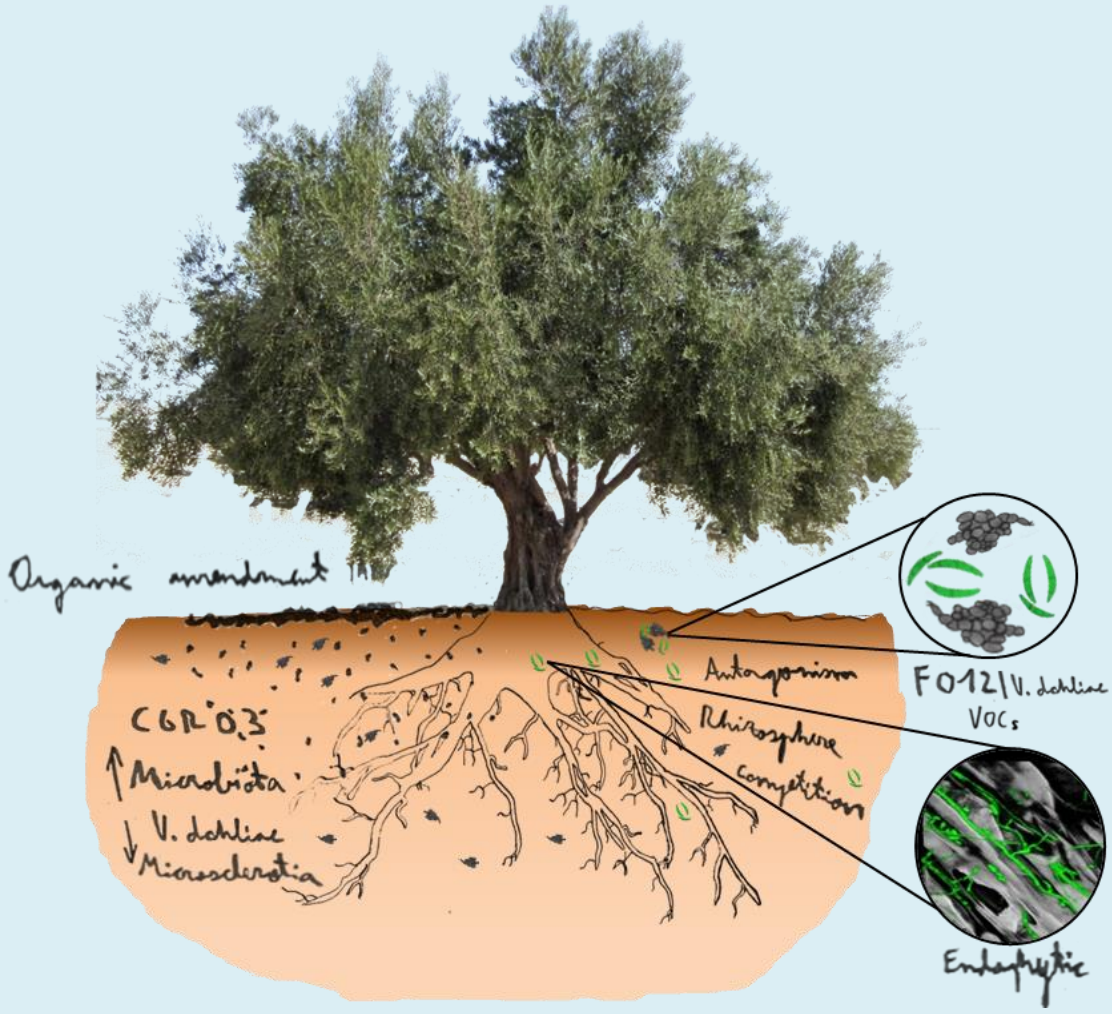
CONTROL BIOLÓGICO DE LA VERTICILOSIS DEL OLIVO CON LA CEPA NO PATOGÉNICA DE *Fusarium oxysporum* FO12 Y CON EL COMPOST DE ORUJO DE VID CGR03

Biological control of Verticillium wilt of olive with the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03



PhD THESIS Antonio Mulero Aparicio

UNIVERSIDAD D CÓRDOBA

ETSIAM

TU Graz

Institute of Environmental Biotechnology
Graz University of Technology

ESCUELA TÉCNICA SUPERIOR DE INGENIERÍA AGRONÓMICA Y DE MONTES, DEPARTAMENTO DE AGRONOMÍA, UNIVERSIDAD DE CÓRDOBA

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

INSTITUTE OF ENVIRONMENTAL BIOTECHNOLOGY, GRAZ UNIVERSITY OF TECHNOLOGY

Doctoral program in Natural Sciences; Area of concentration: Biotechnology, Biochemistry and Food Chemistry

CONTROL BIOLÓGICO DE LA VERTICILOSIS DEL OLIVO CON LA CEPA NO PATOGÉNICA DE *Fusarium oxysporum* FO12 Y CON EL COMPOST DE ORUJO DE VID CGR03

(BIOLOGICAL CONTROL OF VERTICILLIUM WILT OF OLIVE WITH THE NON-PATHOGENIC STRAIN OF *Fusarium oxysporum* FO12 AND THE GRAPE MARC COMPOST CGR03)

Memoria de Tesis doctoral por compendio de artículos realizada en régimen de Cotutela para optar al grado de Doctor con Mención Internacional por la Universidad de Córdoba y por la Graz University of Technology realizada por el Graduado en Ingeniería Agrícola Antonio Mulero Aparicio.

PhD Thesis by compendium of articles conducted in Cotutelle regime to qualify for the International Mention Doctor degree by the University of Córdoba and by the Graz University of Technology conducted by the Graduate in Agricultural Engineering Antonio Mulero Aparicio.

Director: Dr. Antonio Trapero Casas
Codirector: Dr. Francisco Javier López Escudero
Cotutelle supervisor: Dr. rer. nat. Gabriele Berg

Córdoba, octubre de 2019

TÍTULO DE LA TESIS:

Control biológico de la Verticilosis del olivo con la cepa no patogénica de *Fusarium oxysporum* FO12 y con el compost de orujo de vid CGR03.

DOCTORANDO: Antonio Mulero Aparicio

1. INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS

El doctorando **Antonio Mulero Aparicio** ha realizado satisfactoriamente y en los plazos previstos el trabajo presentado en esta Tesis Doctoral. A lo largo de su investigación, el doctorando ha contribuido con diversas aportaciones de interés para la comunidad científica respecto al control biológico de la Verticilosis del olivo, así como la identificación de potenciales tratamientos biológicos y sus mecanismos de acción. Además de la novedad e importancia científica, este trabajo aporta información aplicable en el control de esta enfermedad siendo a su vez de gran relevancia para la continuidad de la línea de investigación sobre el control biológico de la Verticilosis del olivo en el grupo de Patología Agroforestal de la Universidad de Córdoba.

El doctorando ha realizado una estancia internacional de 3 meses y un traslado temporal de 6 meses en el Institute of Environmental Biotechnology de la Graz University of Technology (TU Graz, Austria) bajo la cotutela de la Profesora Gabriele Berg. Estas estancias han permitido al doctorando la obtención de resultados relevantes para el desarrollo de la presente Tesis, como son profundizar en los mecanismos de acción de los agentes de control biológico estudiados. Además el doctorando ha ampliado su formación mediante el aprendizaje de técnicas de microscopía confocal y de espectrofotometría de gases. Como fruto de estas estancias, se ha publicado un artículo en la revista internacional *Frontiers in microbiology*, incluida en la presente Tesis. Además, la realización de una estancia de al menos 6 meses de manera continuada ha permitido la realización de esta Tesis en régimen de cotutela, dando un valor internacional añadido a ésta.

El trabajo realizado en el Departamento de Agronomía de la Universidad de Córdoba por Antonio Mulero Aparicio queda reflejado en varias contribuciones relacionadas con el control biológico de la Verticilosis en el olivo en las cuales consta como primer autor: tres artículos en revistas de prestigio científico indexadas en la base de datos JCR, dos de ellos ya publicados y un tercero aceptado y en prensa. Además, han sido enviados para su publicación en revistas científicas indexadas en JCR dos artículos más. Así mismo, ha presentado cuatro comunicaciones a tres congresos internacionales y seis a tres congresos nacionales. Además, ha participado en otras actividades de extensión y divulgación de los resultados obtenidos, así como en dos publicaciones científicas y una comunicación a un congreso internacional de las que es coautor.

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

Córdoba a 21 de Octubre de 2019

Fdo.: Dr. Antonio Trapero Casas
Primer director de la Tesis Doctoral

Fdo.: Dr. Francisco Javier López Escudero
Segundo director de la Tesis Doctoral

Graz, October 21st, 2019

Fdo.: Dr. rer. nat. Gabriele Berg
Cotutelle supervisor

TITLE OF THE THESIS:

Biological control of Verticillium wilt of olive with the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03.

PhD STUDENT: Antonio Mulero Aparicio

1. REASONED REPORT OF THESIS´ SUPERVISORS

The PhD student **Antonio Mulero Aparicio** has satisfactorily completed the work presented in this PhD Thesis. Throughout his research, the PhD student has contributed with several novelty studies of interest to the scientific community regarding the biological control of Verticillium wilt of olive. In addition, this work provides information applicable to the control of this disease, which is also of great relevance for the continuity of the research this research line within the Group of Agroforestry Pathology of the University of Cordoba.

During his PhD, the student has made a 3-month international stay and a 6-month temporary transfer to the Institute of Environmental Biotechnology of the Graz University of Technology (TU Graz, Austria) under the co-supervision of Proffesor Gabriele Berg. These stays have allowed him to obtain relevant results for the development of this Thesis, such as elucidating the modes of action of the biological control agents evaluated. In addition, the PhD student has expanded his formation by learning techniques of confocal microscopy and gas spectrophotometry. As a result of these stays, a joint article has been published in the international journal *Frontiers in Microbiology*, included in this Thesis. In addition, the realization of a stay of at least 6 continuous months has allowed the realization of this Thesis in a cotutelle regime, giving an added international value.

The works conducted in the Department of Agronomy of the University of Córdoba by Antonio Mulero Aparicio have been published in several papers related to the biological control of Verticillium wilt of olive in which he appears as the first

author. Three articles in journals of scientific prestige indexed in the JCR database, two of them already published and a third in press. In addition, two more articles have been submitted for publication in scientific journals indexed in JCR. Likewise, he has presented four communications to three international conferences and six to three national conferences. Additionally, he is co-author of two additional scientific publications and of a communication to an international conference.

Therefore, the supervisors authorize the defense of this PhD Thesis.

Córdoba, October 21st, 2019

Fdo.: Dr. Antonio Trapero Casas
Primer director de la Tesis Doctoral

Fdo.: Dr. Francisco Javier López Escudero
Segundo director de la Tesis Doctoral

Graz, October 21st, 2019

Fdo.: Dr. rer. nat. Gabriele Berg
Cotutelle supervisor

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 (30 de mayo del 2017 – 30 de agosto del 2017) en la Universidad Tecnológica de Graz (TU Graz, Austria) Supervisor: Dr. rer. nat Gabriele Berg, head of the Institute of Environmental Biotechnology, Section of Plant Pathology and Antagonistic microorganisms.

- La tesis cuenta con el informe previo de dos doctores externos con experiencia acreditada pertenecientes a alguna institución de educación superior o instituto de investigación distinto de España:

    - Dr. Ana Borrego Benjumea, Brandon Research and Development Center, Agriculture and Agri-Food Canada, Canada.

    - Dr. José A. Pereira, Escola Superior Agrária de Bragança (ESA), Instituto Politécnico de Bragança, Portugal.

- Un doctor perteneciente a alguna institución de educación superior o centro de investigación no español forma parte del tribunal evaluador de la tesis:

    - Dr. rer. nat. Christina Andrea Müller. Institute of Environmental Biotechnology, Graz University of Technology. Graz, Austria.

- Parte de la Tesis Doctoral, de acuerdo a la normativa y al convenio de cotutela, se ha redactado y se presentará en dos idiomas, castellano e inglés.

El doctorando

Fdo: Antonio Mulero Aparicio

Tesis por compendio de artículos

Esta tesis cumple el requisito establecido por la Universidad de Córdoba para su presentación por compendio de artículos, consistente en un mínimo de 3 artículos publicados o aceptados 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 Journal Citations Report (SCI):

1. **Mulero-Aparicio, A.,** Agustí-Brisach, C., Varo, A. López-Escudero, F.J., Trapero, A., 2019. A non-pathogenic strain of *Fusarium oxysporum* as a potential biocontrol agent against Verticillium wilt of olive. Biological Control. doi.org/10.1016/j.biocontrol.2019.104045. Datos JCR (2018): índice de impacto 2.607, posición 12/98 y 1er cuartil en el área temática de Entomology.

2. **Mulero-Aparicio, A.,** Cernava, T., Turra, D., Schaefer, A., Di Pietro, A., López-Escudero, F.J., Trapero, A., Berg, G., 2019. The role of volatile organic compounds and rhizosphere competence in the mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt. Frontiers in Microbiology. 10:1808. doi.org/10.3389/fmicb.2019.01808. Datos JCR (2018): índice de impacto 4.259, posición 31/133 y 1er cuartil en el área temática de Microbiology.

3. **Mulero-Aparicio, A.,** Varo, A., Agustí-Brisach, C., López-Escudero, F.J., Trapero, A., 2019. Biological control of Verticillium wilt of olive in the field. Crop Protection. doi.org/10.1016/j.cropro.2019.104993. Datos JCR (2018): índice de impacto 2.172, posición 20/89 y 1er cuartil en el área temática de Agronomy.

El doctorando

Fdo: Antonio Mulero Aparicio

Financiación

Los trabajos incluidos en esta Tesis Doctoral han sido parcialmente subvencionados por el proyecto AGL 2016-76240-R "Gestión Integrada de la Verticilosis del Olivo mediante resistencia genética, prácticas agronómicas y control biológico" del Ministerio de Ciencia, Innovación y Universidades (MICINN) y cofinanciado con fondos FEDER de la UE; por el proyecto AGR08-3635 de la Junta de Andalucía; el proyecto de compra pública INNOLIVAR Línea 8 "Formulados pre-comerciales de microorganismos antagonistas para el control de la Verticilosis del olivo" del MICINN; y por la Interprofesional del Aceite de Oliva Español.

Además, la beca del doctorando Antonio Mulero Aparicio ha sido financiada por el programa de Formación del Profesorado Universitario (FPU), del Ministerio de Educación, Cultura y Deportes.

Funding

The studies included in this PhD Thesis have been partially subsidized by the AGL 2016-76240-R project "Integrated Management of Verticillium wilt of olive through genetic resistance, agronomic practices and biological control" of the Ministry of Science, Innovation and Universities (MICINN, in Spanish) and co-financed with FEDER funds; by the AGR08-3635 project of the Junta de Andalucía; the INNOLIVAR public purchase project Line 8 "Pre-commercial formulations of antagonistic microorganisms for the control of Verticillium wilt of olive" of MICINN; and by the Interprofessional of the Spanish Olive Oil.

In addition, Antonio Mulero Aparicio PhD scholarship has been funded by the University Teacher Training Program (FPU, in Spanish) of the Ministry of Education, Culture and Sports.

Agradecimientos/Acknowledgements

Quisiera agradecer a todas las personas que de alguna forma u otra hayan participado en la realización de esta Tesis Doctoral.

Primero de todo, quería expresar mi agradecimiento a mis directores de tesis, los profesores Antonio Trapero Casas, por ser el primero que me informó de la existencia de la beca FPU, gracias a la cual tuve financiación durante el periodo de realización de la tesis y Francisco Javier López Escudero. Agradecer a ambos compartir conmigo vuestros conocimientos y experiencias que han sido esenciales para mi formación como doctor.

Thanks to Professor Gabriele Berg and Dr. Tomislav Cernava for allowing me to join their group during my two stays in Graz. Thank you for teaching me new techniques that have been an important part of this thesis. Thank you Tomi for your unconditional help, and overall, for your wise advice and unforgettable moments in Graz, it has been a pleasure working with you.

Agradecer al grupo del profesor Antonio Di Pietro del Departamento de Genética por su colaboración en la obtención de los transformantes de *Fusarium oxysporum* y *Verticillium dahliae*. Especialmente dar las gracias a David Turrà y a Stefania Vitale por su ayuda y por enseñarme técnicas que de otra forma no hubiera aprendido pero sobre todo, por sus consejos y por hacerme sentir uno más del grupo.

Gracias a todas las entidades y personas por su colaboración y participación en los ensayos de campo: la empresa "Castillo de Canena" (Peal de Becerro, Jaén), a Cándido (Villanueva de la Reina), a la Comunidad de Regantes "Ntra. Sra. De los Dolores" (Arjona, Jaén), a la empresa "Cotobajo" S.A. (Guadalcázar, Córdoba), y a Rafael Jiménez del Río (Arjona, Jaén).

Gracias Carlos Agustí Brisach por haberme enseñado y ayudado a escribir mis primeros artículos y por tu paciencia y colaboración en sus correcciones y revisiones. Además, gracias por haber compartido conmigo buenos ratos de laboratorio y también de trabajo de campo.

A Luis Roca por ser un compañero ideal y haberme enseñado mundo evaluando ensayos en mis primeros meses de doctorado. A ver si ahora tengo más tiempo de compartir contigo algunas aficiones como la fotografía.

A Mª Carmen Raya por estar siempre ahí cuando me ha hecho falta, por aguantar mi inicial torpeza con asuntos moleculares y por colaborar en algún que otro arduo ensayo con cientos de muestras. Gracias también por haberme escuchado cuando necesitaba algo de desahogo.

A Paqui por su amabilidad y ayuda en el laboratorio siempre que lo he necesitado. Gracias por hacerme sentir que no estaba lejos de casa.

A Mª Ángeles Fernández, por haber sido la persona más eficaz cada vez que le he pedido ayuda/favor. No sé cómo va la lista de favores pero seguro que me te quedan muchos por tachar, poco a poco iré recuperando.

A los técnicos de laboratorio Paco, Francisco y Juan Antonio. Muchas gracias por vuestra colaboración, ya que sin vosotros gran parte de esta tesis no hubiera sido posible. Gracias Paco por haber sido un compañero de batallitas a la vez que de trabajo.

A todos mis compañeros que siguen o han terminado el doctorado: a Ana por su ayuda y colaboración en los ensayos de campo durante las últimas fases de la tesis, estoy para lo que necesites en los inicios de la tuya; A Ángela por ser quien me formó y enseñó a trabajar como doctorando en mis comienzos y por ser una estupenda colega; A Eduardo por compartir muchos momentos de risa en el despacho; A Mario Pérez por haber sido el padre de los nuevos doctorandos; a Mª Ángeles Romero, la mami, por haber sido la sonrisa que todo el mundo necesita ver cada día; a Tere porque aunque he compartido menos tiempo contigo, ese tiempo ha sido genial y sé que eres una estupenda compañera.

Thanks to all Graz laboratory technicians: María, Angelika, Barbara, and Monika for their unconditional help.

Thanks to all my friends and mates in Graz: Pascal, Julian, Peter, Alessandro, Manu, Mel, Birgit, Lisa 1, Lisa 2 and Wisnu, for receiving me as one more and for making me feel at home during my two stays Graz

A Nuria por haber sido mi compi española durante mi última estancia en Graz, ha sido un placer haberte conocido y saber que en ella tengo compi para rato.

A mi compañero y amigo Joaquín, por haber sido el ente bipolar que todo el mundo necesita en su vida y por haberme acogido y aguantado durante estos casi 5 años en tu despacho, seguro que nos quedan muchos buenos momentos juntos.

A medio camino entre compañeros y familia te coloco a ti, Mario, por haber sido mi amigo y compañero total de tesis. Por haberme aguantado tanto mis días buenos como los malos, por haberme enseñado valores muy importantes de la vida, por los innumerables buenos momentos que hemos compartido juntos y por los que están por llegar, vamos lo que viene siendo un hermano mayor en Córdoba.

A mis abuelos, que aunque siempre les tenía que explicar en qué consiste un doctorado, seguían semana a semana mis avances deseándome siempre lo mejor.

A mi padre, por haberme inculcado desde pequeño el amor hacia el campo y las ganas de querer a aprender siempre desde la humildad y honestidad. A mi madre y a mi hermana por creer siempre en mí y por su confianza y ánimos.

A Marina, por darme sus ánimos y su apoyo durante todos los días sin los cuales me hubieran faltado fuerzas para terminar la tesis. Por haberme aconsejado cada vez que le he pedido opinión respecto a algo y sobre todo, por su paciencia en los numerosos días de agobio y estrés durante la tesis.

A todos vosotros, sin los cuales no hubiera ni disfrutado ni aprendido como lo he hecho durante todos estos años.

Resumen

La Verticilosis del olivo (VO) causada por el hongo de suelo *Verticillium dahliae* Kleb. es considerada la enfermedad más importante que afecta a los olivares en la mayoría de los países productores. Hasta la fecha, no existe un método de control que, aplicado de forma individual, sea realmente efectivo. Por ello, el manejo de la enfermedad mediante una estrategia de control integrado es el enfoque más aconsejable para reducir tanto su incidencia como su dispersión. En este contexto, el control biológico surge como una de las medidas más prometedoras para implementar dentro de esta estrategia de control integrado. Debido a la escasez de tratamientos biológicos efectivos contra la VO, el Grupo de Patología Agroforestal de la Universidad de Córdoba ha llevado a cabo recientemente una evaluación masiva de más de 220 productos naturales contra *V. dahliae*. Como resultado de esta evaluación, se seleccionaron varios tratamientos biológicos potenciales por su efectividad demostrada frente a la VO en condiciones controladas.

Por ello, el primer objetivo de esta Tesis fue evaluar la efectividad de 16 tratamientos biológicos, seleccionados en la anterior evaluación, contra la VO en condiciones de campo. Para ello, se realizaron tres experimentos en campos infestados con diferentes densidades de inóculo de *V. dahliae* y en tres escenarios diferentes para la infección natural del patógeno situados en diferentes zonas de Andalucía. Los resultados de esos experimentos mostraron que un aceite esencial comercial de *Thymus* sp., la cepa no patógena de *Fusarium oxysporum* FO12 y el compost de orujo de vid CGR03 fueron los tratamientos más efectivos para reducir la densidad del inóculo del patógeno en el suelo, consiguiendo hasta un 100% de reducción. Además, los tratamientos con FO12 y CGR03 redujeron significativamente la incidencia de la enfermedad tanto en plantas de olivo jóvenes como en olivos adultos en un experimento realizado en condiciones de un olivar comercial. Por lo tanto, la cepa no patógena de *F. oxysporum* y el compost de orujo CGR03 se seleccionaron como dos de los tratamientos de biocontrol más prometedores contra la VO.

Un segundo paso fue evaluar el potencial completo de biocontrol así como dilucidar los modos de acción tanto de FO12 como de CGR03. Para ello, se realizaron varios experimentos *in vitro* e *in vivo* para evaluar el efecto de diferentes formulados a base de FO12 contra *V. dahliae*. El tratamiento con extracto crudo de FO12 resultó en una reducción total tanto de la densidad de inóculo del patógeno

en suelo, como en el progreso de la enfermedad en condiciones controladas. Además, los tratamientos con la suspensión tanto conidial como de clamidosporas también fueron efectivos para reducir tanto la densidad del inóculo (95 y 85 %, respectivamente), como el progreso de la enfermedad en plantones de olivo.

En experimentos *in vitro* e *in vivo* complementarios, se estudió el papel que desempeñan las sustancias inhibidoras y la competencia en la rizosfera en el efecto antagonista de FO12 contra *V. dahliae*. Los compuestos orgánicos volátiles y las sustancias solubles producidas por FO12 causaron una inhibición significativa en el crecimiento micelial y en la viabilidad de los microesclerocios. Tras el estudio de microscopía utilizando una cepa de FO12 marcada con sGFP se confirmó el comportamiento endofítico de esta cepa, demostrando que FO12 es un colonizador eficaz de la rizosfera, pudiendo competir por los sitios de infección con *V. dahliae*.

Paralelamente, se evaluó el efecto de diferentes tés y extractos de compost obtenidos a partir de CGR03 para identificar los mecanismos de acción involucrados en su efecto contra *V. dahliae*. Los resultados obtenidos sugirieron que tanto factores bióticos como abióticos están involucrados en el efecto supresivo de este compost. Así, el extracto natural y dos extractos esterilizados en autoclave fueron los tratamientos más efectivos para reducir la densidad del inóculo de *V. dahliae* en el suelo (62.0, 72.1 y 66.6% de reducción, respectivamente). Además, el té de compost natural y el té de compost esterilizado en autoclave lograron reducir la enfermedad en un 67.4 y 96.5%, respectivamente.

Finalmente, se realizó un experimento en condiciones semi-controladas para evaluar la efectividad de la cepa no patógena de *F. oxysporum* FO12 y el compost de orujo de vid CGR03 en la reducción tanto de la densidad de inóculo de *V. dahliae* en un suelo naturalmente infestado como del progreso de la enfermedad y su interacción con dos cultivares de olivo con diferentes niveles de resistencia (Picual y Arbequina). Tanto FO12 como CGR03 fueron altamente efectivos en la reducción de la densidad del inóculo alcanzando valores mínimos de 0.53 y 0.8 propágulos g^{-1}, respectivamente, mientras que en el testigo tuvo un valor mínimo de 39.2 propágulos g^{-1}. Además, CGR03 redujo significativamente el progreso de la enfermedad, mientras que FO12 logró un control total de la VO ya que ninguna planta tratada con esta cepa mostró síntomas de la enfermedad.

El conocimiento generado de los estudios incluidos en esta Tesis, proporciona una información útil sobre el efecto de FO12 y CGR03 contra la VO, así como sus principales modos de acción. Además, este trabajo proporciona un

nuevo enfoque integrado para estudiar el potencial completo de tratamientos de control biológico contra *V. dahliae* como parte esencial del desarrollo de futuros tratamientos para controlar la VO. En conclusión, los resultados obtenidos en la presente Tesis representan un avance en el desarrollo de alternativas prometedoras para el control de esta enfermedad en condiciones de campo, donde los olivareros necesitan urgentemente medidas que sean viables, efectivas y respetuosas con el medio ambiente para controlar esta importante enfermedad.

Summary

Verticillium wilt of olive (VWO) caused by the soil-borne plant pathogen *Verticillium dahliae* Kleb. is currently considered the most important disease infecting olive groves in most producing countries. To date, there is no truly effective individual method of control, so following an integrated control strategy is the most advisable approach to reduce both its incidence and dispersal. In this context, biological control emerges as one of the most potentially effective measures to implement within this integrated control strategy. Given the scarce of effective biological treatments, the Agroforestry Pathology Group of the University of Córdoba has recently carried out a massive screening of more than 220 natural products against *V. dahliae*. From that screening, several promising biological treatments were selected given their effectiveness in controlling VWO under controlled conditions.

Therefore, the first objective of this PhD thesis was to evaluate the effectiveness of 16 biological treatments, selected in the previous screening, against VWO under field conditions. For that purpose, three experiments were conducted in infested fields with different inoculum densities of *V. dahliae* and under three different scenarios for the natural infection of the pathogen in the Andalusian Region. Results from those experiments showed that a commercial essential oil from *Thymus* sp., the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 were the most effective treatments in reducing the inoculum density of the pathogen in soil up to 100%. Additionally, FO12 and CGR03 treatments significantly reduced the disease incidence in young and old olive plants in an experiment conducted in a commercial olive orchard. Thus, the non-pathogenic strain of *F. oxysporum* and the grape marc compost CGR03 were selected as two of the most promising biocontrol treatments against VWO.

A second step was evaluating the complete potential of biocontrol as well as expanding the knowledge about the modes of action of FO12 and CGR03. Several *in vitro* and *in vivo* experiments were carried out to evaluate the effect of different culture fractions of FO12 against *V. dahliae*. Treatments with crude culture broth of FO12 resulted in a total reduction of viable propagules of *V. dahliae* in naturally infested soils as well as in the progression of VWO under controlled conditions. Moreover, treatments with conidial suspension or chlamydospores were also

effective in reducing the inoculum density in the soil and the disease severity in olive plants, reaching values of 95 and 85% of inoculum reduction, respectively.

In complementary *in vitro* and *in vivo* experiments, the implications of inhibitory substances and rhizosphere competence in antagonistic effects of FO12 against *V. dahliae* were studied. Volatile organic compounds and soluble substances produced by FO12 caused significant inhibition of mycelial growth and microsclerotia viability. Microscopic analysis using a generated sGFP-tagged strain of FO12 confirmed the endophytic behavior of FO12 showing that this biocontrol strain is an efficient root colonizer which could compete with *V. dahliae* in the same ecological niche.

In parallel, the effects of different compost teas and extracts obtained from the grape marc compost CGR03 were evaluated by means of *in vitro* and *in vivo* experiments to identifying the mechanisms of action involved in their effect against *V. dahliae*. The results suggested that both biotic and abiotic factors are involved in the suppressive effect of this compost, since the natural extract and two extracts sterilized by autoclaving were the most effective aqueous treatments for reducing the inoculum density of *V. dahliae* in soil (62.0, 72.1 and 66.6% reductions, respectively). Furthermore, the natural compost tea and the compost tea sterilized by autoclaving achieved significant disease suppression rates of 67.4 and 96.5%, respectively.

Finally, a long-term experiment under semi-controlled conditions was carried out to evaluate the effectiveness of the non-pathogenic strain of *F. oxysporum* FO12 and the grape marc compost CGR03 in reducing both the inoculum density of *V. dahliae* in naturally infested soil and the disease progress in two olive cultivars with different resistance levels (Picual and Arbequina). Both FO12 and CGR03 were highly effective in reducing the inoculum density of the pathogen reaching minimum values of 0.53 and 0.8 propagules g^{-1}, respectively, whereas the minimum value in untreated control was 39.2 propagules g^{-1}. Additionally, CGR03 significantly reduced the progression of the disease whereas FO12 achieved total control of VWO since no plants treated with this strain showed VWO symptoms.

The generated knowledge from the studies included in this PhD Thesis, provides useful information about the efficacy of FO12 and CGR03 against VWO and their main modes of action. Furthermore, this work provides new insights of an integrated way to study the complete potential of biocontrol treatments against

V. dahliae as an essential part of the development of eco-friendly treatments for controlling VWO. In conclusion, the outcomes obtained in the present study represent an advance in the development of feasible alternatives for the control of this disease under field conditions, where olive farmers are in urgent need of attainable and effective measures to controlling this important disease.

Zusammenfassung

Verticillium-Welke, die vom bodenbürtigen Pflanzenpathogen *Verticillium dahliae* Kleb. Verursacht wird, gilt als die weltweit wichtigste Erkrankung in Olivenhainen. Derzeit stehen keine effizienten Gegenmaßnahmen zur Verfügung um die Inzidenz und Ausbreitung der Krankheit zu reduzieren. Integrativer Pflanzenschutz bietet eine vielversprechende Alternative um Krankheitsausbrüche einzudämmen. In diesem Zusammenhang wurde biologischer Pflanzenschutz als ein essentieller Bestandteil alternativer Behandlungsmöglichkeiten identifiziert. Um das Portfolio von möglichen Gegenmaßnahmen zu erweitern hat die *Agroforestry Pathology Group* der Universität von Córdoba zuletzt mehr als 220 natürliche Produkte auf ihre Wirkung gegen *V. dahliae* untersucht. Im Rahmen dieses Screenings wurden mehrere vielversprechende biologische Behandlungsmöglichkeiten der Verticillium-Welke identifiziert.

Aus diesem Grund war die erste Zielsetzung dieser Dissertation 16 biologische Behandlungen, die zuvor unter Freilandbedingungen als vielversprechend identifiziert wurden, tiefergehend zu untersuchen. Es wurden Versuche in drei Olivenhainen mit unterschiedlichem Pathogendruck durchgeführt um die derzeitigen Bedingungen in Andalusien zu simulieren. Im Rahmen dieser Versuche wurde gezeigt, dass ein kommerzieller *Thymus* sp. Extrakt, der nicht-pathogene *Fusarium oxysporum* Stamm FO12 und CGR03 (Kompost aus Traubentrester) die Pathogendichte in der Erde um bis zu 100% senken konnten. Zusätzlich wurde in einem kommerziellem Olivenhain gezeigt, dass der Stamm FO12 und CGR03 die Erkrankungsinzidenz in alten als auch in jungen Olivenbäumen senken konnten. Diese beiden Behandlungen wurden in zusätzlichen Versuchen tiefergehend untersucht.

Um den Wirkmechanismus der beiden Behandlungen näher zu untersuchen, wurden mehrere *in vitro* und *in vivo* Experimente durchgeführt. Zudem wurden unterschiedliche Kulturfraktionen des FO12 Stamms hinsichtlich ihrer Wirkung gegen *V. dahliae* näher untersucht. Behandlungen mit der ungefilterten Kultur führten sowohl zu einer vollständigen Reduktion von lebenden Verbreitungseinheiten des Pathogens in natürlich kontaminierten Böden als auch zu einer Eindämmung der Verticillium-Welke unter kontrollierten Bedingungen. Zudem konnte auch mit Konidiensuspensionen und Chlamydosporen die Pathogendichte in der Erde sowie der Schweregrad der Erkrankung in Olivenbäumen um jeweils 95 und 85% reduziert werden.

In komplementären *in vitro* und *in vivo* Experimenten wurde der Einfluss von inhibierenden Substanzen und Rhizosphärenkompetenz von FO12 im Zusammenhang mit dessen Antagonismus untersucht. Es konnte gezeigt werden, dass sowohl flüchtige organische Verbindungen als auch lösliche Substanzen die von diesem Stamm produziert werden, an der Inhibierung vom Wachstums des Pathogens und dessen Mikrosklerotien beteiligt sind. Mittels sGFP-markierten Stämmen wurde zudem gezeigt, dass FO12 als Endophyt im Pflanzeninneren vorkommt und den Wurzelraum effizient kolonisiert. Diese Eigenschaften lassen ihn mit *V. dahliae* um die gleiche ökologische Nische konkurrieren.

Parallel zu den angeführten Versuchen wurde der Traubentrester Kompost CGR03 näher untersucht und insbesondere unterschiedliche Extrakte hinsichtlich ihrer *in vitro* und *in vivo* Wirksamkeit gegen *V. dahliae*. Die Versuche deuteten darauf hin, dass sowohl biotische als auch abiotische Faktoren im suppressiven Effekt involviert sind. Mittels dem natürlichen Extrakt und zwei durch Autoklavieren sterilisierte Extrakte konnte die Pathogendichte um jeweils 62,0, 72,1, und 66,6% reduziert werden. Natürliche Auszüge die aus CGR03 gewonnen wurden, reduzierten die Pathogendichte um 67,4% (nicht autoklaviert) und 96,5% (autoklaviert).

In einem Langzeitversuch unter zum Teil kontrollierten Bedingungen wurde die Effizienz hinsichtlich *V. dahliae* Reduktion in natürlich kontaminierter Erde und in zwei Olivenkultivaren (Picual und Arbequina) von FO12 und CGR03 untersucht. Beide Behandlungen konnten das Vorkommen von Verbreitungseinheiten auf 0,53 g^{-1} (FO12) sowie 0,8 g^{-1} (CGR03) reduzieren im Gegensatz zur unbehandelten Kontrolle die 39,2 g^{-1} aufwies. Zusätzlich wurde gezeigt, dass CGR03 signifikanten Einfluss auf den Fortschritt der Krankheit hatte während mit FO12 die Verticillium-Welke vollständig kontrolliert werden konnte.

Die in dieser Dissertation gewonnenen Erkenntnisse liefern wichtige Hinweise bezüglich der Effizienz und Wirkweise von *F. oxysporum* FO12 und CGR03. Zusätzlich zeigt sie komplementäre Wege mit denen biologische Behandlungen ganzheitlich erfasst werden können um *V. dahliae* auf eine natürliche Weise entgegenwirken zu können. Zusammengefasst liefern die Ergebnisse dieser Arbeit eine Weiterentwicklung verfügbarer Alternativen um Verticillium-Welke unter Feldbedingugen einzudämmen, die vor allem Olivenanbauern in Zukunft zu Gute kommen wird.

Abbreviations

ACCB: adjusted crude culture broth;

ANOVA: analysis of variance;

BCA(s): biological control agent(s)

CCB: crude culture broth;

CE(s): compost extract(s)

CFU: colony forming units

CLSM: confocal laser scanning microscopy

CT(s): compost tea(s)

D: defoliating pathotype

DAI: days after inoculation

DAP: days after planting

DI: disease incidence

DI_{50}: time in weeks from planting until 50% of the plants were affected

GC-MS: gas chromatography-mass spectrometry

HID: high inoculum density

HSD: honestly significant difference test

ID: inoculum density

LID: low inoculum density

LSD: least significant difference

MS: microsclerotia

MSPA: modified sodium polypectate agar medium

ND: non-defoliating pathotype

OA(s): Organic amendment (s)

PDA: potato dextrose agar

PDB: Potato dextrose broth

RAUDPC: relative area under the disease progress curve

RAUIPC: relative area under the inoculum progress curve

SDW: sterile distilled water

s-GFP: super-folder green fluorescent protein

VOC(s): volatile organic compound (s)

VWO: Verticillium wilt of olive

Contents

CHAPTER 1

General Introduction

1. General Introduction and Objectives

1.1. Olive cultivation: history and relevance

Olive (*Olea europaea* subsp. *europaea*) was one of the earliest tree species to be domesticated and cultivated. This woody crop is native from the Mediterranean basin where Phoenicians, Greeks and Romans first began its cultivation and diffusion more than 5000 years ago (Connor, 2005), becoming one of the most ancient tree crops of the Mediterranean region, together with date, fig, grape and pomegranate (Janick, 2005). After the spread of this crop within the Mediterranean Basin, olive cultivation was distributed to other regions with similar climate conditions situated between latitudes 30°-45° at both Northern and Southern Hemispheres. Since then, it has played a vital role for humans for being the axis of the development of culture and food in those regions.

Nowadays, the olive tree is the most cultivated non-tropical fruit woody crop and one of the most important cultivated species worldwide due to the economical relevance of its main commodities (olive oil and olives). Additionally, as a result of the recognized olive oil benefits for human health (Amiot, 2014), the spread of this crop has gradually grown over the last two centuries, being cultivated in regions of South Africa, Asia and Australia, among others (Connor, 2005).

As regards world production and surface of olive cultivation, they produce an annual average of 20 million tons of olives and grow in a cultivated area of more than 10 million ha (97% located in the Mediterranean basin) in more than 20 countries worldwide (FAO, 2017). From olives, the two main commodities of this crop are obtained: olive oil (90% of total production) and table olives (10% of total production). Spain is the main producer of both commodities accounting for 6.5 x 10^6 tons of harvested olives (32% of the world olives production) (FAO, 2017; MAPA, 2017) in an area of 2.5 x 10^6 ha (25% of the world acreage) in 2017. In Spain, the largest production of olives is concentrated in the region of Andalusia (Southern Spain) with a cultivated area of 1.5 x 10^6 ha (60% of the Spanish cultivation area) (MAPA, 2017), being the only area in Europe where there is a concentration of a single tree species cultivated in such a large area (Guzmán-Álvarez et al., 2009).

During the expansion process, olive cultivation has become more and more intensive leading to an increasing of both cultivated area and yield by surface unit. This yield increase is partially explained by an improvement in the use of agrochemicals (fertilizers, herbicides, insecticides and fungicides) that, together with the crop intensification and homogenization, has allowed minimizing the production costs. However, the intensification of the olive cropping system entails parallel negative environmental consequences such as loss of genetic variability (Díez et al., 2016), contamination due to the excessive use of agrochemicals and the explosive appearance and development of pests and diseases.

In this context, a phytosanitary challenge for the olive groves has emerged, which is the great development of a vascular disease, called Verticillium wilt of olive, affecting both new plantations and areas where it was traditionally not a serious problem to date (Barranco et al., 2017).

1.2. Verticillium wilt of olive

Verticillium wilt of olive (VWO) is currently considered the most worrisome and devastating disease affecting both old and adults olive groves in Spain and in most of the olive-growing countries (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012). This disease was first reported in Italy in 1946 (Ruggieri, 1946) and nowadays it has been detected in almost every country of the Mediterranean basin and in many other olive growing areas around the world. In Spain, VWO was first described in 1975 (Caballero et al., 1980) and its dispersion has increased progressively reaching a mean incidence of 0.4%, reaching values of up to 9% in some areas, with more than 50% of affected olive orchards (Ruiz-Torres, 2010). Within the Andalusian region, surveys conducted between 1980 and 1983 in 122 adult olive groves from Córdoba, Seville and Jaén provinces, detected a disease incidence of 38.5%, reaching values of 90% in some olive plantations (Blanco-López et al., 1984). Similar values were recorded later in surveys conducted between 1994 and 1996 when VWO was present in 39.5% out of 122 young olive groves prospected (López-Escudero and Mercado-Blanco, 2011).

Since then, the dispersion and importance of VWO has increased markedly due to five main factors: i) the establishment of new olive orchards with an intensive cropping system in infested fields previously cropped with other susceptible hosts, ii) the use of infected plant material, iii) the use of susceptible

olive cultivars such as 'Picual', iv) the introduction of irrigation in olive orchards in intensive cultivation systems and v) irrigation with infested water (Thanassoulopoulos, 1993; Levin et al., 2003; López-Escudero and Blanco-López, 2007; López-Escudero et al., 2010; Rodríguez et al., 2011; García-Cabello et al., 2012; Pérez-Rodríguez et al., 2015; Roca et al., 2015;). In this context, López-Escudero et al. (2010) observed an average of disease incidence of 20% in VWO affected olive orchards from the tree main provinces growing olive of Andalusia (Jaén, Córdoba and Seville). Consequently, this has resulted in major economic losses in the main olive growing areas during recent decades generating a justified concern within the olive oil sector (Blanco-López et al., 1984; López-Escudero and Mercado-Blanco, 2011).

In addition to the factors discussed above, the lack of a truly effective individual method of control, the lasting persistence of the pathogen in the soil and its wide host range, has made the VWO to become a major threat to olive production whose control supposes a tough challenge for growers in most of the Mediterranean basin countries (López-Escudero and Mercado-Blanco, 2011).

1.2.1. Symptomatology

Symptomatology of VWO has been described by several authors worldwide (Zachos, 1963; Cirulli, 1976; Blanco-López et al., 1984). Under Guadalquivir Valley conditions, VWO traditionally comprises two symptoms complex known as apoplexy (acute form) and slow decline (chronic form).

Apoplexy, which occurs mainly from late winter to early spring, is characterized by a quick death of branches or of the whole plant. Initially, leaves lose their intense green colour, turning to light-brown and self they roll back, remaining attached to the branches (Figure 1A). The early onset and the severity of this symptom complex seem to be related to intense rains and moderate temperatures during autumn and winter. When apoplexy is observed in young olive plants, the death of the whole tree is a common phenomenon (Blanco-López et al., 1984; Rodríguez-Jurado, 1993; Jiménez-Díaz et al., 2012).

On the other hand, slow decline takes place mainly during spring and early summer, and depending on climatic conditions, during autumn. In spring, the main symptoms are necrosis of inflorescences, whereas in autumn fruit mummification

is very common (Figure 1B). Nevertheless, the most important symptom during slow decline is the intense defoliation of green leaves (Figure 1C). Frequently, both symptom complexes may appear in the same tree and partially or completely affecting the whole plant. Infected trees may show the so-called phenomenon of natural recovery, and new healthy suckers can emerge from the base of the trunk or infected branches (López-Escudero and Blanco-López, 2005a; Markakis et al., 2009; Bubici et al., 2014).

Due to the non-specificity of VWO symptoms, they may be confused with those caused by others pathogens such as *Phytophthora* spp. which causes root rot and whose aerial symptoms can be easily confused with apoplexy (Sánchez-Hernández et al., 1998). For that reason, the re-isolation from the affected tissue and the identification of the pathogen by means of microbiological or molecular methods are crucial to make an accurate diagnosis (Barranco et al., 2017).

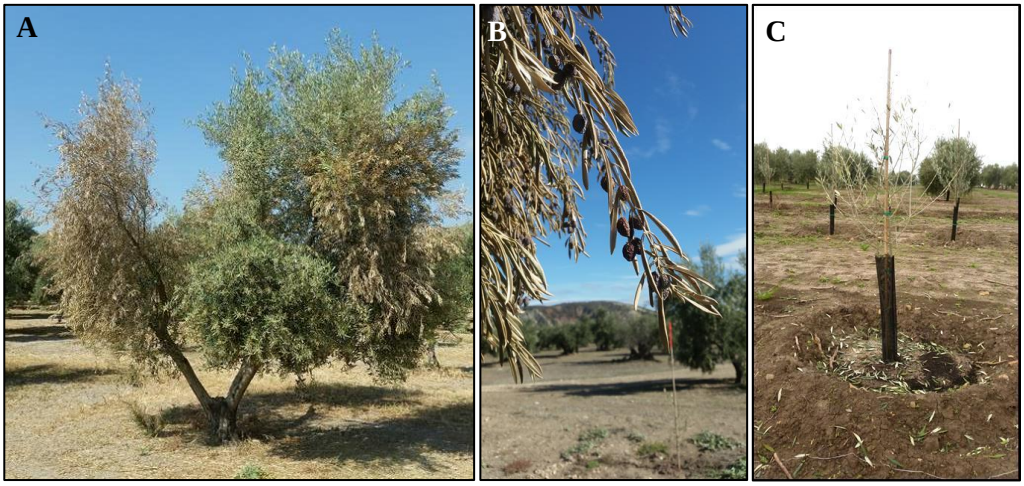


Figure 1. Symptomatology complex of Verticillium wilt of olive. A) Wilted branches affected by apoplexy complex. B) Mummified olive fruits. C) Intense defoliation of green leaves in a young olive tree.

1.2.2. Causal agent

The causal agent of VWO, *Verticillium dahliae* Kleb. (Klebahn, 1913), is a mitosporic, soil-borne, hemibiotrophic, haploid and asexually reproducing fungus included in division Ascomycota, subdivision Pezizomycotina, class Sordariomycetes and family *Plectosphaerellaceae* (Inderbitzin and Subbarao,

2014). This pathogen is worldwide distributed, and it is able to produce vascular infections in a wide range of host species, including annuals, herbaceous crops and weeds, as well as fruit, landscape and ornamental trees and shrubs (Pegg and Brady, 2002; Agrios, 2006; Klosterman et al., 2009).

This species can be morphologically differentiated from other plant pathogenic species within the genus, and particularly from *V. albo-atrum*, by the formation of microsclerotia. These are small, multicellular and heavily melanized resting structures (Isaac, 1967; Goud et al., 2003) which are formed within senescent or dead tissues of infected plants at the end of the parasitic phase of its life cycle or under adverse environmental conditions. Dormant microsclerotia can remain viable in soil for more than 14 years (Wilhelm, 1955).

In *V. dahliae* populations that infect olive trees, two virulence groups or pathotypes have been identified. One is highly virulent and is referred to as defoliating, and the other is moderately virulent and is referred to as non-defoliating (Jiménez-Díaz et al., 2012). The defoliating pathotype is able to completely kill an olive tree, especially when it is young (Sánchez Hernández et al., 1998; López-Escudero and Mercado-Blanco, 2011). On the other hand, the non-defoliating pathotype is moderate or low virulent, although it has also been associated with wilting and death of olive trees (López-Escudero and Mercado-Blanco, 2011). Although the defoliating pathotype was firstly detected in the eighties infecting cotton in a restricted area of the southern Guadalquivir valley (Blanco-López et al., 1984), it has spread during the following decades being currently present in many soils all over Andalusian region due to its higher infection capacity if compared with the non-defoliating pathotype (López-Escudero et al., 2010; Jiménez-Díaz et al., 2012).

1.2.3. Disease cycle

Due to the low saprophytic activity of *V. dahliae*, its life cycle could be divided in two main phases: i) extensive parasitic phase within the vascular system of the host; and ii) dormant phase by means of its resting structure (microsclerotia) (Figure 2) (Powelson and Rowe, 1993).

Microsclerotia can remain viable embedded in the plant tissue or in the soil for more than 14 years (Wilhelm, 1955). Microsclerotia germinate in response to

root exudates from host and/or non-host plants and under favourable environmental conditions, giving rise to infective hyphae (Butterfield and DeVay, 1977; Mol and Scholte, 1995). After germination, hyphae infect the root through infecting points such as formation of lateral roots and/or natural micro-injuries (Schnathorst, 1981). Hyphae penetrate into the root colonizing epidermal cells and cortex (Klosterman et al., 2009). Within the xylem vessels, the pathogen forms conidiophores and conidia which can move through the transpiration flux colonizing the aerial part of the plant. The growth of the pathogen within the xylem vessels induces physical and/or biochemical defensive mechanisms, such as the formation of tyloses or gel plugs inside infected xylem vessels, contributing to the obstruction of the xylem lumen (Baídez et al., 2007), or the production of proteins involved in cell death (Xie et al., 2013). Altogether, these factors contribute to the onset of symptoms, causing extensive dieback and/or heavy defoliation of twigs and branches in olive (López-Escudero and Mercado Blanco, 2011).

At the end of the parasitic phase, the cycle is complete when the pathogen forms microsclerotia within dying tissue of the plant such as leaves, branches and sprouts, which are released to the soil upon degradation of plant debris (Mol and Scholte, 1995), increasing the inoculum density of the pathogen in the soil (Navas-Cortés et al., 2008).

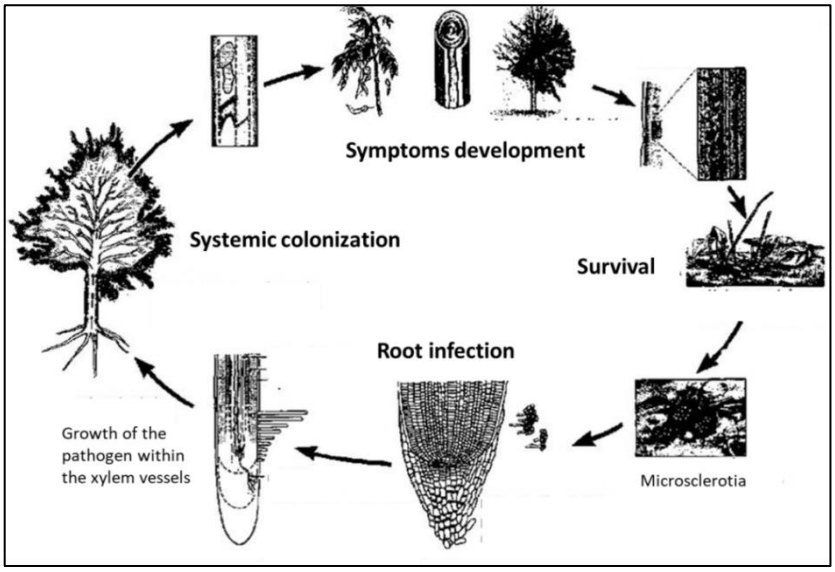


Figure 2. *Verticillium dahliae* life cycle in woody plants (adapted from Hiemstra and Harris, 1998).

1.2.4. Epidemiology

There is a wide range of factors which, single or in combination, promote and increase the dispersion of *V. dahliae* in the soil as well as the incidence of VWO. Among relevant factors affecting the correct management of the disease highlight the use of infected plants, the use of susceptible olive cultivars, the inappropriate management of soil, irrigation and fertilization, wrong cultivation techniques as well as climatic and edaphic factors (López-Escudero and Mercado-Blanco, 2011). Nevertheless, the establishment of new plantations in infested fields due to prior cultivation of susceptible hosts, such as cotton, is considered the most important cause of the current dispersion and incidence of VWO (Wilhelm and Taylor, 1965; Blanco-López et al., 1984; Bejarano-Alcázar et al., 1996; López-Escudero and Blanco-López, 2001). Additionally, epidemic development of VWO is favoured by the long-term viability of microsclerotia during the dormant phase. In the same way, *V. dahliae* is able to persist and even increase the inoculum density in soil due to its capability to infect a wide range of alternative hosts from herbaceous to woody crops as well as wild flora (Pegg and Brady, 2002) inhabiting commercial olive groves.

With regard to the dispersion of *V. dahliae*, the use of infected olive plants from commercial nurseries has been reported to be one of the main causes (Thanassoulopoulos, 1993; Hiemstra, 2015). In addition, microsclerotia constitute the structure of dispersion of this pathogen. In this context, the use of infected pruning debris as soil coverture or organic amendment can contribute to the increasing of inoculum density of *V. dahliae* in soil (López-Escudero et al., 2008; Jiménez-Díaz et al., 2012). Microsclerotia embedded into plant debris can also be spread by means of wind (Klosterman et al., 2009; Tsor, 2011), runoff water (López-Escudero and Mercado-Blanco, 2011), irrigation (García-Cabello et al., 2012; Rodríguez-Jurado and Bejarano-Alcázar, 2007) contaminated vehicles and tillage tools (Wilhelm and Taylor, 1965; López-Escudero et al., 2008) and/or human transport of infected plant residues or crops (Tjamos and Botseas, 1987; Tjamos and Tsougriani, 1990; Navas-Cortés et al., 2008).

The influence of irrigation management on the onset and development of Verticillium wilts in woody crops has been scantly studied (Hiemstra and Harris, 1998), although it has been reported in some woody hosts such as apricot (Vigouroux, 1984), cocoa (Emechebe, 1980), maple (Shulfelt and Linderman,

1986) or tulip tree (Morehart and Melchior, 1982). In olive groves, economic losses caused by VWO are apparently associated with high irrigation dosses (Al-Ahmad and Mosli, 1993; López-Escudero and Blanco-López, 2005a; López-Escudero et al., 2010). Several studies confirm that an increase of the disease incidence is produced when irrigation is incorporated into traditional dry regime olive groves (Rodríguez et al., 2008; 2011; López-Escudero et al., 2010). In addition, irrigated olive groves in combination with high densities plantations favour the increase of the disease incidence (Rodríguez et al., 2008). More recently, a study conducted under semi-controlled conditions reported that a daily irrigation schedule strongly encouraged the onset and development of VWO in the susceptible cultivar 'Picual' when compared with weekly, biweekly and deficit water regimes (Pérez-Rodríguez et al. 2015). Similar results were observed under field conditions when disease progression in daily irrigated 'Picual' trees was clearly exacerbated (Pérez-Rodríguez et al., 2016).

On the other hand, the influence of fertilization on the development of fungal diseases has been mostly studied in herbaceous crops. Thus, several studies reported that low nitrogen levels may decrease the susceptibility to Verticillium wilt disease (Isaac, 1957; Sewell and Wilson, 1967; Talboys, 1987). Nevertheless, a high level of nitrogen together with a deficit of potassium causes an increase of disease severity (Presley, 1950; Young et al., 1959; Pegg and Brady, 2002). Conversely, ammonia and nitrous acid inputs were shown to produce a decrease in the number of *V. dahliae* propagules in soil (Tenuta and Lazarovits, 2002) and, in addition, an increase of the biological activity of pathogen antagonists (Pegg and Brady, 2002).

The optimal temperature for *V. dahliae* growth oscillates from 22 to 25°C, being a favourable temperature range for most of Verticillium wilts caused by *Verticillium* spp. (Garber and Presley, 1971). In the case of VWO, disease severity is stimulated by air temperatures of 20-25°C during spring season, followed by summers with moderate temperatures never exceeding 30-35°C (Wilhelm and Taylor, 1965; López-Escudero and Blanco-López, 2001).

Finally, although some edaphic characteristics can also influence the development of VWO, little is known in this regard. Acidity degree can affect to *V. dahliae* growth and survival in soil. This pathogen is favoured in neutral to alkaline soils (pH 6-9). The mycelial growth of the pathogen, as well as the production and

survival of microsclerotia are significantly inhibited at acidic pH´s levels (<5.5) (López-Escudero and Mercado-Blanco, 2011). Nevertheless, the real effect of the above mentioned factors on the pathogen can be further influenced by soil texture, organic matter content and soil microbiota, among others (Lazarovits et al., 2000; Goicoechea, 2009; López-Escudero et al., 2010).

1.3. Integrated control strategy of Verticillium wilt of olive

All above mentioned agronomic aspects, together with the inefficacy of chemical fungicides due to the location of *V. dahliae* within the xylem vessels and in the soil, make the control of VWO one of the major phytopathological challenges that olive growers currently have to face (López-Escudero and Mercado-Blanco, 2011). In diseases such as VWO, the combination of all available control measures is crucial for its control. In this context, an integrated control strategy should be implemented as the most advisable approach for an effective control of VWO.

The available measures included within the integrated control strategy can be divided in: i) pre-planting (preventive); and ii) post-planting (palliative or curative) measures (Figure 3). The scientific knowledge that has been generated during years of research about the pathogen biology, the host plant, and the epidemiological factors contributing to the development of the disease must be the basis to apply all preventive and curative measures within an integrated disease management context.

On the one hand, pre-planting measures are the most efficient, economical and plausible strategy in the olive groves. Within this group, two of the most advisable approaches are the use of non-infected plant material as well as the establishment of new plantations in *V. dahliae*-free soils. Obviously, a mandatory initial step for an effective control of this disease is the implementation of a certification program to produce healthy olive plants (López-Escudero and Mercado-Blanco, 2011).

Subsequently, the presence of the pathogen in the soil should be estimated by means of microbiological methods such as wet sieving (Butterflied and DeVay, 1977) or molecular techniques such as PCR (Moradi et al., 2014). In the case that the pathogen is present in the soil, it is advisable to use physical methods to

eradicate or to reduce the inoculum density. Soil solarization in sectors or in stands close to the planting point is one of the most efficient methods for that purpose (López-Escudero and Blanco-López, 2001).

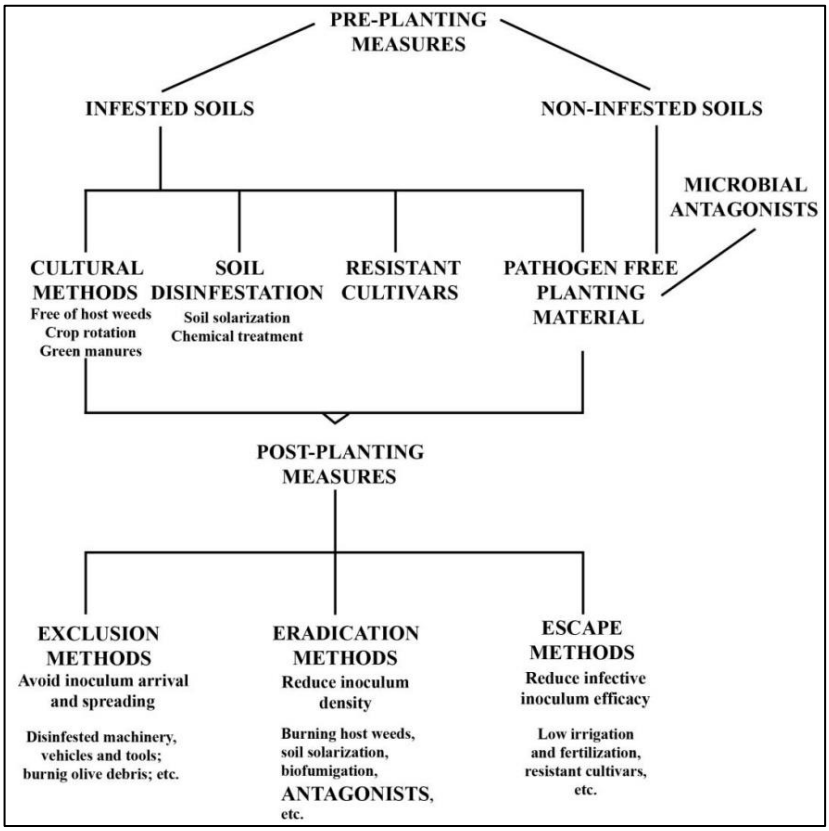


Figure 3. Scheme for an integrated control strategy of Verticillium wilt of olive (based on López-Escudero and Mercado-Blanco, 2011).

In addition to the above mentioned pre-planting measures, the use of resistant plant material is broadly accepted as the least expensive, easiest, eco-friendly and most effective preventive control method (Agrios, 2005; Klosterman et al., 2009; López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012). The evaluation of olive cultivars for Verticillium wilt resistance has been conducted mostly under controlled conditions as the first step of a breeding program. Results from these studies have classified most of the tested olive

cultivars as susceptible or extremely susceptible (López-Escudero and Mercado-Blanco, 2011; Mercado-Blanco and López-Escudero, 2012). More recently, studies conducted under field conditions have classified several olive cultivars widely used in olive groves from Andalusia region into highly susceptible, susceptible, moderately resistant and highly resistant (tolerant). Trapero et al. (2013a) reported that cultivar 'Bodoquera', 'Picual', and 'Manzanilla de Sevilla' were highly susceptible under field conditions. In contrast, cultivar 'Arbequina' showed moderate resistance level under the same conditions (Trapero et al., 2013a; Roca et al., 2015). On the other hand, cultivars 'Changlot Real', 'Empeltre' and 'Frantoio' showed high resistance level under field conditions (Martos-Moreno 2003; Trapero et al., 2013a; López-Escudero et al., 2004). Nevertheless, these cultivars are not completely resistant when they are planted in highly infested soils by *V. dahliae* (Trapero et al., 2013a). Thus, there is no olive cultivar reported as totally resistant to VWO to date.

When preventive measures are not applicable due to both the olive trees and the pathogen is already established in the field, post-planting measures should be used to avoid the dispersion of the pathogen along with to prevent or reduce the progression of the disease. This approach includes measures of exclusion, eradication and evasion.

The main points of exclusion methods are to limit and to prevent the pathogen from entering to the field and its subsequent dispersion. For that purpose is necessary the use of clean and disinfested machinery, vehicles and tools, as well as reducing the tillage to avoid the dispersion of the pathogen towards *V. dahliae*-free areas within the field (Trapero et al, 2017).

The purpose of eradication measures is to reduce the inoculum density of *V. dahliae* and to limit its growth by means of cultural, physical (solarization), chemical or biological control methods. Cultural practices, such as the removal of both potential *V. dahliae*-host weeds and infested plant debris are highly recommended within a routinely field management as they are *V. dahliae* inoculum sources (Tjamos and Botseas, 1987; Navas-Cortés et al., 2008). Solarization is recommended as physical method to reduce the inoculum density of the pathogen in specific areas within the plot such as the place of replacement of a dead tree or in localized areas with a high inoculum level (López-Escudero et al., 2001). In contrast, chemical control measures against VWO have been demonstrated to be

inefficient due to several limitations such as the localization of the pathogen within the xylem vessels and the durability of MS in soil. Several studies evaluating some systemic fungicides, which have been effective under *in vitro* experiments, were no effective when tested under field conditions (López-Escudero and Mercado-Blanco, 2011; Tsor, 2011; Jiménez-Díaz et al., 2012; Trapero et al., 2017).

1.3.1. Biological control measures

In the context of an integrated control strategy against VWO and due to the inexistence of a truly effective control method when it is applied individually, biological control emerges as an alternative approach to be implemented within the group of applicable measures mentioned above (Hiemstra and Harris, 1998; Pegg and Brady, 2002). Moreover, biological control is acquiring more relevance in recent years given its potential effectiveness against plant pathogens, its low environmental impact and its compatibility with the others available control measures.

Nevertheless, biological control of soil-borne plant pathogens in woody crops such as olive has been scantly studied due to the difficulty added in this kind of plant species (woody long-lived species, with a complex anatomy and large root system). VWO has not been an exception, and this difficulty has notably limited this type of research (Trapero et al., 2008; López-Escudero and Mercado-Blanco, 2011).

In general, the available biological control measures against plant pathogens can be divided into three main groups:

1.3.1.1. Essential oils and plant extracts

The use of essential oils and plant extracts is considered an alternative method to the use of chemical products. These compounds have many known advantages in terms of sustainability, mode of action and efficacy against plant pathogens such as *V. dahliae* within an integrated control strategy (Nega, 2014). Two interesting advantages are their high rate of degradation in the environment and their low toxicity towards non-target microorganisms (Thakore, 2006). Essential oils are volatile liquid fractions obtained generally from plant material.

Several studies have focused on obtaining secondary metabolites from plant extracts and essential oils extracts as substances with high potential against pathogens. Different compounds such as carvacrol (5-isopropyl-2-methylphenol) and thymol (2-isopropyl-5-methylphenol) are produced by some plants as a chemical defense mechanism against plant pathogens (Vázquez et al., 2001; Falcone et al., 2005). Secondary metabolites cause alterations in the morphology of hyphae of certain pathogens such as *Sclerotinia sclerotiorum*, resulting in a lysis of the fungus walls. In addition, they have a germination inhibitory effect of the resistance structures of *S. sclerotorium* (Soylu et al., 2007). There are several studies evaluating the effect of plant extracts against *V. dahliae* (Uppal et al., 2008; Arslan and Dervis, 2010; Yohalem and Passey, 2011). However, the antifungal effect of plant extracts and essential oils against VWO has been scantly studied (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012; Varo et al., 2017).

1.3.1.2. Organic amendments

The use of crop debris and composted organic amendments is considered a promising category of measures in controlling VWO (Bailey and Lazarovits, 2003). Composting is a controlled biological process in which organic matter is degraded by different groups of microorganisms (Dees and Ghiorse, 2001), resulting in a biologically stable organic amendment (Adani et al., 1995). After composting, the product is free of pathogens and its structure is favorable for hosting antagonistic microorganisms. The organic amendments have a direct effect on the nutritional balance of crops and influence the equilibrium of beneficial microorganisms and pathogens inhabiting the rhizosphere (Uppal et al., 2008). In addition, their use contributes to reducing residues caused by agro-industry. Organic composted amendments can protect the plants against pathogens by improving nutritional status and/or by direct chemical or biological toxicity. During the last phase of composting process, the temperature of the compost falls and it is colonized by mesophilic microorganisms, mainly bacteria of the *Bacillus* and *Pseudomonas* genera. In this phase the compost is also colonized by many fungal species from different genera such as *Alternaria*, *Aspergillus*, *Bipolaris*, *Fusarium*, *Mucor*, *Rhizopus*, *Peziza*, *Phoma*, and *Trichoderma* (Mehta et al., 2014). Despite the potential use of this type of control measure in olive groves, there are very few studies on biological control of VWO with organic amendments (Avilés et al., 2011) since most of research has been conducted in herbaceous crops (Lazarovits et

al., 2000; Termorshuizen et al., 2006;Bonanomi et al., 2007; Goicoechea, 2009; Alfano et al., 2011; Castaño and Avilés, 2013; Papasotiriou et al., 2013; Markakis et al., 2016).

Inhabiting composted organic amendments can be found different biological control agents (BCAs) as well as undetermined chemical substances, which are responsible for the antagonist effect against plant pathogens (Cronin et al., 1996). A possible use to this natural fact could be the enhancement of compost by mixing it with BCAs. Several studies have demonstrated the synergistic effect of *Trichoderma* sp. and non-pathogenic strains of *F. oxysporum* when they are mixed with compost, showing better results in the suppression of the disease than when they are individually applied (Postma et al., 2003; Trillas et al., 2006). This strategy can be considered for its potential use in field, due to the improvement of the microorganisms' diversity in the soil.

In olive, as far as we know, only three studies have been carried out to evaluate the use of organic amendments against *V. dahliae*. The study conducted by Wilhelm et al. (1962), emphasized the need to look for effective control methods against VWO such as the use of dry wood shavings as an organic amendment. Other study evaluated the effect of olive mill liquid wastes on nursery-grown olive plants (Vitullo et al., 2013). Finally, the last work studying the effectiveness of organic amendments against VWO was a screening of 35 organic amendments from different sources, 15 compost mixtures and five aqueous compost extracts conducted by Varo-Suárez et al. (2018).

Even though the use of organic amendments seems to be a very promising biological control strategy, a greater knowledge about the role of microorganisms in their effectiveness, and to their feasible application under field conditions is needed.

1.3.1.3. Biological control agents

The use of antagonistic microorganisms as BCAs against *V. dahliae* in numerous herbaceous crops has been widely reported (Berg et al., 1994, 2005; Tjamos et al., 2004, 2005; Dayan et al., 2009; Ownley et al., 2009; Kaewchai et al., 2009; Erdogan and Benlioglu, 2010; El Hadrami et al., 2011; Bubici et al., 2013;

Xue et al., 2013; Yang et al., 2013, 2014; Angelopoulou et al., 2014; Pane et al., 2014; Rybakova et al., 2015).

In olive, certain strains of endophytic bacteria such as the strain HRO-C48 of *Serratia plymuthica* have been broadly studied (Müller et al., 2008). It has been shown that when applied under controlled and semi-controlled conditions, this strain shows prolonged rhizosphere colonization in olive trees of cv. Arbequina and an effective control of VWO. Another example is the strain K165 belonging to *Paenibacillus alvei* (Tjamos et al., 2004; Markakis et al., 2016), that is able to control the disease in olive trees both under semi-controlled and under field conditions. Besides the biocontrol effect, bacterial strains such as those within the genus *Bacillus* and *Pseudomonas* have been reported to exhibit plant growth promoting effects (Mercado-Blanco and Bakker, 2007; Höfte and Altier, 2010). In the particular case of the olive tree, several strains of *Pseudomonas* spp. have emerged as very promising BCAs (Mercado-Blanco et al., 2004; Debode et al., 2007; Sanei and Razavi, 2011; Triki et al., 2012). The endophytic strain PICF7 of *P. fluorescens* is characterized by its prolonged root colonization being considered as an effective BCA against VWO (Mercado-Blanco et al., 2004; Mercado-Blanco, 2015a; Prieto et al., 2009; Maldonado-González et al., 2015).

With regard to the use of fungi as BCAs, several genera play a very important role in the biological control of VWO. Among them, different strains of *Trichoderma* sp. have been studied (Lima et al., 2007; Otero et al., 2012). This genus is widely distributed in many ecological niches and several strains within this genus have been evaluated against VWO. However the only commercial formulation that is currently marketed in Spain against this disease is the mixture of two species, *T. asperellum* and *T. gamsii*, but the effectiveness of this mixture has been demonstrated only in nursery plants (Jiménez-Díaz et al., 2009). Other mixture was studied by Lima et al. (2007) in which the potential of *T. viride* mixed with a composted amendment in the elimination of microsclerotia in artificially infested soil was demonstrated.

The use of non-pathogenic isolates of *Fusarium oxysporum* against Fusarium wilts has been extensively studied (Benhamou et al., 2002; Shishido et al., 2005; Edel-Hermann et al., 2011; Aimé et al., 2013). Moreover, non-pathogenic strains of *F. oxysporum* have been recently identified as promising BCAs against Verticillium wilts in different crops (Fravel et al., 2003; Pantelides et al., 2009;

Angelopoulou et al., 2014; Veloso et al., 2016). To date, only two studies conducted by Varo et al. (2016a) and Mulero-Aparicio et al. (2019a) (included in chapter 3 of this Thesis) have demonstrated the effectiveness of a non-pathogenic strain of *F. oxysporum* in olive trees.

Regardless of the high potential of these biological control measures in controlling plant diseases, one of their biggest drawbacks is the absence of correlation between the results obtained under controlled conditions (*in vitro* and *in vivo*) and under field conditions (Hall et al., 1986; Fravel, 1988; Paulitz et al., 1992). Thus, there are many factors that can influence the effectiveness of any BCA under field conditions. The antagonist activity of the BCAs against any soil-borne pathogen could be influenced by the soil (pH, texture, temperature, water content, etc.), the host plant (root exudates, cultivar, nutricional status, etc.), and the microbiome inhabiting the rhizosphere (Berg et al., 2006; Costa et al., 2006; Mercado-Blanco, 2015b). Therefore, it is essential to obtain a deeper knowledge of the interactions occurring in the rhizosphere (O'Sullivan and O'Gara, 1992; Lugtenberg et al., 2001; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Raaijmakers et al., 2009).

1.4. Massive screening of natural compounds against *Verticillium dahliae*

In spite of all studies on biological control of VWO described above, olive farmers still in the urgent need for feasible and effective control methods against this disease in field. This fact makes the identification of potential biological treatments a priority objective for developing an integrated control strategy. In this sense, since 2011 the Agroforestry Pathology Group of the University of Córdoba is developing a research line on biological control of VWO. The general objective of that research line was to carry out a massive screening of more than 220 natural compounds against *V. dahliae* under controlled conditions (Lozano-Tovar et al., 2013; Varo et al., 2016a, 2017; Varo-Suárez et al., 2018). For that purpose, a wide range of *in vitro* and *in vivo* experiments were carried out.

The natural compounds evaluated in the screening were divided in the same main groups of biological control measures indicated above: i) essential oils and plant extracts, ii) organic amendments, and iii) biological control agents (fungi, bacteria and their microbial extracts). The main findings obtained from the massive screening for each group of natural compounds are summarized below.

A total of 84 plant extracts and essential oils were evaluated against *V. dahliae*. Generally, essential oils were more effective than plant extracts. Thus, essential oil obtained from *Thymus* sp., *Citrus* sp., *Malaleuca cajeputi, Satureja* sp., and *Verbena officinalis* totally inhibited the mycelial growth of the pathogen. Nevertheless, only the essential oil from *Thymus* sp. achieved a significant control of the disease and a reduction of the inoculum density in naturally infested soils by *V. dahliae* (Varo et al., 2017).

Regarding organic amendments, a screening of 35 organic amendments from different sources, 15 compost mixtures and five aqueous compost extracts against VWO was conducted by Varo-Suárez et al. (2018). Although several composts achieved a significant reduction of mycelial growth, viable inoculum and disease progress, the grape marc compost labelled CGR03 was one of the most effective solid organic amendments, showing a total suppression of *V. dahliae* in both *in vitro* and *in vivo* experiments. In this study, Varo-Suárez et al. (2018) indicated that phenols and volatile organic compounds could be involved in the suppressive effect of CGR03, but the effect of aqueous extracts obtained from this compost as well as most of the mechanisms responsible for its suppression of *V. dahliae* are not yet understood.

The effectiveness of a wide range of microorganisms against VWO was also evaluated in the frame of the massive screening. A total of 60 microbiological compounds, ranging from fungal and bacterial strains and their microbial extracts to consortia of various microorganisms, were evaluated (Lozano-Tovar, 2013; Varo et al., 2016a). Among all compounds evaluated, several fungal species within the genus *Rhizopus* and *Trichoderma*, one strain of *Beauveria bassiana* and one strain of *Metarhizium brunneum* (the last two used as entomopathogenic fungi), several non-pathogenic strains of *F. oxysporum*, and some bacteria and fungi consortia inhibited the mycelial growth of *V. dahliae* in more than 90%. On the other hand, several non-pathogenic isolates of *F. oxysporum*, a strain of *Gliocladium roseum*, two microorganisms consortia and a commercial formulation based on *T. asperellum* and *T. gamsii* reduced the inoculum density of *V. dahliae* in naturally infested soil. Among all microorganisms evaluated in the screening, the non-pathogenic isolate of *F. oxysporum* named as FO12 was described as one of the most effective BCAs against the pathogen, allowing a total control of the disease (Varo et al., 2016a).

The outcomes obtained from the massive screening described above represent the starting point of this PhD Thesis. Thus, among all natural compounds selected at the end of the screening, the non-pathogenic strain of *F. oxysporum* FO12 and the grape marc compost CGR03 were selected as two of the most promising treatment against VWO. Consequently, expanding the knowledge about their effectiveness under field conditions as well as elucidating their principal modes of action against the pathogen is crucial to developing an effective integrated control strategy in field, where olive farmers are in urgent need of feasible and effective measures to controlling this important disease.

1.4. Objectives of this Thesis

In view of all above explained, the general objective of this Thesis is to study the real potential against VWO of the natural compounds selected in the massive screening.

Thus, this general objective can be divided into the following specific objectives:

1. To evaluate the efficacy of different natural products, selected in previous studies, against VWO under field conditions. This objective is addressed in **chapter 2**.

2. To elucidate the modes of action of the non-pathogenic strain of *F. oxysporum* FO12 in its effect against *V. dahliae*. This objective is approached in **chapters 3** and **4**.

3. To elucidate the modes of action of the grape marc compost CGR03 in its effect against *V. dahliae*. This objective is approached in **chapter 5**.

4. To study the combined effect of two of the most effective biocontrol treatments (FO12 and CGR03) together with the genetic resistance level of two olive cultivars in soils infested with different inoculum density levels of *V. dahliae*. This objective is addressed in **chapter 6**.

1.5. Outline of the Thesis

In **chapter 2** of this thesis the effectiveness of 16 natural biological compounds, including biological control agents, organic amendments and one essential oil, against VWO under field conditions. Three experiments were conducted in infested fields with different inoculum densities of *V. dahliae* and under three different scenarios for the natural infection of the pathogen in the Andalusian Region. Results from this study were useful to confirm the effect of potential biocontrol products against VWO in field.

Based on the results of previous work, **chapter 3** deals with the evaluation of different culture fractions of FO12 to determine the overall potential of this strain as a biological control agent against *V. dahliae*, optimizing its mode of application. For that purpose, several *in vitro* and *in vivo* experiments were carried out. This work provides useful information about the efficacy of several FO12-based culture fractions against VWO, which could be the basis for the development of effective commercial formulations in the coming future.

The study of the modes of action of any biocontrol agent is crucial to develop an effective control strategy against any pathogen. Therefore, **chapter 4** aims to elucidate the main modes of action of the non-pathogenic strain *F. oxysporum* FO12. In this chapter, complementary *in vitro* and *in vivo* experiments were conducted in order to explore the implications of inhibitory substances and rhizosphere competence in antagonistic effects of FO12 against *V. dahliae* and *V. longisporum*. The findings obtained in this study provide new insights into the mode of action of this potential biocontrol agent, which are relevant to develop effective strategies against VWO.

The grape marc compost CGR03 was reported in previous studies as one of the most effective organic amendments for controlling VWO, but its whole potential and its mechanisms of action remain unclear. For that purpose **chapter 5** deals with the evaluation of several compost teas and extracts obtained from CGR03 for its effect against *V. dahliae* by means of *in vitro* and *in vivo* experiments. The effectiveness of composts teas and extracts and the implication of biotic and abiotic factors in their effect against *V. dahliae* are discussed.

Finally, **chapter 6** addresses the evaluation under semi-controlled conditions of the efficacy of two of the most effective biocontrol treatments (FO12 and CGR03) against VWO, with the combined effect of the genetic resistance level of two olive cultivars, in soils with two different inoculum density levels of *V. dahliae*. Results from this study highlight the effectiveness of these biocontrol treatments, increasing the knowledge about the use of combined eco-friendly approaches for effective control of VWO.

Chapters 2, 3, 4 have been published in peer-reviewed journals. Although chapters 5 and 6 have not been published yet, a similar format has been applied for them since they are pending acceptance in two peer-reviewed journals. At the end of most of the chapters, several figures are displayed in order to illustrate each study. An abbreviated title is displayed in the first page of each chapter (see “Contents”).

CHAPTER 2

Biocontrol of VWO in the field

2. Biological control of Verticillium wilt of olive in the field

ABSTRACT

Verticillium wilt of olive (VWO) caused by *Verticillium dahliae* is considered one of the most serious diseases in olives. Effective control of this disease requires an integrated management strategy in which implementing biological control is a potential tool. Thus, the aim of this study was to evaluate the effect of 16 natural biological compounds, including biological control agents, organic amendments and one essential oil, under field conditions. The compounds were selected out of 220 treatments previously evaluated against *V. dahliae* under controlled conditions in both *in vitro* and *in planta* experiments. Three experiments were conducted under three different scenarios for the natural infection of *V. dahliae* in the Andalusian Region (southern Spain): under semi-controlled conditions on potted plants grown with naturally infested soil (*Experiment I*); new plantation in a field with high inoculum density (ID) of *V. dahliae* in the soil (*Experiment II*); replanting a commercial olive orchard affected by Verticillium wilt with 1 or 30 year-old trees (*Experiment III)*. In the *Experiment I*, the non-pathogenic strain of *Fusarium oxysporum* (FO12) and a commercial essential oil from *Thymus* sp. were the most effective treatments, achieving a total reduction of the ID two months after planting. In *Experiment II*, the grape marc compost (CGR03) was the most effective treatment in reducing the ID of the pathogen in soil up to 100%. In *Experiment III*, both FO12 and CGR03 treatments reduced significantly the disease incidence in both 1 or 30 year-old olive plants in comparison with the untreated control plants (P = 0.05). This work provides a practical basis for the use of these selected treatments and a useful methodology for the establishment of future field experiments for research on the biological control of VWO.

This chapter has been published in:

Mulero-Aparicio A, Varo A, Agustí-Brisach C, López-Escudero FJ, Trapero A. 2019. Biological control of Verticillium wilt of olive in the field. *Crop Protection* **139.** doi.org/10.1016/j.cropro.2019.104993

2.1. INTRODUCTION

Verticillium wilt of olive (VWO), caused by the widespread soil-borne fungus *Verticillium dahliae* Kleb., is currently considered the most important disease affecting olive (*Olea europaea* L.) and causes severe economic losses due to plant death (López-Escudero and Mercado Blanco, 2011). *V. dahliae* survives in soil by means of microsclerotia, which are also the primary inoculum of the pathogen in natural conditions and its main dispersal propagule. Microsclerotia germinate, giving rise to infective hyphae that penetrate the plant roots and grow toward the xylem vessels, producing mycelium and conidia (Pegg and Brady, 2002). The occlusion caused by fungus mycelia together with the plugging caused by the production of gels and tyloses in the cells of the xylem consequently reduces the water flow and leads to water stress (Ayres, 1978; Trapero et al., 2017).

During recent decades, VWO has increased markedly mainly due to the following causes: i) the use of pathogen-infested soils and/or infected plant material to establish new olive orchards; ii) the introduction of irrigation in olive orchards in an intensive cultivation system (Pérez-Rodríguez et al., 2015); and iii) the widespread distribution of highly virulent isolates of the pathogen (i.e., defoliating pathotype) (López-Escudero and Mercado-Blanco, 2011). Furthermore, among all factors that hinder the control of this disease, the endophytic behavior of the pathogen growing in the xylem, the wide host range, and the longevity of its propagules in soil have turned this disease into one of the major threats to the olive crop worldwide (Alström, 2001).

Due to the difficulties mentioned above and the lack of a truly efficient method to control VWO, an integrated management strategy is needed to reduce both pathogen dispersal and disease incidence in olive orchards (López-Escudero and Mercado Blanco, 2011). Within this strategy, the use of natural or biological compounds arises as a potential complementary and eco-friendly control tool against VWO. Likewise, several studies conducted during the last 15 years have reported the use of antagonist microorganisms as biological control agents (BCAs) against *V. dahliae* in herbaceous and horticultural crops such as rape, tomato, pepper and cotton (Rybakova et al., 2015; Tjamos et al., 2004; Veloso et al., 2016; Xue et al., 2013) as well as in olive (Jiménez-Díaz et al., 2009; Markakis et al., 2016; Mercado-Blanco et al., 2004; Mulero-Aparicio et al., 2019a; Triki et al., 2012; Varo et al., 2016a).

Biological control of *V. dahliae* by means of treatments with organic amendments (OAs) have been extensively studied on horticultural crops (Castaño and Avilés, 2013; Papasotiriou et al., 2013; Vitullo et al., 2013). Although several preliminary studies on the use of OAs against VWO have been conducted (Varo-Suárez et al., 2018; Vitullo et al., 2013; Wilhelm et al., 1962), the effect of OAs against the disease is still uncertain. Although the mechanisms of action of OAs in plants against disease infection are unclear, Avilés and Borrero (2017) have recently reported the potential suppressive effects of different olive mill compost types on Verticillium wilt of cotton, elucidating the primary mechanisms that explain the suppressive effects. In addition, Vitullo et al. (2013) reported that a mixture of OAs with BCAs effectively controlled VWO when olive mill waste was combined with the bacteria *Bacillus amyloliquefaciens* and *Burkholderia cepacia*. The use of extracts of compost from olive mill waste against VWO has also been evaluated recently by Varo-Suárez et al. (2018), showing promising results in reducing the viability of *V. dahliae* microsclerotia. Finally, although very few studies have focused on the antifungal activity of plant extracts and essential oils against *V. dahliae*, *Thymus*-based essential oils were reported to efficiently reduce both microsclerotia viability and VWO under controlled conditions (López-Escudero et al., 2007; Varo et al., 2017).

To date, most of the research related to biological control of *V. dahliae* has been performed mainly using *in vitro* sensitivity tests and *in vivo* experiment under controlled conditions (Mercado-Blanco et al., 2004; Pantelides et al., 2009; Rybakova et al., 2015; Veloso et al., 2016; Veloso and Díaz, 2012). However, to our knowledge, studies evaluating the efficacy of natural compounds such as OAs or essential oils and BCAs against VWO under field conditions in commercial olive orchards have not yet been conducted. The application of these kinds of products at the nursery stage could have practical, economical and efficiency advantages in comparison with field treatments. Moreover, organic alternatives for managing the disease both in established or new plantings are needed for olive growers when other strategies, such as genetic resistance, are not applicable. Altogether, effective practical management strategies to control VWO, including biocontrol, should aim to eradicate microsclerotia or prevent their germination in soil (Antonopoulos et al., 2008). This strategy could only be achieved in field conditions by applying natural compounds or BCAs in the planting holes to inhibit

microsclerotia viability around the rhizosphere and protect the plants during the first years of planting.

Recently, a massive screening was conducted to evaluate the effectiveness of 220 natural compounds, including microorganisms (Lozano et al., 2016; Varo et al., 2016a), OAs (Varo-Suárez et al., 2018) and plant extracts (Varo et al., 2017), against *V. dahliae* by *in vitro* and *in planta* experiments under controlled conditions. However, further studies evaluating the most effective of these products in natural field conditions are essential to demonstrate their effectiveness against VWO. Thus, the aim of this study was to evaluate the effect of 16 natural compounds selected from the previously mentioned studies against VWO in three different scenarios of the natural infection of *V. dahliae*: i) under semi-controlled conditions on potted plants grown in naturally infested soil collected from commercial olive orchards affected by VWO; ii) in a new planting established in a field with a high inoculum density of *V. dahliae*; and iii) replanting a commercial olive orchard affected by VWO.

2.2. MATERIALS AND METHODS

2.2.1. Biological control agents and natural compounds

A total of 16 compounds were evaluated, including OAs, essential oils, BCAs and several mixtures of BCAs and/or OAs (Table 1). All the compounds were selected according their antagonistic effects as demonstrated in previous screenings of a broad range of natural compounds and BCAs against VWO under controlled conditions (Varo et al., 2016a, 2017; Varo-Suárez et al., 2018).

2.2.1.1. Organic amendments

An olive waste compost (CAL03), a grape marc compost (CGR03), a compound provided by an agroindustry based on dairy wastes with high content in lactic acid (2.5%, vol) (LAC02), a poultry manure (MAN01) and a compost tea (TEA01) consisting of fermented aqueous extracts obtained from the solid olive oil waste compost CAL03 were evaluated. All OAs were collected from commercial and experimental composting plants using agro-industry waste from different sources and from different areas of southern Spain (Table 1). To solve possible phytotoxicities, the maturity and stability of all the OAs tested were checked prior

being used in the experiments of this study. To this end, the temperatures of each OA were measured until they reached values below 30-35°C (standard temperature values for mature composts) (Mehta et al., 2014; Varo-Suárez et al., 2018). Additionally, the commercial copper product Folicupro 70 (COPP; 47% copper oxychloride + 4% organic nitrogen; Nufol) was included as a positive control treatment.

2.2.1.2. Microorganisms (biological control agents)

The following three BCAs were evaluated: *i*) a non-pathogenic strain of *Fusarium oxysporum* (FO12) (Varo et al., 2016a; Mulero-Aparicio et al., 2019a); *ii*) the mycorrhiza *Glomus intraradices* (MYCO); and iii) a strain of *Pseudomonas fluorescens* (PICF04) (Mercado-Blanco et al., 2004). The fungal strains FO12 and MYCO were prepared from single-spore stock cultures maintained on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants at 4ºC. The fungal strains were grown on PDA, and conidial suspensions were obtained from 4-day-old cultures and adjusted to 10^6 conidia ml^{-1} using a hematocytometer. When an aqueous inoculum was required, conidial suspensions of each strain were used to inoculate 2 L Erlenmeyer flasks containing 1 L of sterile potato dextrose broth (PDB; Difco Laboratories®) and incubated in an orbital shaker (Grant bio PSU-20i, Grant Instruments, Cambridge, UK) at 100 rpm, 25ºC and 24 h light for 7 days to obtain a final concentration of 10^7 conidia ml^{-1}. The bacterial strain PICF04 was cryopreserved with 30% glycerol at -80ºC, and its inoculum was prepared from colonies grown on nutrient agar (Difco Laboratories®) plates incubated at 25ºC for 48 h. The density of the strain was adjusted to 10^8 cell ml^{-1} (Mercado-Blanco et al., 2004).

2.2.1.3. Compost and microorganism mixtures

To evaluate the combined use of compost with other OAs or BCAs, the olive waste compost CAL03 was combined with FO12 or with LAC2. These two compounds were added to CAL03 as an aqueous treatment applied by direct watering (1:5, v:v) before mixing. Each compost mixture (CALFO12 and MBLAC02, respectively) was aerobically incubated at room temperature in a plastic bag for one week before its use to allow compost colonization by mesophilic bacteria and fungi. In addition, three commercial microorganism mixtures (Bioten®, MO1 and MO2) and a mixture of the non-pathogenic *F.*

oxysporum strain FO12 and *P. fluorescens* strain PICF04 (FO12+PICF04) were evaluated (Table 1).

2.2.1.4. Essential oil

A commercial essential oil from *Thymus* sp. (Oleatbio, Trabe S.A.) was also included in this study (Table 1).

2.2.2. Inoculum density

The initial inoculum density (ID) of the different experimental field soils used in this study was measured before setting up each experiment. A total of three soil samples were randomly collected from three different points of each experimental field. They were collected using a cylindrical (3.5 cm x 22 cm) auger at a depth from 25 to 30 cm and were mixed to obtain a homogenous sample from each zone (Trapero et al., 2013a). Subsequently, soil samples were air-dried at room temperature for two weeks. The ID of *V. dahliae* in each sample (experimental field) was estimated by wet sieving (Huisman and Ashworth, 1974) onto-modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977). Briefly, 25 g of each sample was suspended in 100 ml of distilled water, shaken at 270 rpm for 30 minutes at room temperature and filtered through 150 and 35 µm sieves. Subsequently, the residue retained on the 35 µm sieve was recovered in 100 ml of sterile distilled water (SDW). Finally, 1 ml of this suspension was plated onto MSPA. There were 10 replicated Petri dishes per sample, with 30 Petri dishes per soil sample, and they were incubated for 14 days at 24 ± 2ºC in the dark. Then, soil residues were removed from the agar surface under running tap water, and colonies of *V. dahliae* were counted by means of a stereoscope microscope (Nikon SMZ-2T, Tokyo, Japan). The ID in soil was estimated from the number of *V. dahliae* colonies counted per sample and expressed as colony forming units (microsclerotia) per gram of soil (CFU g^{-1}) (López-Escudero and Blanco-López, 2005b).

2.2.3. Plant material

Twelve-month-old rooted olive plants of the cvs. Picual, Arbequina, and Frantoio (susceptible, moderately susceptible, and moderately resistant, respectively) (López-Escudero et al., 2004; Trapero et al., 2013a) were used for the

different experiments. Healthy olive cuttings were obtained from a commercial nursery. At planting time, plants were 1.0-1.1 m high with a single trunk and three or four secondary branches.

2.2.4. Experimental fields

Three experiments were carried out covering different favorable scenarios for the natural infection of *V. dahliae* across the representative olive growing areas of the Andalusian Region (southern Spain) with different edapho-climatic conditions and ID levels of the pathogen (Table 2): i) *Experiment I*, under semi-controlled conditions on potted plants grown with naturally infested soil; ii) *Experiment II*, new plantation in a field with high ID of *V. dahliae* in the soil; and iii) *Experiment III*, replanting a commercial olive orchard affected by VWO. The climatic conditions were monitored over time in the three experimental fields by means the Andalusian weather station network, which belongs to the Andalusian Institute for Research and Formation in Agriculture and Fishery (IFAPA in Spanish). The data of the different weather stations and the average of the daily mean, minimum and maximum temperatures recorded during each experimental period are shown in Table 2.

2.2.4.1. *Experiment I*: under semi-controlled conditions experiment on potted plants grown with naturally infested soil.

The *Experiment I* was conducted from May 2013 to June 2015 under semi-controlled conditions using potted olive plants of cv. Picual, which were protected from rain and excessive sun by a shading structure with a plastic mesh cover. Plastic pots (20 L volume) were filled with a soil naturally infested by *V. dahliae* (5.5 CFU g^{-1}) from a commercial horticultural orchard located in the municipality of Utrera (Sevilla Province, lower Guadalquivir Valley, southern Spain) used in previous studies (Pérez-Rodríguez et al., 2015). The soil from the horticultural orchard was annually cultivated with typical *V. dahliae* hosts such as cotton (*Gossypium hirsutum*), tomato (*Solanum lycopersicum*) or eggplant (*Solanum melongena*). These kinds of soils are usually heavily infested with highly virulent strains of *V. dahliae* (cotton-defoliating pathotype), and VWO progresses quickly in this growing region (Trapero et al., 2013a). A total amount of 3,000 kg of soil was needed, and solid and liquid treatments were tested in this experiment. Regarding solid treatments (OAs and compost mixtures), evaluated compounds

were applied by mixing them into the soil at a dose of 1:9 (compound:soil; w:w; Table 1). Subsequently, one olive plant per pot was planted and irrigated with 5 L of tap water. Aqueous treatments (BCAs, mixtures of BCAs, and essential oil) were applied after planting olive cuttings in the pots by drenching each pot with 5 L at the appropriate doses of each product, except MYCO, which was applied 21 days before planting (Table 1). All treatments were applied at the beginning of spring and at the beginning of autumn in the first year of the experiment. The experiment was carried out in a completely randomized design, with treatments (10 treatments) as the independent variables and potted plants (14 potted plants per treatment; 140 potted plants in total) as replicates. Additionally, 10 olive plants were grown in pots filled with the same naturally infested soil and watered only with tap water as the control. To determine the ID progress of the pathogen in the soil of each pot, one soil sample (100 g) per pot was collected at 2, 12 and 24 months after inoculation. Soil samples from the same treatments were homogeneously mixed, and three subsamples per treatment were obtained and processed as described above. The relative area under the inoculum progress curve (RAUIPC) was calculated from ID values by the trapezoidal integration method (Campbell and Madden 1990).

2.2.4.2. *Experiment II*: new planting established in an experimental field with a high inoculum density of *V. dahliae* in soil.

A new planting was established in May 2014 in an experimental field previously cultivated with cotton over the last 50 years, and it was surrounded by commercial olive orchards seriously affected by VWO. Consequently, the initial ID of the pathogen in the soil of this experimental field was high (35 CFU g^{-1}), and the *V. dahliae* isolates recovered from this soil were mainly the highly virulent type (defoliating pathotype) (Trapero et al., 2013b).

In this experimental field, there were four olive rows (4 × 1.5 m row and plant spacing, respectively; 1,667 olive/ha) in a randomized block design with five blocks. Each block consisted of 12 treatments [11 biological treatments (Table 1) and one control] with three replicated olive plants (cv. Picual) per treatment (36 plants per block; 180 plants in total). In addition, in each block, olive plants of cvs. Arbequina, Frantoio and Picual were planted between treatments in order to avoid interference between adjacent treatments (four plants per cv. and per block).

The first applications of the treatments evaluated in this experiment were conducted just after planting in May 2014. Solid treatments (OAs and compost mixtures) were applied at a dose of 4 L $tree^{-1}$ by spreading into the planting hole and incorporated manually into the first layers of the soil by using a hoe. Subsequently, plants were irrigated with 10 L of tap water. For aqueous treatments (BCAs and mixtures of them), planting holes were treated by drenching with 10 L of the treatment previously adjusted at the recommended dose (Table 1). Treatments were applied twice a year at the beginning of each spring and autumn season. Control plants and plants between treatments (cvs. Arbequina, Frantoio and Picual) were not subjected to any treatment, and they were irrigated only with 10 L of tap water at each application time. The experimental field was maintained using reduced tillage management, and herbicide applications were performed when needed. Plants were irrigated biweekly with 20 L per tree during the dry season (late spring-summer).

The ID of the soil of each treated planting hole was recorded twice a year. To this end, a total of three soil samples (100 g) were collected per treatment and block combination, homogeneously mixed and split into five subsamples per treatment, which were processed as described above. RAUIPC was calculated by the trapezoidal integration method (Campbell and Madden 1990).

2.2.4.3 *Experiment III*: replanting a commercial olive orchard affected by VWO.

This experiment was conducted from May 2015 to January 2018, and was established in several stands around a commercial olive orchard highly affected by VWO. Although the presence of stands with an active disease progression was clear, the level of initial inoculum of *V. dahliae* in soil was low (0.03 CFU g^{-1}). Two types of olive plants were used in this experiment: i) one-year-old olive plants of cv. Picual obtained from a commercial nursery and ii) 30-year-old olive trees of cv. Picual transplanted from noninfested soil of a commercial olive orchard. All olive trees were planted within the stands in the same place where an olive plant had previously died of VWO. In this experiment, treatments included the non-pathogenic *F. oxysporum* strain FO12 and grape marc compost CGR03 (Table 2). Their applications were performed as described for *Experiment II* for aqueous and solid treatments, respectively. Untreated plants irrigated with tap water were included as controls. This field experiment was performed in a complete block

design with 15 blocks. In each block, there were three treatments (two biocontrol treatments and one control) and three replicated olive plants per treatment and age combination (45 olive plants per age category; 90 olive plants in total). Due to the low population of the pathogen in this soil, ID was not assessed in this trial during the experiment. The experimental field was maintained with reduced tillage, and herbicide applications were performed according to the traditional management of commercial olive groves in the area. Automatic daily drip irrigation lines of 16 L $tree^{-1}$ were used for watering the whole field plot.

2.2.5. Disease assessment

All experiments were surveyed every two weeks from disease onset for wilt symptoms assessment. Disease severity was estimated based on a 0 to 16 rating scale according to the percentage of plant tissue affected by any of the following symptoms: chlorosis, necrosis or defoliation. The scale estimated the percentage of affected tissue using four main categories or quartiles (≤25, 26-50, 51-75, and 76-100%) with four values per category. Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) were linearly related to the percentage of affected tissue (Y) by the equation $Y = 6.25X – 3.125$ (Varo-Suárez et al., 2018). At the end of the disease assessment, the relative area under the disease progress curve (RAUDPC) was calculated from the disease severity values by the trapezoidal integration method (Campbell and Madden 1990). In addition, the disease incidence, i.e., the percentage of symptomatic plants and percentage of dead plants (mortality), was recorded (López-Escudero et al., 2004; Wilhelm and Taylor, 1965).

Plant infection was confirmed by isolating the fungus from the affected shoots or leaf petioles of diseased plants by microbiological methods, as described by López-Escudero and Blanco-López (2001).

2.2.6. Data analysis

Analyses of variance (ANOVA) of the disease parameters (final disease severity and RAUDPC) and of the RAUIPC were performed. Values of these parameters met the assumptions of normality and homogeneity of variances for this analysis. Final disease severity and RAUDPC were arranged in a randomized block design. The results from ID assessment were analyzed in a completely randomized

experimental design. When ANOVA showed significant differences among treatments, final disease severity and RAUDPC mean values were compared using Fisher's protected least significant difference (LSD) test for experiments with independent variables with less than six levels and Tukey's honestly significant difference (HSD) test for experiments with independent variables with six or more levels, both at $P = 0.05$ (Steel and Torrie, 1985). Both incidence and mortality data were analyzed by Chi-squared test for multiple comparisons for the proportions at $P = 0.05$ (Zar, 1999). Means from relative inoculum reduction were compared according to Tukey's HSD test at $P = 0.05$, and means from RAUIPC were compared according to Dunnett´s test (*$P = 0.05$, **$P = 0.01$, ***$P = 0.001$). All data of this study were analyzed using Statistix 10 (Analytical Software, 2013)

2.3. RESULTS

2.3.1. *Experiment I*: Under semi-controlled conditions on potted plants grown into naturally infested soil.

Disease symptoms were not observed during the 24 months of the experiment (Figure 1A, B). However, significant differences ($P < 0.0001$) in ID were observed between treatments. The initial ID was 5.5 MS g^{-1}, and it decreased steeply during the evaluation period in all treatments evaluated. The most effective treatments reducing ID in soil were FO12 and THYM01, a BCA and an essential oil, respectively. Both treatments showed the most consistent results over time at 2, 12 and 24 months after planting, showing an ID reduction of 80.0 and 66.6% for FO12 and THYM01, respectively, at the end of the experiment. Two months after planting, soil treated with CAL03 or MAN01 did not show ID reduction, while FO12 and THYM01 were the most effective treatments. The remaining treatments showed an intermediate effect of reducing the ID of *V. dahliae* at this evaluation point. At 12 months after planting, all of the OAs tested (CAL03, LAC02, MAN01, and TEA01) reduced the ID of *V. dahliae*, which ranged from 68.1 to 15.8% for THYM01 and MAN01, respectively. At this evaluation point, FO12 and THYM01 were also the most effective products in inhibiting the ID, with 81.8 and 86.3% ID reductions of *V. dahliae*, respectively. Overall, at the end of the experiment, the relative ID inhibition of the pathogen decreased for all treatments tested. FO12 was the most effective treatment on ID inhibition (80.0%), whereas THYM01, LAC02, MO1 and CAL03 showed an intermediate effect in reducing the ID of *V. dahliae* (66.6, 43.3, 40.0 and 30.0%, respectively) (Table 3). On the other hand, TEA01,

MO2 and Bioten® reached a low level of ID inhibition (8.3, 3.3 and 3.3%, respectively). In contrast, MYCO and MAN01 showed negative values of ID inhibition at 24 months after planting (-8.3 and -16.7%, respectively) (Table 3). These negative values indicate a relative increase of the ID of the treatments over control.

Regarding RAUIPC, FO12 and THYM01 showed significantly lower values of RAUIPC compared to the control (14.5 and 16.7%, respectively; $P < 0.0001$). In addition, Bioten®, MYCO, LAC02 and MO1 were also able to significantly reduce the ID of *V. dahliae*, showing an intermediate effect on the inhibition of ID in comparison with the control ($P < 0.001$). The remaining treatments did not show significant differences from the control on the ID inhibition of *V. dahliae* (Figure 2A).

2.3.2. *Experiment II*: new planting in a field with high inoculum density of *V. dahliae* in soil.

In this experiment, VWO symptoms were first observed 14 weeks after planting. Typical symptoms of the disease, such as wilting, dieback, and/or defoliation, were observed on affected plants (Figure 1C, D). Wilting primarily started at the lower branches and developed as generalized green leaf defoliation that spread to the entire tree canopy. Flower mummification occurred during spring and early summer. Occasionally, affected plants exhibited wilt, chlorosis and rolling of leaves that remained attached to the shoots until plants became completely wilted.

Only plants treated with COPP and untreated plants of cvs. Frantoio and Arbequina showed a significant reduction in disease incidence compared to the control cv. Picual (40.0, 30.0 and 25.0%, respectively; $P < 0.001$) (Table 4). In contrast, plants treated with CGR03 and CAL03 showed a significantly higher disease incidence in comparison with the control (both 93.3%) (Table 4). Olive cuttings of cvs. Arbequina and Frantoio showed a significantly lower percentage of dead plants than those observed in all treated as well as in control plants at 24 months after planting (10.0 and 5.0%, respectively; $P < 0.0001$). The remaining treatments did not differ compared with the control (cv. Picual), except for CGR03 and CALFO12, whose mortality values were higher in comparison with the control (80.0 and 73.3%, respectively). The time elapsed from the planting time to 50% of

plants being affected by *V. dahliae* (DI_{50}) ranged from 37 to 96 weeks depending on the evaluated product. TEA01 treatment showed the lowest value of DI_{50} (34 weeks), while COPP01 showed the highest DI_{50} (96 weeks). Regarding disease severity, at the end of the experiment, there were significant differences (P = 0.0107) among cultivars, but no biological treatments significantly differed from the control cv. Picual. Thus, the moderately susceptible cv. Arbequina and the resistant cv. Frantoio differed significantly from the control treatment cv. Picual (14.8 and 18.3%, respectively). Finally, cvs. Arbequina and Frantoio showed significant differences in RAUDPC in comparison with the control cv. Picual (5.6 and 4.3%, respectively; $P < 0.0001$), but no significant differences were found between treatments and the control cv. Picual 24 months after planting (P = 0.1429) (Table 4).

Among all biological and natural treatments tested, MO1 and CGR03 were the most effective treatments at reducing ID of *V. dahliae* in comparison with the control (RAUIPC= 12.1 and 13.8%, respectively; $P < 0.0001$) at the end of the experiment. In addition, FO12, COPP, CALFO12 and FO12+PICF4 were also significantly effective in inoculum inhibition (RAUIPC = 23.0, 23.8, 29.4 and 32.1%, respectively; $P < 0.0001$) (Figure 2B). All biological products mentioned above were able to achieve an ID reduction close to 100% between 14 months after planting and the end of the experiment (Supplementary Table 1). In contrast, TEA01 showed a significantly higher RAUIPC (85.5%; $P < 0.0001$) in comparison with the control (RAUIPC = 56.5%) (Figure 2B).

2.3.3. *Experiment III*: Replanting a commercial olive orchard affected by VWO.

In this experiment, the first symptoms were observed in early spring, just nine months after planting. Affected plants exhibited wilt, chlorosis and rolling of leaves or defoliation of green leaves (Figure 1E, F, G).

No significant differences for disease suppression were found in plants of the two age categories treated with both CGR03 and FO12 treatments in comparison with the control (P = 0.5349 and P = 0.2975 for one-year- and 30-year-old plants, respectively). However, when comparing the final disease severity, there were no significant differences in disease severity between treatments and control in one-year-old plants (P = 0.9687), but 30-year-old plants treated with CGR03 showed a

significant reduction in the final disease severity in comparison with the control (18.1%; $P = 0.0476$) (Table 5).

Treatments with CGR03 and FO12 significantly reduced the disease incidence in plants of the two age categories in comparison with their respective control plants. In one-year-old plants, there were no significant differences between the treatments showing the same disease incidence (50%), whereas the control showed a significantly higher DI (71.4%; $P = 0.0017$) (Table 5). CGR03 and FO12 were also effective in reducing disease incidence in 30-year-old plants, showing disease incidences of 33.3% and 53.3%, respectively, compared to the control (80%; $P < 0.0001$), with CGR03 being significantly more effective than FO12. No differences were found in the percentage of dead plants between treatments in 1-year-old olive plants ($P = 0.0517$). Nevertheless, 30-year-old plants treated with CGR03 and FO12 showed a significant reduction in mortality compared to the untreated control plants (13.3% mortality for treated plants compared to 33.3% for control plants; $P = 0.0002$) (Table 5).

Table 1. Biological control agents and natural compounds evaluated in this study to control Verticillium wilt of olive under field conditions.

Treatment code	Composition[a]	Origin[b] (reference)	Doses [*Experiment*[d]]
Organic amendments (OAs)			
CAL03	Olive waste compost	Almodóvar 1[c]/Córdoba	1:9 (w/w) [*Exp. I*]; 4 L tree^{-1} (amendment) [*Exp. II*]
CGR03	Grape marc compost	Montemayor/Córdoba	1:9 (w/w) [*Exp. II*]; 4 L tree^{-1} (amendment) [*Exp. III*]
COPP	47% copper oxychloride + 4% organic nitrogen	Folicupro 70, Nufol	0.2:9.8 (v:v) [*Exp. I, II*]
LAC02	Dairy waste (Lactic acid 2.5%, vol)	Jaén	1:9 (v:v) [*Exp. I*]
MAN01	Poultry manure	Almodóvar 2/Córdoba	1:9 (w/w) [*Exp. I*]
TEA01	Compost tea obtained from CALP03	Almodóvar 2/Córdoba	1:9 (v:v) [*Exp.s I, II*]
Microorganisms (BCAs)			
FO12	Non-pathogenic strain of *Fusarium oxysporum* (10^7 conidia ml^{-1})	*Quercus suber*	1:9 (v:v) [*Exp. I, II, III*]
MYCO	*Glomus intraradices*	MYCOSTAR®	1:9 (v/v) [*Exp. I*]
PICF04*	*Pseudomonas fluorescens* 10^8cell ml^{-1}	*Olea europaea* cv. Picual (rhizosphere)	1:9 (v:v) [*Exp. II*]
Compost mixtures			
CALFO12	CAL03 + FO12 (20% volume 10^7 conidia mL^{-1})	Almodóvar 1/Córdoba-*Quercus suber*	4 L tree^{-1} (amendment) [*Exp. II*]
MBLAC02	CALP03 + LAC02 (20% volume 10^7 conidia mL^{-1})		4 L tree^{-1} (amendment) [*Exp. II*]

Table 1. (Continued)

Treatment code	Composition[a]	Origin[b] (reference)	Doses [*Experiment*[d]]
Microorganism mixtures			
Bioten®	*Trichoderma asperellum* + *T. gamsii*	Bioten® Isagro	100 g L^{-1} [*Exp.s I, II*]
FO12 + PICF04	FO12+PICF04		1:9 (v:v) [*Exp. II*]
MO1	*Rhodopseudomonas palustris*, *Rhodobacter sphacrodes*, *Lactobacillus plantarum*, *Lactobacillus casei* and *Streptococcus lactis*, *Saccharomyces* spp. and *Streptomyces* spp.	EM®	1:9 (v:v) [*Exp. I, II*]
MO2	MO1 under continuous fermentation	EM®	1:9 (v/v) [*Exp. I*]
Essential oils			
THYM01	Essential oil from *Thymus* sp.	Oleatbio, Trabe S.A.	1:9 (v:v) [*Exp. I*]

[a] The percentages of ingredients in the mixtures are volume-based.

[b] Origin refers geographical origin (Location/Province), host or substrate from which the microorganism was isolated, or brand and company in the case of commercial products.

[c] Numbers after each location refer to products with different origins within this municipality.

[d] Experiments in which each product was evaluated.

* This strain was characterized and supplied by Dr. Jesús Mercado-Blanco from CSIC-IAS, Córdoba, Spain (Mercado-Blanco et al. 2004).

Table 2. Location and edapho-climatic conditions of each experiment conducted in this study, and the weather stations monitored.

Experiment[a]	Locality (Province)	UM (X; Y)	Soil	ID[b] (CFU g^{-1})	Weather Station[c]					
					Name (Location)	Altitude (m)	UTM (X; Y)	Temperature[d] (ºC)		
								T_{av}	T_{max}	T_{min}
I	Córdoba (Córdoba)	37.919056; -4.724306	Clay	5.5	Córdoba	94	34.1399; -4.191480	17.7	25.3	10.9
II	Villanueva de la Reina (Jaén)	38.012827; -3.909571	Clay	35.0	Mengíbar	293	43.0790; -4.199560	18.2	26.1	10.5
III	Peal de Becerro (Jaén)	37.909526; -3.232036	Clay	0.03	Úbeda	343	47.3599; -4.199520	16.6	26.3	8.9

[a] *Experiment I*: under semi-controlled conditions on potted plants grown with naturally infested soil; *Experiment II*: new plantation in a field with high ID of *V. dahliae* in the soil; *Experiment III*: replanting a commercial olive orchard affected by Verticillium wilt of olive.

[b] ID: Inoculum density of *V. dahliae* in the soil (CFU: Colony forming units).

[c] All the weather stations belong to the Andalusian Institute for Research and Formation in Agriculture and Fishery ('Instituto de Investigación y Formación Agraria y Pesquera de Andalucía' in Spanish, IFAPA).

[d] Data represents the average of the daily mean (T_{av}), minimum (T_{min}) and maximum (T_{max}) temperatures recorded during each experimental period (*Experiment I*: from May 2013 to June 2015; *Experiment II*: from May 2014 to July 2016; *Experiment III*: from May 2015 to January 2018).

Figure 1. Symptoms development of Verticillium wilt of olive in the different experiments conducted in this study: (A, B) *Experiment I*, asymptomatic plants at two (A) and 24 (B) months after planting; (C, D) *Experiment II*, plants showing different levels of disease severity (C) and severe defoliation (D) at the end of the experiment; (E-G) *Experiment III*, dead control plant (E), slightly affected plant treated with FO12 (F), and healthy plant treated with CGR03 (G) at the end of the experiment.

Table 3. Relative inhibition (%) of inoculum density of *Verticillium dahliae* in soils of potted olive plants treated with organic amendments (OAs), biological control agents (BCAs) and essential oil of *Thymus* spp. under semi-controlled conditions (*Experiment I*).

	Inhibition (%) of inoculum density[a]		
Treatment	**2 months[c]**	**12 months**	**24 months**
Control[b]	**5.1 ± 0.2**	**2.8 ± 0.5**	**2.0 ± 0.5**
FO12	100 ± 0.0 a	81.8 ± 4.6 a	80.0 ± 0.0 a
THYM01	100 ± 0.0 a	86.3 ± 7.9 a	66.6 ± 6.7 ab
LAC02	28.2 ± 5.1 bcd	36.3 ± 4.6 bcd	43.3 ± 8.8 bc
MO1	29.5 ± 3.4 bc	56.8 ± 8.2 abc	40.0 ± 10.0 bc
CAL03	0.0 ± 0.0 e	68.1 ± 9.1 ab	30.0 ± 5.8 cd
TEA01	5.1 ± 5.1d	44.3 ± 8.9bcd	8.3 ± 7.3 d
MO2	10.2 ± 6.8 cd	26.1 ± 4.1cd	3.3 ± 3.3 d
Bioten®	51.3 ± 3.4 b	22.6 ± 5.0 cd	3.3 ± 3.3 d
MAN01	0.0 ± 0.0 e	15.8 ± 2.3 d	-8.3 ± 6.0 f
MYCO	46.8 ± 8.0 b	36.3 ± 12.0 bcd	-16.7 ± 8.8 f

[a] Relative reduction of inoculum density of *V. dahliae* in soil of potted plants compared with the control (potted plants irrigated with water only). Data represent the average of three soil subsamples per treatment and evaluation time ± standard error of the means.

[b] Absolute values of inoculum density (CFU g^{-1}) of *V. dahliae* in soil samples taken from the control. Data in this row represent the average of three soil subsamples per evaluation time ± standard error of the means.

[c] Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at $P = 0.05$.

Table 4. Effect of the biological and natural treatments on the progress of Verticillium wilt of olive along the two-experimental years in field conditions (*Experiment II*).

Treatment	Incidence (%)[a]	Mortality (%)[a]	DI_{50}[b]	Disease severity[c]	RAUDPC[d]
CGR03	93.3[e] a	80.0[e] a	39	90.2[e] ± 5.8 a	35.6[e] ± 5.1 a
CAL03	93.3 a	53.3 bcd	38	78.3 ± 8.5 ab	25.4 ± 8.3 a
CALFO12	86.7 ab	73.3 ab	37	86.3 ± 6.8 a	42.7 ± 5.1 a
TEA01	80.0 abc	66.7 abc	34	88.8 ± 6.6 a	38.7 ± 6.2 a
FO12	80.0 abc	66.7 abc	48	61.7 ± 11.6 ab	33.5 ± 6.4 a
MBLAC	73.3 bcd	53.3 bcd	50	67.5 ± 10.0 ab	28.5 ± 6.0 a
PICF04	73.3 bcd	46.7 cde	53	72.3 ± 9.5 ab	26.3 ± 5.1 a
cv. Picual	71.4 bcd	48.6 cde	44	70.8 ± 6.7 ab	24.3 ± 3.5 a
Bioten®	66.7 cd	40.0 de	39	83.8 ± 7.1 a	26.2 ± 6.3 a
FO12+PICF04	60.0 cde	40.0 de	48	63.3 ± 11.3 ab	24.4 ± 6.1 a
MO1	53.3def	40.0 de	55	50.0 ± 11.3 ab	19.5 ± 6.1 a
COPP	40.0 ef	26.7 e	96	36.9 ± 11.6 b	32.4 ± 13.3 a
cv. Frantoio	30.0 g	5.0 f	+96	18.3 ± 7.6 c	4.3 ± 1.9 b
cv. Arbequina	25.0 g	10.0 f	+96	14.8 ± 6.7 c	5.9 ± 2.1 b

[a] Percentage of plants showing symptoms of VWO (Incidence, %) or dead plants by VWO (Mortality, %) 24 months after planting.

[b] DI_{50} = Time in weeks from planting to the 50% of the plants were affected.

[c] Final disease severity ± standard error 24 months after planting based on a 0-16 rating scale (0 = no lesions, 16 = 94-100% of canopy with symptoms).

[d] Relative area under the disease progress curve (RAUDPC) developed over the assessment period.

[c, d] Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at $P = 0.05$.

[e] In each column, data represent the mean of 15 replicated plants per treatment (20 plants for cvs. Arbequina and Frantoio). Mean values in a column followed by the same letter are not significantly different according to the multiple comparisons Chi-squared proportion test at $P = 0.05$ (Zar, 1999).

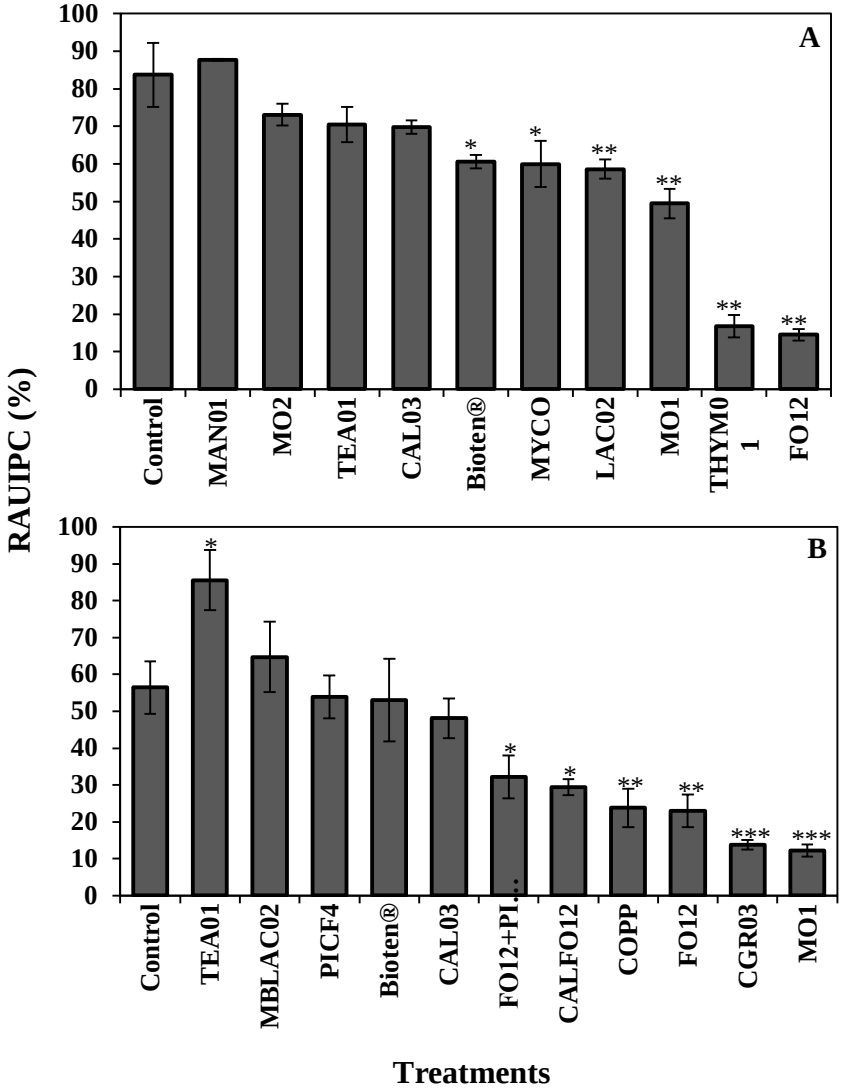


Figure 2. Effect of biological and natural treatments evaluated in *Experiment I* (A) and in *Experiment II* (B) on the inoculum reduction of *Verticillium dahliae* (Relative area under inoculum progress curve, RAUIPC, %) in naturally infested soils over the assessment period (24 months). For each trial, columns represent the means of three and five assessments per treatment and experiment combination, respectively. Means in a column followed by an asterisk are significantly different from the mean for the control according to Dunnett´s test ($^{*}P = 0.05$, $^{**}P = 0.01$, $^{***}P = 0.001$). Vertical lines in each column are the standard error of the mean.

Supplementary Table 1. Relative inhibition (%) of inoculum density of *Verticillium dahliae* in soil of planting holes treated with organic amendments (OAs) and biological control agents (BCAs) under field conditions.

	Inhibition (%) of soil inoculum density[a]				
Treatment	**2 months[c]**	**7 months**	**14 months**	**19 months**	**27 months**
Control[b]	**39.7 ± 6.5**	**26.5 ± 5.9**	**4.2 ± 0.1**	**18.4 ± 3.4**	**12.8 ± 3.5**
CGR03	42.3 ± 12.5 a	97.4 ± 2.1 a	100 ± 0.0 a	94.8 ± 3.0 a	100 ± 0.0 a
COPP	58.7 ± 11.1 a	41.9 ± 35.8 ab	88.4 ± 11.5 a	69.2 ± 7.5 abc	100 ± 0.0 a
MO1	71.3 ± 11.3 a	94.0 ± 4.0 a	77.9 ± 9.8 a	94.4 ± 4.1 ab	97.4 ± 1.9 ab
FO12	1.5 ± 20.7 a	85.2 ± 11.5 a	100 ± 0.0 a	96.5 ± 2.5 a	95.4 ± 2.5 ab
CALFO12	-7.3 ± 18.9 a	86.7 ± 4.7 a	83.2 ± 10.6 a	33.6 ± 10.4 cd	91.4 ± 1.7 ab
FO12+PICF04	44.6 ± 18.2 a	40.4 ± 31.1ab	65.4 ± 8.8 a	40.1 ± 11.7 bcd	82.1 ± 8.4 ab
Bioten®	3.8 ± 37.4 a	-2.2 ± 19.2 ab	19.2 ± 40.8 abc	4.8 ± 15.3 de	51.7 ± 11.3 bc
PICF04	-6.8 ± 14.3 a	-4.3 ± 21.0 ab	32.7 ± 19.2 ab	17.6 ± 10.2 cde	13.2 ± 43.8 bc
CAL03	37.5 ± 23.2 a	5.4 ± 25.9 ab	-23.1 ± 46.8 abc	14.1 ± 13.0 de	13.2 ± 17.8 bc
MBLAC02	42.2 ± 18.0 a	-7.6 ± 13.3 ab	-53.8 ± 36.7 bc	-61.4 ± 14.0 f	-15.2 ± 33.7 bc
TEA01	-25.2 ± 31.0 a	-64.4 ± 51.4 b	-478.9 ± 67.0 c	-24.1 ± 19.7 ef	-63.6 ± 48.0 c

[a] Relative reduction of inoculum density of *V. dahliae* in soil of planting holes compared with the control (olive plants irrigated with water only). Data represent the average of five soil samples per treatment and evaluation time ± standard error of the means.

[b] Absolute values of inoculum density (CFU g^{-1}) of *V. dahliae* in soil samples taken from the control. Data in this row represent the average of five soil samples per evaluation time ± standard error of the means.

[c] Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at $P = 0.05$.

Table 5. Effect of the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 on the progress of Verticillium wilt of olive in olive plants of two different age categories along the three-experimental years in field conditions (*Experiment III*).

Treatments	**Age of plants (years)**	**Incidence (%)[a]**	**Mortality (%)[a]**	**Disease severity[b]**	**RAUDPC[c]**
Control	1	71.4[e] a	21.4[e] a	31.3 ± 10.1[e] a	18.3 ± 5.6[e] a
	30	80.0 A	33.3 A	53.3 ± 11.5 A	22.6 ± 6.3 A
FO12	1	50.0 b	28.6 a	31.5 ± 11.7 a	30.3 ± 7.5 a
	30	53.3 B	13.3 B	39.6 ± 11.1 AB	11.7 ± 4.7 A
CGR03	1	50.0 b	14.3 a	26.6 ± 10.4 a	24.0 ± 9.7 a
	30	33.3 C	13.3 B	18.1 ± 8.7 B	16.3 ± 8.0 A

[a] Percentage of plants showing symptoms of VWO (Incidence, %) or dead plants by VWO (Mortality, %) 32 months after plantation establishment.

[b] Final disease severity ± standard error 32 months after planting based on a 0-16 rating scale (0 = no lesions, 16 = 94-100% of canopy with symptoms)

[c] Relative area under the disease progress curve (RAUDPC) developed over the assessment period (three years).

[e] In each column, data represent the mean of 15 replicated plants per treatment and age category combination. For each age category, mean values in a column followed by the same letter are not significantly different according to the multiple comparisons Chi-squared proportion test[a] at $P = 0.05$ or to LSD test[b,c] at $P = 0.05$.

2.4. DISCUSSION

In this study, a total of 16 natural products, including BCAs, OAs and one essential oil, were selected from previous studies conducted under controlled conditions. These products were selected among the 220 treatments previously evaluated (Lozano et al., 2016; Varo et al., 2016a, 2017; Varo-Suárez et al., 2018) since they were the most effective at suppressing *V. dahliae* growth *in vitro* in naturally infested soil as well as *in planta*. Since discrepancies between the antagonistic effects demonstrated *in vitro* and the corresponding effect in microsclerotia and *in planta* have been repeatedly reported (Reddy et al., 1994; Weller, 1988), the suppressive effect of these selected 16 natural products was carefully evaluated under field conditions in this current work. To our knowledge, this study represents the first report evaluating natural compounds and BCAs under field conditions.

Experimental fields were selected according to their edaphic and climatic characteristics, crop history and level of inoculum of *V. dahliae* in soil to test the selected products in different real scenarios within olive growing areas. Likewise, soil from *Experiment I* was selected because it was annually cultivated with typical *V. dahliae* hosts such as cotton, tomato or eggplant. Therefore, the soil was heavily infested with highly virulent strains of *V. dahliae* (cotton-defoliating pathotype), markedly favoring the development of the disease in this olive growing region (Trapero et al., 2013a). In addition, the clayey texture of the soil could also favor disease development since it has been indicated as a significant physical property inducing disease development (Pegg and Brady, 2002). *Experiment II* was established in a plot previously cultivated with cotton and surrounded by an olive orchard seriously affected by VWO. In fact, the inoculum of the pathogen in this soil was much higher than that observed in the soil of *Experiment I*. Finally, *Experiment III* was conducted in stands within a commercial olive orchard seriously affected by VWO. Despite the high incidence of the disease in this orchard, the ID in its soil was the lowest of all experimental field soils included in this study.

In *Experiment I*, no disease symptoms were observed on potted plants, probably because the volume of the plastic pots used in this study (20 L) was not enough to induce disease development in plants (Figure 1A, B). This experiment

was established in large volume pots (20 L) because previous studies have shown that VWO does not develop when plants are grown in naturally infested soil in regular pots with moderate or low inoculum densities, and extremely high inoculum densities (111.9 CFU g^{-1} soil) were necessary to achieve a very low incidence of the disease in these conditions (Varo et al., 2016a). In contrast, the disease developed adequately in large capacity containers (1,000 L) even at low inoculum densities (López-Escudero and Blanco-López, 2007; Pérez-Rodríguez et al., 2015). The minimum volume of soil and the minimum density of inoculum for the development of VWO in potted olive plants growing in naturally infested soil is therefore still a pending objective that is of great interest for studies that need to be carried out under the natural conditions for infection.

Our results using potted plants are in contrast with those obtained in olive of cv. Picual under field conditions (Trapero et al., 2013a; Roca et al., 2015). These authors observed severe symptoms of VWO on olive plants of cv. Picual planted in soils with only 0.8-4 CFU g^{-1} of inoculum. This difference may be due to differences in virulence between isolates. Conversely, in herbaceous crops such as cotton or eggplant, the disease progress develops consistently when the crops are potted with naturally infested soil (Trapero et al., 2013b; Varo et al., 2016b; Xiao and Subbarao, 1998). Despite these difficulties, significant differences were observed in the RAUIPC between treatments, with FO12, THYM01, MO1, LAC02, MYCO and Bioten® being the most effective treatments. These results agree with those reported by Mulero-Aparicio et al. (2019a), Varo et al. (2017, 2016b) and Varo-Suárez et al. (2018), who demonstrated that these natural products were also able to effectively reduce the ID of *V. dahliae* in naturally infested soils. In contrast, an increase in ID detection in potted soil treated with MYCO and MAN01 was observed. These results contrast with those obtained by Varo-Suárez et al. (2018), who indicated that MAN01 effectively reduced the ID of the pathogen in naturally infested soil.

Due to the lack of information about the effectiveness of the treatments on disease progress obtained in *Experiment I*, *Experiment II* was carried out under field conditions using olive cuttings planted directly in soils naturally infected by *V. dahliae*. In this experiment, several natural products effectively reduced RAUIPC, with MO1, CGR03, FO12, COPP, CALFO12 and FO12+PICF4 being the most effective treatments. The treatments CGR03 and COPP achieved a reduction of 100 in ID, whereas MO1 and FO12 were able to almost completely

reduce the ID of *V. dahliae* in soil at the end of the experiment (Supplementary Table 1). These results confirm those reported by Mulero-Aparicio et al. (2019a) and Varo-Suárez et al. (2018), who indicated that these same products were able to inhibit or reduce microsclerotia of *V. dahliae* in *in vitro* experiments. Thus, our results suggest that the effect of these biocontrol treatments against *V. dahliae* shown in *in vitro* experiments could also be achieved under field conditions. The capacity to reduce the inoculum of *V. dahliae* shown by some of the natural products tested in this experiment may result in lower *V. dahliae* infection pressure and, consequently, in a reduction of VWO incidence. Conversely, as happened in *Experiment I*, some treatments achieved an increase in ID detection in soil at the end of the experiment. In this case, MBLAC02 and TEA01 showed increases in ID of 15.2 and 63.6%, respectively. These results also disagree with those obtained by Varo-Suárez et al. (2018), where TEA01 was effective in reducing the ID in naturally infested soils. Indeed, Termorshuizen et al. (2006) demonstrated that the effectiveness and consistency of OAs in disease suppression were influenced, among other factors, by the variability due to the original source, chemical characteristics, and year of production of the OAs.

Nevertheless, these promising results on ID inhibition did not guarantee a reduction or a delay of the disease. Indeed, no significant differences were detected in RAUDPC between treatments at the end of the experiment. The incidence of the disease increased quickly, causing a high increment of RAUDPC, where almost 50% of plants were affected seven months after the plantation establishment. This effect could be due to the high pressure of ID at the beginning of the plantation establishment and also to the moderate temperatures that occurred during summer 2014; these temperatures allowed an intensive activity of the pathogen at the first stages of olive plant growth. We think that all these factors did not allow consistent disease reduction since plant infection would have occurred before treatments to reduce the inoculum of the pathogen in soil. In fact, only COPP was able to reduce the disease incidence in comparison with the control of cv. Picual. These results support the idea that an application at the nursery stage in olive plants should be done before planting establishment in the field, as suggested by Varo et al. (2016b), to protect the plants until the biocontrol treatments achieve an inoculum reduction in the field. This method should be considered for future biological control experiments under field conditions to prevent an early infection of the olive plants.

In *Experiment II,* the effect of cultivar resistance of 'Arbequina' and 'Frantoio' had more relevance to controlling VWO than the applied biological treatments. This fact indicates the importance of the proper selection of olive cultivars to establish plantings in *V. dahliae*-infested soils. The level of resistance of the three cultivars evaluated under natural conditions (cvs. Arbequina, Frantoio and Picual) is supported by previous studies that showed the low ability of *V. dahliae* to colonize cvs. Arbequina and Frantoio in relation to cv. Picual (López-Escudero and Mercado-Blanco, 2011; Pérez-Rodríguez et al., 2016; Trapero et al., 2013a). However, 'Frantoio' and 'Arbequina' did not exhibit complete resistance when they were planted in the highly infested soil in the current field study. This same situation was previously observed by Trapero et al. (2013a).

Finally, *Experiment III* was conducted to evaluate the effectiveness of two treatments (FO12 and CGR03) in a commercial olive orchard seriously affected by VWO but with a low ID of the pathogen in soil. Despite the low level of *V. dahliae* in the soil shown in this trial, the disease progress developed consistently, possibly due to the presence of virulent isolates of the pathogen. In fact, one strain isolated from a diseased olive shoot from this experiment was molecularly characterized as a defoliating pathotype of *V. dahliae*. Both treatments were able to significantly reduce disease incidence in both plant age categories, although the effect was even more consistent when they were applied to 30-year-old plants. Nevertheless, no differences in RAUDPC were found between treatments. This could be because the first symptoms of the disease were found in plants treated with FO12 and CGR03, although at the end of the experiment, untreated control plants were more severely affected than those treated with the natural products. Our results are in accordance with those obtained by Varo-Suárez et al. (2018), who demonstrated the effectiveness of grape marc compost (CGR03) in controlling VWO under controlled conditions. Compost suppressiveness to plant pathogens has been attributed to abiotic and/or biotic factors (Noble and Coventry, 2005). The effect of FO12 on disease reduction agrees with the results reported by Mulero-Aparicio et al. (2019a), who demonstrated that applications of FO12 by irrigation were effective in reducing VWO progress under controlled conditions. Non-pathogenic strains of *F. oxysporum* have already been identified as potential BCAs for Verticillium wilt diseases (Angelopoulou et al., 2014; Veloso et al., 2016). One of the main advantages of the protective strain FO12 is that strains of *F. oxysporum* were much more efficient in establishing suppressiveness in soil than other fungi or other species of *Fusarium* (Lemanceau and Alabouvette 1991).

However, this work also demonstrates the difficulties in evaluating natural products against VWO and their application under field conditions. The results obtained in this study suggest that grape marc compost (CGR03) or the nonpathogenic *F. oxysporum* FO12 could be useful for reducing both the ID of *V. dahliae* and VWO progress in field conditions. This work is relevant because it provides a practical basis for the use of these eco-friendly treatments and a useful methodology to consider as a potential tool within an integrated management strategy against VWO in commercial olive orchards. The application of these treatments in the substrate material used during the nursery olive propagation process as well as in the newly established plantations could be a promising strategy for reducing the ID of *V. dahliae* in the soil and consequently the percentage of affected plants. In addition, it is interesting to note that our results suggest the use of natural and biological products as potential alternatives to other classic eco-friendly strategies, such as solarization, which have been reported as effective management strategies in reducing the ID of *V. dahliae* (López-Escudero and Blanco-López, 2001). Finally, we think that further research is needed on the mode and time of application of these treatments to improve their efficiency under field conditions and on the mechanisms responsible for the suppressive effect of these compounds.

2.5. CONCLUSIONS

In conclusion, the results of the present study demonstrate that some natural products, such as grape marc compost (CGR03) and the non-pathogenic strain of *F. oxysporum* FO12, are highly effective in inoculum inhibition of *V. dahliae* in the field and may exert a suppressive effect against VWO under field conditions. Thus, this study represents, as far as we know, the first report of biological control of VWO under field conditions. This study could lead to a substantial advance in the control of *V. dahliae* in commercial orchards, particularly for woody plants such as olive for which no chemical control treatments are currently available.

ACKNOWLEDGMENTS

This research was funded by the Spanish Ministry of Science, Innovation and Universities (MICINN; project AGL2016-76240-R) co-financed by the European Union FEDER Funds; and by the Spanish Interprofessional Olive Oil Association. A.M.A. and C.A.B. are holders of 'Formación de Profesorado Universitario' (FPU) and 'Juan de la Cierva-Incorporación' fellowships from the Spanish Ministry of Education, Culture and Sports (MECD) and MICINN, respectively. The authors also thank M. Reva, C. Medina and F. Vañó for their assistance in the experimental fields. We thank J. Mercado-Blanco (IAS-CSIC, Córdoba) for kindly supplying the *Pseudomonas fluorescens* strain PICF04.

SUPPLEMENTARY FIGURES

Experiment II. A) Experimental plot. B) Olive trees with different severity level of Verticillium wilt of olive.

Experiment II. A) Treatments application. B) Soil sampling with a soil auger.

Experiment III. A) Highly affected stands Verticillium wilt of olive used for the experiment establishment. B) Replanting a dead olive tree by *Verticillium dahliae* to plant a young olive tree used in the experiment.

CHAPTER 3

FO12 as a potential biocontrol agent

3. A non-pathogenic strain of *Fusarium oxysporum* as a potential biocontrol agent against Verticillium wilt of olive

ABSTRACT

The non-pathogenic *Fusarium oxysporum* FO12 strain isolated from cork (*Quercus suber*) was one of the most effective treatments against *Verticillium dahliae* from among more than 200 natural compounds evaluated in previous studies. Based on the results of previous work, the present study aims to determine the potential of FO12 as a biological control agent against Verticillium wilt of olive (VWO). To this end, several *in vitro* and *in vivo* experiments were carried out to evaluate the effectiveness of FO12 against *V. dahliae*. Dual cultures were conducted, showing a significant reduction in the mycelial growth of *V. dahliae*. However, inhibition zones were not observed *in vitro*. Four different culture fractions of FO12 were prepared to evaluate their effect on the reduction of viable propagules of *V. dahliae* in soil and on the disease progression in inoculated plants. Treatments with crude culture broth of FO12 resulted in a total reduction of the inoculum density of *V. dahliae* in naturally infested soils as well as in the progression of VWO under controlled conditions. Treatments with conidial suspension or chlamydospores were also effective in reducing the inoculum density in the soil and the disease severity in olive plants. This work provides useful information about the efficacy of several FO12-based culture fractions against VWO under controlled conditions, which could be the basis for the development of effective commercial formulations in the coming future.

This chapter has been published in:

Mulero-Aparicio A, Agustí-Brisach C, Varo A, López-Escudero FJ, Trapero A. 2019. A non-pathogenic strain of *Fusarium oxysporum* as a potential biocontrol agent against Verticillium wilt of olive. *Biological Control* **139.** doi.org/10.1016/j.biocontrol.2019.104045

1. INTRODUCTION

Olive (*Olea europaea* subsp. *europaea*) is broadly cultivated throughout the Mediterranean basin as well as in other regions worldwide with similar climatic conditions. Among diseases affecting olive trees, Verticillium wilt of olive (VWO), caused by the soil-borne fungus *Verticillium dahliae*, is the most threatening disease of this crop (López-Escudero and Mercado-Blanco, 2011). The importance of VWO has increased markedly due to three main factors: i) the establishment of new olive orchards in fields previously cropped with other susceptible hosts, ii) the use of infected plant material, and iii) the introduction of irrigation in olive orchards in intensive cultivation systems (López-Escudero and Mercado-Blanco, 2011). Consequently, this has resulted in major economic losses in the main olive growing areas during recent decades (Blanco-López et al., 1984; López-Escudero and Mercado-Blanco, 2011).

Verticillium dahliae produces resting structures called microsclerotia, which can remain viable in the soil for many years. Microsclerotia germinate in response to root exudates and favorable environmental conditions, giving rise to infective hyphae that penetrate the plant roots and grow towards the vascular system, producing mycelia and conidia (Pegg and Brady, 2002). The growth of the pathogen within the xylem vessels induces physical and/or biochemical defensive mechanisms, such as the formation of tyloses or gel plugs inside infected xylem vessels, contributing to the obstruction of the xylem lumen (Baídez et al., 2007), or the production of proteins involved in cell death (Xie et al., 2013). Together, these factors contribute to the onset of symptoms, causing extensive dieback and/or heavy defoliation of twigs and branches in olive (López-Escudero and Mercado Blanco, 2011).

The pathogen is distributed worldwide, and it is able to produce vascular infections in a wide range of host species, including annuals, herbaceous crops and weeds, as well as fruit, landscape and ornamental trees and shrubs (Pegg and Brady, 2002). In the *V. dahliae* populations that infect olive trees, two virulence groups or pathotypes have been identified; one is highly virulent and is referred to as defoliating (D), and the other is moderately virulent and is referred to as non-defoliating (ND) (Jiménez-Díaz et al., 2012). To date, there is not one single truly effective method to control VWO. Therefore, pathogen dispersal and the severity of infections can only be controlled using an integrated management strategy (López-Escudero and Mercado-Blanco, 2011). Within this strategy, the use of

biological control agents (BCAs) has been considered to be an important eco-friendly approach. Indeed, several studies have been conducted to evaluate the use of BCAs against *V. dahliae* in different hosts, such as *Arabidopsis thaliana* (Tjamos et al., 2005), rape (Rybakova et al., 2015), cotton (Xue et al., 2013) and tomato (Tjamos et al. 2004). However, to date, few studies on the use of BCAs against VWO have been reported. Endophytic bacteria such as *Serratia plymuthica* (isolate HRO-C48) (Müller et al., 2008), *Paenibacillus alvei* (isolate K165) (Markakis et al., 2016) and *Pseudomonas* spp. (Mercado-Blanco et al., 2004; Triki et al., 2012) are the most studied BCA against *V. dahliae* in olive. Moreover, *Trichoderma* spp. have also been reported as promising BCAs against VWO (Lima et al., 2007; Jiménez-Díaz et al., 2009).

The use of non-pathogenic isolates of *Fusarium oxysporum* against Fusarium wilts has been extensively studied (Benhamou et al., 2002; Shishido et al., 2005; Edel-Hermann et al., 2011; Aimé et al., 2013). Furthermore, studies on the interaction between non-pathogenic *F. oxysporum* strains and *V. dahliae* isolates have been performed in herbaceous crops with promising results on the control of disease (Pantelides et al., 2009; Angelopoulou et al., 2014; Veloso et al., 2016). In olive, Varo et al. (2016b) recently performed a large-scale screening of different microorganisms for the biocontrol of VWO in which the non-pathogenic *F. oxysporum* isolate FO12 was described as one of the most effective BCAs against the pathogen. This strain was able to completely reduce the viability of *V. dahliae* microsclerotia in naturally infested soil and to effectively suppress VWO under controlled conditions. Although these previous results have indicated a significant advance in the biocontrol of VWO, further research is needed to increase our knowledge of the potential of this strain as a BCA against the disease. In this way, the main goal of this study was to evaluate the potential of the non-pathogenic FO12 strain as a BCA against *V. dahliae* isolates associated with VWO by means of *in vitro* and *in vivo* experiments under controlled conditions. Therefore, the specific objectives of this study were as follows: i) to evaluate the effect of FO12 against *V. dahliae* isolates by *in vitro* sensitivity tests; ii) to evaluate the effect of FO12 on the inoculum density of *V. dahliae* in naturally infested soils; and iii) to evaluate the effect of FO12 against VWO by *in vivo* tests using potted plants of olive inoculated with *V. dahliae* defoliating pathotypes. This current study could be the basis for the development of effective commercial formulations in the coming future.

3.2. MATERIALS AND METHODS

3.2.1. Fungal isolates and culture conditions

Two *V. dahliae* isolates were used in this study: the mildly virulent isolate V004, classified as a non-defoliating pathotype (Blanco-López et al., 1989), and the highly virulent isolate V024, classified as a defoliating pathotype (Varo et al., 2016b). The non-pathogenic *F. oxysporum* isolate FO12 was used as a BCA (Varo et al., 2016a). All isolates belonging to the fungal collection of the Department of Agronomy at the University of Córdoba (Spain). Single-spore isolates were prepared prior to use by means of the serial dilution method (Dhingra and Sinclair, 1995) and were maintained on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants at 4ºC. Seven-day-old single spore cultures of *V. dahliae* and *F. oxysporum* isolates incubated on PDA at 25ºC in the dark and with a 12-h photoperiod of fluorescent light, respectively, were used as inoculum sources.

3.2.2. In vitro effect of FO12 against *V. dahliae*

3.2.2.1. Dual culture assay

Mycelial plugs (7-mm diameter) of each isolate were obtained from the margin of 7-day-old colonies grown on PDA as described above. For each *V. dahliae* isolate (V004 and V024), a mycelial plug was placed 1 cm from the border of a Petri dish (9 cm in diameter). Subsequently, one mycelial plug of *F. oxysporum* isolate FO12 was placed in the same position on the other side of the Petri dish. Additionally, Petri dishes inoculated only with mycelial plugs of each *V. dahliae* isolate were used as a control. All Petri dishes were incubated at 25ºC for 13 days with a 12-h photoperiod of fluorescent light. There were three replicated Petri dishes per treatment, and the experiment was conducted twice. After 13 days of incubation, the largest and smallest diameters of the colonies of each *V. dahliae* isolate were measured using a digital caliper, and the mean data were converted to radial growth rate (mm day^{-1}). The inhibition percentage of mycelial growth was calculated using the following formula: Mycelial growth inhibition (MGI) (%) = [(RGR-rgr)/RGR] × 100, where rgr is the radial growth of *V. dahliae* in dual cultures with *F. oxysporum* FO12, and RGR is the radial growth rate of the control treatment.

3.2.2.2. Effect of FO12 crude culture broth on mycelial growth of *V. dahliae*

To check the presence of chemical compounds produced by FO12 during the incubation period (i.e., secondary metabolites) with antifungal activity against *V. dahliae*, a crude culture broth (CCB) of *F. oxysporum* FO12 was evaluated. To produce this CCB, a 2-l Erlenmeyer flask containing 1 l of potato dextrose broth (PDB; Difco Laboratories®) was inoculated with a conidial suspension from a 7-day-old PDA mycelial culture of FO12. The conidial concentration of the inoculated PDB flask was adjusted to 2×10^5 conidia ml^{-1} based on hemocytometer counts, and the conidial suspension was incubated at 25°C in an orbital shaker (Grant bio PSU-20i, Grant Instruments, Cambridge, UK) at 90 rpm for 7 days. Equal volumes of FO12 CCB were sterilized by two different methods: (i) by autoclaving at 120ºC for 20 min to kill the living structures of the fungus and (ii) by filtration to remove living structures from the extract, preventing the destruction of possible active antifungal metabolites. To filter the extract, 5-ml samples were filter sterilized using a 0.2-µm pore size syringe filter (Sartorius Stedium Biotech; Goettingen, Germany).

To achieve final concentrations of 50, 500 and 5,000 mg l^{-1} of CCB, appropriate volumes of the respective sterile CCB were added to 2-l Erlenmeyer flasks filled with sterilized PDA (approximately 50ºC) and homogenized for 2 min using a magnetic rotor (Agimatic-N, JP-Selecta, Barcelona, Spain). Subsequently, the medium was poured into Petri dishes (9 cm in diameter; 25 ml $dish^{-1}$). For each *V. dahliae* isolate, mycelial plugs (7 mm in diameter) obtained from the margin of 7-day-old actively growing cultures on PDA were transferred to the center of the sterile CCB-amended plates. Control PDA plates were prepared similarly by adding sterile PDB instead of the CCB. The dishes were incubated as previously described for 2 weeks. There were five replicated plates for each *V. dahliae* isolate, sterilization method and CCB concentration, and the experiment was conducted twice. Colony measurements, radial growth rate and inhibition (%) were determined as described for the dual culture assay.

3.2.3. Effect of FO12 on inoculum density of *V. dahliae* in naturally infested soil

3.2.3.1. Soil samples

Samples of soils naturally infested with *V. dahliae* were collected from two commercial olive orchards that were severely affected by VWO, located in Villanueva de la Reina (soil 1) (UTM coordinates X: 38.012845; Y: 3.909219) and Lebrija (soil 2) (UTM coordinates X: 36.918400; Y: -6.077705) from Jaen and Seville provinces, respectively (Andalucía, southern Spain). Five soil sub-samples of ≈500 g were collected in each orchard 1 m from the trunk and from the upper 30 cm using a cylindrical soil auger. Sub-samples from each orchard were mixed, air-dried at room temperature until completely dry, and sieved (0.8 mm diameter) to remove large particles (Trapero et al., 2013a).

3.2.3.2. Preparation of FO12-based culture fractions

A total of four different culture fractions of *F. oxysporum* isolate FO12 were obtained: (i) *Adjusted crude culture broth* (ACCB). The ACCB consisted in the CCB prepared as described above, which wasadjusted at 10^6 conidia ml^{-1} with sterile PDB using a hemocytometer; (ii) *Supernatant*. A 200-ml sample of this CCB was centrifuged for 15 min at 10,000 rpm (Sigma 6-16K centrifuge, SciQuip, London, UK), and the supernatant was separated and diluted to 20% with sterile PDB; (iii) *Conidial suspension*. The pellets obtained during the centrifugation process to obtain the supernatant were re-suspended with sterile PDB, and the conidial suspension was adjusted at 10^6 conidia ml^{-1} using a hemocytometer; and (iv) *Chlamydospore suspension*. A chlamydospore suspension was prepared using a soil extract medium according to Bennet and Davis (2013), and adjusted at 10^5 chlamydospore ml^{-1} with sterile distilled water.

3.2.3.3. Soil treatment and quantification of *V. dahliae* inoculum density

Three 100-ml plastic pots with holes drilled in the base for drainage were filled with 60 g of the two naturally infested air-dried soils and were irrigated with 30 ml of the FO12-based culture fractions described above. Three additional plastic containers filled with 60 g of the two naturally infested soils and watered only with sterile distilled water were used as controls. Subsequently, all containers were hermetically covered and incubated for 24 hours at 25ºC. After incubation, soil samples were removed from the plastic pots and air-dried at room temperature. There were three replicated plastic pots for each soil and the FO12-based

compound or control treatments, resulting in 30 plastic pots in total. The experiment was conducted twice.

The inoculum density of *V. dahliae* in each soil sample was estimated by wet sieving (Huisman and Ashworth, 1974) by splitting each soil sample across 10 plates of a modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977). MSPA plates were incubated for 14 days at 24ºC in the dark. After incubation, soil residues were removed with tap water, and colonies of *V. dahliae* were counted under a stereoscopic microscope (Nikon SMZ-2T, Tokyo, Japan). The inoculum density in each soil sample was estimated from the number of *V. dahliae* colonies and was expressed as the number of propagules per gram of air-dried soil (ppg), and the percentage of inoculum density reduction was calculated.

3.2.4. Effect of FO12 on Verticillium wilt of olive

3.2.4.1. Inoculum preparation, plant material and fungal inoculation

The inoculum of *V. dahliae* was produced in a cornmeal sand mixture prepared by mixing dry sand, cornmeal and distilled water at a 9:1:2 weight proportion, respectively. Subsequently, 2-l flasks were filled with 1 kg of cornmeal sand mixture and inoculated with 50 mycelial plugs (5 mm in diameter) of *V. dahliae* isolate V024 grown on PDA at 25ºC for 10 days in the dark. The flasks were shaken once a week to favor the homogeneous colonization of the cornmeal sand mixture by the fungus (Varo et al., 2016b). After 4 weeks, the inoculum density in the cornmeal sand mixture flasks was calculated as colony-forming units (CFUs) by plating several serial dilutions of the cornmeal sand mixture on PDA plates. The average of the CFU values was obtained from 15 PDA replicated plates.

Six-month-old rooted cuttings of the olive cv. Picual (highly susceptible) (López-Escudero et al., 2004), obtained from a commercial nursery, were used as the host. Olive plants were transplanted from their original substrate in plastic pots (0.8 l) containing sterile peat moss with 20% (weight/weight) corn meal sand mixture infested with the *V. dahliae* isolate V024. The inoculum density of the pathogen in the final potting mixture was 10^7 CFU g^{-1}, as previously described.

3.2.4.2. Plant treatment and experimental design

All the treatments using FO12-based culture fractions (ACCB, supernatant, conidial suspension, and chlamydospore suspension) evaluated against VWO in the following *in planta* experiments are described in Table 1.

Experiment I. The following five treatments were conducted: (i) plants irrigated with 300 ml of ACCB twice, 14 days and one day before inoculation with *V. dahliae* (T1); (ii) olive roots treated by dipping in ACCB immediately after inoculation (T2); (iii) plants irrigated with 300 ml of ACCB immediately after inoculation (T3); (iv) plants irrigated with 300 ml of supernatant immediately after inoculation (T4); and (v) plants irrigated with 300 ml of conidial suspension immediately after inoculation (T5). Untreated plants grown in inoculated peat moss with *V. dahliae* were used as the inoculated control (positive control). In addition, plants grown in non-inoculated sterile peat moss and irrigated with 300 ml of sterile distilled water were used as negative control.

Experiment II. In this experiment, a total of six treatments were tested, comprising the T1, T3, T4 and T5 described in *Experiment I*, and two additional treatments: (i) plants irrigated with 300 ml of chlamydospore suspension immediately after inoculation (T6) and, (ii) foliar treatment by spraying the plants with ACCB 14 days and the day before inoculation with *V. dahliae* (T7). Positive and negative control treatments were included as in *Experiment I*. After inoculation, all plants were incubated in a growth chamber at 22 ± 2°C in the dark and 100% relative humidity for 4 days. Subsequently, light and humidity parameters were modified, i.e., a 12-h photoperiod of fluorescent light [10,000 lux] and 70%, respectively, and maintained until the end of the experiment. Plants were irrigated three times per week. In each experiment, completely randomized design was used with FO12-based compound/treatments and controls (positive and negative) as independent variables and ten olive plants per treatment as replications. Both experiments were conducted twice.

3.2.4.3. Disease severity assessment

Disease severity was evaluated based on the percentage of affected plant tissues such as leaves and shoots showing symptoms of chlorosis, necrosis and/or defoliation. To this end, plants were assessed weekly for 16 weeks after inoculation

using a 0 to 16 rating scale. This scale estimated the percentage of affected tissue using four main categories or quarters (≤25, 26–50, 51–75, and 76–100%) with four values per category. Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) are linearly related to the percentage of affected tissue (Y) by the equation Y = 6.25X - 3.125 (Varo-Suárez et al., 2018). At the end of the disease assessment, the relative area under the disease progression curve (RAUDPC) was calculated from the disease severity values by the trapezoidal integration method (Campbell and Madden, 1990). In addition, disease incidence (DI) and mortality were recorded as the percentage of symptomatic or dead plants, respectively, to assess the intensity of the response (López-Escudero et al., 2004).

At the end of the experiments, three symptomatic plants per experiment and treatment combinations were selected to perform re-isolations to confirm the fungal infection. For this purpose, affected roots and stems were selected, washed under running tap water, and surface sterilized by immersing them in 0.5% solution of commercial bleach (Cl at 50 g L^{-1}) for 1 min. Subsequently, small wood fragments were plated on PDA acidified with lactic acid (2.5 ml of 25% [vol/vol] per liter of medium) to avoid bacterial contamination and were incubated at 24°C in darkness for 6 days.

3.2.5. Data analysis

Analysis of variance (ANOVA) was performed according to the experimental design of each trial. In all experiments, data were tested for normality, homogeneity of variances, and residual patterns, which proved their suitability for the statistical analysis. Treatments that did not show symptoms (mean values of 0.0) were not included in the analysis. Treatment means were compared using Fisher´s protected least significant differences (LSD) at $P = 0.05$. Final DI and mortality values were analyzed by a Chi-squared test for multiple comparisons for the proportions at $P= 0.05$. All data in this study were analyzed using Statistix 10 (Analytical Software, Tallahassee, FL).

3.3. RESULTS

3.3.1. In vitro effect of FO12 against *V. dahliae*

3.3.1.1. Dual culture assay

The ANOVA results showed that *F. oxysporum* FO12 was able to significantly reduce the radial growth rate of both *V. dahliae* isolates V004 (P = 0.0005) and V024 ($P < 0.0001$) in comparison with their respective controls (Figure 1). For *V. dahliae* isolate V004, the mycelial growth rate was 2.0 and 3.2 mm day^{-1} in the FO12 and control treatments, respectively; for *V. dahliae* isolate V024, the corresponding values were 2.2 and 4.0 mm day^{-1} (Figure 1). At the end of the experiment, although the reduction of *V. dahliae* mycelial growth was observed before the FO12 mycelium contacted the colony of the pathogen, the FO12 colony subsequently overgrew the *V. dahliae* colony (Figure1).

3.3.1.2. Effect of FO12 crude culture broth on mycelial growth of *V. dahliae*

The autoclaved-sterilized CCB only significantly reduced the mycelial radial growth rate for *V. dahliae* isolate V004 (2.8 mm day^{-1}; P = 0.0363) at 500 mg l^{-1} in comparison with the control (0 mg l^{-1}), which showed a 3.1 mm day^{-1} radial growth rate (Figure 2A). However, this type of extract did not significantly reduce the growth rate of *V. dahliae* isolate V024 at any of the tested doses (P = 0.1654). Concerning the filtered-sterilized extract, this significantly increased (P = 0.0004) the mycelial growth of *V. dahliae* isolate V004 at all doses tested (Figure 2B). In this case, the mycelial radial growth rate was 1.6 and 2.5 mm day^{-1} for the control and FO12 treatments, respectively, at 500 mg l^{-1} (Figure 2B). In contrast, this type of extract had no effect on the mycelial growth rate of *V. dahliae* isolate V024 at any of the doses tested (Figure 2B).

3.3.2. Effect of FO12 on the inoculum density of *V. dahliae* in naturally infested soil

The inoculum densities of *V. dahliae* in the control soil samples from Villanueva de la Reina and Lebrija orchards were 150 and 18 CFU g^{-1}, respectively. The treatment with the ACCB of *F. oxysporum* FO12 was the most effective at reducing the inoculum density of the pathogen when it was applied to naturally infested soils. The reduction of viable propagules reached 100% for both soils treated with the FO12 extract. Conidia and chlamydospore suspension treatments also effectively inhibited survival propagules without significant differences from the ACCB treatment. In contrast, a lower reduction of inoculum

density was observed in both soils when they were drenched with supernatant treatment in comparison with the other FO12-based culture fractions ($P < 0.0001$). In this last case, the treatment reduced the inoculum density by 23 and 28% in soil 1 and 2, respectively (Figure 3).

3.3.3. Effect of FO12 on Verticillium wilt of olive

Untreated control plants inoculated with *V. dahliae* isolate V024 showed typical Verticillium wilt symptoms caused by infection by the D pathotype on olive plants of cv. Picual in the two experiments (Figure 7). In *Experiment I*, the first symptoms developed on the inoculated control plants 30 days after inoculation, reaching a final DI of 100% (Figure 4A). Both T3 and T2 were the most effective against VWO (0% DI). T1, T4 and T5 significantly reduced the final DI (43.0, 70.0, and 88.0%, respectively; $P < 0.0001$) compared with the control. Nevertheless, regarding RAUDPC, T4 and T5 were not significantly different from the control, being the least effective treatments in this experiment (61.0 and 48.0%, respectively) (Figure 6A). Concerning mortality, all plants were killed by the pathogen in the inoculated control treatment, while no plants were killed when T3and T2 were applied. The remaining treatments resulted in plant mortality ranged between 14 and 60% (Figure 4A).

In *Experiment II*, the first symptoms on the inoculated control plants appeared 35 days after inoculation. In general, this experiment showed lower disease incidence and severity than those observed in *Experiment I* (Figure 4B; Figure 5B). Control plants inoculated with the pathogen showed 50% DI. T3 and T5 were the most effective treatments (0% DI) (Figure 4B). The T1 significantly reduced the final DI (20%; $P = 0.0001$). T4 and T6 did not reduce the DI compared with the control (63.0 and 33.0%, respectively), although they produced a delay in the symptom onset (42 and 55 days after inoculation, respectively). In contrast, T7 increased the final DI compared with the control treatment (81.8%). Concerning the RAUDPC, T1 and T6 significantly reduced the disease compared with the positive control ($P = 0.0289$; 1.4 and 5.6%, respectively), whereas T4 and T7 did not differ from the positive control (Figure 6B; Figure 7). Regarding mortality, only T4 and T7 had dead plants at the end of the experiment (27% for both treatments) (Figure 4B). In both experiments, T3 was the most effective treatment, whereas T4 was the least effective ($P < 0.0001$). Nevertheless, there were differences between both experiments regarding T5 treatment; it was the least

effective treatment in *Experiment I*, whereas it was one of the most effective treatments in *Experiment II*.

The pathogen was successfully re-isolated from all selected symptomatic plants, confirming the infection by *V. dahliae.*

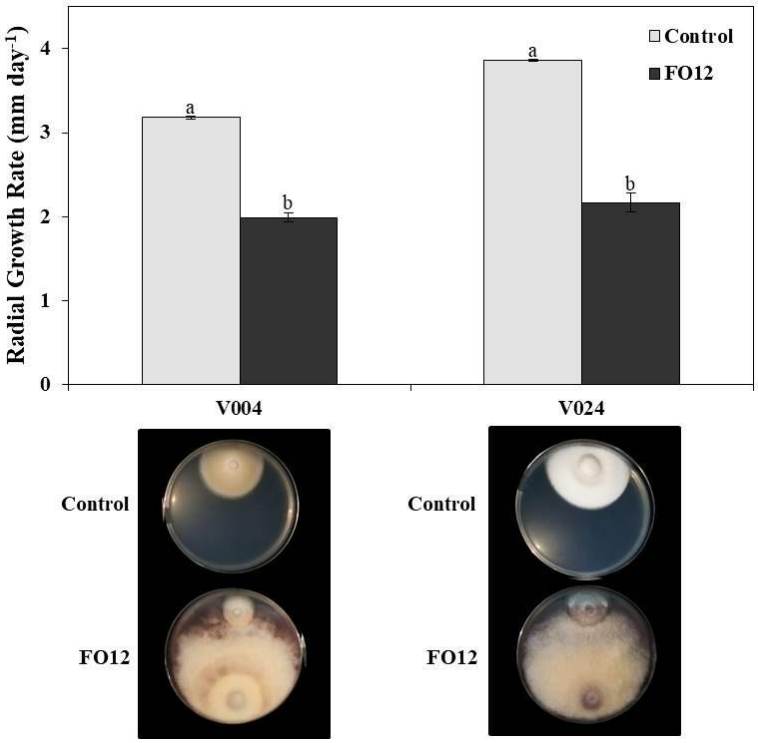


Figure 1. *In vitro* effect of *Fusarium oxysporum* FO12 against *Verticillium dahliae* isolates V004 (non-defoliating pathotype) and V024 (defoliating pathotype) after 10 days of growth on PDA at 25ºC with a 12-h photoperiod of fluorescent light. Bars represent the mean values of six replicated Petri dishes. For each *V. dahliae* isolate, bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean. Pictures below illustrate the mycelial growth of each *Verticillium* isolate in dual cultures against *F. oxysporum* FO12.

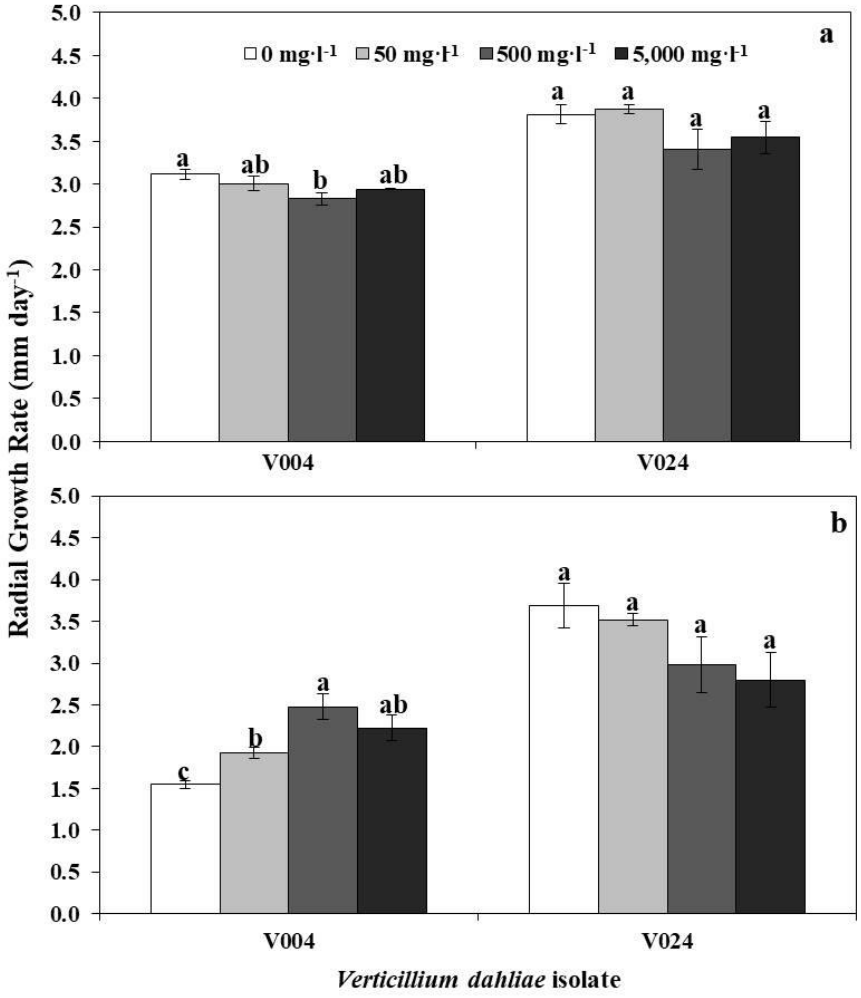


Figure 2. Effect of crude culture broth (CCB) of *Fusarium oxysporum* FO12 sterilized (A) by autoclaving and (B) by filtration, on mycelial growth of *V. dahliae* isolates V004 (non-defoliating pathotype) and V024 (defoliating pathotype) grown for 2 weeks on PDA with 0, 50, 500 and 5,000 mg l^{-1} of CCB. Bars represent the mean values of ten replicated Petri dishes. For each *V. dahliae* isolate and extract type, bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

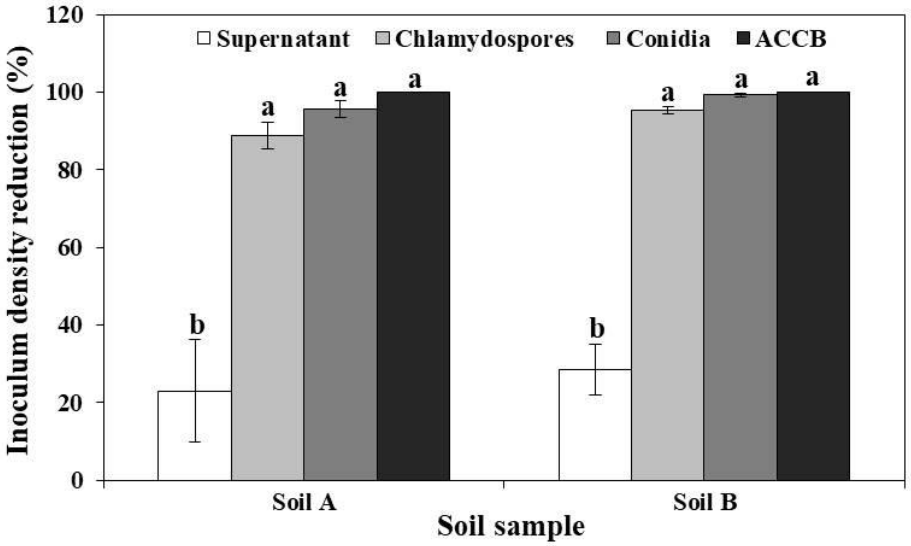


Figure 3. Effect of the four evaluated *Fusarium oxysporum* FO12-culture fractions [Adjusted crude culture broth (ACCB); Supernatant; Conidial suspension; Chlamydospore suspension] on reducing the inoculum density in two naturally infested soils by *Verticillium dahliae*. Treatments were conducted using 100-ml plastic pots filled with 60 g of naturally infested air-dried soil and irrigated with 30 ml of each FO12 treatment. For each treatment, bars represent the mean values of six replicated plastic pots. Soil 1 was obtained from Villanueva de la Reina, and soil 2 was obtained from Lebrija. For each soil, bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

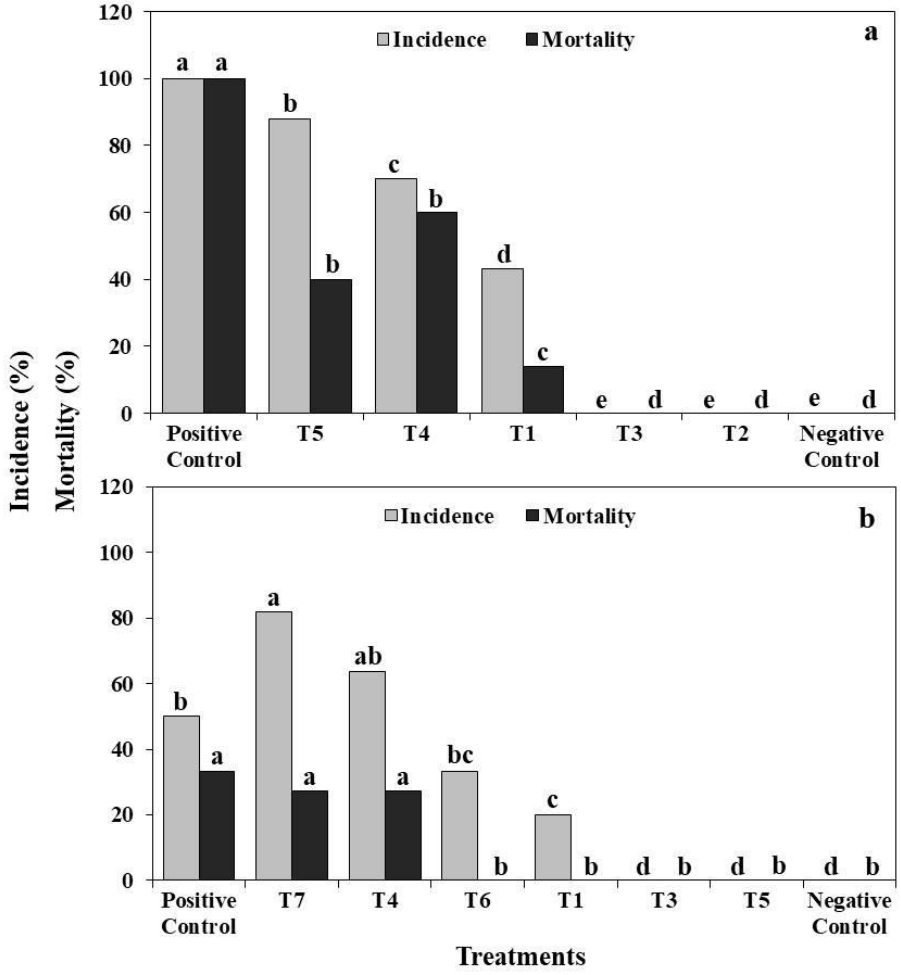


Figure 4. Percentage of disease incidence and mortality *in planta* caused by *V. dahliae* isolate V024 at 16 weeks after inoculation in the *Experiment I* (A) and in the *Experiment II* (B). For each experiment, bars are the means of ten replicated olive plants per treatment (see Table 1). For each disease parameter, bars with a common letter do not differ significantly according to the multiple comparisons for the proportions Chi-square test at $P = 0.05$.

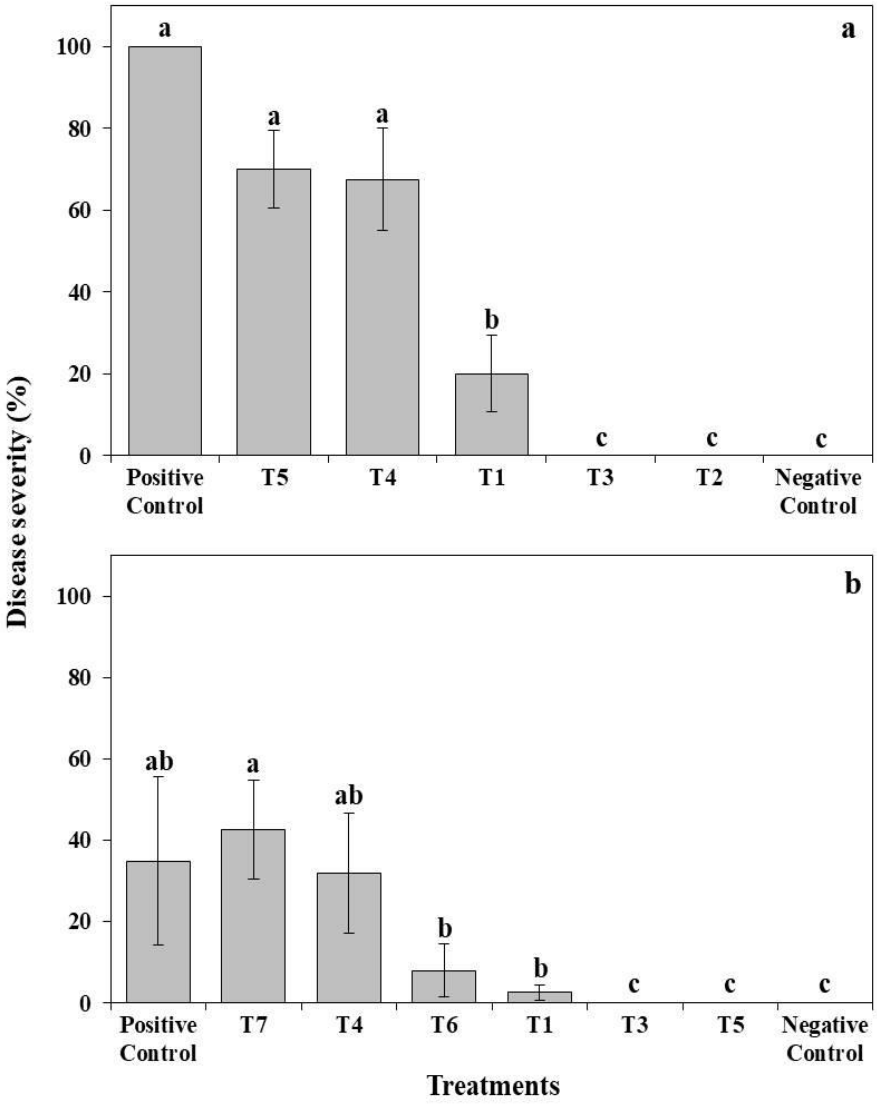


Figure 5. Final disease severity (%) in olive plants at 16 weeks after inoculation with *V. dahliae* isolate V024 in the *Experiment I* (A) and in the *Experiment II* (B). Disease severity was evaluated using a rating scale of 0 to 16 (0 = no lesions, 16 = 94-100% of canopy with symptoms. For each experiment, bars represent the means of ten replicated plants per treatment (see Table 1). Bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on the bars are the standard error of the mean.

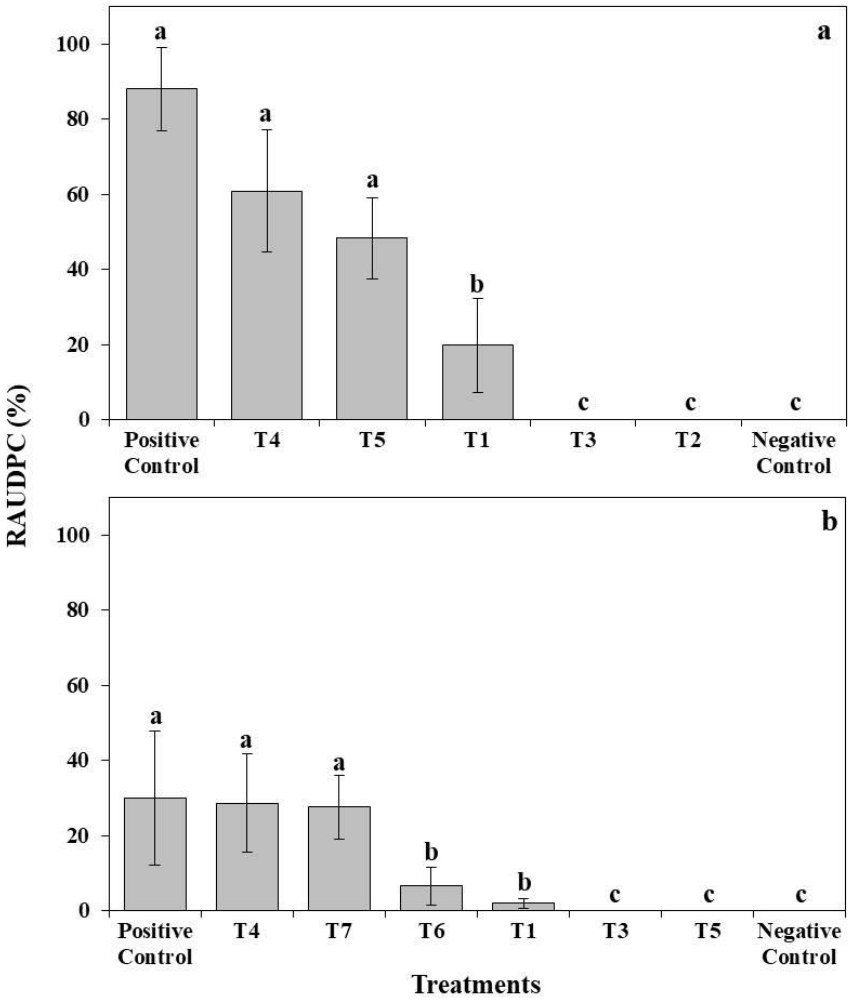


Figure 6. Relative area under the disease progression curve (RAUDPC, %) on olive plants at 16 weeks after inoculation with *V. dahliae* isolate V024 in the *Experiment I* (A) and in the *Experiment II* (B). For each experiment, bars represent the means of ten replicated plants per treatment (see Table 1). Bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

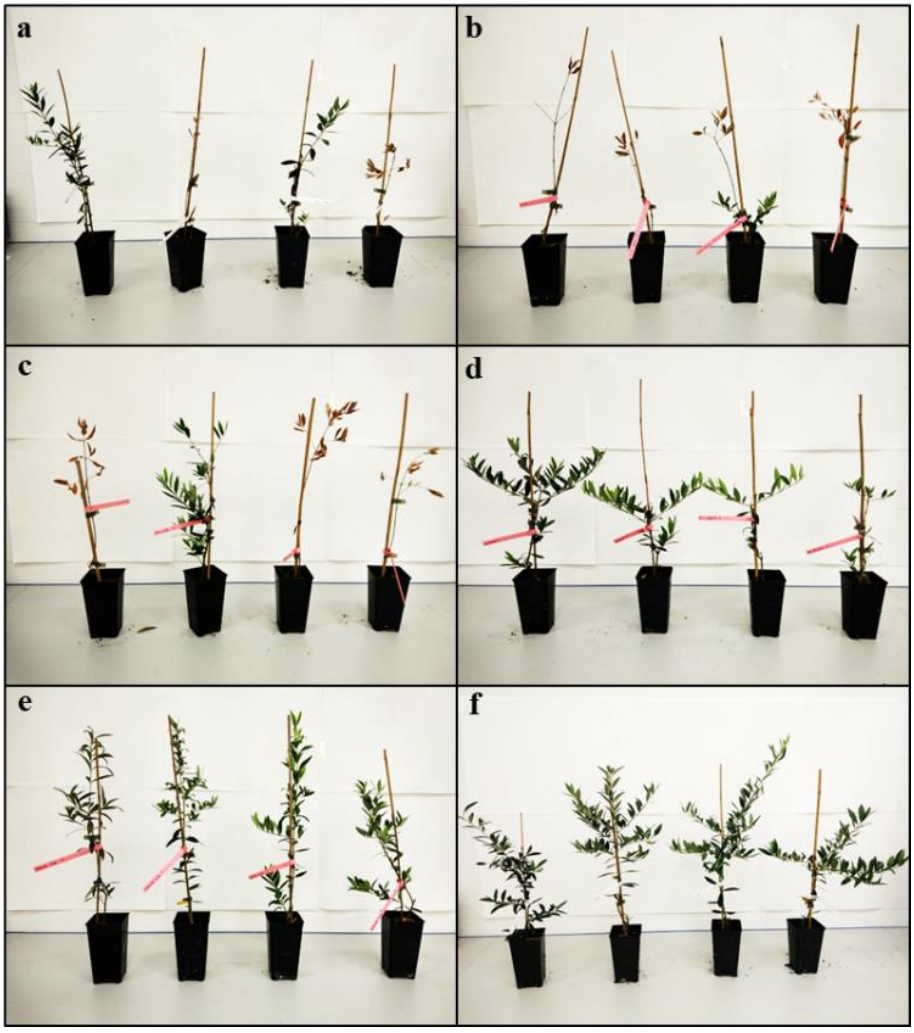


Figure 7. Differences in symptoms development of VWO in the different lots of olive potted plants at 16 weeks after inoculation with *V. dahliae* isolate V024. Plants were subjected to the following treatments: (A) positive control plants; (B) foliar treatments with 300 ml of ACCB, 14 and one day before inoculation (T7); (C) plants irrigated with 300 ml of supernatant (T4); (D) plants irrigated with 300 ml of chlamydospore suspension (T6); (E) plants irrigated with 300 ml of ACCB immediately after inoculation (T3); and (F) negative control (see Table 1).

Table 1. Treatments with FO12-based culture fractions evaluated against VWO by means of *in planta* experiments.

Experiment[a]	Code	Treatment
I	T1	Plants irrigated with 300 ml of ACCB, 14 and one day before inoculation
	T2	Plants treated by root dipping in ACCB immediately after inoculation
	T3	Plants irrigated with 300 ml of ACCB immediately after inoculation
	T4	Plants irrigated with 300 ml of supernatant
	T5	Plants irrigated with 300 ml of conidial suspension (10^6 conidia ml^{-1})
II	T1, T3, T4 and T5	Same treatments described for the *Experiment I*
	T6	Plant irrigated with 300 ml of chlamydospore suspension (10^5 chlamydospores ml^{-1})
	T7	Foliar treatment with ACCB, 14 and one day before inoculation

[a] In both experiments, positive and negative control were included. Positive control: non-treated and inoculated plants; Negative control: non-treated and non-inoculated plants.

3.4. DISCUSSION

The non-pathogenic FO12 strain isolated from cork (*Quercus suber*) was one of the most effective treatments against VWO from among more than 200 natural compounds evaluated in previous studies conducted by our research group (Varo et al. 2016b, 2017; Varo-Suárez et al., 2018). Because these previous studies were based on qualitative assessments to enclose more effective compounds against the target disease, the next step should be to quantify the effect of FO12 against pathogen development and disease progression. In this way, the main goal of this study was to evaluate the potential of the non-pathogenic FO12 strain as a BCA against *V. dahliae* isolates associated with VWO by means of *in vitro* and *in vivo* experiments under controlled conditions. The experiments in this study were based on previous work in which non-pathogenic isolates of *F. oxysporum* against *V. dahliae* were evaluated in several pathosystems, such as eggplant (Pantelides et al., 2009) or pepper (Veloso et al., 2016).

In the dual culture experiment, the reduction in the mycelial growth of the defoliating and non-defoliating *V. dahliae* isolates in the presence of FO12 was 62 and 56%, respectively (Figure 1). At the end of the experiment, FO12 overgrew the *V. dahliae* colony and covered the plate completely, preventing the growth of the pathogen. In this regard, Varo et al. (2016b) also indicated that FO12 mycelia grew over the *V. dahliae* colony at the end of their dual culture experiments. Although no inhibition zones were observed, the initial reduction of mycelial growth observed before FO12 overgrew the colonies of *V. dahliae* suggests that antibiosis should be considered one of the potential modes of action involved in its antagonistic effect. The mycelial overgrowth of FO12 over colonies of *V. dahliae* suggests that competition for space and nutrients could be the main mode of action involved in the mycelial growth reduction of the pathogen. In this way, Malandraki et al. (2008) obtained similar results because the non-pathogenic *F. oxysporum* strain F2 did not show inhibition zones in dual cultures against *V. dahliae*. In spite of these results, further research should be conducted to determine whether parasitism behavior could be associated with the effect of FO12 when its mycelia overgrow over the colonies of the pathogen.

When CCB was added to PDA plates, the mycelial radial growth rate was significantly reduced only in the treatment in which autoclaved-sterilized CCB was applied at 500 mg l^{-1} for the *V. dahliae* isolate V004 (Figure 2). Moreover, the

mycelial radial growth rate of this isolate was significantly increased when filtered-sterilized CCB was added at any of the doses tested (Figure 2). Several studies have reported the efficacy of different BCA secondary metabolites against pathogenic *Fusarium* spp. (Kavitha and Nelson, 2013; Petrisor et al., 2017) and Verticillium wilts (El-Hadrami et al., 2011; Lozano-Tovar et al., 2017). Nevertheless, this is the first study testing secondary metabolites from a non-pathogenic *F. oxysporum* strain against *V. dahliae*. Our results indicate that metabolites produced by FO12 have little effect on the mycelial growth inhibition of *V. dahliae* isolates. This observation suggests that the FO12 strain should be alive and in simultaneous presence with the pathogen to develop all its potential as a BCA to achieve a high antagonistic effect against the pathogen. This result is in accordance with our observations in the dual culture experiment of this same study. According to Alabouvette et al. (2009), the metabolites produced by a given BCA may have different efficacies depending on the type of metabolite. Thus, although we only tested the effect of FO12 *in vitro* on two isolates of *V. dahliae,* our results suggest that the metabolites produced by FO12 are not toxic against *V. dahliae* when they are produced without the presence of the pathogen. Consequently, as we indicated above, the mode of action of FO12 against pathogen development could be linked with an antagonistic effect based, for example, on nutrient or space competition in the ecosystem. However, we cannot discard the efficacy of the metabolites when they are directly produced as a result of the BCA activity in the environment or in the presence of the pathogen.

Fusarium oxysporum FO12 ACCB applied to naturally infested soils was significantly the most effective treatment tested, being able to completely reduce the inoculum density of the pathogen. In addition, both chlamydospores and conidial suspension treatments were also highly effective in reducing the inoculum density in both soils (Figure 3). In contrast, the supernatant treatment showed little effect. These results support the theory that the secondary metabolites produced by FO12 in the absence of the pathogen have a low effect on the inhibition and that the greatest control effect of FO12 is only achieved when viable culture fractions are incorporated into the soil. Our results suggest that the reduction of inoculum density of the pathogen observed when ACCB, chlamydospores or conidial suspensions were applied to the soil is probably due to the competition between FO12 and *V. dahliae* for space and nutrients.

Regarding the effect of the FO12-based culture fractions in controlling VWO, the most effective treatment was the ACCB applied by irrigation just after

inoculation (T3). These results are similar to those obtained by Varo et al. (2016b), who demonstrated that ACCB of FO12 reduced disease progression by 100%. The treatment with chlamydospore suspension (T6) had an intermediate effect, which may be due to the lower concentration of inoculum in this treatment in comparison with ACCB. The treatment with the conidial suspension (T5) showed contradictory results between the experiments. In *Experiment I*, this treatment was less effective than in *Experiment II*, possibly because fungal structures from the pellet in *Experiment I* were damaged and could not achieve effective control. Another reason for this fact could be that the pressure of the disease in *Experiment II* was lower than that in *Experiment I*, as was shown in the final level of disease severity reached in positive control plants (Figure 5). However, the results obtained in *Experiment II* are more in accordance with the effect observed in the remaining experiments carried out in this study with the same culture fraction. The lowest control of VWO was observed when olive plants were treated with the FO12 supernatant (T4) which has no living structures of the BCA. These results suggest that the presence of viable BCA propagules is required to achieve effective control of the disease. In addition, these results are in agreement with those obtained in our *in vitro* experiments in which FO12 was most effective against *V. dahliae* in dual cultures when inoculated as actively growing mycelial plugs compared with the incorporation of sterilized CCB.

As far as we know, this work represents the first report testing the effect of different culture fractions obtained from a non-pathogenic strain of *F. oxysporum* against *V. dahliae* in naturally infested soils and on the onset and progression of VWO. Our results showed that the *F. oxysporum* FO12 strain has great potential as a BCA against *V. dahliae* by reducing the inoculum density of the pathogen in naturally infested soils and subsequently reducing the possibilities of root infections. Therefore, in this study, FO12 was able to prevent *V. dahliae* infections of olive under controlled conditions. Chlamydospores are known to be a viable resting structure under stress conditions which suppose a beneficial trait for its use under natural field conditions. Interestingly, the capacity of this strain to form chlamydospores and the effectiveness of this fungal structure in reducing the inoculum density and consequently, the disease progression, supports the real potential of this BCA for its use in commercial olive orchards. However, the evaluation of different FO12-based formulations for its commercial use under natural field conditions should be widely evaluated in future studies.

Although defining the modes of action of the FO12 strain was not the main goal of this study, our results suggest that some well-known mechanisms involved in the antagonistic effect of FO12 could play an important role against *V. dahliae*. Fravel et al. (2003) reported that non-pathogenic strains of *F. oxysporum* used as BCA can present several modes of action, such as competition for space and nutrients, antibiosis and the induction of resistance of plant defenses. To summarize, this study suggests that part of the inhibition effect of FO12 could be due to competition for space and nutrients, as was shown in the dual culture and soil experiments. Similar to our results, Pantelides et al. (2009) reported that the non-pathogenic *F. oxysporum* isolate F2 was able to compete for nutrients and space on the root surface of eggplants. On the other hand, although our results showed that secondary metabolites from FO12 had little effect against *V. dahliae*, an antibiosis effect of secondary metabolites produced by FO12 during the colonization of the soil or in simultaneous presence with the pathogen should not be discarded. Thus, there is potential that inhibitory metabolites are not constitutively produced, but they could be produced only in the presence of the pathogen, so they would not be produced in the liquid media tested in our experiments. On the other hand, the use of a different culture media could have allowed the production of different secondary metabolites. However, our results suggest that the inhibition effect observed in both soil and *in planta* experiments does not depends on the culture medium used since the antagonistic effect was also observed when the conidial and chlamydospore suspensions were applied separately from the secondary metabolites which are present only in CCB. Likewise, parasitic behavior of FO12 or its effect on the induction of resistance of plant defenses cannot be discarded since previous studies with the non-pathogenic *F. oxysporum* strain Fo47 have demonstrated its effect inducing the systemic resistance on pepper plants against *V. dahliae* (Veloso et al., 2016). Nevertheless, further specific research is needed to determine the modes of action of FO12 in olive plants against VWO, and these should be the goal for future research studies on this strain and pathosystem.

Finally, it is important to discuss the non-pathogenic character of the FO12 strain, which has already been evaluated in pathogenicity tests for a wide range of hosts (i.e. cotton, olive, pepper, tomato, melon, eggplant, lettuce, peas, sunflower, etc.) inoculated with FO12. In all cases, no symptoms associated with Fusarium wilt were observed several weeks after inoculation (Mulero-Aparicio et al. *unpublished results*). In addition, previous studies have demonstrated that the non-

pathogenic strains of *F. oxysporum* lack accessory chromosomes that regulate pathogenicity (Ma et al., 2010; van der Does et al., 2016; van Dam et al., 2017). Therefore, molecular characterization of the FO12 strain needs to be conducted to determine whether this strain contains packages of pathogenicity genes in its genome and to consequently discard the possibility that this isolate becomes pathogenic over time.

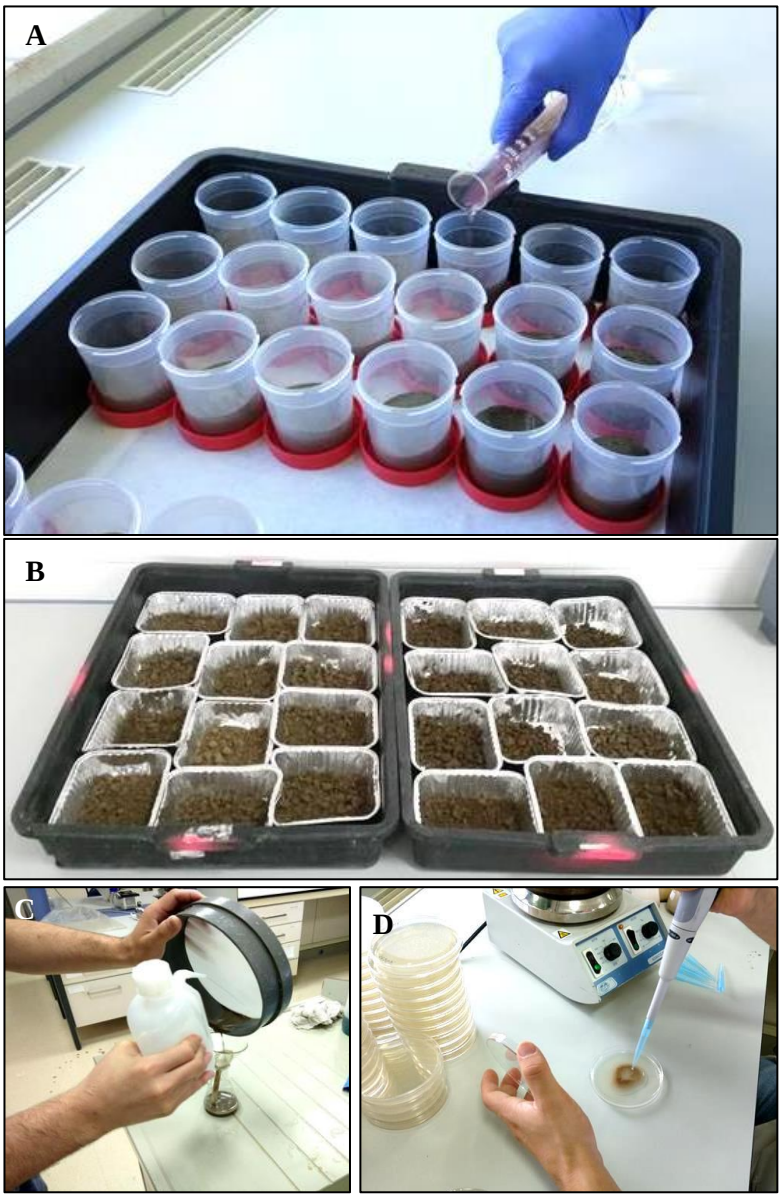
3.5. CONCLUSIONS

This work provides useful information about the efficacy of several FO12-based culture fractions against VWO under controlled conditions. Our results demonstrate the potential effect of FO12 to control the disease. The conclusions obtained here are essential to planning future experiments to evaluate its effect against VWO under semi-controlled and under natural field conditions. Furthermore, this work provides a new insight of an integrated way to study the complete potential of a BCA against *V. dahliae* in naturally infested soils through *in vitro* and *in vivo* experiments as an essential part of the development of an eco-friendly treatment for the control of VWO. In fact, if we succeed in further studies expected in this research to increase our knowledge about the modes of action of FO12 and the determination of its non-pathogenic character, this strain could be a putative candidate as a commercial BCA in the coming future.

ACKNOWLEDGMENTS

This research was funded by the Spanish Ministry of Economy, Industry and Competitivity (MINECO; project AGL2016-76240-R), co-financed by the European Union FEDER Funds. A.M.A. and C.A.B. are holders of 'Formación de Profesorado Universitario' (FPU) and 'Juan de la Cierva-Incorporación' fellowships from the Spanish Ministry of Education, Culture and Sports (MECD) and MICINN, respectively.

SUPPLEMENTARY FIGURES



Evaluation of FO12 culture fractions against *Verticillium dahliae* in naturally infested soil. A) Soil treatment with FO12 culture fractions. B) Incubation of treated soil at room temperature until complete drying. C) Wet sieving. D) Transference of soil suspension into MSPA plates.

Evaluation of FO12 culture fractions against Verticillium wilt of olive in potted olive plants artificially inoculated with *Verticillium dahliae*. A) Solid inoculum of *V. dahliae* prepared in corn meal sand mixture. B) Final potting mixture preparation by mixing the inoculum with sterile peat moss. C) Plant treatment by irrigation with FO12 culture fractions.

CHAPTER 4

Modes of action of FO12

4. The role of volatile organic compounds and rhizosphere competence in mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt

ABSTRACT

Verticillium wilts caused by *Verticillium* spp. are among the most challenging plant diseases to control and affect numerous hosts worldwide. Due to the lack of effective, conventional control methods, integrated control strategies provide a promising approach to manage these diseases. The non-pathogenic *Fusarium oxysporum* strain FO12 was reported in previous studies to be an effective biocontrol agent against *Verticillium dahliae*, however its mode of action remains to be elucidated. In this study, complementary *in vitro* and *in vivo* experiments were conducted in order to explore the implications of inhibitory substances and rhizosphere competence in antagonistic effects of FO12 against *V. dahliae* and *V. longisporum*. Volatile organic compounds and soluble substances produced by FO12, which caused significant inhibition of mycelial growth and microsclerotia viability in the two tested *Verticillium* species, were identified by means of gas and liquid chromatography-mass spectrometry. We showed that the antagonistic effect of *F. oxysporum* FO12 is partially due to the production of bioactive compounds such as 3-methyl-1-butanol and 2-methyl-1-butanol, among others. Several metabolic pathways of FO12 were altered upon contact with *V. dahliae* ELV22 volatiles. The reduced production of alpha, alpha-trehalose, a metabolite used in starch and sucrose metabolism, suggests that the biocontrol agent activates its stress response in the presence of the phytopathogen. Microscopic analysis using sGFP-tagged FO12 on oil seed rape as a model plant suggests that the biocontrol strain is an efficient root colonizer, which could compete with *V. dahliae* in the same ecological niche. The findings obtained in this study provide new insights into the mode of action of this potential biocontrol agent, which are relevant for controlling Verticillium wilt through an ecologically friendly approach.

This chapter has been published in:

Mulero-Aparicio A, Cernava T, Turrà D, Schaefer A, Di Pietro A, López-Escudero FJ, Trapero A, Berg G. 2019. The role of volatile organic compounds and rhizosphere competence in mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt. *Frontiers in Microbiology* **10**:1808. doi.org/10.3389/fmicb.2019.01808

4.1. INTRODUCTION

Verticillium species are generally widely distributed in soil, and are common plant endophytes (Pegg and Brady, 2002; Barbara and Clewes, 2003; Klosterman et al., 2009). However, distinct *Verticillium* species represent a devastating group of plant pathogens that cause wilt disease in a large number of hosts worldwide. Representative species within this genus such as *Verticillium albo-atrum* (Reinke and Berthold, 1879), *Verticillium dahliae* Kleb. (1913), and *Verticillium longisporum* C. Stark (Karapapa et al., 1997) among others (Inderbitzin et al., 2011), are commonly found in agricultural soils. However, their abundance was drastically enhanced in various plant cultivation areas due to short crop rotations and monocultures in intense agriculture. Increasing soil temperatures due to global warming further aggravate their capacity to infect host plants (Tjamos et al., 2000). Moreover, changes in their genomes resulting from inter-kingdom horizontal events have enhanced their adaptability and pathogenicity (Shi-Kunne et al., 2019). Currently, these pathogenic species cause losses in many herbaceous and woody crops with important economic impact (Hiemstra, 1998; Pegg and Brady, 2002). While *V. dahliae* and *V. albo-atrum* can infect a high number of host species, *V. longisporum* has a more limited host range, primarily infesting *Brassicaceae* crops (Daebeler et al., 1988; Zeise and von Tiedemann, 2002; Depotter et al., 2016). Verticillium wilts caused by *V. dahliae* have a high economic impact causing severe yield losses crops such as cotton and olive in temperate and subtropical regions (Pegg and Brady, 2002). During recent years, Verticillium wilt has become a major challenge for olive growing in the Mediterranean basin countries, due to the lack of an effective control method (López-Escudero and Mercado-Blanco, 2011). In Spain, a disease occurrence of 39% in affected orchards was reported in the last decades (Blanco-López et al., 1984; Sánchez-Hernández et al., 1998).

The control of Verticillium wilts is one of the most difficult challenges for growers due to the broad range of hosts that can be colonized by the pathogens. Other aggravating factors are the location of the pathogen within the xylem vessels of the infected plants, the long-lasting viability of their microsclerotia (resting structures), the genetically heterogeneous and polyphyletic character of *Verticillium* isolates and the lack of effective fungicide treatments, among others (Fradin et al., 2009; López-Escudero and Mercado-Blanco, 2011; Jiménez-Gasco et al., 2014). However, the dispersal, incidence and severity of Verticillium wilts can be partially reduced by means of integrated disease management and enhanced

biodiversity (López-Escudero and Mercado-Blanco, 2011; Berg et al., 2017). In this context and due to the increased concern about environmental and human health, the use of eco-friendly alternatives such as biological control measures, have become potential tools to improve the efficiency of integrated disease management (Berg, 2009; Berg et al., 2017). These approaches are perceived as safe and have a minimal environmental impact. Several studies have reported the use of biocontrol agents (BCAs) such as *Serratia plymuthica* HRO-C48 (Müller and Berg, 2008), non-pathogenic *Verticillium* strains (Tyvaert et al., 2014) or strains of *Paenibacillus* and *Serratia* (Kurze et al., 2001; Rybakova et al., 2016) against *V. longisporum*. Likewise, studies conducted during the last 15 years have reported the use of different antagonistic microorganisms as BCAs against *V. dahliae* in herbaceous and horticultural crops such as oilseed rape, tomato, pepper or cotton (Tjamos et al., 2004; Xue et al., 2013; Rybakova et al., 2016; Veloso et al., 2016). The most studied BCAs against *V. dahliae* in olive are *S. plymuthica* (Müller et al., 2008), *Paenibacillus alvei* (Markakis et al., 2016), *Pseudomonas* spp. (Mercado-Blanco et al., 2004; Triki et al., 2012) and *Trichoderma* spp. (Jiménez-Díaz et al., 2009).

Recently, a large-scale screening of potentially beneficial microorganisms for the biocontrol of Verticillium wilt of olive (VWO) yielded a non-pathogenic *Fusarium oxysporum* isolate (FO12) as one of the most effective BCAs against the pathogen (Varo et al. 2016b). However, there is no knowledge related to the underlying antagonistic effects of non-pathogenic strains of *F. oxysporum* against *V. longisporum*. In contrast, several studies on the interaction between non-pathogenic *F. oxysporum* strains and *V. dahliae* have been performed in herbaceous crops, with promising results (Pantelides et al., 2009; Angelopoulou et al., 2014; Veloso et al., 2016). Previous studies suggest that non-pathogenic isolates of *F. oxysporum* have different modes of action (Fravel et al., 2003), including competition, antibiosis and/or induction of systemic resistance in plants (Pantelides et al., 2009; Zhang et al., 2015; Veloso et al., 2016). One specific mode of action reported for some non-pathogenic strains of *F. oxysporum* is the production of volatile organic compounds (VOCs) with antifungal activity against pathogenic *formae speciales* of *F. oxysporum* (Minerdi et al., 2009) and against *V. dahliae* in cotton (Zang et al., 2015). A major advantage of VOCs when compared to larger molecules is their capacity to diffuse over large distances. Cumulative data suggest that volatiles play a more important role for microbial interactions than non-volatile substances (Kanchiswamy et al., 2015). Various studies have

demonstrated that microbial volatiles can significantly reduce the viability and proliferation of devastating plant pathogens such as *Botrytis cinerea*, *F. oxysporum* or *Magnaporthe oryzae* (Cernava et al., 2015a; Minerdi et al., 2009). Moreover, it was shown that exchange of aerial signals such as VOCs between microorganisms can induce a change in the recipient's metabolism (Rybakova et al., 2017). This response can enhance or reduce the production of specific soluble metabolites to guarantee the recipient's survival in the environment. The mode of interaction is often strain-specific; therefore, a detailed understanding of the specific mode of action of a BCA is crucial for the development of an efficient biocontrol strategy.

The objective of this study was to contribute to the understanding of the mode of action of non-pathogenic *Fusarium oxysporum* FO12 towards pathogenic Verticillium species in the rhizosphere. FO12 was able to reduce the mycelial growth of the phytopathogenic *V. dahliae*, the viability of its microsclerotia in naturally infested soils and demonstrated a significant reduction of VWO in *in vivo* experiments (Varo et al., 2016a). Therefore, we elucidate the modes of action by (i) testing the effect of VOCs produced by FO12 on mycelial growth and microsclerotia viability of *V. dahliae* and *V. longisporum* (ii) identifying the chemical nature of the VOCs produced by FO12; (iii) assessing changes in the metabolism of FO12 after exposure to *V. dahliae* VOCs, and (iv) studying root colonization by FO12 in a model plant by means of confocal laser scanning microscopy (CLSM).

4.2. MATERIALS AND METHODS

4.2.1. Fungal strains and growth conditions

The fungal pathogens used in this study were *V. longisporum* (C. Stark) (Karapapa et al. 1997) strain ELV25 and *V. dahliae* Kleb. strains ELV22, V004 and V024. The strains ELV22 and ELV25 from the collection of the Institute of Environmental Biotechnology (Graz University of Technology), were described by Messner et al. (1996). The mild-virulent strain V004 was classified as non-defoliating pathotype (Blanco-López et al., 1989), and the high-virulent strain V024 was classified as defoliating pathotype (Varo et al., 2016b). Both were obtained from the fungal collection of the Agronomy Dpt. of the University of Córdoba. The non-pathogenic *F. oxysporum* strain FO12, also from the fungal collection of the Agronomy Dpt. of the University of Córdoba, was applied as

BCA. Single-spore cultures of all isolates were prepared prior to use by means of the serial dilution method and maintained on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants at 4ºC. Seven-day-old single spore cultures incubated on PDA at room temperature were used as an inoculum source.

4.2.2. Generation of sGFP-tagged *F. oxysporum* FO12 transformants

GFP-labelled strains of FO12 were obtained by co-transforming fungal protoplasts with the hygromycin resistance and the super-folder green fluorescent protein (sGFP) expression cassette, as previously described (Di Pietro et al., 2001; López-Berges et al., 2012). Cytoplasmic sGFP expression was analyzed in at least twenty independent transformants using a Zeiss Axio Imager M2 microscope (Zeiss, Barcelona, Spain) equipped with a GFP (BP 450/490, FT 510, LP 515) filter set and an Evolve Photometrics EM512 digital camera (Photometrics Technology, Tucson, AZ, USA). Transformants exhibiting the highest sGFP expression and retaining efficient biocontrol activity against *Verticillium* isolates were used in subsequent live-cell microscopy studies.

4.2.3. Effects of volatile metabolites from *F. oxysporum* FO12 against *V. longisporum* and *V. dahliae*

The antagonistic effect of the VOCs produced by *F. oxysporum* FO12 and by the transformant FO12-sGFP against all *Verticillium* isolates used in this study was tested by means of the "Two Clamp VOCs Assay" as described in Cernava et al. (2015a). Mycelial plugs (3 mm ø) of each isolate were obtained from the margin of 7-day-old colonies grown on PDA as described above. For each *Verticillium* isolate, a mycelial plug was placed in the centre of the wells of a 6-well plate (Greiner Bio-One, Frickenhausen, Germany) previously filled with 3 mL of PDA per well. Subsequently, one mycelial plug of *F. oxysporum* isolate FO12 and FO12-sGFP was transferred to the same position of a 6-well plate placed opposite to the plate with the pathogen. A perforated (0.5 cm ø) 1mm silicone foil was placed between both 6-well plates for tightening connected wells and usual clamps for fixation. Additionally, six wells with plugs of the pathogens connected to a plate only with PDA were used as a control. Plates with ELV25 and ELV22 and with V004 and V024 were incubated at room temperature for 4 and 5 days, respectively. The assay was performed in six replicates (six wells for each *Verticillium* isolate, FO12 strain, and control combination) randomly distributed in

three 6-well plates (two wells per treatment and plate) to avoid a possible effect of the position of each well on the mycelial growth. The experiment was conducted twice. After 5 days of incubation, the largest and smallest diameters of the colonies of each *Verticillium* isolate were measured using a ruler and the mean data represented total growth (mm).

The potential of FO12-produced VOCs to reduce the viability of the microsclerotia produced by *V. longisporum* ELV25 and *V. dahliae* ELV22 was separately evaluated. For that purpose, microsclerotia from both *Verticillium* species were obtained as described in Varo et al. (2016a). The microsclerotia were produced in Czapek Dox liquid culture (Sigma-Aldrich) prepared in Erlenmeyer flask of 300-ml capacity each containing 100 mL of the medium. For each *Verticillium* isolate, a conidial suspension (10^6 conidia mL^{-1}) obtained from 7-day-old colony was used to inoculate the flasks. Liquid cultures were incubated at room temperature in an orbital shaker (Grant bio PSU-20i, Grant Instruments, Cambridge, UK) at 90 rpm for 28 days in the dark. The obtained microsclerotia suspensions were homogenized by using a FastPrep-24 device (MP Biomedicals, Santa Ana, California, USA) for 8 s at 4 m s^{-1}. Subsequently, microsclerotia suspensions were adjusted with sterile distilled water by using a haemocytometer to 10^6 microsclerotia mL^{-1}. The experiment was carried out with a modified "Two Clamp VOCs Assay". For each *Verticillium* isolate, wells of one 6-well plate were filled with 300 µL of the microsclerotia suspension and dried in sterile conditions until the remaining water was evaporated. The initial number of microsclerotia per well was 3 x 10^5. One 6-well plate with mycelial plugs (3 mm ø) of *F. oxysporum* FO12 was placed opposite to the plate with the microsclerotia, separated by the perforated silicon foil and fixed with two clamps. The assay was performed in six replicates (six wells for each *Verticillium* isolate and FO12 combination) and the experiment was conducted twice. Additionally, six wells with microsclerotia connected to a plate only with PDA were used as a control. The plates were incubated for 7 days at room temperature. After the period of incubation, the microsclerotia from each well were recovered with 700 µL of sterile distilled water in 1 ml tubes. The viability of the microsclerotia was tested by plating several serial dilutions of 100 µL of each recovered microsclerotia suspension on PDA plates incubated for 3 days at room temperature in the dark. After 3 days of incubation, the number of *Verticillium* colonies per PDA plate was counted in order to obtain the total number of viable microsclerotia (Colony forming units (CFU)). The average number of CFU per well was obtained from three PDA plates,

resulting a total of 18 PDA plates for each treatment combination (3 PDA plates/well x 6 wells/treatment combination).

4.2.4. Analysis of VOCs produced by *F. oxysporum* FO12

The identification of FO12-emitted VOCs was conducted in Gas chromatography-mass spectrometry (GC-MS) headspace solid phase micro extraction experiments with minor adaptions as described by Cernava et al. (2015a). For samples preparation, one mycelial plug (3 mm ø) of FO12 was transferred into a 20 mL headspace vial (75.5 × 22.5 mm; Chromtech, Idstein, Germany) previously filled with 8 mL of PDA. In order to test the VOCs produced by FO12 in presence of the pathogens, additional vials with mycelial plugs of ELV25 and ELV22 were prepared. Vials with the BCA were co-incubated together with those with *V. dahliae* or *V. longisporum* in a sterile glass jar (0.5 L) hermetically closed in order to exchange their VOCs without direct contact with one another. FO12 vials incubated without the presence of the pathogens were added as a control. All vials were incubated at room temperature for three days and the glass jars were opened every 12 h to ensure aerobic conditions. Following three days of incubation, vials were aerated under sterile conditions for 2 h to avoid the presence of VOCs produced by *Verticillium* isolates in the vials inoculated with FO12. Subsequently, vials were separately sealed with adequate crimp seals and incubated for additional 3 h for VOCs accumulation. Three replicated vials were used per each pathogen/FO12 combination. Vials containing only PDA were analyzed under the same conditions and used to subtract compounds originating from the medium. Identification of the volatile compounds was performed with NIST MS Search 2.2 included in the Software-Package of the NIST 2014 database. Further verification was done by calculation of the Kovats index (KI) followed by comparisons to database entries of NIST Search 2.2 and the entries in the Online Database maintained by NIST (http://webbook.nist.gov/).

4.2.5. Soluble metabolite analyses of *F. oxysporum* FO12

The identification of the soluble metabolites from *F. oxysporum* FO12 was carried out as described by Rybakova et al. (2017). *Verticillium dahliae* ELV22 and *F. oxysporum* FO12 were co-incubated in order to exchange their VOCs without direct contact with one another. A petri dish with *V. dahliae* ELV22 was placed on top of the *F. oxysporum* FO12 plate both transferred in the groove by

means of a sterile handle just before the incubation and sealed to facilitate the accumulation of VOCs. Plates with FO12 in co-incubation with non-inoculated PDA plates were included as a control. The experiment was conducted in three replicates. Cell lysis was performed by using a FastPrep-24 device (MP Biomedicals, Santa Ana, California, USA) for two times 30 s at 6m s−1 in 90% methanol. The cell-free extract was stored at −70ºC. The *F. oxysporum* FO12 metabolite extracts were analyzed with a combined HPLC hybrid quadrupole-orbitrap mass spectrometer (Q Exactive; Thermo Scientific, Bremen, Germany). To separate different metabolites from the cell extracts, an Atlantis dC18, 3 µm, 2.1x100mm column (Waters GesmbH, Phenomenex, Vienna, Austria) was used as described by Cernava et al. (2015b). Identification of the soluble compounds was performed with the XCalibur 2.2 and Compound Discoverer 2.1 (Thermo Scientific, Bremen, Germany) and manual comparison of the spectra with corresponding spectra from literature as well as such from mzCloud (HighChem LLC, Bratislava, Slovakia).

4.2.6. *In situ* visualization of *F. oxysporum* FO12 in oilseed rape

In order to study root colonization of the non-pathogenic *F. oxysporum* strain FO12, a colonization assay was conducted following the modified protocol described by Rybakova et al. (2016). A total of 16 surface-sterilized oilseed rape (*Brassica napus* L. "Traviata H 605886"; KWS Saat Einbeck, Germany) seeds were aseptically placed into two germination pouches (Mega International, Minneapolis, USA) (8 seeds per pouch) previously filled with 15 mL of sterile distilled water. The pouches were placed into sterilized plastic containers and incubated under gnotobiotic conditions in a greenhouse at 22ºC and a 12 h photoperiod. After four days of incubation, germinated seedlings were inoculated in germination pouches by roots drenching with 200 µL of a conidial suspension (10^6 conidia mL^{-1}) from a 5-day-old colony of the sGFP-labeled *F. oxysporum* FO12-sGFP. After the inoculation, the seedlings were kept in the greenhouse for 14 days at the conditions described above.

For fluorescence microscopy visualization, two oilseed rape seedlings were sampled at 4, 6, 8, 10, 14 and 17 days after inoculation. The root and stem of the seedlings were cut into small pieces with a sterile razor blade. Seedlings samples were additionally stained with calcofluor white (CFW; 1 g/l; Sigma-Aldrich) for improved imaging of host structures. Subsequently, samples were transferred on

optical slides. To study colonization patterns a Leica TCS SPE confocal laser scanning microscope (CLSM) (Leica Microsystems, Mannheim, Germany) was used. sGFP and calcofluor staining were sequentially excited with 635 and 405 nm laser beams, respectively. The confocal stacks were acquired with a LeicaACS APO 40x oil CS objective lens (NA, 1.30) and for each field of view, an appropriate number of optical slices were acquired with a Z-step ranging from 0.15 to 0.5 µm. Laser settings were adjusted to maximize signal to noise ratio of both fluorescent signals (sGFP and CFW) . The software Imaris 7.3 (Bitplane, Zurich, Switzerland) was used for imaging and post-processing of the confocal stacks and maximum projections. Additionally, at the end of the experiment, three seedlings were harvested to perform re-isolations to confirm FO12-sGFP colonization. For this purpose, stem and root of each seedling were cut into six small pieces and plated on PDA-hygromycin B. Subsequently, plates were incubated at room temperature for 5 days and positive isolations were recorded.

4.2.7. Statistical analysis

Analysis of variance of the mycelial growth (mm), microsclerotia viability (CFU) and abundance of metabolites produced by FO12 were performed according to a completely randomized design. The data from replicated experiments were combined after assessment of the homogeneity of the experimental error variances by the *F* test. Furthermore, data were tested for normality, homogeneity of variances, and residual patterns, which proved their suitability for the statistical analysis. When analysis of variance showed significant differences among treatments, means were compared according to Fisher´s protected least significant differences (LSD) test at $P = 0.05$. All data of this study were analyzed using Statistix 10 (Analytical Software, Tallahassee, FL).

4.3. RESULTS

4.3.1. Effect of *F. oxysporum* FO12 VOCs on mycelial growth of *V. longisporum* and *V. dahliae*

The effect of VOCs emitted by *F. oxysporum* FO12 and FO12-sGFP on mycelial growth of different *Verticillium* isolates was assessed in co-incubation experiments. VOCs produced by both FO12 and FO12-sGFP reduced the mycelial growth of the phytopathogenic fungi when they shared the same headspace.

Mycelial growth of *V. longisporum* ELV25 was significantly reduced ($P = 0.0029$) with a final growth diameter of 10.5, 8.1 and 13.5 mm for FO12, FO12-sGFP and control treatments, respectively (Figure 1A). Moreover, VOCs emitted by FO12 and FO12-sGFP were also able to significantly reduce the mycelial growth of *V. dahliae* isolates ELV22 ($P < 0.0001$), V004 ($P = 0.0003$) and V024 ($P = 0.0016$) in comparison with their respective controls. The final growth diameter for the ELV22 strain was 3.25, 4.83 and 11.67 mm; for strain V004 it was 11.79, 12.04 and 18.04 mm; and for strain V024, it was 9.54, 10.88 and 16.63 mm for FO12, FO12-sGFP and control treatments, respectively (Figure 1A). The VOCs-mediated reduction of mycelial growth by the *F. oxysporum* strain and the sGFP mutant was more pronounced in *V. dahliae* isolates than in *V. longisporum* ELV25 (Figure 1B). The effectiveness of VOCs emitted by *F. oxysporum* FO12 and FO12-sGFP against mycelial growth of *Verticillium* isolates was similar and no significant differences were found between the strains. .

4.3.2. Effect of VOCs on microsclerotia of *V. longisporum* and *V. dahliae*

A modified version of the "Two Clamp VOCs Assay" (Cernava et al., 2015a) was used to evaluate the efficacy of the VOCs from the non-pathogenic FO12 strain to reduce the viability of microsclerotia. After exposure of *V. longisporum* ELV25 and *V. dahliae* ELV22 microsclerotia to FO12-emitted VOCs, their viability was significantly decreased. The incubation of recovered microsclerotia on PDA after the treatment resulted in lower CFU numbers for treated samples. In detail, the exposure resulted in a significant reduction of the viability ($22.7*10^3$ CFU; $P = 0.0252$) of *V. longisporum* ELV25 in comparison with the control ($62.9*10^3$ CFU) (Figure 2). VOCs produced by FO12 were also able to significantly reduce the viability of microsclerotia from *V. dahliae* ELV22 ($62.8*10^3$ CFU; $P = 0.0282$) in comparison with the control ($188.5*10^3$ CFU) (Figure 2).

4.3.3. Identification of VOCs produced by *F. oxysporum* FO12

A total of 21 VOCs produced by FO12 were identified by means of GC-MS analysis based on their mass spectra (Table 1). These VOCs belong to different chemical groups and included terpenes, alcohols, esters, cyclic carbon compounds, as well as alkanes. VOCs belonging to the terpene group were the most abundant ones (6/21) and distinct compounds (cedr-8-ene, cembrene, β-acorenol) were

produced both when FO12 was incubated either alone or after the exposure to *Verticillium*. Contrarily, β-cedrene was produced only after exposure to *V. longisporum*. Different alcohols and esters were also abundant among the identified compounds (4/21). They were constantly produced by FO12 and only 2-methyl-1-propanol (alcohol) and 2-methylbutyl acetate (ester) were produced specifically after exposure to both *Verticillium* species or of *V. dahliae*, respectively (Table 1). We identified three volatiles which included cyclic carbons in their structure as main chemical group; two of them were produced only when FO12 was exposed to both *Verticillium* species and the other one was constantly emitted by FO12 (Table 1). In terms of alkanes, tridecane and hexane, 2, 3,-dimethyl were detected and both were constantly produced by FO12. Finally, volatiles assigned to the aromatic compounds group, such as 1-ethyl-4-methoxybenzene and pyrocatechol, were emitted by FO12 constantly or after exposure to the two *Verticillium* species, respectively.

4.3.4. Changes in soluble metabolites of *F. oxysporum* FO12 after exposure to *V. dahliae* VOCs

The composition of soluble metabolites produced by *F. oxysporum* FO12 grown in the presence of VOCs produced by *V. dahliae* ELV22 was assessed by means of high-resolution LC-MS analyses. Relative abundance of the 26 compounds produced by FO12 was affected in the presence of the pathogen. These metabolites are involved in distinct pathways and have different metabolic functions as shown in Table 2. The interaction with *V. dahliae* mostly affected the pathways associated with amino acids metabolism. Within this group, some compounds as pantothenic acid related to the metabolism and synthesis of carbohydrates, proteins, and fats showed a significant upregulation (2.42 fold). In contrast, L-ergothioneine showed a significant downregulation (-2.40 fold) (Table 2). The abundance of metabolites associated with carbohydrate metabolism pathways was also highly affected by the interaction with the pathogen. Thus, gluconic acid and alpha,alpha-trehalose showed a downregulation by ELV22 VOCs (-2.22 and -3.24 fold, respectively). Beauvericin was also significantly downregulated (-1.89 fold) when FO12 was co-incubated with the pathogen (Table 2). Finally, we also detected a significant decrease in indole-3-lactic acid production (-1.95 fold), following exposure to ELV22.

4.3.5. Root colonization of FO12 in oil seed rape

After root inoculation of oilseed seedlings with the sGFP-labelled *F. oxysporum* FO12-sGFP strain, roots and stem of the seedlings were sampled for CLSM visualization. FO12-sGFP was able to extensively colonize the roots of oilseed seedlings. sGFP-labelled hyphae were observed growing between root hairs of oilseed seedlings (Figure 3A) as well as attached to the surface of the main root following preferably the root growth direction (Figure 3C, D). Germinating microconidia attached to the main root surface were observed at 6 days after inoculation (DAI) (Figure 3B). Several infection points were observed at 8 DAI where FO12-sGFP was able to infect the seedlings. Figure 3C shows several micro-injuries on the root surface by which hyphae were directly infecting the plant. Formation of appressoria-like structures on the root surface was also observed as an alternative way to infect the plant. Appressoria were preferably formed in the intercellular space of the main root surface (Figure 3C). Confocal microscopy confirmed the endophytic lifestyle of this strain, since hyphae of FO12-sGFP were found growing inside roots hairs at 6 DAI (Figure 3D, F, G). Figure 3G is a 3D reconstruction of Figure 3F confirming that the fluorescent hypha was growing within a root hair. The spread of microconidia of FO12-sGFP along the xylem vessels of the oilseed stem was observed at 17 DAI, but no hyphal colonization of the stem was found (Figure 3H, I). At 14 DAI, the presence of embedded chlamydospores of FO12-sGFP in root hairs bundles was detected (Figure 3J, K). Chlamydospores were able to germinate in order to continue the root colonization (Figure 3K). Additionally, FO12-sGFP was consistently re-isolated from the stem and root of seedlings harvested at the end of the experiment.

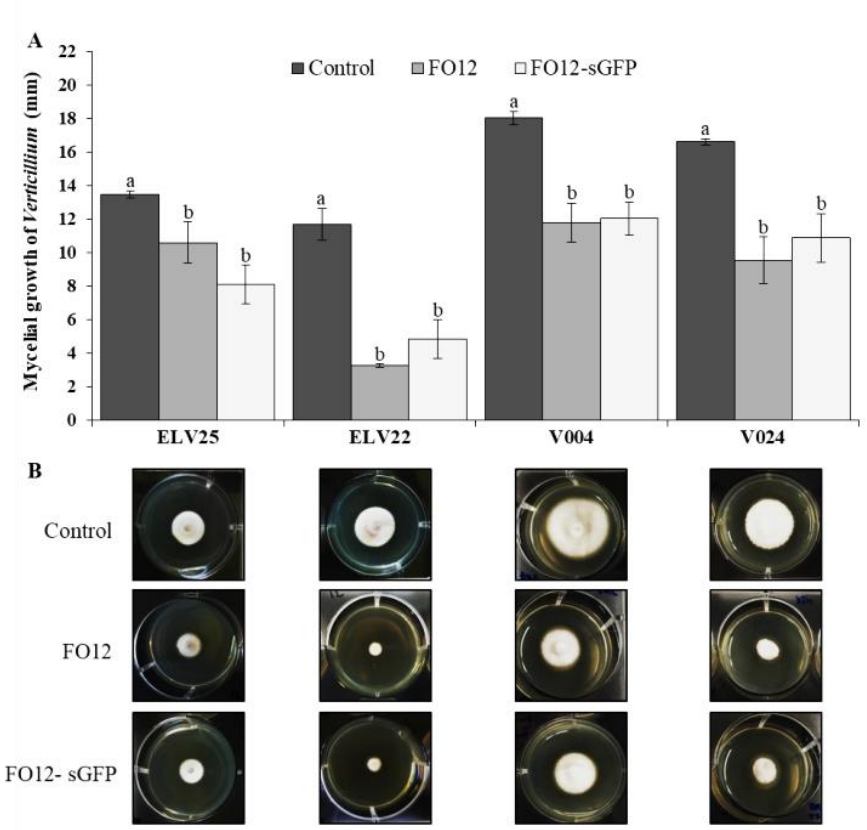


Figure 1. Effect of volatile organic compounds (VOCs) from the non-pathogenic *F. oxysporum* strain FO12 and from the GFP-labelled FO12 (FO12-sGFP) against the mycelial growth of *V. longisporum* ELV25 and *V. dahliae* isolates ELV22, V004 and V024. For each isolate, columns represent the means of 12 replicates per treatment. Vertical lines in each column are the standard error of the mean. For each *Verticillium* isolate, means in a column followed by different letters are significantly different according to Fisher´s protected least significant differences (LSD) test at $P = 0.05$. Pictures below illustrate the mycelial growth of each *Verticillium* isolate according to the different treatments.

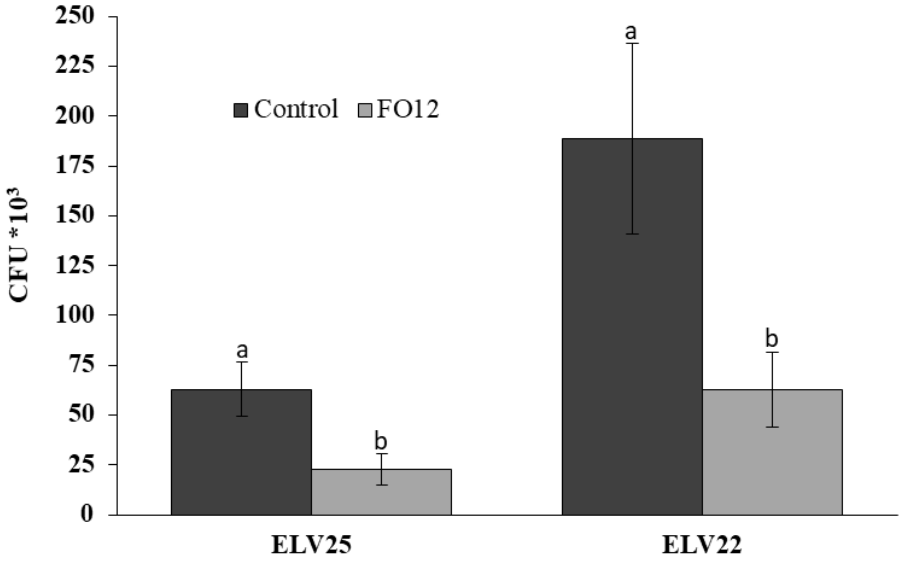


Figure 2. Effect of volatile organic compounds (VOCs) from the non-pathogenic *F. oxysporum* strain FO12 on microsclerotia viability of *V. longisporum* ELV25 and *V. dahliae* ELV22. For each isolate, columns represent the means of 12 replicates per treatment. Vertical lines in each column are the standard error of the mean. For each *Verticillium* isolate, means in a column followed by a different letter are significantly different according to Fisher´s protected least significant differences (LSD) test at $P = 0.05$.

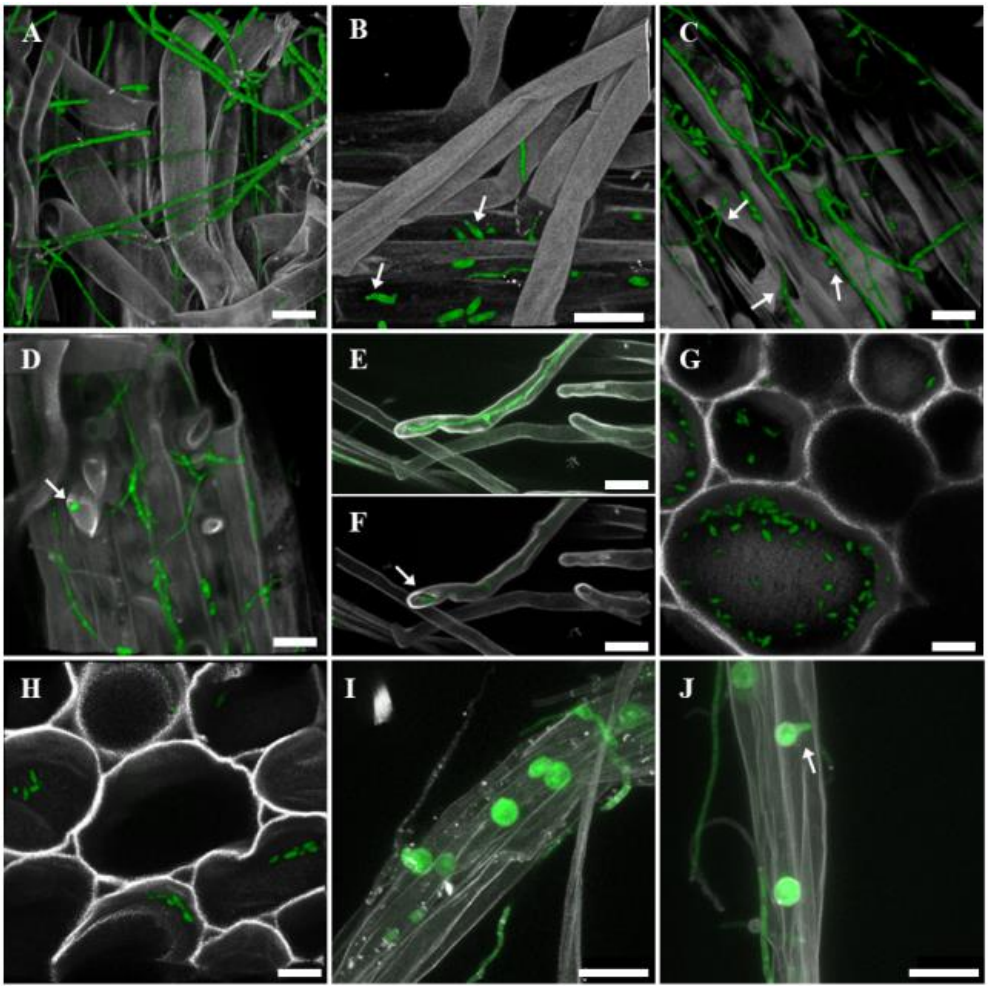


Figure 3. Confocal laser scanning microscopy (CLSM) micrographs showing the colonization pattern of oilseed roots and stem by the strain FO12-sGFP. Green, fungus; grey, host tissues stained with white calcofluor. (A), extensive root hairs colonization by FO12-sGFP, 10 days after inoculation (DAI). (B), germinating microconidia (indicated by arrows) attached to the main root tissue at 6 DAI. (C), extensive main root colonization and root infection by FO12-sGFP towards micro-injuries and by appressorium 8 DAI (arrows indicate micro-injuries and appressorium for FO12-sGFP penetration). (D, F, G), endophytic behaviour of FO12-sGFP growing inside root hairs at 6 DAI (arrows indicate the detail of a hypha inside the root hair). (H, I), conidial spread of FO12-sGFP towards the stem, 17 DAI. (J, K), embedded chlamydospores in root hairs bundles at 14 DAI (arrow indicates a germinating chlamydospore). Scale bars: 25 µm.

Table 1. GC-MS headspace SPME identification of relevant VOCs produced by the non-pathogenic *F. oxysporum* FO12 alone and during co-incubation with *V. longisporum* ELV25 or *V. dahliae* ELV22.

Predicted compound[a]	KI[b]	Match[c]	Predicted funtion[d]
VOCs constantly produced by FO12			
3-methyl-1-butanol	736	966	Antifungal (de Souza et al., 2018)
2-methyl-1-butanol	739	934	Antifungal (Raza et al., 2015)
β-acorenol	1649	922	Antibacterial (Albay et al., 2009)
Ethyl acetate	612	873	Antifungal (Toffano et al., 2017)
1-hexanol	868	928	PGP (Splivallo et al., 2007)
Cedr-8-ene	1411	936	N.a.
3-methylbutyl acetate	876	907	PGP (Amavizca et al., 2017)
Isobutyl acetate	771	838	N.a.
1-ethyl-4-methoxybenzene	1110	887	N.a.
Tridecane	1300	790	PGP (Amavizca et al., 2017)
1,1,2b,6-tetramethyl-2,2a,2b,3,4,6a,7,7a-octahydro-1H-cyclobuta[a]indene	1330	843	N.a.
Hexane, 2,3-dimethyl	760	806	N.a.
VOCs produced by FO12 after exposure to *V. longisporum* or *V. dahliae*			
(1R,4R,5S)-1-isopropenyl-4,8-dimethylspiro[4.5]dec-7-ene	1475	917	N.a.
2-methyl-1-propanol	625	845	Antifungal (Stotzky et al., 1976)
Pyrocatechol	2020	937	N.a.

Table 1. (Continued).

Predicted compound[a]	**KI**[b]	**Match**[c]	**Predicted funtion**[d]
Cembrene	1939	933	N.a.
Aristol-1-ene	1453	862	N.a.
(4R,5R)-1-isopropylidene-4,8-dimethylspiro[4.5]dec-7-ene	1515	906	N.a.
Alloaromadendrene	1461	909	N.a.
VOCs produced by FO12 only after exposure to *V. longisporum*			
β-cedrene	1421	921	N.a.
VOCs produced by FO12 only after exposure to *V. dahliae*			
2-methylbutyl acetate	880	887	Nematicidal (Terra et al., 2018)

[a] Compound names according to International Union of Pure and Applied Chemistry (IUPAC).
[b] Kovats index (KI) of the compounds was calculated with an alkane series.
[c] Match index: Only substances with match index with the NIST MS Search 2.2 included in the Software-Package of the NIST 2014 database over 750 were considered.
[d] Predicted function of the compounds according to referenced literature. N.a.: Not available (unknown function). PGP: Plant growth promotion.

Table 2. Effects of exposure to *V. dahliae* ELV22 on *F. oxysporum* FO12 metabolism detected by LC-MS.

Predicted metabolite[a]	Fold change[b]	Metabolic function[c]
		Amino acid metabolism
N-acetyl-L-methionine	28.88	Cysteine and methionine metabolism
N-acetyl-L-phenylalanine	7.15*	Phenylalanine metabolism
N-acetylvaline	6.85	Valine, leucine and isoleucine degradation
N-acetylornithine	4.74	Arginine biosynthesis
4-acetamidobutanoic acid	2.48*	Arginine and proline metabolism
Pantothenic acid	2.42*	Beta-Alanine metabolism
N-acetyl-DL-tryptophan	2.34*	Tryptophan metabolism
L-glutathione (reduced)	1.53*	Glutathione metabolism
2-isopropylmalic acid	-1.49	Valine, leucine and isoleucine degradation
L-glutamic acid	-1.98	Arginine biosynthesis
L-ergothioneine	-2.40*	Histidine metabolism
L-saccharopine	-2.56*	Lysine biosynthesis
L-aspartic acid	-2.57*	Arginine biosynthesis
L-glutathione oxidized	-13.70	Glutathione metabolism
		Carbohydrate metabolism
D-(+)-maltose	1.93	Starch and sucrose metabolism
Gluconic acid	-2.22	Pentose phosphate pathway
Alpha,alpha-trehalose	-3.24*	Starch and sucrose metabolism
N-acetyl-D-galactosamine	-3.61*	Amino sugar and nucleotide sugar metabolism
α-D-mannose 1-phosphate	-4.61*	Fructose and mannose metabolism

Table 2. (Continued).

Predicted metabolite[a]	Fold change[b]	Metabolic function[c]
α-D-glucose-1,6-bisphosphate	-8.69*	Starch and sucrose metabolism
		Lipid metabolism
(+/-)12(13)-DIHOME	2.79	Linoleic acid metabolism
(15Z)-9,12,13-trihydroxy-15-octadecenoic acid	-1.85*	Fatty acid biosynthesis
		Nucleotide metabolism
Uric acid	8.42	Purine metabolism
		Energy metabolism
Flavin mononucleotide (FMN)	6.51*	Oxidative phosphorylation
		Chemical structure transformation maps
Beauvericin	-1.86*	Fungal toxin
Indole-3-lactic acid	-1.95*	Biosynthesis of plant hormones

[a] Only substances that were up- or down-regulated by *V. dahliae* ELV22 VOCs are shown.
[b] Abundance ratio between compounds from FO12 in co-incubation with ELV22 and from unexposed controls. Positive and negatives values correspond to upregulated and downregulated substances by *V. dahliae* ELV22 VOCs, respectively. Only metabolites with an up- or downregulation higher than 1.5 or lower than -1.5 were added. Values followed by an asterisk (*) indicate that the up- or downregulation was statistically significant according to Fisher´s protected least significant differences (LSD) test at $P = 0.05$.
[c] Metabolic pathways assigned by Kyoto Encyclopedia of Genes and Genomes. N.a. (not available) indicates that the metabolic pathway could not be found.

4.4. DISCUSSION

In the present study, new insights related to the interaction between the non-pathogenic *F. oxysporum* strain FO12 and different pathogenic Verticillium species were obtained. We have identified VOCs, which could play an important role in the antagonistic interactions between the two fungi. Moreover, rhizosphere colonization patterns of the potential competitors showed that they occupy the same niche within the plant, which suggest competition between them. The exposure of *V. longisporum* and *V. dahliae* to VOCs from FO12 resulted in a significant inhibition of mycelial growth in both pathogens. These results indicate that the antagonistic effect reported by Varo et al. (2016b) when *V. dahliae* was confronted with FO12 in dual cultures, was at least partially due to the production of volatile compounds with inhibitory activity against the pathogen. The effect of microbial VOCs from various BCAs against pathogenic fungi was addressed in recent studies (*e.g.* Zhang et al., 2015; Rybakova et al., 2017). The results of the current study are in agreement with those reported by Zhang et al. (2015) in which VOCs produced by the non-pathogenic strain CanR-46 of *F. oxysporum* inhibited the growth of different phytopathogenic fungi, including *V. dahliae*. In addition, we found that VOCs produced by FO12 were able to significantly reduce the viability of microsclerotia of both *V. longisporum* and *V. dahliae*. The effectiveness of FO12 in reducing inoculum density of *V. dahliae* in naturally infested soils was also recently reported (Varo et al., 2016a). It was shown that FO12 was able to completely inhibit the viability of microsclerotia when it was applied to naturally infested soils. Although a total reduction of microsclerotia viability with the VOCs assay was not achieved, our results suggest that a high proportion of the observed inhibition effect can be attributed to VOCs produced by FO12. In addition, after exposure with VOCs, a fraction of microsclerotia from both pathogens was unable to germinate on PDA plates, confirming the fungitoxic effect of the VOCs produced by FO12.

Interestingly, some of the identified VOCs produced by *F. oxysporum* FO12 were short-chain alcohols with known antifungal properties. The biocontrol activity of 3-methyl-1-butanol and 2-methyl-1-butanol has been confirmed in previous studies. Several compounds belonging to the chemical group of alcohols have been reported to have antifungal activity including 3-methyl-1-butanol (de Souza et al., 2018), 2-methyl-1-butanol (Raza et al., 2015) and 2-methyl-1-propanol (Stotzky et al., 1976). De Souza et al. (2018) reported the capability of *Saccharomyces*

cerevisiae to produce 3-methyl-1-butanol and 2-methyl-1-butanol which, among others VOCs, were able to significantly reduce the growth of *Penicillium digitatum.* Additionally, Lopes et al. (2015) observed a total inhibition of *Colletotrichum gloeosporioides* and *C. acutatum* by 3-methyl-1-butanol and 2-methyl-1-butanol produced by *S. cerevisiae*. Interestingly, also distinct *Verticillium* species were shown to produce both of these alcohols (Li et al., 2018). It remains to be elucidated if producers of these compounds are less affected by inhibitory effects of *F. oxysporum* FO12. The inhibitory effects of alcohols seems to affect the organization and stability of the lipid bilayer from the plasma membrane (Fialho et al., 2010; Toffano et al., 2017). Within the alcohol group, 1-hexanol, a commonly produced fungal VOC, was reported to reduce *Arabidopsis thaliana* growth (Splivallo et al., 2007). Among terpenes, β-acorenol is known for its antibacterial activity (Albay et al., 2009). A broad range of biological functions have been found among esters compounds, such as ethyl acetate with antifungal activity against *Sclerotinia sclerotiorum* when it is produced by *S. cerevisiae* (Toffano et al., 2017). The ester 3-methylbutyl acetate has known plant growth promotion activity, enhancing the performance of the microalga *Chlorella sorokiniana* (Amavizca et al., 2017). Additionally, within the ester group, 2-methylbutil acetate was reported to show nematicidal activity (Terra et al., 2018). Finally, among alkanes, tridecane is also known for its plant growth promotion activity (Amavizca et al., 2017). Although some of the VOCs found have been reported for antimicrobial activity, the biological function of compounds such as cembrene, alloaromadendrene or pyrocatechol, among others, and volatiles belonging to the cyclic carbon compounds group, remain unknown. The identification of bioactive volatile compounds in this study supports the hypothesis that the antagonistic effect of FO12 on mycelial growth and microsclerotia viability of *V. dahliae* is mainly due to the production of VOCs with antifungal activity. Most of the VOCs found in this study with biocontrol activity were continuously produced by FO12, which indicates that the production of antifungal volatiles from FO12 could be a rather unspecific strategy of the BCA to shield of competitors.

Recently, Rybakova et al. (2017) reported that microorganisms are able to mutually regulate their metabolism by means of an interchange of aerial signals such as VOCs. This communication between microorganisms may induce a differential metabolic performance in order to enhance or reduce the production of specific soluble metabolites to guarantee the recipient's survival in the environment. Compounds involved in the metabolism of amino acids, carbohydrates, lipids, nucleotide, energy or other chemical structures can be

responsible for an aerial dialogue between microorganisms. This facilitates broad adaptability of the interacting microorganisms to biotic stress. In this study, regulation of FO12's metabolism was observed when the production of several soluble metabolites was up- or down-regulated in presence of *V. dahliae* ELV22. Among the metabolites involved in amino acid metabolism, ergothioneine, which showed a significant downregulation, is biosynthesized exclusively by some fungi and mycobacteria and it has a role as a physiologic cytoprotectant (Paul and Snyder, 2009). The downregulation of alpha,alpha-trehalose, a metabolite used in starch and sucrose metabolism, might indicate that the BCA is improving the stress-resistance of its cells (Wyatt el al., 2015) prior to the interaction with the pathogen. In addition, we found several metabolites with antifungal activity such as gluconic acid (Kaur et al., 2006) and beauvericin (Wang and Xu, 2012), both showing a downregulation in the presence of the pathogen. Some microorganisms are known for the biosynthesis of plant hormones like auxins (Liu et al., 2016), in this context, we also detected downregulation of indole-3-lactic acid, a metabolite involved in the biosynthesis of plant hormones as auxin (Sardar and Kempken, 2018) when FO12 was interacting with the phytopathogen. Although our data indicate an extensive regulation of FO12 metabolic pathways during its interaction with the pathogen, further research is needed in terms of how the regulation of FO12 metabolism interferes with the antagonistic effect of this BCA against *V. dahliae*.

Root colonization patterns of FO12 by means of confocal laser scanning microscopy showed the entire process of colonization, beginning with conidial germination on the root surface until the formation of resting structures (chlamydospores). The extensive root surface colonization by FO12 was consistent with the root colonization patterns of the non-pathogenic isolate Fo47 of *F. oxysporum* in pepper (Veloso et al., 2016). No preferential growth along the intercellular junctions was observed. This is contrary to the observation reported by Pantelides et al. (2009) in which strain F2 grew attached to intercellular space on eggplant roots. This observation confirms the hypothesis by the same author that non-pathogenic *F. oxysporum* strains have their own colonization pattern, as before suggested by Steinberg et al. (1999). After conidial germination, FO12 was able to infect the roots through micro-injuries and appressoria formation on the root surface. This observation indicates that FO12 has similar infection sites preferences as *V. dahliae* as reported by Veloso et al. (2016) after visualization of the interaction between Fo47 and *V. dahliae* in pepper rhizosphere. Thereby

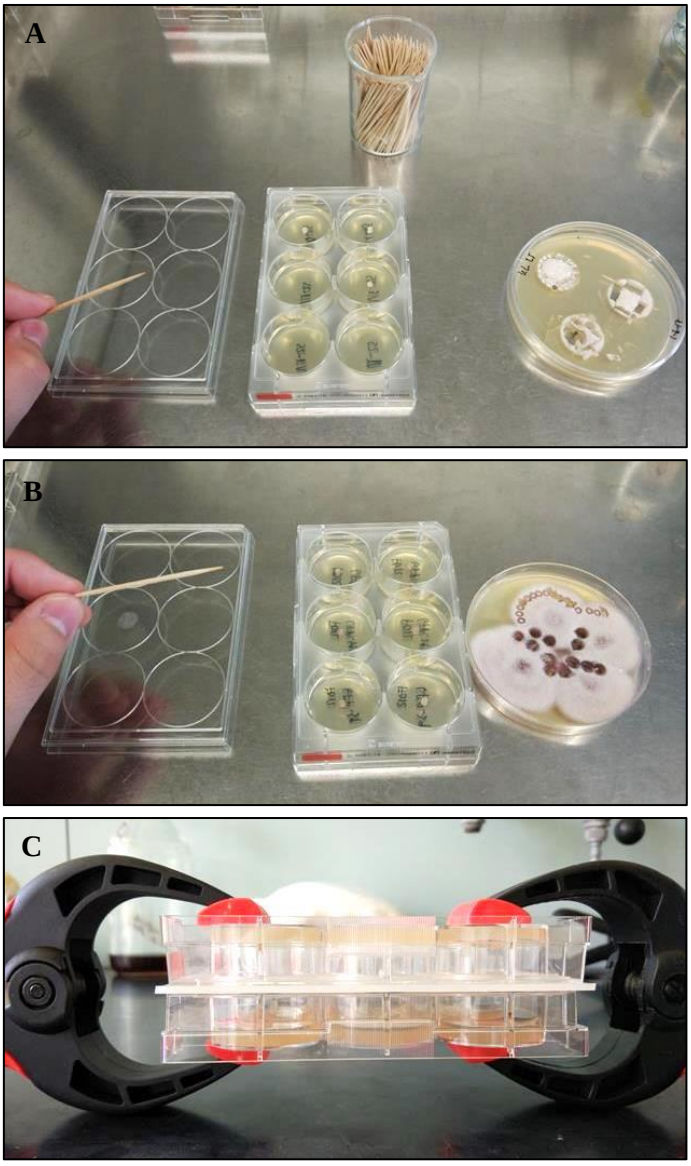
competition for space and infection points could play an essential role in the control of VWO as observed by Varo et al. (2016b). Moreover, the endophytic behaviour of FO12 and conidial spread along the vascular system was confirmed. The systemic plant colonization by the BCA following the xylematic flux is the same strategy used by *V. dahliae*, although no symptomatic plants by FO12 were observed. One of the most interesting observations conducted in this study was the formation of chlamydospores embedded in root hairs bundles. The capability of FO12 to form chlamydospores is considered an important trait of this BCA to ensure a long-term survival and antagonistic effect against the pathogen under field conditions. Moreover, the capacity to form resting structures facilitates the development of future commercial formulations.

Various traits of FO12 are in agreement with those proposed by Deketelaere et al. (2017) that are desirable for a promising BCA towards *Verticillium* because (i) the produced VOCs affect microsclerotia and mycelia, (ii) colonize the same ecological niche than the pathogen, and (iii) compete with the pathogen. Understanding the ecology, interactions and evolution of microbial key players in agricultural microbiomes will have a great potential for food security and safety. In contrast to the pathogenic effects of various species, recent research results indicate a natural function of *Verticillium* for plants: VOCs for auxin signaling and ripening of plants (Li et al., 2018) and their aroma production (Landa et al., 2019). These important findings should be considered for upcoming plant protection strategies as well in order to maintain functioning of non-pathogenic species.

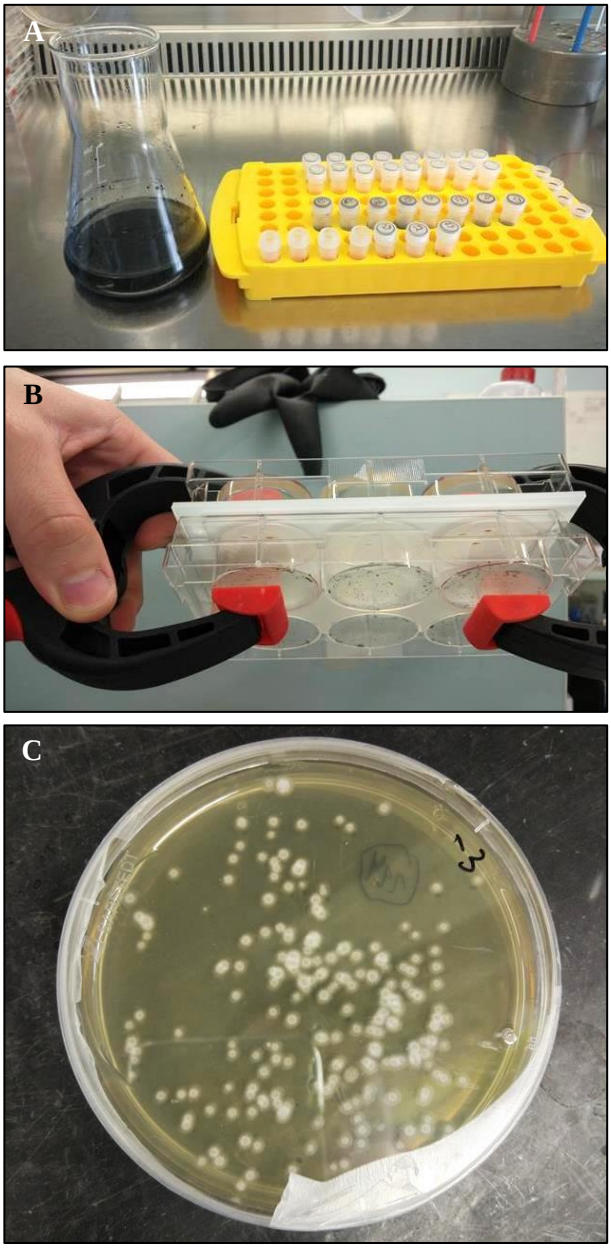
ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministry of Economy, Industry and Competitiveness (MINECO; project AGL2016-76240-R), co-financed by the European Union FEDER Funds. AMA is holder of 'Formación de Profesorado Universitario' (FPU) fellowships from the Spanish Ministry of Education, Culture and Sports (MECD). The authors thank Dr. Armin Erlacher (Graz) for his support during the acquisition of CLSM micrographs.

SUPPLEMENTARY FIGURES

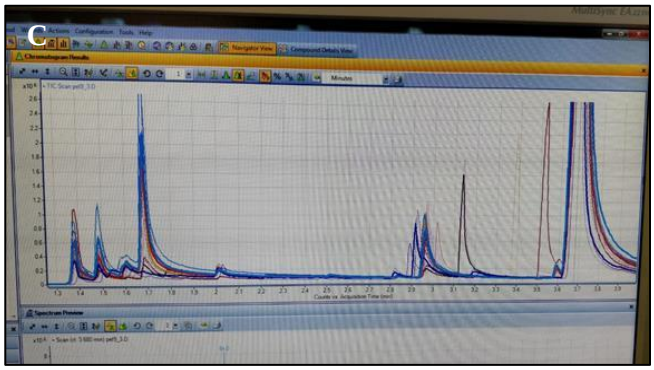


Effect of Volatile organic compounds produced by FO12 on mycelial growth of *Verticillum dahliae* by means of two-clamp VOCs assay. Transferring mycelial plugs of *V.dahliae* (A) or *Fusarium oxysporum* FO12 (B) into a 6-well-plate. C) Co-incubation of *V. dahliae* and FO12 in two 6-well plates fixed with two clamps.



Effect of Volatile organic compounds produced by FO12 on microsclerotia viability by means of two-clamp VOCs assay. A) Artificially production of microsclerotia. B) Co-incubation of *V. dahliae* microsclerotia and FO12 in two 6-well plates fixed with two clamps. C) Counting of viable colony forming units.

Microscopy study of the colonization process of *Fusarium oxysporum* FO12 on oil seed rape. A) Inoculation of oil seed rape plants with a conidial suspension of FO12-sGFP strain. B) Oil seed rape plants used for sampling. C) Confocal Laser Scanning Microscope used for the study. D) Software used for imaging and post-processing of the confocal stacks.



Analysis and identification of Volatile Organic Compounds produced by *F. oxysporum* FO12. A) Headspace vials with mycelial plugs of FO12. B) Gas Chromatography-mass spectrophotometry equipment. C) Identification and verification of volatile compounds with NIST MS Search 2.2.

CHAPTER 5

Suppressiveness of compost teas and extracts against VWO

5. Suppressive effects of compost teas and extracts from the grape marc compost CGR03 on Verticillium wilt of olive

ABSTRACT

Verticillium wilt of olive caused by *Verticillium dahliae* is considered the most important disease affecting olive groves worldwide. Currently, an integrated management strategy is the most advisable approach for controlling this disease. Biocontrol measures such as the use of organic amendments are considered potential methods to be implemented within an integrated management strategy. The grape marc compost CGR03 has been noted for its suppressive effect on Verticillium wilt of olive, but its mechanisms of action remain unknown. Therefore, the objectives of this study were to evaluate the effectiveness of different compost teas and extracts obtained from the grape marc compost CGR03 against *V. dahliae* and to identify the mechanisms of action involved in its suppressive effect by means of *in vitro* and *in vivo* experiments. The results of this study suggest that both biotic and abiotic factors are involved in the suppressive effect of this compost, since the natural extract and two extracts sterilized by autoclaving were the most effective aqueous treatments for reducing the inoculum density of *V. dahliae* in soil (62.0, 72.1 and 66.6% reductions, respectively). Furthermore, natural compost tea and compost tea sterilized by autoclaving achieved significant disease suppression rates of 67.4 and 96.5%, respectively. This study highlights the effectiveness of different compost teas and extracts from CGR03 in reducing both the inoculum density of the pathogen and the disease progress and represents an advance in the development of more feasible methods for controlling this disease under field conditions.

This chapter has been submitted to Annals of Applied Biology:

Mulero-Aparicio A, López-Escudero FJ, Trapero A. Suppressive effects of compost teas and extracts from the grape marc compost CGR03 on Verticillium wilt of olive. *Annals of Applied Biology*.

5.1. INTRODUCTION

In Mediterranean countries where olive (*Olea europaea*) and grapevine (*Vitis vinifera*) are two of the most important crops, the management of residues obtained during olive oil and wine extraction has become an ecological concern due to the large amounts of these wastes that are generated annually (Papasotiriou et al., 2013). Composting has been broadly accepted as a practical method for recycling those wastes for use as organic compost amendments. Organic amendments have a high agronomic value and have been described as improving soil properties (Alburquerque et al., 2007), being a nutrient source for plants, reducing the use of conventional fertilizers and thus contributing to more sustainable agriculture.

Beyond the beneficial agronomic traits of these composts, their use has acquired special relevance within biological control methods, since organic amendments have been reported to have suppressive effects against several major soilborne diseases. In this context, the use of organic amendments such as compost for the control of plant pathogens was first reported by Hoitink et al. (1975). Since then, the potential of composts to suppress several soilborne plant pathogens has been considered one of the most promising options within biocontrol strategies (Noble and Coventry, 2005; Metha et al., 2014).

Verticillium wilt of olive (VWO) is currently one the most concerning diseases affecting olive crops in the Mediterranean basin, since there are no chemical treatments for its control. This vascular disease is caused by the widespread soilborne fungus *Verticillium dahliae* Kleb. and its control represents a challenge for farmers and researchers attributable to several main factors: (i) the long survival of the resting structures of the pathogen (microsclerotia), (ii) the lack of chemical products that are able to reach the pathogen once it colonizes the vascular system and (iii) the intensification of olive production systems in the last decade. Therefore, an integrated management strategy is currently the most suitable approach to reduce the dispersion of the pathogen as well as the incidence and severity of VWO (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012).

Within this strategy, biological control methods against *V. dahliae*, such as the employment of compost, have acquired relevance in the last decade. However,

most of the studies have been conducted on herbaceous hosts (Termorshuizen et al., 2006; Alfano et al., 2011; Castaño and Avilés, 2013; Markakis et al., 2016). In contrast, only three studies have been carried out to evaluate the use of organic amendments against *V. dahliae* in olive. The first study was conducted by Wilhelm et al. (1962), emphasizing the importance of this olive disease and the need to look for effective control methods such as the use of dry wood shavings as an organic amendment. Although the use of compost water extracts has been previously reported to have positive results in controlling several plant pathogens (Yohalem et al., 1994; Trillas et al., 2006; Alfano et al., 2011), Vitullo et al. (2013) evaluated the effect of olive mill liquid wastes on nursery-grown olive plants. Finally, to the authors' knowledge, the last work studying the effectiveness of organic amendments against VWO was conducted by our group by Varo-Suárez et al. (2018), in which a screening of 35 organic amendments from different sources, 15 compost mixtures and five aqueous compost extracts was performed. In that study, the grape marc compost labelled CGR03 was one of the most effective solid organic amendments, showing a total suppression of *V. dahliae* in both *in vitro* and *in vivo* experiments. More recently, this compost has been evaluated for its effectiveness against VWO under field conditions; it is one of the most effective products at reducing both the inoculum density of the pathogen in soil and the progression of the disease (Mulero-Aparicio, et al., 2019b).

Although Varo-Suárez et al. (2018) indicated that phenols and volatile organic compounds could be involved in the suppressive effect of CGR03, the effect of aqueous extracts obtained from this compost as well as most of the mechanisms responsible for its suppression of *V. dahliae* are not yet understood. Traditionally, the suppressive qualities of compost have been related to biotic and/or abiotic factors (Noble and Coventry, 2005). The roles of beneficial microorganisms from composts in the antagonistic activity, including producing antifungal compounds (Whipps, 1997), creating competition for nutrients in the rhizosphere (Hoitink et al., 1997) and inducing plant systemic resistance (Weller, 1988) are widely known. Several studies have highlighted biological control agents (BCAs) that inhabit composts as the main biotic factor explaining their suppression of *V. dahliae* (Malandraki et al., 2008; Castaño et al., 2013; Markakis et al., 2016). This suppressive effect can be reduced after compost sterilisation, verifying that predominantly biological factors are involved in the suppression (Cotxarrera et al., 2002; Weller et al., 2002). However, evidence also suggests that the physicochemical characteristics of composts such as nutrients and phenolic and

bioactive compounds are implicated in disease suppression (Hoitink et al., 1997; Markakis et al., 2016). In addition, pH and electrical conductivity were recently reported by Avilés and Borrero (2017) to be abiotic factors explaining part of the suppressive effect of olive mill compost on Verticillium wilt of cotton. Consequently, reaching a general conclusion about the mechanisms involved in the suppressive effect of composts against different diseases has become a challenge for researchers, since their effectiveness is highly dependent on their physical, chemical and biological properties as well as on the pathosystem tested.

In view of these factors, the potential effect of composts against a certain disease should be specifically evaluated, including a study of its suppression mechanisms, which is essential for the development of effective control strategies that optimize the mode as well as the moment of application in field conditions. Within this context, the use of aqueous extracts from suppressive composts could be a potential method of VWO control due to the advantages of applying the treatment through the irrigation system in field conditions. Furthermore, since the suppression abilities and mechanisms of the solid and aqueous-compost amendments against VWO have been insufficiently studied, further research is vital in order to maximize the efficacy of compost-based treatments for the control of this disease in the field, where farmers urgently need reliable and feasible control strategies. For that purpose, the objectives of this study were (i) to evaluate the effectiveness of several compost teas and extracts obtained from the suppressive grape marc compost CGR03 against *V. dahliae* and (ii) to identify the biotic or abiotic factors involved in the suppressive effect by means of *in vitro* sensitivity tests and *in vivo* experiments.

5.2. MATERIAL AND METHODS

5.2.1. Fungal isolates

The highly virulent isolate V024 of *V. dahliae*, classified as a defoliating pathotype (Varo et al., 2016b), was used in this study. This isolate was obtained from an affected olive tree showing VWO symptoms in a commercial olive orchard in the Andalusia region (southern Spain) and belongs to the fungal collection of the Department of Agronomy of the University of Córdoba (Spain). Single-spore cultures were prepared prior to use by means of the serial dilution method (Dhingra and Sinclair, 1995) and were maintained on potato dextrose agar (PDA; Difco®

Laboratories, MD, USA) slants at 4°C. Seven-day-old single spore cultures of the *V. dahliae* isolate incubated on PDA at 25°C in the dark were used as inoculum sources.

5.2.2. Compost material and preparation of compost teas and extracts

Experiments were conducted using compost teas (CTs) and compost extracts (CEs) obtained from the suppressive grape marc compost CGR03 (Varo-Suárez et al., 2018) provided by the Cooperativa San Acacio, Montemayor, Córdoba (southern Spain). To obtain the compost, grape marc wastes from the alcohol distilling industry consisting of grape skins, seeds and stems of the grapevine cv. Pedro Ximénez were used as feedstock. Grape marc is collected annually from August to September and composted in insulated bins of 15 m^3 for eight months. When the compost temperature falls below 60°C at a depth of 50 cm, the compost was turned to promote aeration and homogeneity and to renew the process. Compost was collected for use when temperatures permanently fell below 40°C (maturation/recolonization phase). The pH value in the compost was 6.89, determined in 1:5 v/v compost/water extract.

Table 1 shows all CTs and CEs obtained from the suppressive grape marc compost CGR03 and a detailed description of the treatment tested in each experiment. The methodology followed to obtain all the different CTs and CEs evaluated in this study was a modification of the method used by Varo-Suárez et al. (2018). CTs were obtained by suspending a compost sample in sterile deionized water (SDW; 1:4, v:v) in a 1 L Erlenmeyer flask and incubating at 25°C (Grant bio PSU-20i, Grant Instruments, Cambridge, UK) at 100 rpm for 7 days. The same methodology described for CTs was used to obtain CEs but used potato dextrose broth (PDB; Difco Laboratories®) instead of SDW. To discern whether the effect against the pathogen is due to the direct action of the microorganisms grown during CT and CE production, portions of both CTs and CEs were sterilized after incubation by two different methods: (i) by autoclaving at 120°C for 20 min and (ii) by filtration to remove living structures, preventing the destruction of possible active antifungal metabolites. To filter CTs and CEs, 5 ml samples were filter-sterilized using a 0.2-μm pore size syringe filter (Sartorius Stedium Biotech; Goettingen, Germany). Additionally, to rule out the effect of both microorganisms and their secondary metabolites produced during the incubation of CTs and CEs, a different version of each CT and CE was prepared by prior sterilization of CGR03

by autoclaving at 120°C for 20 min on two consecutive days (Table 1). Three experiments were conducted to evaluate the effect of all CTs and CEs against *V. dahliae* and on disease suppression.

5.2.3. Experiment I: effect of compost teas and extracts on mycelial growth of *Verticillium dahliae*

The main purpose of this experiment was to verify whether the presence of chemical compounds with antifungal activity against *V. dahliae* was due to the chemical properties of CGR03 or due to the production of secondary metabolites from microorganisms living in CGR03 during the CT and CE incubation period. For that purpose, an *in vitro* experiment was conducted to evaluate five treatments based on sterilized CTs and CEs: CT and CE sterilized by autoclaving (CT2 and CE2), by filtration (CT3 and CE3) and the compost tea obtained by prior sterilization of CGR03 (CT4). To achieve final concentrations of 50, 500 and 5,000 mg L^{-1} for each treatment, appropriate volumes of sterilized CTs and CEs were added to 2-L Erlenmeyer flasks filled with 1 L of sterilized PDA (approximately 50°C) and homogenized for 2 min using a magnetic rotor (Agimatic-N, JP-Selecta, Barcelona, Spain). Subsequently, the medium was poured into Petri dishes (9 cm in diameter; 25 ml $dish^{-1}$). Mycelial plugs (7 mm in diameter) of the isolate V024 of *V. dahliae*, obtained from the margin of 7-day-old growing cultures on PDA, were transferred to the centre of the sterilized CTs and CEs-amended plates. PDA plates amended with similar amounts of SDW instead of sterilized CTs or CEs were used as controls. All Petri dishes were incubated at 24°C in darkness for 18 days. There were four replicated plates for each sterilized CT and CE and concentration, and the experiment was conducted twice. After 18 days of incubation, the largest and smallest diameters of the colonies of *V. dahliae* were measured using a digital calliper, and the mean data were converted to radial growth rates (mm day^{-1}).

5.2.4. Experiment II: effect of compost teas and extracts on inoculum density of *Verticillium dahliae* in naturally infested soil

Different versions of CTs and CEs were evaluated to test their ability to reduce the viable inoculum of *V. dahliae* in naturally infested soil.

5.2.4.1. Soil samples

Samples of soil naturally infested with *V. dahliae* were collected from a commercial olive orchard that was severely affected by VWO located in Villanueva de la Reina (UTM coordinates X: 38.012845; Y: 3.909219) from Jaen Province (Andalucía, southern Spain). Five soil sub-samples of ≈500 g were collected 1 m from the trunk and from the upper 30 cm of soil using a cylindrical soil auger. Sub-samples were mixed, air-dried at room temperature until completely dry, and sieved (0.8 mm diameter) to remove large particles (Trapero et al., 2013a).

5.2.4.2. CTs and CEs-based treatments

All the CTs and CEs that were evaluated in this experiment are indicated in Table 1. Additionally, CTs and CEs were diluted to 50% (v:v) by adding SDW and sterile PDB, respectively. Therefore, a total of six CTs, CT1, CT1-50, CT2, CT2-50, CT4 and CT4-50, and six CEs, CE1, CE1-50, CE2, CE2-50, CE4 and CE4-50, were evaluated.

5.2.4.3. Soil treatment and estimation of *Verticillium dahliae* inoculum density

Three 100-ml plastic pots with holes drilled in the base for drainage were filled with 60 g of naturally infested air-dried soil and were watered with 30 ml of the CT- and CE-based treatments described above. Two additional treatments, including soil amendment with the grape marc compost CGR03 and with its sterile version SCGR03, were used as references. The treatments of infested soil with both solid compost amendments were conducted as described by Varo-Suárez et al. (2018). Thus, 60 g of naturally infested soil was amended with each CGR03 amendment at a dose of 20% (v:v). Subsequently, the mixture was homogenized, introduced into a 100-ml plastic pot and irrigated with 30 ml of SDW. Additional plastic containers filled with 60 g of naturally infested soil and watered only with 30 ml of SDW or sterile PDB were used as controls. Subsequently, all containers were hermetically sealed and incubated for 24 hours at 25°C. After incubation, soil samples were removed from the plastic pots and air-dried at room temperature. There were three replications per each CGR03, CTs, CEs and control treatments, resulting in 48 plastic pots in total. The experiment was conducted twice.

The inoculum density (ID) of *V. dahliae* in each soil sample was estimated by wet sieving (Huisman and Ashworth, 1974) by splitting each soil sample across 10 plates of a modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977). MSPA plates were incubated for 14 days at 24°C in the dark. After incubation, soil residues were removed with tap water, and colonies of *V. dahliae* were counted under a stereoscopic microscope (Nikon SMZ-2T, Tokyo, Japan). The ID in each soil sample was estimated from the number of *V. dahliae* colonies and was expressed as the number of propagules per gram of air-dried soil (ppg), and the relative percentage of ID reduction was calculated.

5.2.5. Experiment III: effect of compost teas and extracts on VWO suppression

5.2.5.1. Preparation of *Verticillium dahliae* inoculum

The inoculum of *V. dahliae* was produced in a cornmeal-sand mixture prepared by mixing dry sand, cornmeal and distilled water at a 9:1:2 weight proportion. Subsequently, 2-L flasks were filled with 1 kg of the cornmeal-sand mixture and inoculated with 50 mycelial plugs (5 mm in diameter) of *V. dahliae* isolate V024 grown on PDA at 25°C for 10 days in the dark. The flasks were shaken once a week to promote the homogeneous colonization of the cornmeal-sand mixture by the fungus (Varo et al., 2016b). After 4 weeks, the inoculum density in the cornmeal-sand mixture flasks was calculated as colony-forming units (CFUs) by plating several serial dilutions of the cornmeal-sand mixture on PDA plates. The average of the CFU values was obtained from 15 PDA replicate plates.

5.2.5.2. Plant material and fungal inoculation

Six-month-old rooted cuttings of the olive cv. Picual (highly susceptible) (López-Escudero et al., 2004) obtained from a commercial nursery were used as a susceptible host. Olive plants were transplanted from their original substrate to plastic pots (0.8 L) containing sterile peat, which was mixed with 20% (w:w) of the cornmeal-sand mixture infested with *V. dahliae* isolate V024, resulting in an inoculum density of 10^7 CFU g^{-1} in the final potting mixture.

5.2.5.3. Plant treatment and experimental design

Treatments using CTs and CEs prepared as previously described were evaluated *in planta* by means of plant irrigation with 300 ml of each treatment just after transplanting. Four CTs, CT1, CT1-20, CT2 and CT2-20, and four CEs, CE1, CE1-20, CE2 and CE2-20, were used. Additionally, two solid compost amendments, CGR03 and SCGR03, were evaluated. The solid compost amendments were added by mixing the final potting mixture with 20% (v:v) of each compost and subsequently watering with 300 ml of SDW after transplanting. Untreated plants grown in peat inoculated with *V. dahliae* were used as the untreated control. In addition, plants grown in non-inoculated sterile peat and irrigated with 300 ml of SDW were used as sterile controls. After inoculation, all plants were incubated in a growth chamber at 22 ± 2°C in the dark at 100% relative humidity for 4 days to prevent the plants from suffering high stress after transplanting. Subsequently, the light and humidity parameters were modified to a 12-h photoperiod of fluorescent light [10,000 lux] and 70% relative humidity and maintained until the end of the experiment. Plants were irrigated three times per week. In this experiment, a completely randomized design was used, with CTs, CEs, solid compost amendments and controls (untreated and sterile) as independent variables and twelve olive plants per treatment as replications; the experiment was conducted twice.

5.2.5.4. Disease assessment

Disease severity was evaluated based on the percentage of affected plant tissues, such as leaves and shoots showing symptoms of chlorosis, necrosis and/or defoliation. To this end, plants were assessed weekly using a 0 to 16 rating scale until the development of the disease stopped in each treatment. This scale estimated the percentage of affected tissue using four main categories or quarters (≤25, 26–50, 51–75, and 76–100%) with four values per category. Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) are linearly related to the percentage of affected tissue (Y) by the equation $Y = 6.25X - 3.125$ (Varo-Suárez et al., 2018). Disease incidence (DI) and mortality were recorded as the percentage of symptomatic or dead plants, respectively, to assess the intensity of the response (López-Escudero et al., 2004). At the end of the disease assessment, the area under the disease progression curve (AUDPC) was calculated from the disease severity values by the trapezoidal integration method

(Campbell and Madden, 1990), and the percentage of disease suppression was calculated as:

$$100\text{-}100 \times (AUDPC_{treatment}/AUDPC_{inoculated\ control})$$

At the end of the experiment, olive plants were harvested and the aerial fresh weight (i.e., branches and leaves) per treatment was recorded.

Finally, three symptomatic plants per treatment were selected after harvesting to perform re-isolations to confirm the fungal infection. For this purpose, affected stems were selected, washed under running tap water, and surface-sterilized by immersing them in a 0.5% solution of commercial bleach (Cl at 50 g L^{-1}) for 1 min. Subsequently, small wood fragments were plated on PDA acidified with lactic acid (2.5 ml of 25% [v:v] per litre of medium) to avoid bacterial contamination and were incubated at 24°C in darkness for 6 days.

5.2.6. Data analyses

In experiment I, a two-way analysis of variance (ANOVA) was performed according to a factorial design with treatment, dose and the interaction treatments x dose as factors. In experiments II and III, ANOVA was performed according to a completely randomized design. In all experiments, data were tested for normality, homogeneity of variances, and residual patterns, which proved their suitability for the statistical analysis. When ANOVA showed significant differences among treatments, mean values were compared using Fisher´s protected LSD test at P = 0.05. In experiment III, treatments in which plants did not show symptoms (mean values of 0.0) were not included in the analysis. All data in this study were analysed using Statistix 10 (Analytical Software, 2013).

5.3. RESULTS

5.3.1. Experiment I: effect of compost teas and extracts on mycelial growth of *Verticillium dahliae*

The ANOVA showed that there were no significant differences between the three doses tested (F = 2.08; P = 0.1348) and the interaction treatment x dose (F = 1.91; P = 0.0652), but significant differences between treatments were found (F =

6.32; P = 0.0001). Therefore, the mean of the three doses for each treatment was used to compare them. For CTs, treatments with sterilized tea, by autoclaving (CT2) or by filtration (CT3) significantly reduced the mycelial growth rate in comparison with that of the control (2.9, 3.1 and 3.7 mm day^{-1} for CT2, CT3 and control, respectively) (Figure 1). Nevertheless, the treatment with sterilized compost tea (CT4) was not able to significantly reduce the mycelial growth rate of *V. dahliae* in comparison with that in the control. On the other hand, regarding CEs, only the treatment with sterilized extract by filtration (CE3) achieved a significant reduction in the mycelial growth of the pathogen compared with that in the control (2.7 and 3.7 mm day^{-1} for CE3 and control, respectively) (Figure 1). In contrast to the results for CTs, significant differences were found between sterilization methods for CEs; the sterilized extract by filtration (CE3) was more effective than the sterilized extract by autoclaving (CE2) in reducing the mycelial growth rate of the pathogen (2.7 and 3.8 mm day^{-1}, respectively) (Figure 1).

5.3.2. Experiment II: effect of compost teas and extracts on inoculum density of *Verticillium dahliae* in naturally infested soil

The ID of *V. dahliae* estimated by wet sieving of the SDW and PDB controls resulted in 124 and 69 ppg, respectively. Significant differences in terms of ID reduction were found between CT treatments (F = 10.87; P < 0.0001). The amendments with both sterilized and non-sterilized CGR03 achieved the highest ID reduction of the pathogen (76.1 and 61.2%, respectively), but no significant differences were found between them (Figure 2). For CTs, the highest ID reduction was observed in soil treated with sterilized compost tea at the higher dose (CT4), which achieved a reduction of 54.8%, although no differences in ID reduction were found between CT4 and CT2-50. However, sterilized compost tea applied at a lower dose (CT4-50) achieved a significantly lower ID reduction (16.7%) than CT4. In the case of the teas sterilized by autoclaving (CT2 and CT2-50), no significant differences were found between the doses, which reached reduction values of 33.4 and 47.8%, respectively. The natural teas (CT1 and CT1-50) were the least effective treatments among all CTs tested in this experiment, with ID reductions of 24.5 and 17.4%, respectively (not significantly different) (Figure 2A).

In the CEs, significant differences were found between treatments (F = 7.48; P = 0.0004). The natural extract (CE1) and the extracts sterilized by autoclaving

(CE2 and CE2-50) were the most effective treatments among CEs in reducing the ID of *V. dahliae* (62.0, 72.1 and 66.6%, respectively) and were as effective as the solid compost amendments used as reference (Figure 2B). In contrast, CE1-50, CE4 and CE4-50 were the least effective treatments among CEs in reducing the ID of the pathogen, with values of 21.3, 24.4 and 19.2%, respectively (Figure 2B).

5.3.3. Experiment III: effect of compost teas and extracts on VWO suppression

The efficacy of CTs and solid compost amendments with CGR03 on disease progression is reported in Table 2. The disease progression was assessed for 13 weeks from the day of inoculation with the pathogen. Olive plants treated with all types of CEs at both tested doses showed phytotoxicity symptoms four days after treatment application, so these treatments were excluded from the experiment. Non-inoculated (sterile control) plants remained healthy throughout the experiment. Untreated control plants inoculated with the *V. dahliae* isolate V024 showed typical Verticillium wilt symptoms caused by infection with the defoliating pathotype in olive plants of cv. Picual such as defoliation, wilting and necrosis of shoots. The first symptoms were observed in the inoculated control plants 34 days after inoculation, reaching a DI of 91.7% at the end of the experiment.

Plant showing VWO symptoms were observed in all treatments tested, but treatments with CT1 and CT1-50 and with both solid compost amendments were able to significantly reduce the final DI in comparison with that in the inoculated control ($P < 0.0001$). Although the treatment with natural tea (CT1) showed a final DI of 40%, no dead plants were observed at the end of the assessed period; this was the only treatment in which there was no plant mortality. In addition, treatments with CT2, SCGR03 and CGR03 also achieved a significant reduction in mortality in comparison with that in the inoculated control ($P < 0.0001$) (Table 2).

In terms of final disease severity and suppression, significant differences were found between treatments ($F = 11.8$; $P < 0.0001$ and $F = 15.6$; $P < 0.0001$ for disease severity and suppression, respectively). Treatments with CTs at the lower doses tested (CT1-20 and CT2-20) were not able to reduce disease progression compared with that in the untreated controls. Conversely, the same CTs treatments at the higher doses (CT1 and CT2) and both solid compost amendments showed significant disease suppression, with values ranging from 67.4 to 96.5% for

SCGR03 and CT1, respectively (Table 2). Finally, plants treated with CT1, CT2, CGR03 and SCGR03 showed similar aerial weight values as plants from the sterile control, which were significantly higher than those of plants treated with CT1-20 and CT2-20 and of the untreated control plants ($F = 9.0$; $P < 0.0001$) (Table 2). The pathogen was re-isolated from all selected symptomatic plants, confirming infection by *V. dahliae*.

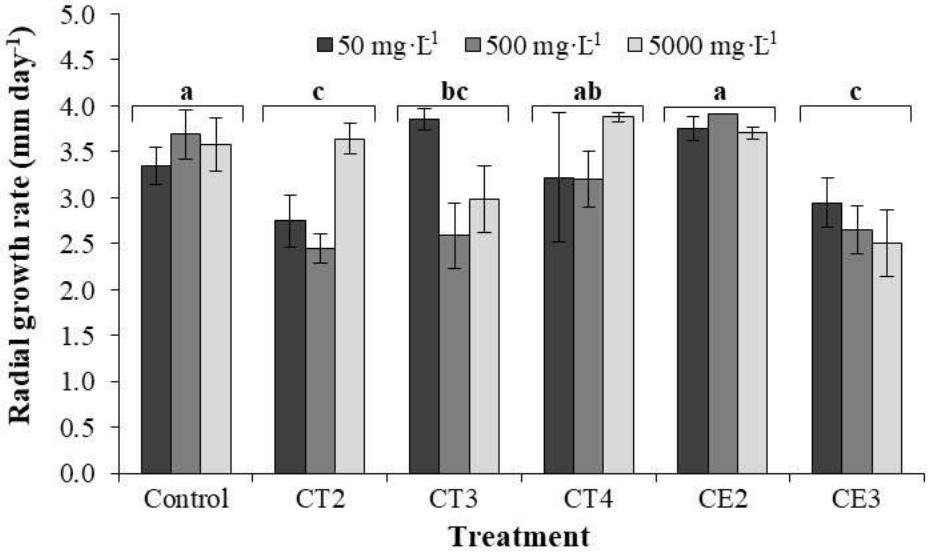


Figure 1. Effect of sterilized compost teas (CTs) and compost extracts (CEs) obtained from CGR03 on mycelial growth of *Verticillium dahliae* isolate V024 after 18 days of growth on PDA. Bars represent the mean values of eight replicated Petri dishes. Bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

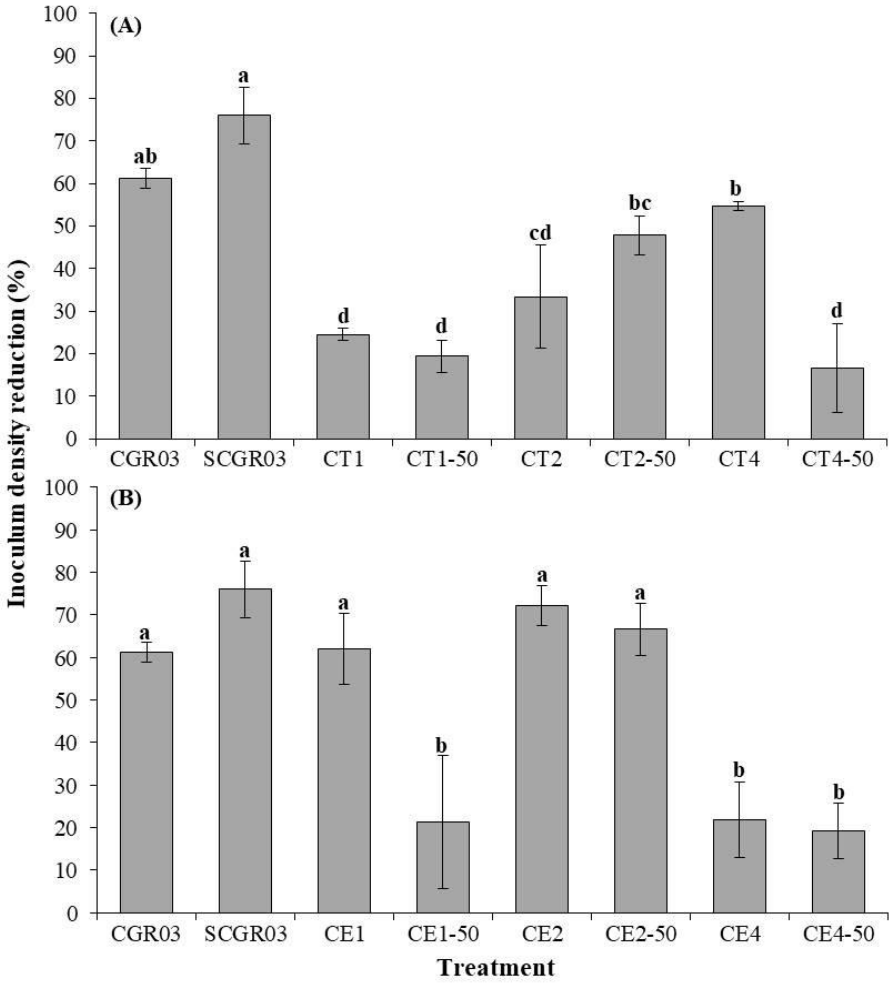


Figure 2. Effect of (A) compost teas (CTs) and (B) compost extracts (CEs), obtained from CGR03 on reducing the inoculum density of *Verticillium dahliae* in a naturally infested soil. For each treatment, bars represent the mean values of six replicated plastic pots. Bars with a common letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

Table 1. Compost teas (CTs) and extracts (CEs) obtained from the suppressive grape marc compost CGR03 and tested in this study.

Treatment code	Treatment description	Experiment[a]
Solid compost amendments		
CGR03	Natural grape marc compost	Exp. II, III
SCGR03	Sterilized grape marc compost by autoclaving	Exp. II, III
Compost teas (CTs)		
CT1	Natural tea	Exp. II, III
CT1-50	CT1 diluted to 50% with SDW	Exp. II
CT1-20	CT1 diluted to 20% with SDW	Exp. III
CT2	Sterilized tea by autoclaving	Exp. I, II, III
CT2-50	CT2 diluted to 50% with SDW	Exp. II
CT2-20	CT2 diluted to 20% with SDW	Exp. III
CT3	Sterilized tea by filtration	Exp. I
CT4	Compost tea by prior sterilization of CGR03	Exp. I, II
CT4-50	CT4 diluted to50 % with SDW	Exp. II
Compost extracts (CEs)		
CE1	Natural extract	Exp. II, III
CE1-50	CE1 diluted to 50% with PDB	Exp. II
CE1-20	CE1 diluted to 20% with PDB	Exp. III
CE2	Sterilized extract by autoclaving	Exp. I, II, III
CE2-50	CE2 diluted to 50% with PDB	Exp. II
CE2-20	CE2 diluted to 20% with PDB	Exp. III
CE3	Sterilized extract by filtration	Exp. I
CE4	Sterilized extract by prior sterilization of CGR03	Exp. II
CE4-50	CE4 diluted to 50% with PDB	Exp. II

[a] Experiments in which each product was evaluated.

Table 2. Effect of compost teas (CTs) and solid amendments with grape marc compost CGR03 against Verticillium wilt of olive.

Treatments[a]	Incidence[b] (%)	Mortality[c] (%)	Disease Severity[d] (%)	Disease Suppression[e] (%)	Aerial weigh[f] (g)
Untreated control	91.7 ± 8.3	75.0 ± 13.1	81.3 ± 11.0	0.0 ± 14.4	6.4 ± 1.5
Sterile control	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	21.3 ± 1.6
CT1	40.0 ± 16.3	0.0 ± 0.0	7.5 ± 3.8	96.5 ± 1.6	28.4 ± 2.2
CT2	28.6 ± 18.4	14.3 ± 14.3	24.6 ± 16.1	76.9 ± 18.9	21.7 ± 4.0
CGR03	50.0 ± 15.1	8.3 ± 8.3	21.1 ± 10.1	78.8 ± 12.0	22.0 ± 2.6
SCGR03	33.3 ± 14.2	16.7 ± 11.2	24.5 ± 12.3	67.4 ± 16.6	22.7 ± 3.8
CT2-20	91.7 ± 8.3	66.7 ± 14.2	81.5 ± 9.9	1.8 ± 12.4	9.5 ± 2.3
CT1-20	100 ± 0.0	91.7 ± 8.3	91.7 ± 8.3	-16.9 ± 11.5	8.2 ± 3.4
LSD 5%	32.8	29.6	28.2	35.8	7.8
d.f.	76	76	76	76	88

[a] Treatments code are described in Table 1. Untreated control: inoculated with *V. dahliae* and non-treated plants. Sterile control: non-inoculated with *V. dahliae* and non-treated plants. LSD 5%, least significant difference between two means at the 5% level; d.f., degrees of freedom associated with LSD.

[b,c] Percentage of olive plants showing symptoms[b] or dead[c] caused by *V. dahliae* 13 weeks after inoculation.

[d] Final disease severity (%) evaluated 13 weeks after inoculation of *V. dahliae*.

[e] Percentage of disease suppression at the end of the experiment.

[f] Fresh weigh of the aerial part of olive plants harvested at the end of the experiment.

[b, c, d, e, f] Values represent the means of twelve replicated plants per treatment ± standard error of the means.

5.4. DISCUSSION

The sustainable management of agro-industrial residues such as olive mill and grape marc wastes is becoming increasingly important for olive and wine-producing countries. Thus, the composting of those residues has been widely proposed as the most sustainable approach for that purpose. The use of compost as an organic amendment is known to improve soil health and structure. In addition to the beneficial results for soil properties, the application of compost is also considered an environmentally friendly strategy for controlling soilborne plant pathogens.

In a recent study, Varo-Suárez et al. (2018) reported the suppressive effect of the grape marc compost CGR03 against *V. dahliae* in *in vitro* and *in vivo* tests using olive plants. The suppressive effect as well as the potential use of the aqueous extracts obtained from CGR03 as a feasible strategy to control VWO is further explored in the present study in an attempt to elucidate the biotic or abiotic mechanisms of action of this suppressive compost. Thus, a total of 9 CTs and 9 CEs obtained from the suppressive grape marc compost CGR03 were evaluated in this study for their ability to reduce the mycelial growth, inoculum density and disease progression of *V. dahliae* in olive plants by means of *in vitro* and *in vivo* experiments under controlled conditions.

The use of sterilized compost teas and extracts in Petri dish experiments can allow the elucidation of the contribution of secondary metabolic compounds produced by the microorganisms living in the compost during CTs and CEs production. When CTs were added to PDA to verify their effect on the mycelial growth of *V. dahliae*, sterilized teas by both autoclaving and filtration achieved a significant reduction in mycelial growth. This result suggests that the compounds involved in this effect were not affected by the sterilization method in the case of the CTs. Interestingly, when compost tea was obtained from previously sterilized CGR03 (CT4), the inhibition effect disappeared, and no differences were found in comparison with the control. This observation indicates that part of the mycelial reduction observed in CT2 and CT3 is due to the secondary metabolites produced by microorganisms living in the compost during the incubation period; therefore, biotic factors seem to be involved in the mycelial reduction. In contrast, in the case of CEs, there were significant differences in the effects of the CEs produced by both sterilization methods. A significant reduction in mycelial growth was found in the case of the sterilized extract by filtration (CE3), but no differences were found

between the effect of the extract sterilized by autoclaving (CE2) and that of the control. Different types of secondary metabolite could be produced during CEs incubation compared with those in CTs, which may be due to the different media used for the incubation (PDB and SDW for CEs and CTs, respectively). This could explain the differences observed between the sterilization methods, since some types of secondary metabolite produced in CEs could be affected by the autoclaving sterilization process. This finding agrees with those reported by Alfano et al. (2011), who observed that filter sterilization of compost water extracts obtained from olive mill wastes preserves the metabolic compounds produced by the active microflora, which could have led to the growth inhibition of *V. dahliae*. Although the stimulation of mycelial growth was not observed in this experiment in any of the treatments tested, Vitullo et al. (2013) reported differences between sterilization methods, in which the pre-sterilization by filtration of a water extract from olive mill wastes even stimulated the fungal growth of *V. dahliae*. Although a significant mycelial growth reduction was observed in CT2, CT3 and CE3, our results indicate that secondary metabolites produced during CTs or CEs incubation have little effect on the mycelial growth inhibition of *V. dahliae*, since a reduction of more than 50% was not achieved in any of the treatments tested. Similar results were obtained by Gea et al. (2009), who reported that compost teas obtained from several agricultural wastes lose much of their activity and have little effect on the mycelium growth of *Verticillium fungicola* when they are obtained by autoclaving or microfiltration, highlighting the importance of the presence of living microorganisms involved in pathogen inhibition.

The eradication of microsclerotia or their inability to germinate is considered essential to an effective management strategy for controlling Verticillium wilt diseases (Antonopoulos et al., 2008). The study conducted by Varo-Suárez et al. (2018) is the only work in the literature that reported the effectiveness of compost teas in inhibiting the natural microsclerotia of *V. dahliae.* More recently, Mulero-Aparicio et al. (2019b) reported a significant ID reduction when CGR03 was applied in field conditions. Although the authors evaluated several organic amendments and compost teas that were able to reduce microsclerotia viability in those studies, the effects of compost teas and extracts obtained from the suppressive grape marc compost CGR03 were not tested. In our study, when CTs and CEs obtained from CGR03 were applied to a naturally infested soil, significant differences in ID reduction were observed. Interestingly, there were no significant differences between the natural and the sterilized versions of the solid grape marc compost. This result indicates that the ID reduction previously observed by Varo-

Suárez et al. (2018) and Mulero-Aparicio et al. (2019b) as well as in the current study can be attributed, at least to some extent, to abiotic factors such as the chemical and/or physical properties of the compost. Furthermore, only CT2-50 and CT4, which are the sterile versions of the CTs, were able to reduce the ID of the pathogen at a similar rate as the natural solid organic amendment (CGR03) used as a positive reference for inhibition, whereas natural versions of CTs were the least effective at reducing the ID of *V. dahliae*. In contrast, in the case of CEs, the natural and sterilized compost extracts (CE1, CE2 and CE2-50, respectively) were able to effectively reduce the ID, and no significant differences were found in comparison with the reductions from CGR03 and SCGR03. However, a partial loss of effectiveness in reducing the ID of *V. dahliae* was found when CGR03 was sterilized prior to the generation of the extract (CE4). This result suggests that, in the case of CEs, the effect on ID reduction was predominantly biotic rather than abiotic, since the production of secondary metabolites by microorganisms during the incubation period for CEs could be higher than that of CTs, possibly due to the higher nutrient richness of the medium used in the generation of these compost extracts (PDB). Since compost teas and extracts are liquid versions of the original solid compost (Ingham, 2003), the biological changes that take place during compost tea and extract production should be taken into account because their suppressive effects could be substantially different from those of the original compost. In conclusion, the results obtained from the ID reduction experiment conducted in this study indicate that the effectiveness of the pathogen of the grape marc compost and its CTs and CEs in reducing the ID could be attributable to both biotic and abiotic factors, and their respective relevance could vary depending on the characteristics of the product tested.

Bonanomi et al. (2007) reported that a significant reduction in the pathogen population is correlated in more than 80% of cases with efficient control of the diseases caused by *V. dahliae*. In the current study, after the ID reduction of the pathogen achieved by some versions of the CTs and CEs and by the solid grape marc composts, a significant control of VWO was also observed. In this study, both the natural and sterilized compost teas (CT1 and CT2, respectively) achieved significant control of the disease. The same level of disease suppression was achieved by CGR03 and SCGR03, confirming that heat sterilization did not influence the suppressive ability of this grape marc compost. This result indicates that the effect of biocontrol observed in this experiment was predominantly abiotic. These results are in agreement with those obtained in the microsclerotia

experiment, in which both natural and sterile solid grape marc compost achieved the same ID reduction.

The application of organic amendments such as crop residues, organic wastes or compost can also have a negative effect on plants (i.e., phytotoxicity) depending on the composition of the material and the rate of application (Bonanomi et al., 2007). However, partially decomposed materials such as compost generally lose their phytotoxicity during the composting process (Zucconi et al., 1981). In this study, plants treated with both versions of the grape marc compost (CGR03 and SCGR03) at a rate of 20% (v:v) did not show phytotoxicity symptoms. On the other hand, plants showed phytotoxicity symptoms and died four days after being treated with all versions of the CE. The phytotoxicity effect of the CEs is probably due to the higher concentrations of substances toxic to the plants that were produced by microorganisms inhabiting the compost during CE incubation. This effect did not occur with the CTs, which were produced in a nutrient-poor medium (SDW). Disease suppression and phytotoxicity are related to the rate of application (Bonanomi et al., 2007). The minimum application rate of CTs, CEs and CGR03 used in this study was 20% (v:v), since several studies reported that, for composts, an application rate of at least 20% (v:v) is necessary to achieve significant disease suppression (Diab et al., 2003; Serra-Whittling et al., 1996; Tuitert et al., 1998). Due to the phytotoxic effects of some treatments (CEs), more research is needed to adjust the application doses to avoid phytotoxicity while maintaining the suppressive effect.

As Termorshuizen et al. (2006) stated, a compost of a given composition or made at one factory should be consistent over time in terms of disease suppression. To this end, Varo-Suárez et al. (2018) reported total ID reduction and disease suppression when plants were amended with the same grape marc composts produced in two consecutive years (CGR02 and CGR03) with the same products and at the same place, confirming the consistency of the compost in terms of suppressive ability. In the current study, although CGR03 did not achieve total ID reduction or total control of the disease, it was able to effectively reduce both the ID of *V. dahliae* and the disease progression. This consistency against the pathogen is considered an interesting trait of this compost, which could be produced on a large scale in the future by means of a standardized method of production.

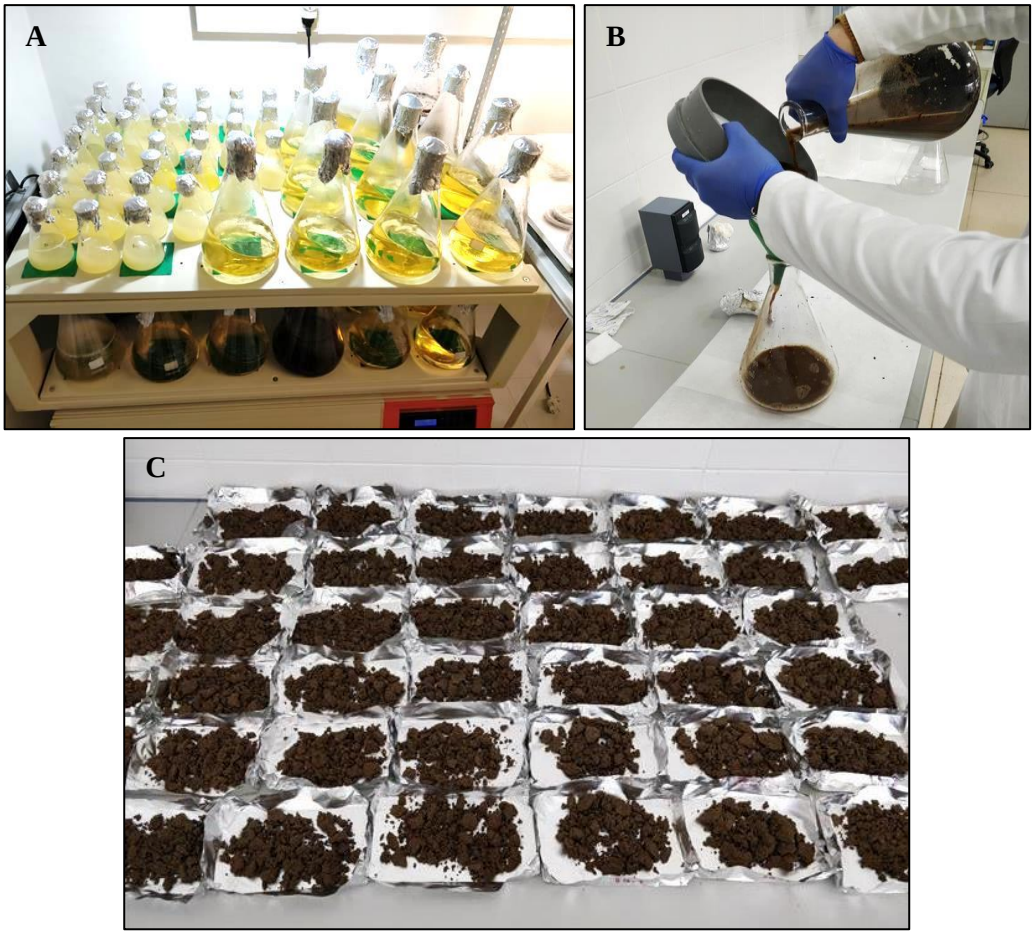
In conclusion, the outcomes obtained in the present study highlight the effectiveness of different compost teas and extracts obtained from the suppressive

grape marc compost CGR03 in reducing the inoculum density of the pathogen in soil and in VWO suppression under controlled conditions. Although more research is needed to elucidate the role of the microorganisms in the effect of the grape marc compost CGR03 against the pathogen, this study highlights the possible modes of action of this compost, which represents an advance in the development of feasible alternatives for the control of this disease under field conditions. Furthermore, since aqueous versions of the compost could be applied through irrigation systems, the evaluation of CTs and CEs for use under field conditions is crucial to optimizing the dose and mode of application for the purpose of achieving effective control of the disease in commercial olive orchards where farmers are in urgent need of feasible control strategies.

ACKNOWLEDGEMENTS

This research was funded by the Spanish Ministry of Economy, Industry and Competitiveness (MINECO; project AGL2016-76240-R) and co-financed by the European Union FEDER Funds. A.M.A. is the holder of a 'Formación de Profesorado Universitario' (FPU) fellowship from the Spanish Ministry of Education, Culture and Sports (MECD).

SUPPLEMENTARY FIGURES



Compost teas and extracts obtained from the suppressive grape marc compost CGR03. A) Incubation of compost teas and extracts. B) Filtration of compost teas with a sieve to delete compost particles. C) Samples of naturally infested soil after treating with compost teas and extracts to evaluate their effect on reducing the inoculum density of *Verticillium dahliae*.

CHAPTER 6

Efficacy of FO12 and CGR03 under semi-controlled conditions

6. Effectiveness of the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 in controlling Verticillium wilt of olive under semi-controlled conditions.

ABSTRACT

Verticillium wilt of olive (VWO), caused by the widespread soil-borne fungus *Verticillium dahliae* Kleb., is currently the most serious disease affecting olive trees (*Olea europaea* L.) in all production areas worldwide. An integrated management strategy using eco-friendly approaches such as genetic resistance and biological control is considered the most advisable approach to controlling the disease in commercial olive orchards. Therefore, the main objective of this study was to evaluate the effectiveness of the non-pathogenic strain of *F. oxysporum* FO12 and the grape marc compost CGR03 in reducing both the inoculum density of *V. dahliae* and the disease progress in two olive cultivars with different resistance levels (Picual and Arbequina). The experiment was conducted under semi-controlled conditions using a naturally infested soil with two inoculum densities of *V. dahliae*. The biocontrol treatments (FO12 and CGR03) were previously selected out of 220 natural products as two of the most effective treatments against the pathogen. Both FO12 and CGR03 significantly reduced the inoculum density of the pathogen in comparison with that of the control ($P = 0.05$), with minimum values of 0.13 and 0.53 MS g^{-1}, respectively, during the experimental period. Additionally, CGR03 significantly reduced the progression of the disease compared with that in the control ($P = 0.05$), and FO12 achieved total control of VWO, since no plants treated with this biological control agent showed VWO symptoms. This study highlights the effectiveness of these biocontrol treatments under semi-controlled conditions and increases the knowledge about the potential use of eco-friendly approaches for effective control of VWO in the future.

This chapter has been submitted to Phytopathologia Mediterranea:

Mulero-Aparicio A, Trapero A, López-Escudero FJ. Effectiveness of the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 in controlling Verticillium wilt of olive under semi-controlled conditions. *Phytopathologia Mediterranea*.

6.1. INTRODUCTION

The widespread soil-borne fungus *Verticillium dahliae* Kleb. is known to cause vascular diseases in several crops with relevant agronomic value (Pegg and Brady, 2002). Verticillium wilt of olive (VWO) is currently the most serious disease caused by this pathogen affecting olive trees (*Olea europaea* L.) in all producing areas worldwide, causing significant economic losses and plant death (López-Escudero and Mercado Blanco, 2011). In the infected olive groves of the Guadalquivir valley (Andalusia, Spain), this disease has reached an average incidence of 20% (López-Escudero et al., 2010).

Since there is no truly efficient control of VWO when control measures are individually applied, an integrated management strategy is considered the most advisable approach to reducing the disease incidence in commercial olive crops (López-Escudero and Mercado Blanco, 2011). Within this strategy, and due to current environmental concerns, eco-friendly approaches such as cultural practices, genetic resistance and biological control have acquired more relevance in recent years. The genetic resistance levels of olive cultivars (cvs.) against VWO have been assessed in previous studies. López-Escudero et al. (2004) reported that cvs. Picual and Arbequina are highly susceptible to the defoliating pathotype of *V. dahliae* when they are artificially inoculated by root dipping. Additionally, the relationship between the inoculum density (ID) of *V. dahliae* in infested soils and the resistance showed among olive cultivars was confirmed in several studies. López-Escudero and Blanco-López (2007) reported that 'Picual' was extremely susceptible to *V. dahliae* after being planted in soils with low ID, while 'Arbequina' was moderately resistant under the same conditions (Trapero et al., 2013b). This relationship was recently confirmed in a study carried out in commercial olive orchards (Roca et al., 2015). Although there are numerous studies related to the biological control of *V. dahliae*, very few studies have addressed the use of biocontrol treatments against VWO. Therefore, the use of *Pseudomonas* spp. (Mercado-Blanco et al., 2004; Triki et al., 2012; Gómez-Lama et al., 2017) and *Trichoderma* spp. (Lima et al., 2007; Jiménez-Díaz et al., 2009) have been reported as promising biological control agents (BCAs) against VWO. In addition, preliminary studies on the use of organic amendments against VWO have been conducted (Vitullo et al., 2013). Recently, a massive screening was conducted to evaluate the effectiveness of 220 natural compounds, including microorganisms (Lozano et al., 2016; Varo et al., 2016a), organic amendments

(Varo-Suárez et al., 2018) and plant extracts (Varo et al., 2017), against *V. dahliae* by *in vitro* and *in planta* experiments under controlled conditions. From that screening, a non-pathogenic strain of *Fusarium oxysporum*, named FO12, and a grape marc compost, labelled CGR03, were selected as two of the most effective treatments against the pathogen. However, further studies evaluating these products under semi-controlled and field conditions are essential for demonstrating their effectiveness against VWO.

Knowledge about the effect of the interaction between different approaches, such as genetic resistance and biological control, used for an integrated control strategy of VWO is of crucial importance in achieving efficient control of the disease. However, to date, the combined effect of these approaches remains unknown. Thus, the main objective of this study was to evaluate the effect of the non-pathogenic strain of *F. oxysporum* FO12 and the grape marc compost CGR03 on the progression of ID of *V. dahliae* and on the development of VWO in two olive cultivars with different levels of genetic resistance in an experiment carried out under semi-controlled conditions.

6.2. MATERIALS AND METHODS

6. 2.1. Plant material

One-year-old rooted olive plants of cvs. Picual and Arbequina (susceptible and moderately susceptible, respectively) (Trapero et al., 2013a) were used for the experiment. Rooted olive plants were obtained from a commercial nursery producing plants that were certified free of *V. dahliae* and other olive pathogens. At planting time, plants were one year old and 1.0-1.1 m high, with a single trunk and three or four secondary branches.

6.2.2. Naturally infested soil and inoculum density estimation

The soil used in this study was collected from a commercial field previously cultivated with cotton over the last 50 years located in Villanueva de la Reina (UTM coordinates X: 38.012845; Y: 3.909219) in Jaen Province (Andalusia, southern Spain).

To estimate the ID of *V. dahliae* in this soil, five soil sub-samples of ≈500 g were collected from the upper 30 cm of soil using a cylindrical soil auger. Sub-samples were mixed, air-dried at room temperature until completely dry, and sieved (0.8 mm diameter) to remove large particles. Subsequently, the inoculum density of *V. dahliae* was estimated by wet sieving (Huisman and Ashworth, 1974) onto modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977). Briefly, three samples of 25 g of the naturally infested soil were suspended in 100 ml of distilled water, shaken at 270 rpm for 30 min at room temperature and filtered through 150 and 35 µm sieves. Subsequently, the residue retained on the 35 µm sieve was recovered in 100 ml of distilled water. Finally, this suspension was plated across ten plates of MSPA (1 ml/plate). Subsequently, plates were incubated for 14 days at 24 ± 2 °C in the dark. Afterward, soil residues were removed from the agar surface under running tap water, and colonies of *V. dahliae* were counted by means of a stereoscope microscope (Nikon SMZ-2T, Tokyo, Japan). The ID in soil was estimated from the number of *V. dahliae* colonies counted per sample and was expressed as microsclerotia (MS) per gram of soil (MS g^{-1}) (López-Escudero and Blanco-López, 2005b).

6.2.3. Biocontrol treatments

Two biocontrol treatments were evaluated in this study. The grape marc compost CGR03 and the non-pathogenic strain of *Fusarium oxysporum* FO12 were selected due to their suppressive effects on VWO demonstrated in previous *in vitro* and *in vivo* experiments (Varo et al., 2016a; Varo-Suárez et al., 2018; Mulero-Aparicio et al., 2019a).

6.2.3.1 Grape marc compost CGR03

The organic amendment CGR03 was provided by the agri-food cooperative "Cooperativa San Acacio", Montemayor, Córdoba (southern Spain). To obtain the compost, grape marc wastes consisting of grape skins, seeds and stems of the grapevine cv. Pedro Ximénez from the wine industry were used as feedstock. Grape marc is collected annually from August to September and composted in insulated bins of 15 m^3 for eight months. When the compost temperature fell below 60 °C at a depth of 50 cm, the compost was turned to promote aeration and homogeneity and to renew the composting process. Compost was collected for use when the temperature permanently fell below 40 °C (maturation/recolonization

phase). The pH value of the compost was 6.9, determined in 1:5 (v:v) compost/water extract. Before use, the compost was proven to be mature and stable by measuring its temperature (30-35 °C for mature compost) to avoid phytotoxicity (Mehta et al., 2014; Varo-Suárez et al., 2018).

6.2.3.2 Non-pathogenic strain of *Fusarium oxysporum* FO12

The non-pathogenic *F. oxysporum* strain FO12, from to the fungal collection of the Department of Agronomy at the University of Córdoba (Spain), was used as a BCA. It was prepared from a single-spore stock culture maintained on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants at 4 °C. Seven-day-old cultures of *F. oxysporum* incubated on PDA at 25 °C under a 12-h photoperiod of fluorescent light were used as the inoculum source. To prepare the aqueous inoculum of FO12 needed for the treatment, a 2 L Erlenmeyer flask containing 1 L of potato dextrose broth (PDB; Difco Laboratories®, MD, USA) was inoculated with a conidial suspension from a seven-day PDA-mycelial culture of the BCA. The conidial concentration of the inoculated PDB flask was adjusted to 2×10^5 conidia ml^{-1} by means of a haemocytometer and incubated at 25 °C in an orbital shaker (Grant bio PSU-20i, Grant Instruments, Cambridge, UK) at 90 rpm for seven days. After incubation, the aqueous inoculum of FO12 was adjusted to 10^6 conidia ml^{-1} prior to application.

6.2.4. Semi-controlled conditions and experimental design

The experiment was conducted under semi-controlled conditions in a set of microplots located at the 'Campus de Rabanales' at the University of Córdoba (UCO, Córdoba Province, Andalusia region, southern Spain. UTM coordinates X: 37.919056; Y: -4.724306) from March 2015 to January 2017. The setup consisted of a line of 40 cement and brick containers built on the ground. Each container had 1 m^3 of capacity and was open at the bottom, oriented north to south and protected from rain and excessive sun by a metal rooftop structure. This microplot system has been used previously in epidemiological studies of VWO (López-Escudero and Blanco-López, 2007; Pérez-Rodriguez et al., 2015).

The ID of *V. dahliae* in the original naturally infested soil described before and used in this experiment was estimated at 168 MS g^{-1}. To study the influence of the initial ID of *V. dahliae* on the effectiveness of both biocontrol treatments, the

soil was diluted to obtain two different ID levels. For that purpose, the original naturally infested soil was mixed with *V. dahliae*-free sand at two rates: 1:2 and 1:10 (v:v; infested soil:sand). Both mixtures were separately homogenized by continuous crumbling using a motor hoe (Zeppelin® 111 7HP, Zaragoza, Spain) at the experimental site. The initial ID of the two mixtures was estimated as explained before and resulted in 83.6 and 23.6 MS g^{-1} for the high inoculum density (HID) and low inoculum density (LID) mixtures, respectively.

Each microplot was filled with 800 kg of the corresponding soil mixture (bulk density = 1,300 kg/m3), and eight olive plants were planted in each microplot, four of each of the two cultivars. A total of 18 microplots were used in this experiment, which was carried out in a split-split-plot design with three blocks, each block composed of six microplots, two levels of ID (HID and LID) as the main plot, three treatments (FO12, CGR03 and water treated control) as the subplot and two olive cvs. (Arbequina and Picual) as the sub-subplot, with a total of 144 olive plants and 72 plants of each cultivar.

6.2.5. Planting establishment and treatment application

Olive plants were transplanted in March 2015. Microplots were correspondingly treated with the grape marc compost (CGR03) or with the non-pathogenic strain of *F. oxysporum* (FO12). To prevent damage to the roots of the olive plants, CGR03 was incorporated into the soil just before planting as an organic amendment by tillage (30 cm) with a manual hoe at a rate of 20% (v:v) (i.e., 60 L/microplot). Subsequently, microplots treated with CGR03 were planted and watered with 30 L of tap water. The treatment with FO12 was applied just after planting by watering each microplot with 30 L of the aqueous inoculum previously prepared and adjusted to 10^6 conidia ml^{-1}. Additionally, microplots watered with 30 L of tap water were used as the untreated control. Treatments were applied twice a year at the beginning of each spring and autumn season until the end of the experiment in January 2017. The subsequent treatments with CGR03 were applied at a dose lower than the initial dose (i.e., 30 L/microplot) by superficial tillage to prevent damage to the plants. Microplots were irrigated weekly during spring, summer and autumn and biweekly during the winter season according to Pérez-Rodríguez et al. (2015).

6.2.5. Assessment of inoculum density progress

The ID of *V. dahliae* in the soil of each microplot was periodically quantified to evaluate the ID progress over time in each treatment. In detail, a total of nine soil samples times were collected during the experiment. Initially, soil samples were collected at 15, 30 and 60 days after planting (DAP). From this moment until the end of the experiment, samples were collected approximately at the beginning of each season, which corresponded to 100, 180, 250, 390, 470 and 570 DAP. At each sampling time, four soil sub-samples (100 g) per microplot were collected using a cylindrical (3.5 cm x 22 cm) auger at a soil depth from 20 to 30 cm. Subsequently, the sub-samples of each microplot were mixed to obtain a homogenous sample, which was processed to estimate the ID by means of the wet sieving method described above. Additionally, the randomized area under the inoculum progression curve (RAUIPC) of each treatment was calculated by the trapezoidal integration method (Campbell and Madden, 1990) from all ID values obtained from the nine soil samplings.

6.2.6. Disease evaluation

The first symptoms of VWO were observed four months after planting, in July 2015. The experiment was surveyed weekly for wilt symptoms from disease onset until the end of the experiment in January 2017. Disease severity was estimated based on a 0 to 16 rating scale according to the percentage of plant tissue affected by any of the following symptoms: chlorosis, necrosis or defoliation. The scale estimated the percentage of affected tissue using four main categories or quarters ($\leq$ 25, 26-50, 51-75, and 76-100%) with four values per category. Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) were linearly related to the percentage of affected tissue (Y) by the equation $Y = 6.25X - 3.125$ (Varo-Suárez et al., 2018). At the end of the disease assessment, the relative area under the disease progression curve (RAUDPC) was calculated from the disease severity values by the trapezoidal integration method (Campbell and Madden, 1990). In addition, plant infection by *V. dahliae* was confirmed by isolating the fungus from the affected shoots of diseased plants by microbiological methods, as described by Varo-Suárez et al. (2018).

6.2.7. Data analysis

Analyses of variance (ANOVA) of the disease parameters (final disease severity and RAUDPC) and of the inoculum progression data (final ID and RAUIPC) were performed. Values of these parameters met the assumptions of normality and homogeneity of variances for this analysis. Final disease severity and RAUDPC data were analysed according to a split-split-plot design with the initial ID level as the main plot, treatments as the subplot and the cultivars as the sub-subplot factor. The data on final ID and RAUIPC were arranged in a split-plot design with the initial ID level as the main plot and treatments as the subplot. When the ANOVA showed significant differences among treatments, values were compared using Fisher's protected least significant difference (LSD) at P = 0.05. All data in this study were analysed using Statistix 10 (Analytical Software, 2013).

6.3. RESULTS

The ID progression of *V. dahliae* in naturally infested soils after the application of CGR03 and FO12 is represented in Figure 1. The effectiveness of CGR03 in reducing the ID of *V. dahliae* was observed at the first sampling time (15 DAP) for both initial inoculum densities (HID and LID). At this time, in the case of a high initial ID of the pathogen (HID), CGR03 significantly reduced the ID of the pathogen in comparison with that in the control and in the FO12 treatment ($P = 0.0006$), with an ID of 2.3 MS g^{-1}. Thus, a more pronounced effect of CGR03 compared with that of FO12 was observed at the first stages of the experiment (Figure 1A). On the other hand, FO12 achieved a significant reduction in ID at 100 DAP ($P = 0.0059$) compared with the control, with a concentration of 23.9 MS g^{-1} (Figure 1A). Fluctuations in the ID of *V. dahliae* during the sampling period were observed in all treatments tested, reaching minimum values of 39.2, 0.8 and 0.53 MS g^{-1} for the control (15 DAP), CGR03 (470 DAP) and FO12 (470 DAP) treatments, respectively. In the last sampling (570 DAP), significant differences were found among treatments ($P < 0.0001$), with CGR03 being the treatment that achieved the greatest reduction with a final ID of 1.9 MS g^{-1}.

When the initial ID was lower (LID), both biocontrol treatments were able to significantly reduce the ID of *V. dahliae* in comparison with that in the control at 15 DAP, with IDs of 13.6, 3.4 and 0.5 MS g^{-1} for the control, FO12 and CGR03 treatments, respectively ($P = 0.0005$) (Figure 1B). The ID of *V. dahliae* in soil

treated with the two biocontrol products remained significantly lower than that in the control ($P = 0.0003$) until the end of the experiment, reaching final IDs of 17.2, 3.6 and 1.2 MS g^{-1} for the control, FO12 and CGR03 treatments, respectively. Therefore, CGR03 also achieved a greater reduction in the viable inocula of *V. dahliae* in this case (Figure 1B). In LID soil, fluctuations in ID during the experiment were also observed in all tested treatments, with minimum ID values of 13.6, 0.53 and 0.13 MS g^{-1} for the control (15 DAP), CGR03 (15 DAP) and FO12 (470 DAP) treatments, respectively (Figure 1B).

The initial level of ID had no effect in terms of RAUIPC ($P = 0.9224$), but significant differences were found between treatments (HID: $P = 0.0004$ and LID: $P < 0.0001$). In both cases, CGR03 was the most effective treatment on reducing the ID, showing significantly lower values of RAUIPC than those of FO12 and the control (7.6 and 7.4% for HID and LID, respectively) (Figure 2).

With regard to the onset and progression of the disease, VWO symptoms were first observed 17 weeks after planting. Microbiological reisolations from the shoots of diseased plants confirmed their infection by *V. dahliae*. In this study, there were no significant differences between the two initial ID levels of the pathogen concerning final disease severity ($P = 0.4557$) or RAUDPC ($P = 0.1993$). Similarly, no significant differences were found between cvs. Picual and Arbequina in terms of final disease severity ($P = 0.2632$) or RAUDPC ($P = 0.5483$). Thus, data for each disease parameter (severity and RAUDPC) of both cultivars were grouped for the statistical analysis (Table 1) and significant differences between treatments were observed. The plants grown in the microplots treated with CGR03 or FO12 showed a significant reduction in both disease severity ($P = 0.0190$) and RAUDPC ($P = 0.0054$) in comparison with those of the control (Table 1). The treatment with FO12 achieved complete control of the disease, since no plants treated with this BCA showed VWO symptoms (Table 1).

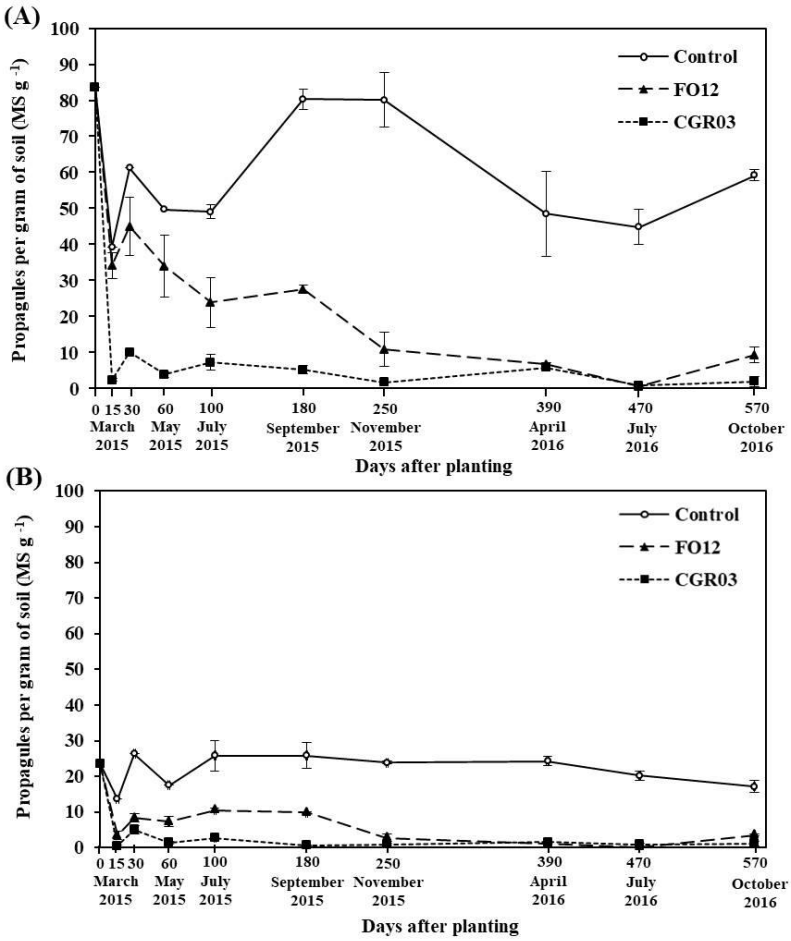


Figure 1. Progress of inoculum density (ID) of *Verticillium dahliae* in naturally infested soils with (A) High inoculum density (HID) and (B) Low inoculum density (LID), after the treatment with FO12 and CGR03 over nine sampling times (from 15 to 570 days after planting). For each sampling time, points represent the mean of three soil samples per treatment. Bars on each point are the standard error of the mean.

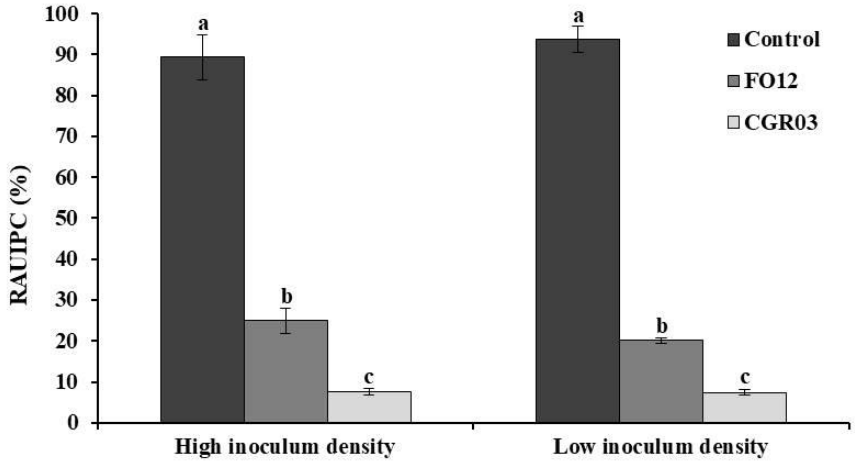


Figure 2. Relative area under inoculum progression curve (RAUIPC, %) in naturally infested soils with high inoculum density (HID) and low inoculum density (LID) for each treatment at 570 days after planting. For each treatment, bars represent the mean of three replications. Different letters indicate significant differences according to Fisher´s protected LSD test at $P = 0.05$. Vertical lines on bars are the standard error of the mean.

Table 1. Effect of the non-pathogenic strain of *Fusarium oxysporum* (FO12) and the grape marc compost (CGR03) on the progress of Verticillium wilt of olive along two-experimental years under semi-controlled conditions.
[a] Initial inoculum density (ID) of *V. dahliae* in the two soil mixtures used in the experiment.

Initial ID[a]	Treatment	Disease severity (%)[d]	RAUDPC (%)[e]
High inoculum density (HID, 83.6 MS g^{-1})	Control	23.0 ± 15.2 a	16.1 ± 11.8 a
	FO12	0.0 ± 0.0 c	0.0 ± 0.0 c
	CGR03	8.1 ± 5.2 b	5.0 ± 3.2 b
Low inoculum density (LID, 23.6 MS g^{-1})	Control	37.1 ± 15.1 a	36.2 ± 14.5 a
	FO12	0.0 ± 0.0 c	0.0 ± 0.0 c
	CGR03	8.6 ± 5.2 b	6.2 ± 3.9 b

[b] Final disease severity (%) ± standard error 22 months after planting based on a 0-16 rating scale (0 = no lesions, 16 = 94-100% of canopy with symptoms).
[c] Relative area under the disease progression curve (RAUDPC, %) ± standard error developed over the assessment period (22 months).
[b, c] In each column, data represent the mean of 24 replicated plants per treatment. Mean values followed by the same letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

6.4. DISCUSSION

Verticillium wilt of olive is considered the most important disease affecting olive groves in Mediterranean countries and, to date, there is no effective control measure when it is individually applied. In this study, the non-pathogenic strain of *F. oxysporum* FO12 and the suppressive grape marc compost CGR03 have been evaluated for its effectiveness in controlling this major disease under semi-controlled conditions. These biocontrol products were selected from previous studies conducted under controlled conditions since they were the most effective at suppressing *V. dahliae* growth *in vitro* and *in planta* (Varo et al., 2016a; Varo-Suárez et al., 2018; Mulero-Aparicio et al., 2019a). This study conducted under semi-controlled conditions supposes the final step for understanding the real potential of these treatments before their evaluation under field conditions, where farmers are urgently in need of feasible control strategies.

Results obtained in this study agree with those reported by Mulero-Aparicio et al. (2019a) and Varo-Suárez et al. (2018), where CGR03 and FO12 were two of the most effective products on reducing the ID of *V. dahliae* tested in two different soils in an *in vitro* experiment. These results highlight that the CGR03 treatment, compared with FO12, achieved a greater reduction in ID at the first stages of the experiment when the initial ID was higher (HID). However, when the initial ID was lower, both biocontrol treatments showed a similar efficacy in ID reduction at the first sampling time (15 DAP). In addition, the minimum values of ID reached during the assessment period were similar for both CGR03 and FO12. This observation confirms the effectiveness of these two biological treatments in reducing the viable inoculum of *V. dahliae* in naturally infested soils regardless of the initial ID of the pathogen. Nevertheless, neither of the two biocontrol treatments tested in this study achieved a total reduction in the ID of *V. dahliae* in soil, and the presence of the pathogen was detected at all sampling times. In fact, fluctuations in the ID of *V. dahliae* during the sampling period were observed in this study. The increase in ID observed in July 2015 and July 2016 in the HID control microplots is in accordance with the results of López-Escudero and Blanco-López (2001). These authors attributed these ID changes in July to a probable drop in the superficial soil microbiota due to the high temperatures at this time of year under our experimental conditions. This change in the microbiota could lead to a higher ID of *V. dahliae* due probably to a lower microbial competition against the pathogen in the natural soil or in the MSPA Petri dishes used for the assessment.

The similarity in the level of resistance of cvs. Picual and Arbequina observed in this study has been previously reported. Thus, previous studies under controlled conditions reported that the genetic resistance level of cv. Arbequina was overcome when plants were artificially inoculated by means of root dipping with a high dose of the pathogen (10^6 conidia ml^{-1}), showing the same level of resistance to VWO as the susceptible cv. Picual (López-Escudero et al., 2004). Nevertheless, in field studies carried out in naturally infested soils with ID levels ranging from 5 to 21 MS g^{-1} cv. Arbequina showed a higher level of resistance to VWO than cv. Picual (Trapero et al., 2013a). Similarly, an earlier study conducted under our experimental conditions (semi-controlled conditions in brick containers) using a naturally infested soil with moderate inoculum densities of *V. dahliae* (9.8 MS g^{-1}) confirmed the higher resistance level to VWO shown by cv. Arbequina in comparison with cv. Picual (Pérez-Rodríguez et al., 2015). In the current study, with much higher initial ID levels of the pathogen (83.6 and 23.6 MS g^{-1} for HID and LID, respectively), both cultivars may have shown the same resistance to VWO due to the high inoculum pressure of the pathogen at the beginning of the experiment that overcame the genetic resistance level of cv. Arbequina.

Plants treated with FO12 did not show VWO symptoms over the experimental period. These results agree with those reported by Varo et al. (2016) and Mulero-Aparicio et al. (2019a), in which this strain showed a total control of VWO in experiments conducted under controlled conditions. The treatment with CGR03 showed a significant reduction in disease development in comparison with that in the control, but complete control of VWO was not achieved, probably due to the presence of enough remaining inoculum of the pathogen to infect the plants. This statement is in agreement with the results of a previous study carried out under the same experimental conditions by López-Escudero et al. (2007), who reported an initial ID of 0.04 MS g^{-1} was enough to infect olive plants of the susceptible cv. Picual and allow the development of the disease.

Even though FO12 was not the most efficient treatment for reducing the ID of the pathogen, its capability to (i) colonize the rhizosphere, (ii) produce volatile organic compounds with antifungal effects against *V. dahliae*, and (iii) produce chlamydospores that remain attached within the root system (Mulero-Aparicio et al., 2019c) could play an essential role in its antagonistic effects against the pathogen by competing in the rhizosphere for the infection points, thus preventing

the infection of olive roots by *V. dahliae* and achieving effective control of the disease.

The effectiveness of these biocontrol products against *V. dahliae* shown in the current study confirms the consistency of these treatments, since they were previously reported as effective biocontrol treatments against *V. dahliae* in studies conducted under controlled conditions (Varo et al., 2016a; Varo-Suárez et al., 2018; Mulero-Aparicio et al., 2019a). Consistency is considered one of the most remarkable traits of any biocontrol product (Deketelaere et al., 2017), since this kind of products are normally known for their contradictory results among laboratory, semi-controlled and field conditions. Finally, further research about the mode and time of application is needed to evaluate the efficacy and consistency of these products under different commercial field conditions as well as further research about the mechanisms responsible for the suppressive effects of these compounds.

In conclusion, the outcomes of the present study highlight the effectiveness of these biocontrol treatments in reducing both the ID of the pathogen and the progress of VWO under semi-controlled conditions. Furthermore, this study expands the knowledge about the use of biocontrol strategies as an eco-friendly approach to the effective control of VWO within an integrated control strategy. Although further research is needed to study the interactions of different biocontrol treatments with olive cultivars with different resistance levels in field conditions, the results obtained in this study suggest the possibility of applying these biocontrol treatments in *V. dahliae*-infested commercial fields both before planting and during cultivation with the aim of reducing the initial inoculum pressure of the pathogen. This strategy, in combination with the use of moderately resistant olive cultivars, could be one of the most effective approaches for the control of VWO to date.

ACKNOWLEDGMENTS

This research was funded by the Spanish Ministry of Science, Innovation and Universities (MICINN; project AGL2016-76240-R), co-financed by the European Union FEDER Funds. A.M.A is holder of 'Formación de Profesorado Universitario' (FPU) fellowship from the Spanish Ministry of Education, Culture and Sports (MECD).

SUPPLEMENTARY FIGURES

Preparation of soil mixture used in the semi-controlled conditions experiment. A) Mixture of naturally infested soil by *Verticillium dahliae* and *V. dahliae*-free sand. B) Homogenization of the soil mixture by means of a motor hoe.

Semi-controlled experiment set-up. A) Filling of microplots with both mixtures of soil. B) Application of grape marc compost CGR03 in the corresponding microplots. C) Incorporation of CGR03 into the soil of the corresponding microplots by mean of a manual motor hoe.

A) Microplots after planting with olive cuttings of cvs. Picual and Arbequina. B) First treatment after planting with the non-pathogenic strain of *Fusarium oxysporum* FO12 and irrigation with tap water of microplots with the grape marc compost CGR03 and untreated control.

A) Soil sampling to estimate the inoculum density progress of *Verticillium dahliae*. B) First symptom of Verticillium wilt of olive observed in a plant corresponding to the untreated control. C) Positive isolation of *V. dahliae* from a stem of a symptomatic plant to confirm the infection by the pathogen.

CONCLUSIONS

Conclusions

1. After evaluating 16 natural products under field conditions, a commercial essential oil from *Thymus* sp., the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 achieved a complete reduction in the inoculum density of *Verticillium dahliae* in soil. (*Chapter 2*)

2. Additionally, *Fusarium oxysporum* FO12 and the grape marc compost CGR03 were able to significantly reduce the disease incidence in young and old olive plants in a commercial olive grove. Therefore, the results obtained have demonstrated that these biological products are highly effective in reducing the inoculum density of *V. dahliae* in the field and may exert a suppressive effect against VWO under field conditions. (*Chapter 2*)

3. The antagonist activity of the strain FO12 and its potential as BCA against *Verticillium dahliae* was confirmed by obtaining four different culture fractions and their evaluation by means of *in vitro* and *in vivo* experiments. The treatment with crude culture broth of FO12 resulted in a total reduction of viable propagules of *V. dahliae* in naturally infested soils as well as in the progression of the disease in inoculated olive cuttings. (*Chapter 3*)

4. Treatments with conidial suspension or chlamydospores were also effective in reducing the inoculum density in the soil and the disease severity in olive plants. The outlines obtained from this study could be the basis for the development of effective commercial formulations against this disease in the coming future. (*Chapter 3*)

5. Volatile organic compounds and soluble substances produced by FO12 caused significant inhibition of mycelial growth and microsclerotia viability in *Verticillium dahliae* and *Verticillium longisporum*. Several metabolic pathways of FO12 were altered upon contact with *Verticillium dahliae* volatiles which suggests that FO12 activates its stress response in the presence of the pathogen. (*Chapter 4*)

6. Confocal Laser Scanning Microscopic analysis using sGFP-tagged FO12 on oil seed rape as a model plant suggests that the biocontrol strain is an

efficient root colonizer, which could compete with *V. dahliae* in the same ecological niche. The findings provide new insights into the mode of action of this biocontrol agent, which should be considered for upcoming control strategies against Verticillium wilt of olive. (*Chapter 4*)

7. Different compost teas and extracts were obtained from the grape marc compost CGR03 to discern the modes of action involved in its effectiveness against *Verticillium dahliae*. Both natural and sterilized compost extracts were the most effective aqueous treatments for reducing the inoculum density of *V. dahliae* in naturally infested soil. Furthermore, natural and autoclave-sterilized compost teas achieved significant disease suppression in olive plants. Although further research is needed to understand the role of microorganisms inhabiting the compost, these results suggest that both biotic and abiotic factors are involved in its suppressive effect against the pathogen. (*Chapter 5*)

8. The long-term effect of the strain FO12 and the grape marc compost CGR03 and their interaction with two olive cultivars with different resistance levels (Picual and Arbequina) were studied in an experiment under semi-controlled conditions. Both FO12 and CGR03 significantly reduced the inoculum density of the pathogen over the experimental period. Additionally, CGR03 significantly reduced the progression of the disease and FO12 achieved total control of the disease regardless the olive cultivar. Although further research is needed to study the interaction between biocontrol treatments and different olive cultivars, this strategy could be one of the most advisable approaches for an effective control of Verticillium wilt of olive. (*Chapter 6*).

REFERENCES

References

Adani, F., Genevini, P.L., Tambone, F., 1995. A new index of organic matter stability. Compost Science and Utilization 3, 25–37.

Agrios GN. **2006**. Plant pathology. Fifth edition. London: Elsevier.

Agrios, G.N., **2005**. Control of plant diseases. In: Plant Pathology. Fifth edition. Elsevier, London, 295–350.

Aimé, S., Alabouvette, C., Steinberg, C., Olivain, C., 2013. The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. Molecular Plant-Microbe Interaction 26, 918–926.

Alabouvette, C., Olivain, C., Migheli, Q., Steinberg, C., 2009. Microbiological control of soil-borne phytopathogenic fungi with special emphasis on wilt-inducing *Fusarium oxysporum*. New Phytologist 184, 529–544.

Al-Ahmad, M.A., Mosli, M.N., 1993. Verticillium wilt of olive in Syria. EPPO Bulletin 23, 521–529.

Albay, C.G., Albay, M., Yayli, N., Yildirim, N., 2009. Essential oil analysis and antimicrobial activities of *Anthemis marschalliana* ssp pectinate and *Anthemis cretica* ssp argaea from Turkey. Asian Journal of Chemistry.21, 1425–1431.

Alburquerque, J.A., Gonzálvez, J., García, D., Cegarra, J., 2004. Agrochemical characterization of "alperujo", a solid by-product of the two-phase centrifugation method for olive oil extraction. Bioresource Technology 91, 195–200.

Alfano, G., Lustrato, G., Lima, G., Vitullo, D., Ranalli, G.. 2011. Characterization of composted olive mill wastes to predict potential plant disease suppressiveness. Biological Control 58, 199–207.

Alström, S., 2001. Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. Journal of Phytopathology 149, 57–64.

Amavizca, E., Bashan, Y., Ryu, C., Farag, M.A., Bebout, B.M., de-Bashan, L.E., 2017. Enhanced performance of the microalga *Chlorella sorokiniana* remotely induced by the plant growth-promoting bacteria *Azospirillum brasilense* and *Bacillus pumilus*. Science Reports 7, 41310.

Amiot, M.J., 2014. Olive oil and health effects: from epidemiological studies to the molecular mechanisms of phenolic fraction. Oilseeds & fats Crops and Lipids 21, D512.

Analytical Software, 2013. Statistix10. User's manual. Tallahassee, FL.

Angelopoulou, D.J., Naska, E.J., Paplomatas, E.J., Tjamos, S.E., 2014. Biological control agents (BCAs) of Verticillium wilt: influence of application rates and delivery method on plant protection, triggering of host defence mechanisms and rhizosphere populations of BCAs. Plant Pathology 63, 1062–1069.

Antonopoulos, D.F., Tjamos, S.E., Antoniou, P.P., Rafeletos, P., Tjamos, E.C., 2008. Effect of *Paenobacillus alvei*, strain K165, on the germination of *Verticillium dahliae* microsclerotia in planta. Biological Control 46, 166–170.

Arslan, M., Dervis, S., 2010. Antifungal activity of essential oils against three vegetative-compatibility groups of *Verticillium dahliae*. World Journal of Microbiology and Biotechnology 26, 1813–1821.

Avilés, M., Borrero, C., 2017. Identifying characteristics of Verticillium wilt suppressiveness in olive mill composts. Plant Disease 101, 568–577.

Avilés, M., Borrero, C., Trillas, M., 2011. Review on compost as an inducer of disease suppression in plants grown in soilless culture. Dynamic Soil, Dynamic Plant 5, 1–11.

Ayres, P.G., 1978. Water relations of diseased plants. In: Kozlowski, T. (Ed.), Water Deficits and Plant Growth. Academic Press, London (U.K.), 1–60.

Baídez, A.G., Gómez, P., del Río, J.A., Ortuño, A., 2007. Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanism. Journal of Agricultural and Food Chemistry 55, 3373–3377.

Bailey, K.L., Lazarovits, G., 2003. Suppressing soil-borne diseases with residue management and organic amendments. Soil and Tillage Research 72, 169–180.

Barbara, D. J., and Clewes, E., 2003. Plant pathogenic *Verticillium* species: How many of them are there. Molecular Plant Pathology 4, 297–305.

Barranco, D., Fernández-Escobar, R., Rallo, L., 2017. El Cultivo del Olivo. 7ª Edición. Mundi-Prensa, Madrid, España.

Bejarano-Alcázar, J., Blanco-López, M.A., Melero-Vara, J.M., Jiménez-Díaz, R.M., 1996. Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. Plant Disease 80, 1233–1238.

Benhamou, N., Garand, C., Goulet, A., 2002. Ability of nonpathogenic *Fusarium oxysporum* strain Fo47 to induce resistance against *Pythium ultimum* infection in cucumber. Applied Environmental Microbiology 68, 4044–4060.

Bennet, R., Davis, R., 2013. Method for rapid production of *Fusarium oxysporum* f. sp. *vasinfectum* chlamydospores. Journal of Cotton Science 17, 52–59.

Berg, G., Knaape, C., Ballin, G., Seidel, D., 1994. Biological control of *Verticillium dahliae* Kleb. by natural occurring rhizosphere bacteria. Archives of Phytopathology and Plant Protection 29, 249–262.

Berg, G., Zachow, C., Lottmann, J., Götz, M., Costa, R., Smalla, K., 2005. Impact of plant species and site on rhizosphere-associated fungi antagonistic to *Verticillium dahliae* Kleb. Applied and Environmental Microbiology 71, 4203–4213.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Gotz, M., Costa, R., Smalla, K., 2006. The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. FEMS Microbiology Ecology 56, 250–261.

Berg, G., 2009. Plant–microbe interactions promoting plant growth and health: perspectives for controlled use of microorganisms in agriculture. Applied Microbiology and Biotechnology 84, 11–18.

Berg, G., Köberl, M., Rybakova, D., Müller, H., Grosch, R., Smalla, K., 2017. Plant microbial diversity is suggested as the key to future biocontrol and health trends. FEMS Microbiology Ecology 93, fix050.

Blanco-López, M.Á., Jiménez-Díaz, R.M., Caballero, J.M., 1984. Symptomatology, incidence and distribution of Verticillium wilt of olive trees in Andalucía. Phytopathologia. Mediterranea 23, 1–8.

Blanco-López, M.A., Bejarano-Alcázar, J., Malero-Vera, J.M., Jiménez-Díaz, R.M., 1989. Current status of Verticillium wilt of cotton in southern Spain: Pathogen variation and population in soil, in: Tjamos, E.C., Beckman,

C.H., (Eds.), Vascular wilt diseases of plants. Springer-Verlag, Berlin, 123–132.

Bonanomi, G., Antignani, V., Pane, C., Scala, F., 2007. Suppression of soilborne fungal diseases with organic amendments. Journal of Plant Pathology 89, 311–324.

Bubici, G., Marsico, A.D., D'Amico, M., Amenduni, M., Cirulli, M., 2013. Evaluation of Streptomyces spp. for the biological control of corky root of tomato and Verticillium wilt of eggplant. Applied Soil Ecology 72, 128–134.

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

Butterfield, E.J., DeVay, J.E., 1977. Reassessment of Soil Assays for *Verticillium dahliae*. Phytopathology 67, 1073–1078.

Caballero, J.M., Pérez-Hernández, J., Blanco-López, M.A., Jiménez-Díaz, R.M., 1980. Olive, a new host of *Verticillium dahliae* in Spain. Proceeding 5th Congress Mediterranean Phytopathology Union, Patras, 50.

Campbell, C.L., Madden, L.V., 1990. Introduction to plant disease epidemiology. New York, NY: John Wiley and Sons Ltd.

Castaño, R., Avilés, M., 2013. Factors that affect the capacity of growing media to suppress Verticillium wilt. Acta Horticulturae 1013, 465–472.

Cernava, T., Aschenbrenner, I.A., Grube, M., Liebminger, S., Berg, G., 2015a. A novel assay for the detection of bioactive volatiles evaluated by screening of lichen-associated bacteria. Frontiers in Microbiology 6, 398.

Cernava, T., Müller, H., Aschenbrenner, I. A., Grube, M., and Berg, G. 2015b. Analyzing the antagonistic potential of the lichen microbiome against pathogens by bridging metagenomic with culture studies. Frontiers in Microbiology 6, 620.

Cirulli, M., Montemurro, G., 1976. A comparison of pathogenic isolates of *Verticillium dahliae* and sources of resistance in olive. Agricultural Conspes Science 39, 469–476.

Connor, D.J., 2005. Adaptation of olive (*Olea europaea* L.) to water-limited environments. Crop and Pasture Science 56, 1181–1189.

Costa, R., Salles, J.F., Berg, G., Smalla, K., 2006. Cultivation independent analysis of *Pseudomonas* species in soil and in the rhizosphere of field-grown *Verticillium dahliae* host plants. Environmental Microbiology 8, 2136–2149.

Cotxarrera, L., Trillas-Gay, M.I., Steinberg, C., Alabouvette, C., 2002. Use of sewage sludge compost and *Trichoderma asperellum* isolates to suppress Fusarium wilt of tomato. Soil Biology and Biochemistry 34, 467–476.

Cronin, M.J., Yohalem, D.S., Harris, R.F., Andrews, J.H., 1996. Putative mechanism and dynamics of inhibition of the apple scab pathogen *Venturia inaequalis* by compost extracts. Soil Biology Biochemistry 28, 1241–1249.

Daebeler, F., Amelung, D., Zeise,. K., 1988. Verticillium-welke an winterraps auftreten und bedeutung. Nachrichtenblatt Pflanzenschutzdienst DDR 42, 71–73.

Dayan, F.E., Cantrell, C.L., Duke, S.O., 2009. Natural products in crop protection. Bioorganic and Medicinal Chemistry 17, 4022–4034.

De Souza, J.R.B., Kupper, K.C., Augusto, F., 2018. *In vivo* investigation of the volatile metabolome of antiphytopathogenic yeast strains against *Penicillium digitatum* using comprehensive two dimensional gas chromatography and multivariate data analysis. Microchemical Journal 141, 204–209.

Debode, J., De Maeyer, K., Perneel, M., De Pannecoucque, J., Backer, G., Höfte, M., 2007. Biosurfactants are involved in the biological control of *Verticillium* microsclerotia by *Pseudomonas* spp. Journal of Applied Microbiology 103, 1184–1196.

Dees, P.M., Ghiorse, W.C., 2001. Microbial diversity in hot synthetic compost as revealed by PCR-amplified rRNA sequences from cultivated isolates and extracted DNA. FEMS Microbiology Ecology 35, 207–216.

Deketelaere, S., Tyvaert, L., França, S.C., Höfte, M., 2017. Desirable traits of a good biocontrol agent against Verticillium wilt. Frontiers in Mricrobiology 8, 1186.

Depotter, J.R., Deketelaere, S., Inderbitzin, P., Tiedemann, A.V., Höfte, M., Subbarao, K.V., et al., 2016. *Verticillium longisporum*, the invisible threat to oilseed rape and other brassicaceous plant hosts. Molecular Plant Pathology 17, 1004–1016.

Dhingra, O.D., Sinclair, J.B., 1995. Basic Plant Pathology Methods, 2nd ed. CRC Press Boca Raton, Florida.

Di Pietro, A., Garcia-MacEira, F.I., Meglecz, E., Roncero, M.I., 2001. A MAP kinase of the vascular wilt fungus *Fusarium oxysporum* is essential for root penetration and pathogenesis. Molecular Microbiology 39, 1140–1152.

Diab, H., Hu, S., Benson, D.M., 2003. Suppression of *Rhizoctonia solani* on impatiens by enhanced microbial activity in composted swine waste amended potting mixes. Phytopathology 93, 1115–1123.

Díez, C.M., Moral, J., Barranco, D., Rallo, L., 2016. Genetic Diversity and Conservation of Olive Genetic Resources. In: Ahuja, M.R., Mohan-Jain, S., (Eds), Genetic Diversity and Erosion in Plants. Springer International Publishing, Switzerland, 337–356.

Edel-Hermann, V., Aimé, S., Cordier, C., Olivain, C., Steinberg, C., Alabouvette, C., 2011. Development of a strain specific real-time PCR assay for the detection and quantification of the biological control agent Fo47 in root tissues. FEMS Microbiology Letters 322, 34–40.

El-Hadrami, A., Adam, L.R., Daayf, F., 2011. Biocontrol treatments confer protection against *Verticillium dahliae* infection of potato by inducing antimicrobial metabolites. Molecular Plant-Microbe Interactions 24, 328–335.

Emechebe, A.M., 1980. The effect of soil moisture and of N, P and on incidence of infection of cacao seedlings inoculated with *Verticillium dahliae*. Plant and Soil 54, 143–147.

Erdogan, O., Benlioglu, K., 2010. Biological control of Verticillium wilt on cotton by the use of fluorescent *Pseudomonas* spp. under field conditions. Biological Control 53, 39–45.

Falcone, P.M., Speranza, B., Del Nobile, M.A., Corbo, M.R., Sinigaglia, M., 2005. A study on the antimicrobial activity of thymol intended as a natural preservative. Journal of Food Protection 68, 1664–1670.

FAO. **2017.** The Statistical Database (FAOSTAT). Rome. http://faostat.fao.org.

Fialho, M.B., Toffano, L., Pedroso, M.P., Augusto, F., Pascholati, S.F., 2010. Volatile organic compounds produced by *Saccharomyces cerevisiae* inhibit the in vitro development of *Guignardia citricarpa*, the causal agent of

citrus black spot. World Journal of Microbiology and Biotechnology 26, 925–932.

Fradin, E.F., Zhang, Z., Ayala, J.C., Castroverde, C.D., Nazar, R.N., Robb, J., et al., 2009. Genetic dissection of Verticillium wilt resistance mediated by tomato Ve1. Plant Physiology 150, 320–332.

Fravel, D.R., 1988. Role of antibiosis in the biocontrol of plant diseases. Annual review of phytopathology 26, 75–91.

Fravel, D.R., Olivain, C., Alabouvette, C., 2003. *Fusarium oxysporum* and its biocontrol. New Phytologist 157, 493–502.

Garber, R.H., Presley, J.T., 1971. Relation of air temperature to development of Verticillium wilt on cotton in the field. Phytopathology, 61, 204.

García-Cabello, S., Pérez-Rodríguez, M., Blanco-López, M.A., López-Escudero, F.J., **2012**. Distribution of *Verticillium dahliae* through watering systems in widely irrigated olive growing areas in Andalucia (southern Spain). European Journal of Plant Pathology 133, 877–885.

Gea, F.J., Navarro, M.J., Tello, J.C. 2009. Potential application of compost teas of agricultural wastes in the control of the mushroom pathogen *Verticillium fungicola*. Journal of Plant Diseases and Protection 116, 271–273.

Goicoechea, N., 2009. To what extent are soil amendments useful to control Verticillium wilt? Pest Management Science 65, 831–839.

Gómez-Lama, C., Sesmero, R., Valverde-Corredor, A., López-Escudero, F.J., Mercado-Blanco, J., 2017. A split-root system to assess biocontrol effectiveness and defense-related genetic responses in above-ground tissues during the tripartite interaction *Verticillium dahliae*-olive-*Pseudomonas fluorescens* PICF7 in roots. Plant and Soil 417, 433–452.

Goud, J.K.C., Termorshuizen, A.J., Gams, W., 2003. Morphology of *Verticillium dahliae* and *V. tricorpus* on semi-selective media used for the detection of *V. dahliae* in soil. Mycological Research 107, 822–830.

Guzmán-Álvarez, J.R., Gómez, J.A., Rallo, L., 2009. El olivar en Andalucía: lecciones para el futuro de un cultivo milenario. In: Gómez-Calero, J.A., (Ed), Sostenibilidad de la producción de olivar en Andalucía. Consejería de Agricultura y Pesca, Junta de Andalucía, Sevilla. 7–19.

Hall, T.J., Schreiber, L.R., Leben, C., 1986. Effects of xylem-colonizing *Bacillus* spp. on Verticillium wilt in maples. Plant disease 70, 521–524.

Hiemstra, J.A., 1998. Compendium of Verticillium Wilts in Tree Species. Wageningen: CPRO.

Hiemstra, J.A., Harris, D.C., 1998. A compendium of Verticillium wilts in tree species. Ponsen and Looijen, Wageningen.

Hiemstra, J., 2015. Guide for Best Practices in managing Verticillium wilt in olive. Projet VERTIGEN. Online publication. Contract No: FP7-SME-2011-2-286140.

Höfte, M., Altier, N., 2010. Fluorescent pseudomonas as biocontrol agents for sustainable agricultural systems. Research in Microbiology 161, 464–471.

Hoitink, H.A.J., Schmitthenner, A.F., Herr, L.J., 1975. Composted bark for control of root rot in ornamentals. Ohio Report 60, 25–26.

Hoitink, H.A.J., Stone, A.G., Han, D.Y., 1997. Suppression of plant disease by composts. Horticulture Science 32, 184–187.

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

Inderbitzin, P., Bostock, R.M., Davis, R.M., Usami, T., Platt H.W., Subbarao, K.V., 2011. Phylogenetics and Taxonomy of the Fungal Vascular Wilt Pathogen *Verticillium*, with the Descriptions of Five New Species. Plos one 6, e28341.

Inderbitzin, P., Subbarao, K.V., 2014. Verticillium systematics and evolution: how confusion impedes Verticillium wilt management and how to resolve it. Phytopathology 104, 564–574.

Ingham, E., 2003. The Compost Tea Brewing Manual. Lismore, Australia: Soil Food Web.

Isaac, I., 1957. Wilt of Lucerne caused by species of *Verticillium.* Annals of Applied Biology 45, 550–558.

Isaac, I., 1967. Speciation in *Verticillium*. Annual Review of Phytopathology 5, 201–222.

Janick, J., 2005. The origins of fruits, fruit growing, and fruit breeding. Plant Breeding Reviews, 255–321.

Jiménez-Díaz, R.M., Trapero-Casas, J.L., Boned, J., Landa, B., Navas-Cortés, J.A., 2009. Uso de Bioten para la protección biológica de plantones de olivo contra la Verticilosis causada por el patotipo defoliante de *Verticillium dahliae*. Boletín de Sanidad Vegetal Plagas 36, 595–615.

Jiménez-Díaz, R.M., Cirulli, M., Bubici, G., 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 Disease 96, 304–329.

Jiménez-Gasco, M.D. M., Malcolm, G.M., Berbegal, M., Armengol, J., Jiménez-Díaz, R.M., 2014. Complex molecular relationship between vegetative compatibility groups (VCGs) in *Verticillium dahliae*: VCGs do not always align with clonal lineages. Phytopathology 104, 650–659.

Kaewchai, S., Soytong, K., Hyde, K.D., 2009. Mycofungicides and fungal biofertilizers. Fungal diversity 38, 25–50.

Kanchiswamy, C.N., Malnoy, M., Maffei, M.E., 2015. Chemical diversity of microbial volatiles and their potential for plant growth and productivity. Frontiers in Plant Science 6, 151.

Karapapa, V.K., Bainbridge, B.W., Heale, J.B., 1997. Morphological and molecular characterization of *Verticillium longisporum* comb. nov. pathogenic to oilseed rape. Mycological Research 101, 1281–1294.

Kaur, R., Macleod, J., Foley, W., Nayudu, M., 2006. Gluconic acid: an antifungal produced by *Pseudomonas* species in biological control of take-all. Phytochemistry 67, 595–604.

Kavitha, T., Nelson, R., 2013. Exploiting the biocontrol activity of *Trichoderma* spp. against root rot causing phytopathogens. ARPN Journal of Agricultural and Biological Science 8, 571–574.

Klebahn, H., 1913. Beiträge zur Kenntnis der Fungi Imperfecti I. Eine Verticillium-Krankheit auf Dahliaen. Mycologisches Zentralblatt 3, 49–66.

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.

Kurze, S., Dahl, R., Bahl, H., Berg, G., 2001. Biological control of fungal strawberry diseases by *Serratia plymuthica* HROC48. Plant Disease 85, 529–534.

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. Frontiers in Plant Science 10, 232.

Lazarovits, G., Conn, K., Tenuta, M., 2000. Control of *Verticillium dahliae* with soil amendments: efficacy and mode of action. In: Tjamos, E., Rowe, R., Heale, J., Fravel, D., (Eds), Advances in Verticillium research and disease management. APS Press: St Paul, MN, 274–291.

Lemanceau, P., Alabouvette, C., 1991. Biological control of *Fusarium* diseases by fluorescent *Pseudomonas* and non-pathogenic *Fusarium*. Crop Protection 10, 279–286.

Levin, A.G., Lavee, S., Tsror, L., **2003.** Epidemiology and effects of Verticillium wilt on yield of olive trees (cvs. Barnea and Souri) irrigated with saline water in Israel. Phytoparasitica 31, 333–343.

Li, N., Wang, W., Bitas, V., Subbarao, K., Liu, X., Kang, S., 2018. Volatile Compounds Emitted by Diverse Verticillium Species Enhance Plant Growth by Manipulating Auxin Signaling. Molecular Plant-Microbe Interactions 31, 1021-1031.

Lima, G., De Curtis, F., D´Onghia, A.M., Nigro, F., 2007. Comparison between real-time PCR and semi-selective medium in monitoring *Verticillium dahliae* microsclerotia in the olive rhizosphere and suppression of the pathogen by compost. IOBC/WPRS Bulletin 30, 221–224.

Liu, Y., Chen, L., Zhang, N., Li, Z., Zhang, G., Xu, Y., et al., 2016. Plant-microbe communication enhances auxin biosynthesis by a root-associated bacterium, *Bacillus amyloliquefaciens*. Molecular Plant-Microbe Interaction 29, 324–330.

Lopes, M.R., Klein, M.N., Ferraz, L.P., da Silva, A.C., Kupper, K.C., 2015. *Saccharomyces cerevisiae*: a novel and efficient biological control agent for *Colletotrichum acutatum* during preharvest. Microbiological Research 175, 93–99.

Lopez-Berges, M.S., Capilla, J., Turra, D., Schafferer, L., Matthijs, S., Jöchl, C., et al., 2012. HapX-mediated iron homeostasis is essential for

rhizosphere competence and virulence of the soilborne pathogen *Fusarium oxysporum*. Plant Cell 24, 3805–3822.

López-Escudero, F.J., Blanco-López, M.A., 2001. Effect of a single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. Plant Disease 85, 489–496.

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., Blanco-López, M.A., 2005a. Effects of drip irrigation on population of *Verticillium dahliae* in olive orchards. Journal of Phytopathology 153, 238–239.

López-Escudero, F.J., Blanco-López, M.A., 2005b. Isolation and morphologic characterization of microsclerotia of *Verticillium dahliae* isolates from soil. Biotechnology 4, 296–304.

Lopez-Escudero, F.J., 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.A., Trapero, A., 2008. Influencia de las cubiertas vegetales en las enfermedades del olivar. Cubiertas vegetales en olivar. Consejería de Agricultura y Pesca, Junta de Andalucía, 1001–1114.

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

Lozano-Tovar, M.D., Ortiz-Urquiza, A., Garrido-Jurado, I., Trapero-Casas, A., Quesada-Moraga, E., 2013. Assessment of entomopathogenic fungi and their extracts against a soil-dwelling pest and soil-borne pathogens of olive. Biological Control 67, 409–420

Lozano-Tovar, M.D., Garrido-Jurado, I., Quesada-Moraga, E., Raya-Ortega, M.C., Trapero-Casas, A., 2017. *Metarhizium brunneum* and *Beauveria*

bassiana release secondary metabolites with antagonistic activity against *Verticillium dahliae* and *Phytophthora megasperma* olive pathogens. Crop Protection 100, 186–195.

Lugtenberg, B.J.J., Dekkers, L.C., Bloemberg, G.V., 2001. Molecular determinants of rhizosphere colonization by *Pseudomonas*. Annual Review of Phytopathology 39, 461–490.

Ma, L.J., van der Does, H.C., Borkovich, K.A., et al., 2010. Comparative genomics reveals mobile pathogenicity chromosomes in *Fusarium*. Nature 464, 367–373.

Malandraki, I., Tjamos, S.E., Pantelides, I., Paplomatas, E.J., 2008. Thermal inactivation of compost suppressiveness implicates possible biological factors in disease management. Biological Control 44, 180–187.

Maldonado-González, M., M., Schilirò, E., Prieto, P., Mercado-Blanco, J., 2015. Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. Environmental Microbiology 17, 3139–3153.

MAPA, 2017. Ministerio de Agricultura, Pesca y Alimentación. España. https://www.mapa.gob.es/es/estadistica/temas/estadisticas-agrarias

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C., 2009. Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. European Journal of Plant Pathology 124, 603–611.

Markakis, E.A., Fountoulakis, M.S., Daskalakis, G.C., Kokkinis, M., Ligoxigakis, E.K., 2016. The suppressive effect of compost amendments on *Fusarium oxysporum* f. sp. *radicis-cucumerinum* in cucumber and *Verticillium dahliae* in eggplant. Crop Protection 79, 70–79.

Markakis, E.A., Tjamos, S.E., Antoniou, P.P., Paplomatas, E.J., Tjamos, E.C., 2016. Biological control of Verticillium wilt of olive by *Paenibacillus alvei*, strain K165. Biocontrol 61, 293–303.

Martos-Moreno, C., 2003. Resistencia de cultivares de olivo al aislado defoliante de *Verticillium dahliae* Kleb. y reducción de la enfermedad por la infección previa con el aislado no defoliante. PhD Thesis, University of Córdoba.

Mehta, C.M., Palni, U., Franke-Whittle, I. H., Sharma, A.K., 2014. Compost: its role, mechanism and impact on reducing soil-borne plant diseases. Waste Management 34, 607–622.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., Jiménez-Díaz, R.M., 2004. Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. Biological Control 30, 474–86.

Mercado-Blanco, J., Bakker, P.A.H., 2007. Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. Antonie van Leeuwenhoek 92, 367–389.

Mercado-Blanco, J., López-Escudero, F.J., **2012.** Verticillium wilt of olive and its control: The heat is on. Plant and Soil 355, 17–21.

Mercado-Blanco, J., 2015a. *Pseudomonas* Strains that Exert Biocontrol of Plant Pathogens. *Pseudomonas*. Springer Netherlands, 121–172.

Mercado-Blanco, J., 2015b. Life of Microbes Inside the Plant. In Principles of Plant-Microbe Interactions. Springer International Publishing, 25–32.

Messner, R., Schweigrofler, W., Ibl, M., Berg, G., Prillinger, H., 1996. Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18S rRNA-gene. Journal of Phytopathology 144, 347–354.

Minerdi, D., Bossi, S., Gullino, M. L., Garibaldi, A., 2009. Volatile organic compounds: a potential direct long-distance mechanism for antagonistic action of *Fusarium oxysporum* strain MSA 35. Environmental Microbiology 11, 844–854.

Mol, L., Scholte, K., 1995. Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. Potato Research 38, 143–150.

Moradi, A., Almasi, M.A., Jafary, H., Mercado-Blanco, J., 2014. A novel and rapid loop-mediated isothermal amplification assay for the specific detection of *Verticillium dahliae*. Journal of Applied Microbiology 116, 942–954.

Morehart, A.L., Melchior, G.L., 1982. Influence of water stress on Verticillium wilt of yellow poplar. Canadian Journal of Botany 60, 201–209.

Mulero-Aparicio, A., Agustí-Brisach, C., Varo, A. López-Escudero, F.J., Trapero, A., 2019a. A non-pathogenic strain of *Fusarium oxysporum* as a potential biocontrol agent against Verticillium wilt of olive. Biological Control, 139. https://doi.org/10.1016/j.biocontrol.2019.104045.

Mulero-Aparicio, A., Varo, A., Agustí-Brisach, C. López-Escudero, F.J., Trapero, A., 2019b. Biological control of Verticillium wilt of olive in the field. Crop Protection. https://doi.org/10.1016/j.cropro.2019.104993.

Mulero-Aparicio, A., Cernava, T., Turrà, D., Schaefer, A., Di Pietro, A., López-Escudero, F.J., Trapero, A., Berg, G., 2019c. The role of volatile organic compounds and rhizosphere competence in mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt. Frontiers in Microbiology, 10:1808.

Müller, H., Berg, G., 2008. Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. Biocontrol 53, 905–916.

Müller, H., Tejedor-González, E., Mercado-Blanco, J., Rodríguez-Jurado, D., Jiménez-Díaz, R., Berg, G., 2008. Effect of the biological control strain *Serratia plymuthica* HRO-C48 on Verticillium wilt of olive trees cv. Arbequina, in: Elad, Y., Ongena, M., Höfte, M., Haïssan, J.M., (Eds.), Fundamental and practical approaches to increase biocontrol efficacy. IOBC/WPRS Bulletin 30, 173–177.

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 *Verticillium dahliae* pathotypes within a high tree density olive orchard in southern Spain. Phytopathology 98, 167–180.

Nega, A., 2014. Review on Concepts in Biological Control of Plant Pathogens. Journal of Biology. Agriculture and Healthcare 4, 33–54.

Noble, R., Coventry, E., 2005. Suppression of soil-borne plant diseases with composts: a review. Biocontrol Science and Technology, 15, 3–20.

O'Sullivan, D.J., O'Gara, F., 1992. Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. Microbiological Reviews 56, 662–676.

Otero, M.L., Roca, M., Zapata, R. Ladux, J.L., Ortiz, J., Zanelli, M., Matias, A.C., Pérez, B.A., 2012. Effect of solarization, organic matter, and

Trichoderma on the severity of Verticillium wilt in olive trees (*Olea europaea* L.) and soil inoculum density. Acta Horticulturae 1057, 121–126.

Ownley, B.H., Gwinn, K.D., Vega, F.E., 2009. Endophytic fungal entomopathogens with activity against plant pathogens: ecology and evolution. Biological Control 55, 113–128.

Pane, C., Celano, G., Zaccardeli, M., 2014. Metabolic patterns of bacterial communities in aerobic compost teas associated with potential biocontrol of soilborne plant diseases. Phytopathologia Mediterranea 53, 277–286.

Pantelides, I.S., Tjamos, S.E., Striglis, I.A., Chatzipavlidis, I., Paplomatas, E.J., 2009. Mode of action of a non-pathogenic *Fusarium oxysporum* strain against *Verticillium dahliae* using Real Time QPCR analysis and biomarker transformation. Biological Control 50, 30–36.

Papasotiriou, F.G., Varypatakis, K.G., Christofi, N., Tjamos, S.E., Paplomatas, E.J., 2013. Olive mill wastes: A source of resistance for plants against *Verticillium dahliae* and a reservoir of biocontrol agents. Biological. Control 67, 51–60.

Paul, B.D., Snyder, S.H., 2009. The unusual anino acid L-ergothioneine is a physiologic cytoprotectant. Cell death and Differentiation 17, 1134–1140.

Paulitz, T.C., Zhou, T., Rankin, L., 1992. Selection of rhizosphere bacteria for biological control of *Pythium aphanidermatum* on hydroponically grown cucumber. Biological Control 2, 226–237.

Pegg, G.F., Brady, B.L., 2002. Verticillium wilts. Wallingford: CAB International.

Pérez-Rodríguez, M., Alcántara, E., Amaro, M., Serrano, N., Lorite, I.J., Arquero, O., Orgaz, F., López-Escudero, F.J., 2015. The Influence of irrigation frequency on the onset and development of Verticillium wilt of olive. Plant Disease 99, 488–495.

Pérez-Rodríguez, M., Serrano, N., Arquero, O., Orgaz, F., Moral, J., López-Escudero, F.J., 2016. The effect of short irrigation frequencies on the development of Verticillium wilt in the susceptible olive cultivar 'Picual' at field conditions. Plant Disease 100, 1880–1888.

Petrisor, C., Paica, A., Constantinescu, F., 2017. Effect of secondary metabolites produced by different *Trichoderma* spp. isolates against *Fusarium*

oxysporum f.sp. *radicis-lycopersici* and *Fusarium solani*. Scientific papers. Series B. Horticulture 61, 407–411.

Postma, J., Montanari, M., Van den Boogert, P.H.J.F., 2003. Microbial enrichment to enhance the disease suppressive activity of compost. European Journal of Soil Biology 39, 157–163.

Powelson, M.L., Rowe, R.C., 1993. Biology and management of early dying of potatoes. Annual Review of Phytopathology 3, 111–126.

Presley, J.T., 1950. Verticillium wilt of cotton with particular emphasis on variation of the causal organism. Phytopathology 40, 497–511.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S.G., Dobinson, K.F., Mercado-Blanco, J., 2009. Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. Microbial Biotechnology 2, 499–511.

Raaijmakers, J.M., Paulitz, T.C., Steinberg, C., Alabouvette, C., Moënne-Loccoz, Y., 2009. The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. Plant and Soil 321, 341–361.

Raza, W., Yuan, J., Ling, N., Huang, Q., Shen, Q., 2015. Production of volatile organic compounds by an antagonistic strain *Paenibacillus polymyxa* WR-2 in the presence of root exudates and organic fertilizer and their antifungal activity against *Fusarium oxysporum* f. sp. *niveum*. Biological Control 80, 89–95.

Reddy, M.S., Hynes, R.K., Lazarovits, G., 1994. Relationship between *in vitro* growth inhibition of pathogens and suppression of preemergence damping-off and postemergence root rot of white bean seedlings in the greenhouse by bacteria. Canadian Journal of Microbiology 40, 113–119.

Reinke, J., Berthold, G., 1879. Die Zersetzung der Kartoffel durch Pilze, Vol. 1.

Roca, L.F., Moral, J., Trapero, C., Blanco-López, M.A., López-Escudero, F.J., 2015. Effect of inoculum density on Verticillium wilt incidence in commercial olive orchards. Journal of Phytopathology 164, 61–64.

Rodríguez, E., García-Garrido, J.M., García, P.A., Campos, M., 2008. Agricultural factors affecting Verticillium wilt in olive orchards in Spain. European Journal of Plant Pathology 122, 287–295.

Rodríguez, E., García-Garrido, J.M., García, P.A., and Campos, M., 2011. Implications of plant material origin, land use history and soil properties in the incidence of Verticillium wilt in olive groves. Journal of Plant Pathology 93, 111-118.

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.

Rodríguez-Jurado, D., Bejarano-Alcázar, J., 2007. Dispersión de *Verticillium dahliae* en el agua utilizada para el riego de olivares en Andalucía. Boletín Sanidad Vegetal Plagas 33, 547–562.

Ruggieri, G., 1946. A new disease of olive. L'Italia Agricola 83, 369–372.

Ruiz-Torres, M.J., **2010**. Situación fitosanitaria y control del olivar andaluz en la pasada campaña. Vida Rural 304, 44–47.

Rybakova, D., Schmuck, M., Wetzlinger, U., Varo-Suarez, A., Murgu, O., Müller, H., et al., 2016. Kill or cure? The interaction between endophytic *Paenibacillus* and *Serratia* strains and the host plant is shaped by plant growth conditions. Plant and Soil 405, 65–79.

Rybakova, D., Rack-Wetzlinger, U., Cernava, T., Schaefer, A., Schmuck, M., Berg, G., 2017. Aerial warfare: a volatile dialogue between the plant pathogen *Verticillium longisporum* and its antagonist *Paenibacillus polymyxa*. Frontiers in Plant Science 8, 1294.

Sánchez-Hernández, M.E, Ruiz-Dávila, A., Pérez de Algaba, A., Blanco-López, M.A., Trapero-Casas, A., 1998. Occurrence and aetiology of death of young olive trees in southern Spain. European Journal of Plant Pathology 104, 347–357.

Sanei, S.J., Razavi, S.E., 2011. Suppression of Verticillium wilt of olive by *Pseudomonas fluorescents*. American Journal of Experimental Agriculture 1, 294–305.

Sardar, P., Kempken, F., 2018. Characterization of indole-3-pyruvic acid pathway-mediated biosynthesis of auxin in *Neurospora crassa*. Plos one 13, e0192293.

Schnathorst, W.C., 1981. Life cycle and epidemiology of *Verticillium*. Fungal Wilt Diseases of Plants, 81–111.

Serra-Wittling, C., Houot, S., Alabouvette, C., 1996. Increased soil suppressiveness to Fusarium wilt of flax after addition of municipal solid waste compost. Soil Biology and Biochemistry 28, 1207–1214.

Sewell, G.W.F., Wilson, J.F., 1967. Verticillium wilt of the hop: field studies on wilt in a resistant cultivar in relation to nitrogen fertilizer applications. Annals of Applied Biology 59, 265–273.

Shi-Kunne, X., van Kooten, M., Depotter, J.R.L., Thomma, B.P.H.J., Seidl, M. F., 2019. The Genome of the Fungal Pathogen *Verticillium dahliae* Reveals Extensive Bacterial to Fungal Gene Transfer. Genome Biology and Evolution 11, 855–868.

Shishido, M., Miwa, C., Usami, T., Amemiya, Y., Johnson, K.B., 2005. Biological control efficiency of Fusarium wilt of tomato by nonpathogenic *F. oxysporum* Fo-B2 in different environments. Phytopathology 95, 1072–1080.

Shufelt, C., Linderman, R.G., 1986. The influence of irrigation on the incidence and severity of Verticillium wilt of Norway maple. In: (Abstr) 4th International Verticillium Symposium. Guelph, Canada.

Soylu, S., Yigitbas, H., Soylu, E. M., Kurt, Ş., 2007. Antifungal effects of essential oils from oregano and fennel on *Sclerotinia sclerotiorum*. Journal of applied microbiology 103, 1021-1030.

Splivallo, R., Novero, M., Bertea, C.M., Bossi, S., Bonfante, P., 2007. Truffle volatiles inhibit growth and induce an oxidative burst in *Arabidopsis thaliana*. New Phytologist 175, 417–424.

Steel, R., Torrie, J., 1985. Bioestadística: Principios y procedimientos. Colombia: McGraw-Hill.

Steinberg, C., Whipps, J.M., Wood, D., Fenlon, J., Alabouvette, C., 1999. Mycelial development of *Fusarium oxysporum* in the vicinity of tomato roots. Mycological Research 103, 769–778.

Stotzky, G., Schenck, S., Papavizas, G.C., 1976. Volatile organic compounds and microorganisms. CRC Critical Reviews in Microbiology 4, 333–382.

Talboys, P.W., 1987. Verticillium wilt in English hops: retrospect and prospect. Canadian Journal of Plant Pathology 9, 68–77.

Tenuta, M., Lazarovits, G., 2002. Ammonia and nitrous acid from nitrogenous amendments kill the microsclerotia of *Verticillium dahliae*. Phytopathology 92, 255–264.

Termorshuizen, A.J., Van Rijn, E., Van Der Gaag, D.J., Alabouvette, C., Chen, Y., Lagerlöf, J., et al., 2006. Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. Soil Biology and Biochemistry 38, 2461–77.

Terra, W.C., Campos, V.P., Martins, S.J., Costa, L.S.A.S., da Silva, J.C.P., Barros, A.F., et al. 2018. Volatile organic molecules from *Fusarium oxysporum* strain 21 with nematicidal activity against *Meloidogyne incognita*. Crop Protection 106, 125–131.

Thakore, Y., 2006. The biopesticide market for global agricultural use. Industrial Biotechnology 2, 194–208.

Thanassoulopoulos, C.C., 1993. Spread of Verticillium wilt by nursery plants in olive groves in the Halkidiki area (Greece). EPPO Bulletin 23, 517–520.

Tjamos, E.C., Botseas, D., 1987. Occurrence of *Verticillium dahliae* in leaves of Verticillium-wilted olive trees. Canadian Journal of Plant Pathology 9, 86.

Tjamos, E.C., Tsougriani, H., 1990. Formation of microsclerotia in partially disintegrated leaves of Verticillium affected olive trees. In 5th International *Verticillium* Symposium, Book of Abstracts, Leningrad, Soviet Union, 20.

Tjamos, E.C., Rowe, R., Heale, J.B., Fravel, D.R., 2000. Advances in *Verticillium*: research and disease management. St. Paul: The APS.

Tjamos, E.C., Tsitsigiannis, D.I., Tjamos, S.E., Antoniou, P.P., Katinakis, P., 2004. Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. European Journal of Plant Pathology 110, 35–44.

Tjamos, S.E., Flemetakis, E., Paplomatas, E.J., Katinakis, P., 2005. Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. Molecular Plant-Microbe Interactions 18, 555–561.

Toffano, L., Fialho, M.B., Pascholati, S.F., 2017. Potential of fumigation of orange fruits with volatile organic compounds produced by *Saccharomyces cerevisiae* to control citrus black spot disease at postharvest. Biological Control 108, 77–78.

Trapero, A., López Escudero, F.J., Blanco, M.A. 2017. Enfermedades. En: Barranco, D., Fernández-Escobar, R., Rallo, L. (Eds), El cultivo del olivo. 7ª edición. Mundi-Prensa, Madrid, 733-798.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., 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.

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

Trapero, C., Alcántara, E., Jiménez, J., Amaro-Ventura, M.A. Romero, J. Koopmann, B., et al., 2017. Starch hydrolysis and vessel occlusion related to wilt symptoms in olive stems of susceptible cultivars infected by *Verticillium dahliae*. Frontiers in Plant Science 9, 72.

Triki, M.A., Hadj-Taieb, S.K., Mellouli, I.H., Rhouma, A., Gdoura, R., Hassairi, A., 2012. Identification and screening of bacterial isolates from Saharan weeds for *Verticillium dahliae* control. Journal of Plant Pathology 94, 305–311.

Trillas, M.I., Casanova, E., Cotxarerra, L., Ordovás, J., Borrero, C., Avilés, M., 2006. Compost from agricultural waste and the *Trichoderma asperellum* strain T-34 suppress *Rhizoctonia solani* in cucumber seedlings. Biological Control 39, 32–38.

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

Tuitert, G., Szczach, M., Bollen, G.J., 1998. Suppression of *Rhizoctonia solani* in potting mixtures amended with compost made from organic household waste. Phytopathology 88, 764–773.

Tyvaert, L., França, S.C., Debode, J., Höfte, M., 2014. The endophyte *Verticillium* Vt305 protects cauliflower against Verticillium wilt. Journal of Applied Microbiology 116, 1563–1571.

Uppal, A.K., El Hadrami, A., Adam, L.R., Tenuta, M., Daayf, F., 2008. Biological control of potato *Verticillium* wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. Biological Control 44, 90–100.

van Dam, P., Fokkens, L., Ayukawa, Y., van der Gragt, M., ter Horst, A., Brankovics, B., et al., 2017. A mobile pathogenicity chromosome in *Fusarium oxysporum* for infection of multiple cucurbit species. Scientific Reports **7**, 9042.

van der Does, H.C., Fokkens, L., Yang, A., Schmidt, S.M., Langereis, L., Lukasiewicz, T.R.H., Rep, M., 2016. Transcription factors encoded on core and accessory chromosomes of *Fusarium oxysporum* induce expression of effector genes. PLOS Genetics 12, e1006401.

Varo, A., Raya-Ortega, M.C., Trapero, A., 2016a. Selection and evaluation of microorganisms for biocontrol of *Verticillium dahliae* in olive. Journal of Applied Microbiology 121, 767–777.

Varo, A., Moral, J., Lozano-Tóvar, M.D., Trapero, A., 2016b. 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., Mulero-Aparicio, A., Adem, M., Roca, L.F., Raya-Ortega, M.C., López-Escudero, F.J., Trapero, A., 2017. Screening water extracts and essential oils from Mediterranean plants against *Verticillium dahliae* in olive. Crop Protection 92, 168–175.

Varo-Suárez, A., Raya-Ortega, M.C., Agustí-Brisach, C., García-Ortíz-Civantos, C., Fernández-Hernández, A., Mulero-Aparicio, A., Trapero, A., 2018. Evaluation of organic amendments from agro-industry waste for the control of Verticillium wilt of olive. Plant Pathology. 67, 860–870.

Vázquez, B.I., Fente, C., Franco, C.M., Vázquez, M.J., Cepeda, A., 2001. Inhibitory effects of eugenol and thymol on *Penicillium citrinum* strains in culture media and cheese. International Journal of Food Microbiology 67, 157–163.

Veloso, J., Díaz, J., 2012. *Fusarium oxysporum* Fo47 confers protection to pepper plants against *Verticillium dahliae* and *Phytophthora capsici*, and induces the expression of defence genes. Plant Pathology 61, 281–288.

Veloso, J., Alabouvette, C., Olivain, C., Flors, V., Pastor, V., García, T., Díaz, J., 2016. Modes of action of the protective strain Fo47 in controlling Verticillium wilt of pepper. Plant Pathology 65, 997–1007.

Vigouroux, A., 1984. Verticilliose et bactériose deux importants facteurs de dépérissment de l´abricotier. Arboriculture Fruitière 31, 31–35.

Vitullo, D., Altieri, R., Esposito, A., Nigro, F., Ferrara, M., Alfano, G., Ranalli, G., De Cicco, V., Lima, G., 2013. Suppressive biomasses and antagonist bacteria for an eco-compatible control of *Verticillium dahliae* on nursery-grown olive plants. International Journal of Environmental Science and Technology 10, 209–220.

Wang, Q., Xu, L., 2012. Beauvericin, a bioactive compound produced by fungi: a short review. Molecules 17, 2367–2377.

Weller, D.M., 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annual Review of Phytopathology 26, 379–407.

Weller, D.M., Raaijmakers, J.M., McSpadden Gardener, B.B., Thomashow, L.S., 2002. Microbial populations responsible for specific soil suppressiveness to plant pathogens. Annual Review of Phytopathology 40, 309–348.

Whipps, J.M., 1997. Developments in the biological control of soilborne plant pathogens. Advances in Botanical Research 26, 1–134.

Wilhelm, S., 1955. Longevity of the Verticillium wilt fungus in the laboratory and field. Phytopathology 45, 180–181.

Wilhelm, S., Kaiser, W.J., Georgopoulus, S.G., Opitz, K.W., 1962. Verticillium wilt of olives in California. Phytopathology 52, 32.

Wilhelm, S., Taylor, J.B., 1965. Control of Verticillium wilt of olive through natural recovery and resistance. Phytopathology 55, 310–316.

Wyatt, T.T., Gerwig, G.J., Kamerling, J.P., Wosten, H.A.B., Dijksterhuis, J., 2015. Structural analysis of novel trehalose-based oligosaccharides from extremely stress-tolerant ascospores of *Neosartorya fischeri* (*Aspergillus fischeri*). Carbohydrate Research 411, 49–55.

Xiao, C.L., Subbarao, K.V., 1998. Relationships between *Verticillium dahliae* inoculum density and wilt incidence, severity, and growth of cauliflower. Phytopathology 88, 1108–1115.

Xie, C.J., Wang, C.Y., Wang, X.K., Yang, X.Y., 2013. Proteomics-based analysis reveals that *Verticillium dahliae* toxin induces cell death by

modifying the synthesis of host proteins. Journal of General Plant Pathology 79, 335–345.

Xue, L., Xue, Q., Chen, Q., Lin, C., Shen, G., Zhao, J., 2013. Isolation and evaluation of rhizosphere actinomycetes with potential application for biocontrol of Verticillium wilt of cotton. Crop Protection 43, 231–240.

Yang, P., Sun, Z., Liu, S., Lu, H., Zhou, Y., Sun, M., 2013. Combining antagonistic endophytic bacteria in different growth stages of cotton for control of Verticillium wilt. Crop Protection 47, 17–23.

Yang, W., Zheng, L., Liu, H-X., Wang, K.B., Yu, Y.Y., Luo, Y.M., Guo, J.H., 2014. Evaluation of the effectiveness of a consortium of three plant-growth promoting rhizobacteria for biocontrol of cotton Verticillium wilt. Biocontrol Science and Technology 24, 489–502.

Yohalem, D., Passey, T., 2011. Amendment of soils with fresh and post-extraction lavender (*Lavandula angustifolia*) and lavandin (*Lavandula intermedia*) reduce inoculum of *Verticillium dahliae* and inhibit wilt in strawberry. Applied Soil Ecology 49, 187–196.

Yohalem, D.S, Harris, R.F, Andrews, J.H., 1994. Aqueous extracts of spent mushroom substrate for foliar disease control. Compost Science and Utilization 2, 67–74.

Young, V.H, Foulton, N.D., Waddle, B.A., 1959. Factors affecting the incidence and severity of *Verticillium* wilt disease of cotton. Bulletin of Arkansas Agricultural Experiment Station 612, 1–26.

Zachos, D.G., 1963. La Verticilliose de l'olivier en Greece. Annales de l'Institut Phytopathologique Benaki 5, 105–107.

Zar, J.H., 1999. Biostatistical analysis. 4th ed. Prentice-Hall. Englewood Cliffs, New Jersey.

Zeise, K., von Tiedemann, A., 2002. Host specialization among vegetative compatibility groups of *Verticillium dahliae* in relation to *Verticillium longisporum*. Journal of Phytopathology 150, 112–119.

Zhang, Q., Yang, L., Zhang, J., Wu, M., Chen, W., Jiang, D., et al., 2015. Production of anti-fungal volatiles by non-pathogenic *Fusarium oxysporum* and its efficacy in suppression of Verticillium wilt of cotton. Plant and Soil 392, 101–114.

Zucconi, F., Forte, M., Monaco, A., De Bertoldi, M., 1981. Evaluating toxicity of immature compost. Biocycle 22, 54–57.

APPENDIX

Scientific production during PhD

Appendix: Scientific production during PhD

PEER-REVIEWED JOURNAL PUBLICATIONS

1. Varo-Suárez, A., **Mulero-Aparicio, A.,** Adem, M., Roca, L.F., Raya-Ortega, M.C., López-Escudero, F.J., Trapero, A., 2017. Screening water extracts and essential oils from Mediterranean plants against *Verticillium dahliae* in olive. Crop Protection. 92, pp. 168 - 175.

2. Varo-Suárez, A., Raya-Ortega, M.C., Agustí-Brisach, C., García-Ortíz-Civantos, C., Fernández-Hernández, A., **Mulero-Aparicio, A.,** Trapero, A., 2018. Evaluation of organic amendments from agro-industry waste for the control of Verticillium wilt of olive. Plant Pathology. 67, 860–870.

3. **Mulero-Aparicio, A.,** Agustí-Brisach, C., Varo, A. López-Escudero, F.J., Trapero, A., 2019. A non-pathogenic strain of *Fusarium oxysporum* as a potential biocontrol agent against Verticillium wilt of olive. Biological Control. doi.org/10.1016/j.biocontrol.2019.104045.

4. **Mulero-Aparicio, A.,** Cernava, T., Turra, D., Schaefer, A., Di Pietro, A., López-Escudero, F.J., Trapero, A., Berg, G., 2019. The role of volatile organic compounds and rhizosphere competence in the mode of action of the non-pathogenic *Fusarium oxysporum* FO12 towards Verticillium wilt. Frontiers in Microbiology. 10:1808. doi.org/10.3389/fmicb.2019.01808.

5. **Mulero-Aparicio, A.,** Agustí-Brisach, C., Raya-Ortega, M.C., Lovera, M., Arquero, O., Trapero, A., 2019. First report of *Fusarium solani* causing stem canker in English walnut in Spain. Plant Disease. doi.org/10.1094/PDIS-06-19-1163-PDN.

6. **Mulero-Aparicio, A.,** Varo, A., Agustí-Brisach, C., López-Escudero, F.J., Trapero, A., 2019. Biological control of Verticillium wilt of olive in the field. Crop Protection. doi.org/10.1016/j.cropro.2019.104993.

7. **Mulero-Aparicio, A.,** Trapero, A., López-Escudero, F.J., 2019. Effectiveness of the non-pathogenic strain of *Fusarium oxysporum* FO12 and the grape marc compost CGR03 in controlling Verticillium wilt of olive under semi-controlled conditions. Phytopathologia Mediterranea. Submitted.

8. **Mulero-Aparicio, A.,** López-Escudero, F.J., Trapero, A., 2019. Suppressive effects of compost teas and extracts from the grape marc compost CGR03 on Verticillium wilt of olive. Annals of Applied Biology. Submitted.

SCIENTIFIC AND EXTENSION JOURNAL PUBLICATIONS

1. Varo-Suárez, A., **Mulero-Aparicio, A.,** Raya-Ortega, M.C., Roca, L.F., Agustí-Brisach, C., Trapero, A., 2017. Selección masiva de productos de origen natural para el control biológico de la Verticilosis del olivo. COAG-Jaén. Revista Técnica de Agricultura y Ganadería de Jaén.16, pp. 20-23.

2. **Mulero-Aparicio, A.,** López-Moral, A., Agustí-Brisach, C., Varo, A., Roca, L.F., Raya-Ortega, M.C., Romero, J., López-Escudero, F.J., Trapero, A., 2019. Avances en el control biológico de la Verticilosis del olivo. Vida Rural. In press.

INTERNATIONAL CONFERENCES PAPERS

1. Varo-Suárez, A., Raya-Ortega, M.C., Roca-Castillo, L.F., **Mulero-Aparicio, A.,** Adem, M., López-Escudero, F.J., Trapero, A. Screening of organic amendments, plant extracts and microorganisms for the control of Verticillium wilt in olive trees. 7th meeting of the IOBC-WPRS Working Group "Integrated protection of olive crops". **11/05/2015**. Kalamata, Greek.

2. **Mulero-Aparicio, A.,** Varo-Suárez, A., Trapero, A. Effectiveness of a non-pathogenic strain of *Fusarium oxysporum* (FO12) against *Verticillium dahliae*. 15th Congress of the Mediterranean Phytopathological Union. **20-23/06/2017**. Córdoba, Andalusia, Spain.

3. **Mulero-Aparicio, A.,** Varo-Suárez, A., Adem, M., Roca, L.F., Raya-Ortega, M.C., López-Escudero, F.J., Trapero, A. Assessment of water extracts and essential oils from Mediterranean plants against *Verticillium dahliae* in olive.15th Congress of the Mediterranean Phytopathological Union. **20-23/06/2017**. Córdoba, Andalusia, Spain.

4. **Mulero-Aparicio, A.,** Cernava, T., López-Escudero, F.J., Berg, G., Trapero, A. Elucidating the mechanisms of action of the non-pathogenic strain of *Fusarium oxysporum* FO12 against Verticillium Wilt of Olive. XV Meeting of the IOBC-WPRS Working Group "Biological and integrated control of plant pathogens" Biocontrol products: from lab testing to product development. **23-26/04/2018**. Lleida, Catalonia, Spain.

5. **Mulero-Aparicio, A.,** López-Escudero, F.J., González, M., Trapero, A. Mechanisms involved in the effectiveness of grape compost in controlling Verticillium wilt of olive. 6th International Conference on the Olive Tree and Olive Products. **15-19/10/2018**. Seville, Andalusia, Spain.

NATIONAL CONFERENCES PAPERS

1. **Mulero-Aparicio, A.,** Varo-Suárez, A., Trapero, A. Efecto supresivo del compost de orujo de vid frente a la Verticilosis del olivo. XVIII Congreso de la Sociedad Española de Fitopatología. 20-23/09/2016. Palencia, Castilla y León, España.

2. **Mulero-Aparicio, A.,** Varo-Suárez, A., Trapero, A. Evaluación de una cepa de *Fusarium oxysporum* como agente de control biológico contra la Verticilosis del olivo. XVIII Congreso de la Sociedad Española de Fitopatología. 20-23/09/2016. Palencia, Castilla y León, España.

3. **Mulero-Aparicio, A.,** Varo-Suárez, A., Agustí-Brisach, C., López-Escudero, F.J., Trapero, A. Selección masiva de productos de origen natural para el control biológico de la Verticilosis del olivo. XVIII Simposio Científico-Técnico de Expoliva 2017. 10-12/05/2017. Jaén, Andalucía, España.

4. **Mulero Aparicio, A.,** Cañizares, M.C., Trapero, A., Pérez-Artés, E., López-Escudero, F.J., García-Pedrajas, M.D. Evaluación del efecto de los micovirus VdPV1 y VdRV1 en la virulencia de *Verticillium dahliae* frente a algodón y olivo. XIX Congreso de la Sociedad Española de Fitopatología. 08-12/10/2018. Toledo, Castilla-La Mancha, España.

5. **Mulero-Aparicio, A.,** Raya-Ortega, M.C., López-Escudero, F.J., Trapero, A. Interacción entre la cepa no patogénica de *Fusarium oxysporum* “FO12” y

Verticillium dahliae en huéspedes herbáceos y en olivo. XIX Congreso de la Sociedad Española de Fitopatología. 08-12/10/2018. Toledo, Castilla-La Mancha, España.

6. **Mulero-Aparicio, A.,** López-Escudero, F.J., González, M., Trapero, A. Mecanismos implicados en la eficacia del compost de orujo de vid para el control de la Verticilosis del olivo. XIX Congreso de la Sociedad Española de Fitopatología. 08-12/10/2018. Toledo, Castilla-La Mancha, España.

SUPERVISION OF FINAL DEGREE PROJECTS

1. **Title**: "Caracterización patogénica de *Verticillium dahliae* en algodón, tomate, lechuga y olivo". Student: Antonio Ochoa Navarrete. Qualification: 9.5. Julio de 2017. Departamento de Agronomía, Universidad de Córdoba.

2. **Title**: "Caracterización morfológica de aislados de suelo de *Verticillium dahliae*". Student: Manuel Romero Perán. Qualification: 9,5. Septiembre de 2017. Departamento de Agronomía, Universidad de Córdoba.

3. **Title**: "Caracterización biológica, morfológica y patogénica de la cepa FO12 de *Fusarium oxysporum*. Student: Ignacio Alarcón de la lastra Vicente. Qualification: 9. Septiembre de 2017. Departamento de Agronomía, Universidad de Córdoba.

4. **Title**: "Control biológico de la Verticilosis del olivo mediante la cepa FO12 de *Fusarium oxysporum* en condiciones controladas". Student: Juan Antonio Davias Moreno. Qualification: 10. Septiembre 2017. Departamento de Agronomía, Universidad de Córdoba.

5. **Title**: "Inducción de resistencia: posible mecanismo de acción de la cepa no patogénica de *Fusarium oxysporum* FO12 en el control biológico de la Verticilosis del olivo". Student: Juan Manuel Sillero Reyes. Qualification: 9. Septiembre de 2018. Departamento de Agronomía, Universidad de Córdoba.

SUPERVISION OF FINAL MASTER PROJECTS

1. **Title:** "Efecto de la cepa no patogénica de *Fusarium oxysporum* FO12 en la colonización vascular de *Verticillium dahliae* en varios huéspedes herbáceos". Student: Juan Antonio Davias Moreno. Qualification: 9.5. Julio de 2019. Departamento de Agronomía, Universidad de Córdoba.

2. **Title:** "Efecto de micovirus en la inducción de hipovirulencia en un aislado virulento de *Verticillium dahliae*". Student: Antonio Ochoa Navarrete. Qualification: 9.5. Septiembre de 2019. Departamento de Agronomía, Universidad de Córdoba.

Córdoba, octubre de 2019