103.

Robb, J.R., 2007. Verticillium tolerance: resistance, susceptibility, or mutualism? Botany.

Ros, E., Martínez-González, M.A., Estruch, R., Salas-Salvadó, J., Fitó, M., Martínez, J.A., Corella, D., 2014. Mediterranean Diet and Cardiovascular Health: Teachings of the PREDIMED Study. Adv Nutr 5, 330S-336S.

Rosa, R. de la, Arias-Calderón, R., Velasco, L., León, L., 2016. Early selection for oil quality components in olive breeding progenies. European Journal of Lipid Science and Technology 118, 1160–1167.

Rosa, R.D. la, Kiran, A.I., Barranco, D., León, L., 2006. Seedling vigour as a preselection criterion for short juvenile period in olive breeding. Aust. J. Agric. Res. 57, 477–481.

Santos-Antunes, F., León, L., Rosa, R. de la, Alvarado, J., Mohedo, A., Trujillo, I., Rallo, L., 2005. The Length of the Juvenile Period in Olive as Influenced by Vigor of the Seedlings and the Precocity of the Parents. HortScience 40, 1213–1215.

Saponari, M., Giampetruzzi, A., Loconsole, G., Boscia, D., Saldarelli, P., 2019. Xylella fastidiosa in Olive in Apulia: Where We Stand. Phytopathology® 109, 175–186.

Saponari, M., Loconsole, G., Cornara, D., Yokomi, R.K., De Stradis, A., Boscia, D., Bosco, D., Martelli, G.P., Krugner, R., Porcelli, F., 2014. Infectivity and Transmission of Xylella

fastidiosa by Philaenus spumarius (Hemiptera: Aphrophoridae) in Apulia, Italy. Journal of Economic Entomology 107, 1316–1319.

Trapero, C., Díez, C.M., Rallo, L., Barranco, D., López-Escudero, F.J., 2013. Effective inoculation methods to screen for resistance to Verticillium wilt in olive. Scientia Horticulturae 162, 252–259.

Trapero, C., Rallo, L., López-Escudero, F.J., Barranco, D., Díez, C.M., 2015. Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the Olea genus. Plant Pathology 64, 890–900.

Valverde, P., Trapero, C., Arquero, O., Serrano, N., Barranco, D., Díez, C.M., López-Escudero, F.J., 2021. Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. Plant Pathology 70, 144–153.

7. CONCLUSIONS

- The development of new bred cultivars suitable for new olive growing conditions and environments are of great interest to ensure the future sustainability of olive crop. To accurately achieve this objective, long-term evaluation under different environmental conditions should be always considered as unavoidable requisite in olive breeding programs (Chapters 2-5).

- The results obtained from artificial inoculation experiments with *V. dahliae* in growth chambers must be always corroborated in different conditions to confirm the disease resistance response. In this regard, the evaluation in semi-controlled artificially infested microplots under natural climatic conditions provided an accurate approximation to the performance in commercial olive orchards. Long-term evaluation in microplots also allows initial characterization for plant growth and bearing traits (Chapter 2).

- The oxidative stability of EVOO could be accurately predicted from its chemical composition, being highly correlated with oleic acid, squalene, 3,4-DHPEA-EA and total phenolics contents. In contrast, sensory properties (fruity, bitter and pungent) were not correlated with specific chemical compounds (Chapter 3)

- EVOO composition, oxidative stability and sensory attributes are highly modulated by the genetic and environmental factors, as well as genotype by environment interaction. Genetic factor was mainly responsible for the variability in oleic acid content, tocopherols, squalene, sterols, oxidative stability and fruity attribute, while environment highly influenced phenols and volatile contents. Significant genotype by environment interaction was also observed for EVOO parameters and particularly remarkable for bitterness and pungency sensorial attributes (Chapters 3 and 4)

- Genetic variability was shown to play a fundamental role to produce differentiated EVOO's, so that breeding works may enlarge the diversity of EVOO's currently available. In that way, 'IFAPA111-2' and 'Picual' EVOO's were clearly differentiated from 'IFAPA117-117' and 'IFAPA117-120' (Chapters 3 and 4).

- Olive disease response against *V. dahliae* infection is regulated by several genes involving different defense mechanism. However, for the genes evaluated in this work, nucleotide variation was no associated to disease response. These results underlined that unscrambling these associations, and therefore developing useful marked assisted selection tools, is far from been solved yet (Chapter 5).

- In summary, works carried out in the olive breeding program for verticillium wilt resistance enabled to obtain three new bred selections that represent new alternatives for olive growing areas infested by *V. dahliae*. These results encourage extending this approach for other potentially important vascular olive diseases, such as the caused by the bacterium *Xylella fastidiosa* currently threatening many olive growing areas in the world.

8. KNOWLEDGE TRANSFER

8.1. Agricultural magazines

León, L.; Serrano, A.; Rodríguez–Jurado, D.; Román, B.; Cano, J.; De la Rosa, R. 2020. Variedades resistentes a la Verticilosis del olivo. Olimerca 34, 78-80. *https://issuu.com/olimerca/docs/olimerca-n34-web-72ppp?fr=sZDk4NDEyOTg4NjY*

Serrano, A; Rodríguez–Jurado, D.; Román, B.; Bejarano–Alcázar, J.; Cano, J.; De la Rosa, R. y León, L. 2019. Evaluación de selecciones de olivo para la resistencia a Verticilosis. Vida Rural Especial Olivar, 36-41. *https://www.agronegocios.es/evaluacion-de-selecciones-de-olivo-para-la-resistencia-a-verticilosis/*

Serrano, A; Arias-Calderón, R.; Rodríguez–Jurado, D.; Bejarano–Alcázar, J.; Belaj, A.; Román, B.; Cano, J.; De la Rosa, R. y León, L. 2017. Resistencia a Verticilosis de nuevos genotipos selecionados en el programa de mejora genética de olivo. Fruticultura ISSN 2013-5742, Especial Olivo 56, 6-13. *https://quatrebcn.es/fruticultura-no-56-especial-olivo-2017*

8.2. Congress communications

Serrano, A.; Rodríguez-Jurado D.; López-Escudero, J.; Román, B.; Belaj, A.; De la Rosa, R.; Luque, F.; Ramírez-Tejero, J.A.; León L. 2020. Verticillium wilt response of a olive core collection. The 7th International Horticulture Research Conference. Online. POSTER *http://www.hortres-conference.org/poster2.php?id=317*

Serrano, A.; León, L.; De la Rosa, R.; Belaj, A.; Montilon, V.; Altamura, G.; Saldarelli, P.; Boscia, D.; Saponari, M. 2019. Screening olive germplasm for resistance to olive quick decline syndrome caused by *Xylella fastidiosa* under field and controlled conditions. 2nd European Conference on Xylella fastidiosa. Ajaccio (France). POSTER

Serrano A.; Rodríguez-Jurado, D.; Román, B.; Bejarano-Alcázar, J.; Cano, J.; De la Rosa, R.; León, L. 2019. Evaluación agronómica de selecciones de olivo resistentes a la Verticilosis. VI Jornada Nacional del grupo de olivicultura de la SECH. Madrid (España). POSTER

De la Rosa, R.; León, L.; Serrano, A.; Moralejo, E.; Serra A.J.; Boscia, D.; Saponari, M.; Belaj, A. 2019. Selección de material vegetal resistente a *Xylella fastidiosa* en olivo. VI Jornada Nacional del grupo de olivicultura de la SECH. Madrid (España). POSTER

Serrano A.; Rodríguez-Jurado, D.; Bejarano-Alcázar, J.; Cano, J.; De la Rosa, R.; León, L. 2019. Selecciones de olivo resistentes a la Verticilosis del programa de mejora del IFAPA. XIX simposium científico-técnico Expoliva. Jaén (España). POSTER

León, L.; Serrano, A.; De la Rosa, R.; Belaj, A.; Montilon, V.; Altamura, G.; Saldarelli, P.; Boscia, D.; Saponari, M. 2019. Evaluación de resistencia a *Xylella fastidiosa* en olivo. XIX simposium científico-técnico Expoliva. Jaén (España). POSTER

Serrano, A.; Arias-Calderón, R.; Rodríguez-Jurado, D.; Belaj, A.; Román, B.; León, L. 2018. Caracterización fenotípica y genotípica de selecciones de olivo para resistencia a

Verticilosis. 1er Congreso de Jóvenes Investigadores en Ciencias Agroalimentarias. Almería (España). POSTER

Serrano, A.; Arias-Calderón, R.; Román, B.; Belaj, A.; Cano, J.; De la Rosa, R.; Bejarano-Alcázar, J.; Rodríguez-Jurado, D.; León, L. 2018. Selection of new olive genotypes resistant to Verticillium wilt in comparative field trials. 6th International Conference on the Olive Tree and Olive Products – OliveBioteq'18. Sevilla (España). POSTER

Serrano, A.; León, L.; Belaj, A.; Román, B. 2018. Nucleotide diversity analysis of candidate resistance genes to Verticillium wilt in olive. 6th International Conference on the Olive Tree and Olive Products – OliveBioteq'18. Sevilla (España). POSTER (Poster Award).

Serrano, A.; Arias-Calderón, R.; Rodríguez-Jurado, D.; Román, B.; Bejarano-Alcázar, J.; Cano, J.; León, L. 2018. Evaluación en campo de genotipos procedentes del programa IFAPA de mejora de olivo para resistencia a Verticilosis. XIX Congreso de la Sociedad Española de Fitopatología. Toledo (España). POSTER.

León, L.; Serrano, A.; De la Rosa, R.; Belaj, A.; Boscia, D.; Saponari, M. 2017. Screening olive plant materials for susceptibility to Xylella fastidiosa and selection of resistant germplasm. European conference on Xylella 2017. Palma de Mallorca (España). POSTER

Serrano, A.; León, L.; Belaj, A.; Román, B. 2017. Allelic diversity analysis for Vertcillium wilt resistance candidate genes in olive (*Olea europarea* L.). 15th Congress of The Mediterranean Phytopathological Union. Córdoba (España). POSTER

8.3. Audiovisual media

The world olive germplasm bank and the olive breeding program of IFAPA 'Alameda del Obispo'. 2019. *https://www.oliveoiltimes.com/world/ifapa/77468?amp&__twitter_impression=true*

Entrevista por la Cadena SER en la XIII Feria del Aceite de Porcuna y ponencia "Aplicación al control de enfermedades vasculares". 2019 *https://cadenaser.com/emisora/2019/11/13/ser_andujar/1573643295_937517.amp.html?ssm=tw&__twitter_impression=true*

Entrevista por la Cadena SER en la XI Feria del Aceite de Porcuna y ponencia "Selección de nuevas variedades resistentes a Verticilosis y evaluación para resistencia a *Xylella fastidiosa*". 2017 https://cadenaser.com/emisora/2017/10/26/ser_andujar/1509019197_069027.html

8.4. Other contributions

Co-directora del Trabajo Fin de Grado: "Evaluación de genotipos de olivo seleccionados para la resistencia a Verticilosis mediante ensayos comparativos en campo". Código del TFG: BL17-125-MCR. Trabajo de Iniciación a la Investigación. ALUMNA: Ángela Mª Muñoz Romero, de la Facultad de Biología de la Universidad de Córdoba. 2018

Co-directora del Trabajo Fin de Máster: “Evaluación de progenies de olivo procedentes del Programa de Mejora Genética de IFAPA”. ALUMNA: Mª Valentina Najt Ruiz, del Máster en Producción, Protección y Mejora Vegetal de la Universidad de Córdoba. 2017

La **Verticilosis del olivo**, causada por el hongo de suelo *Verticillium dahliae,* es una de las enfermedades vasculares más limitantes para este cultivo. El programa de mejora genética de olivo desarrollado en el centro IFAPA 'Alameda del Obispo' tiene como una de sus principales líneas de trabajo la obtención de nuevas variedades que aúnen resistencia a esta enfermedad y características agronómicas mejoradas para la producción de aceite de oliva virgen extra. En esta tesis se presentan las evaluaciones de resistencia a la verticilosis realizadas en etapas avanzadas de dicho programa y la caracterización de la calidad de los aceites extraídos de las selecciones más resistentes. Como resultado final se presentan tres selecciones IFAPA111-2, IFAPA117-117 e IFAPA117-120 que de forma consistente han resultado resistentes tras diversas evaluaciones tanto en cámara de cultivo como en campo. Los ensayos comparativos en campo también han permitido el análisis del efecto de los factores genético, ambiental e interacción de ambos en la composición de los aceites de dichas selecciones. El proceso de obtención de estas nuevas variedades es fruto del trabajo de muchos años, por lo que también se presenta un análisis a nivel genético con el objetivo de identificar polimorfismos en genes candidatos para los mecanismos de respuesta a la enfermedad que puedan contribuir a la mejora asistida por marcadores en futuros trabajos de selección.

El esquema del programa de mejora seguido para la obtención de variedades resistentes a la la verticilosis se plantea también como un procedimiento eficaz para el control de otras enfermedades del olivo como la causada por la bacteria *Xylella fastidiosa*.

UNIVERSIDAD DE CÓRDOBA
INIA Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria
IFAPA Instituto de Investigación y Formación Agraria y Pesquera