EVALUACIÓN DE MICROORGANISMOS, ENMIENDAS ORGÁNICAS Y SUSTANCIAS NATURALES PARA EL CONTROL BIOLÓGICO DE LA VERTICILOSIS EN OLIVO

TESIS DOCTORAL

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UNIVERSIDAD DE CÓRDOBA

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TITULO: *EVALUACIÓN DE ENMIENDAS ORGÁNICAS, MICROORGANISMOS Y PRODUCTOS NATURALES PARA EL CONTROL BIOLÓGICO DE LA VERTICILOSIS EN OLIVO*

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EVALUACIÓN DE ENMIENDAS ORGÁNICAS, MICROORGANISMOS Y PRODUCTOS NATURALES PARA EL CONTROL BIOLÓGICO DE LA VERTICILOSIS EN OLIVO

(EVALUATION OF ORGANIC AMENDMENTS, MICROORGANISMS, AND NATURAL PRODUCTS FOR THE CONTROL OF VERTICILLIUM WILT IN OLIVE)

Memoria de Tesis para aspirar al grado de Doctor con Mención Internacional por la Universidad de Córdoba por la Ingeniera Agrónoma Ángela Varo Suárez

Director: Dr. Antonio Trapero Casas

Córdoba, Noviembre 2016

TÍTULO DE LA TESIS:

Evaluación de enmiendas orgánicas, microorganismos y productos naturales para el control biológico de la Verticilosis en olivo.

DOCTORANDO/A: Ángela Varo Suárez

INFORME RAZONADO DEL/DE LOS DIRECTOR/ES DE LA TESIS

(se hará mención a la evolución y desarrollo de la tesis, así como a trabajos y publicaciones derivados de la misma).

La doctoranda **Ángela Varo Suárez** ha realizado satisfactoriamente y en los plazos previstos el trabajo presentado en esta Tesis Doctoral. A lo largo de su investigación, la doctoranda ha contribuido con diversas aportaciones de interés para la comunidad científica respecto al control de la Verticilosis del olivo mediante control biológico, así como la identificación de potenciales tratamientos biológicos. Además de la importancia científica, este trabajo aporta información aplicable en el control de esta enfermedad y representa el principio de una prometedora línea de investigación en el grupo de Patología Vegetal de la Universidad de Córdoba para el control de esta enfermedad.

La estancia internacional realizada en el Institute of Environmental Biotechnology de la Universidad Tecnológica de Graz (Austria), bajo la tutela de la Profesora Gabriele Berg, ha permitido a la doctoranda ampliar su formación mediante el aprendizaje de técnicas de microscopía confocal para el seguimiento de los agentes de control biológico en distintos tejidos de la planta huésped. Además, la estancia le ha permitido profundizar en el estudio de las bacterias colonizadoras de la rizosfera de plantas. Como fruto de esta estancia, se ha logrado una publicación en la revista internacional *Plant and Soil*.

El trabajo realizado en el Departamento de Agronomía de la Universidad de Córdoba por Ángela Varo Suárez queda reflejado en varias contribuciones relacionadas con el control biológico de la Verticilosis en el olivo en las cuales consta como primera autora: cuatro artículos en revistas de prestigio científico indexadas en la base de datos JCR, tres de ellos ya publicados y el cuarto aceptado y en prensa. Además, de un quinto artículo que ha sido enviado para su publicación. Asimismo, ha presentado cinco comunicaciones a cuatro congresos internacionales y cuatro a tres congresos nacionales. Además, ha participado en otras actividades de extensión y divulgación de los resultados obtenidos, así como en dos publicaciones y dos comunicaciones a congresos de las que es coautora.

Además, la doctoranda ha sido codirectora durante este periodo de un proyecto de fin de carrera. Esta codirección le ha permitido transmitir sus conocimientos a otros estudiantes en formación.

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

Córdoba, 2 de Noviembre de 2016

Fdo.: Antonio Trapero Casas
Director de la Tesis doctoral

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 (25 junio 2014 – 26 Septiembre 2014) en la Universidad Tecnológica de Graz (Austria), Institute of Environmental Biotechnology. 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:
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- Un doctor perteneciente a alguna institución de educación superior o centro de investigación no español forma parte del tribunal evaluador de la tesis:
  - Dra. Paula Baptista, Departamento Biología e Biotecnología, Instituto Politécnico de Bragança, Portugal.
- Parte de la Tesis Doctoral se ha redactado y se presentará en dos idiomas, castellano e inglés.

La doctoranda

Fdo: Ángela Varo Suárez

Tesis por compendio de artículos

Esta tesis cumple el requisito establecido por la Universidad de Córdoba para su presentación como 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 el Journal Citations Report (SCI):

1. **Varo A, Moral J, Lozano-Tóvar MD, Trapero A. 2016a.** Development and validation of an inoculation method to assess the efficacy of biological treatments against Verticillium wilt in olive trees. *BioControl* **61(3): 283-292.** Datos de 2016(JCR): índice de impacto 1.767, posición 20/47 y 1º cuartil en el área temática de Entomología.

2. **Varo A, Raya-Ortega MC, Trapero A. 2016b.** Enhanced production of microsclerotia in recalcitrant *Verticillium dahliae* isolates and its use for inoculation of olive plants**.** *Journal of Applied Microbiology* **121(2):** 473-484. Datos de 2016 (JCR): índice de impacto 2.156, posición 78/161 y 2º cuartil en el área temática de Biotecnología y Microbiología Aplicada.

3. **Varo A, Raya-Ortega MC, Trapero A. 2016c.** Selection and evaluation of microorganisms for biocontrol of *Verticillium dahliae* in olive. *Journal of applied Microbiology* **121:** 767-777. Datos de 2016 (JCR): índice de impacto 2.156, posición 78/161 y 2º cuartil en el área temática de Biotecnología y Microbiología Aplicada.

La doctoranda

Fdo: Ángela Varo Suárez

Los trabajos incluidos en esta tesis doctoral han sido parcialmente subvencionados por el contrato (CON 129/11) entre la Universidad de Córdoba y la Interprofesional del Aceite de Oliva Español, el proyecto de excelencia P08-AGR-03635 de la Junta de Andalucía (cofinanciado con fondos FEDER de la UE).

Web con información y publicaciones de la doctoranda:
https://www.researchgate.net/profile/Angela_Varo2

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Resumen

Uno de los retos más importantes de la olivicultura actual es el control de la Verticilosis causada por el hongo *Verticillium dahliae*. Estimaciones realizadas en varios países de la cuenca mediterránea señalan incidencias medias de 1−5% de olivos afectados, aunque en algunas áreas se ha constatado una incidencia superior al 50% y una elevada mortalidad de árboles. La gran dificultad que presenta el control de esta enfermedad, junto a la falta de productos químicos eficaces, tanto para el tratamiento del suelo como de la planta, ha motivado la búsqueda de métodos alternativos de control. Dentro de este contexto, surge el control biológico como una estrategia eficaz y sostenible. Por esta razón, la identificación de potenciales tratamientos biológicos para el control de la Verticilosis es un objetivo prioritario para el desarrollo de una estrategia integrada de control de la enfermedad.

El primer objetivo de la presente tesis doctoral fue la puesta a punto de un método de infestación del suelo que reprodujese las condiciones naturales de infección. Previamente, para superar la limitación que presentaban determinados aislados recalcitrantes de *V. dahliae* para la producción de las estructuras de resistencia (microesclerocios), se realizó la evaluación y optimización de un medio de cultivo para producir dichas estructuras e infestar el suelo. No obstante, el mejor método de infestación del suelo seleccionado en base a la respuesta severa, consistente y homogénea de las plantas fue el medio arena-harina de maíz-agua (AMA) a una dosis del 20% (peso/peso). Este método de infestación permitió establecer diferentes rangos de eficacia, desde altamente eficaces (>90% de reducción de la enfermedad) hasta levemente eficaces (<10% de reducción de la enfermedad).

El siguiente objetivo fue la evaluación de diferentes microorganismos (hongos, bacterias y su extractos), enmiendas orgánicas (residuos de animales y de la industria agroalimentaria) y sustancias naturales (aceites esenciales y extractos vegetales) para el control de *V. dahliae*, mediante el desarrollado de una metodología de selección de los tratamientos biológicos en cuatro etapas: 1) ***in vitro***, por su efecto sobre el crecimiento micelial o la germinación de esporas del patógeno, 2) **en suelo**, por su efecto sobre la reducción de microesclerocios del patógeno en un suelo naturalmente infestado, 3) **en planta**, por su efecto sobre la infección de plantones de olivo en condiciones controladas, y 4) **en campo**, por su efecto sobre la enfermedad en condiciones de infección natural.

En base a los resultados de la evaluación de 162 tratamientos en condiciones controladas, se seleccionaron 14 tratamientos biológicos para ser evaluados en tres experimentos de campo, uno de estos experimentos, FT3, aún

está en curso. La cepa no patogénica de *Fusarium oxysporum* FO12, y el orujo de vid CGR03, fueron los candidatos más prometedores, alcanzando casi el 100% de la inhibición de la enfermedad en condiciones controladas y la erradicación del patógeno en suelo naturalmente infestado. No obstante, éstos y otros potenciales tratamientos deben ser confirmados en futuros experimentos en campo con diferentes densidades de inóculo y condiciones edafoclimáticas.

Los resultados obtenidos suponen un avance significativo con relación al control de la Verticilosis del olivo en campo, puesto que algunos de los candidatos seleccionados en este trabajo podrían estar disponibles en un futuro próximo como tratamientos biológicos eficaces que ayuden a controlar esta importante enfermedad.

SUMMARY

One of the most important challenges for the present olive growing is the sustainable control of the Verticillium wilt (VW) disease caused by *Verticillium dahliae*. Research in several countries of the Mediterranean basin indicates a mean incidence of 1-5% of affected olives, although in some areas of Andalusia region (Southern Spain) disease reaches more than 50%, with a high mortality of trees. The great difficulty of controlling this disease, along with the lack of effective chemicals for the treatment of soil or plant, has motivated the search for alternative methods of control. In this context, biological control appears as an effective and sustainable strategy. In spite of the importance of the disease, there are very scarce and specific research related to biological control of Verticillium wilt in olive. Thus, identifying of potential biological control treatments is currently a major aim for an effective integrated strategy for the control of Verticillium wilt.

The first objective of this thesis was to develop a screening method of soil infestation that would reproduce the natural conditions of infection. To overcome the fail in the production of microsclerotia in recalcitrant isolates, a culture medium was optimized for the mass production of microsclerotia and successful soil infestation using previously obtained microsclerotia. However, the most effective inoculation method was soil infestation with the corn meal sand medium (CMS) at 20% w/w. This inoculation method allowed separate treatments for their efficacy, since highly effective treatments (>90% reduction in disease severity) until little or no effective treatments (<10% reduction in disease severity). The selected inoculation method was then used to screen biological treatments for their efficacy against VW in potted olive plants.

A second step was conducted to evaluate different microorganisms (fungi, bacteria and their extracts), organic amendments (waste from animals and food industry) and natural substances (essential oils and plant extracts) for the control of *V. dahliae*. This mass screening of candidates was conducted in four stages: i) ***in vitro***, by the effect on the mycelial growth and spore germination of the pathogen; ii) **in natural infested soil**, by the effect on the reduction of microsclerotia of the pathogen; iii) **in plant**, by the effect on the infection of olive plants under controlled conditions and iv), **in field**, by the effect on VW of olive trees grown in highly infested soils.

Based on the results of the assessment of 162 treatments under controlled conditions, we have selected 14 biological treatments which have been tested in three field experiments, one of these experiments, FT3, is still ongoing. The non-pathogenic strain of *Fusarium oxysporum* FO12, and the pomace of grape CGR03 treatments were the most promising candidates, reaching almost the

100% of the inhibition of the disease in controlled conditions and the eradication of the pathogen in naturally infested soil. However, these and other potential treatments must be confirmed in further experiments in field soils with different inoculum densities and soil and climate conditions.

The results represent a significant advance in relation to the control of Verticillium wilt in the field, since some of the candidates selected in this work could be available in the near future as effective biological treatments that will help to control this important disease.

Abreviaturas

ACB: Agentes de control biológico

ANOVA: análisis de varianza

D: defoliante /defoliating

DI: disease incidence

ID: inoculum density

LSD: least significant difference

M: mortality

MS: Microesclerocios / microsclerotia

MSPA: modified sodium polypectate agar

ND: no defoliante / non-defoliant

P-value or *P*: *P* value of ANOVA

Ppg: propagules per gram

PCR: polymerase chain reaction

PDA: potato dextrose agar

RAUDPC: relative area under the disease progress curve

VO = Verticilosis del olivo

WVO = Verticillium wilt of olive

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1

Introducción General

1

Introducción general y Objetivos

EL OLIVO: HISTORIA E IMPORTANCIA

El olivo (*Olea europaea* subsp. *europaea*) y sus principales productos como son el aceite y las aceitunas de mesa, están profundamente arraigados en la historia de las sociedades mediterráneas, dada su relevancia económica y cultural. Desde hace al menos 5000 años, fenicios, griegos y romanos extendieron este cultivo por toda la cuenca mediterránea. Posteriormente, este cultivo fue introducido en otros territorios situados entre las latitudes 30-45º en ambos hemisferios y en otras regiones de clima mediterráneo. Tras la colonización del continente americano, se establecieron plantaciones de olivo en Perú, Argentina, Chile, EEUU y Méjico. Además, en los dos últimos siglos, el olivo ha llegado a Australia, Sudáfrica y otros países orientales como China, Japón y Pakistán, experimentando un constante aumento en superficie total cultivada (Connor, 2005). Este fenómeno ha sido recientemente acrecentado por la multitud de estudios que avalan los beneficios para la salud aportados por el consumo de aceite de oliva virgen extra (Amiot, 2014), habiéndose proclamado este "oro líquido", como un pilar fundamental de la dieta mediterránea.

Actualmente, el olivo se encuentra entre las especies de mayor importancia a nivel mundial debido a la relevancia económica de su aprovechamiento aceitero en zonas templadas, ocupando 10,3 millones de hectáreas en más de 20 países en todo el mundo, de las cuales el 95% se cultiva en la cuenca mediterránea. España es el primer productor de sus principales productos. En 2015, la producción de aceite de oliva español supuso el 44% de la producción mundial y el 21% de la producción de aceituna de mesa (IOC 2014; FAO, 2015).

El olivo es un árbol perennifolio, longevo, que puede alcanzar hasta 15 metros de altura, con copa ancha y tronco grueso, retorcido y a menudo muy corto. Corteza finamente fisurada, de color gris o plateado. Durante milenios, el olivar ha modulado el paisaje de grandes áreas geográficas (Besnard et al., 2013).

Este cultivo supone en gran medida, una excepción geográfica, siendo Andalucía, la única área en Europa donde hay una concentración de una única especie arbórea cultivada en una superficie tan extensa (Guzmán-Álvarez et al., 2009).

En el proceso de expansión, la producción olivarera mundial ha sido incrementada mediante el aumento del área cultivada y del rendimiento por unidad de superficie. Estos incrementos se explican por una mejora en el uso de agroquímicos (fertilizantes, herbicidas, insecticidas y fungicidas) que, junto a la intensificación y homogeneización del cultivo, ha permito la reducción de costes de producción. Sin embargo, este proceso progresivo entraña riesgos como son la irreparable pérdida de variabilidad genética (Díez et al., 2016), contaminaciones debidas al excesivo uso de agroquímicos y el desarrollo epidémico de plagas y enfermedades.

En este contexto, un nuevo desafío fitosanitario ha surgido para el olivar, como es la dispersión y aumento de la incidencia de una enfermedad vascular denominada Verticilosis, tanto en nuevas plantaciones como en zonas donde tradicionalmente no había sido un problema grave (Barranco et al., 2004).

PROBLEMÁTICA DE LA VERTICILOSIS DEL OLIVO

La Verticilosis, causada por el hongo *Verticillium dahliae* Kleb., es la enfermedad vascular más importante del cultivo del olivo (Schnathorst, 1981; López-Escudero y Mercado-Blanco, 2011). Fue observada por primera vez en Italia en 1946 (Ruggieri, 1946). Actualmente, ha sido detectada en casi todos los países de la cuenca mediterránea y en otras zonas olivareras del mundo. Estimaciones realizadas en varios países mediterráneos, señalan incidencias medias de 1−5% de olivos afectados, aunque en algunas áreas, se han constatado incidencias superiores al 50% (RAIF, 2014). En España, y de forma especial en Andalucía, la expansión creciente de la Verticilosis, particularmente en olivares jóvenes, está generando una justificada inquietud en el sector olivarero. Dicha extensión de la enfermedad puede ser debida, entre otros factores, a la dispersión del patógeno mediante el establecimiento de nuevas plantaciones en suelos infestados previamente por otro cultivo susceptible a la enfermedad, como es el algodonero, al uso de material de plantación infectado (Thanassoulopoulos, 1993;

Jiménez-Díaz et al., 2011), o incluso a prácticas culturales como el riego, realizado frecuentemente con agua infestada por el patógeno (Pérez-Rodríguez et al., 2015).

La sintomatología de *V. dahliae* en olivo ha sido descrita por diversos investigadores en todo el mundo (Zachos, 1963; Cirulli, 1975; Blanco López et al., 1984). En las condiciones del Valle del Guadalquivir, la Verticilosis del olivo comprende dos complejos sintomáticos conocidos como "Apoplejía" (forma aguda de la enfermedad) y "Decaimiento lento" (forma crónica).

La "apoplejía" comprende la muerte rápida de ramas o de la planta completa. Suele producirse durante el otoño y el principio de la primavera. Se manifiesta inicialmente por la pérdida de color verde intenso de las hojas, que comienza en los extremos de las ramas. Finalmente, las hojas toman un color marrón pajizo a la vez que se abarquillan, permaneciendo adheridas a las ramas. La precocidad en la aparición y la severidad de este síndrome parece estar asociada a lluvias intensas en otoño y a temperaturas moderadas tanto en otoño como en invierno. Cuando la apoplejía ocurre en plantas jóvenes, la muerte del árbol completo es muy común (Blanco-López et al., 1984; Rodríguez-Jurado, 1993; Jiménez-Díaz et al., 2012).

El "decaimiento lento" ocurre principalmente durante la primavera o principio de verano y a veces durante el otoño, dependiendo de la climatología. En primavera se caracteriza por la necrosis de las inflorescencias y en el otoño, por la necrosis de los frutos. En ambos casos, las flores o frutos en crecimiento quedan momificados en el árbol. Además, las hojas de los brotes adquieren un color verde mate y se desprenden. A veces, esta defoliación en verde es muy intensa. Ocasionalmente, ambos síndromes pueden aparecer en el mismo árbol, y afectar parcial o totalmente a la planta, siendo este último más frecuente en árboles jóvenes, mientras que los de mayor edad suelen mostrar unas ramas afectadas y otras asintomáticas. Por otra parte, también puede producirse la aparición de una coloración violácea-purpúrea en la corteza de las ramas afectadas y ocasionalmente una coloración marrón oscura en los tejidos vasculares.

Se ha detectado que los árboles afectados pueden mostrar una remisión de los síntomas desarrollados. Dichos árboles tienen la capacidad de producir tejido vascular nuevo que puede cubrir completamente los vasos de xilema necrosados, lo que permite el crecimiento de tejido vascular nuevo en tallos y ramas infectados (Tjamos et al., 1991; López-Escudero y Blanco-López, 2001)

Dada la inespecificidad de los síntomas provocados por *V. dahliae* en olivo, éstos pueden ser confundidos con aquellos provocados por otros agentes que ocasionan enfermedades de origen biótico o abiótico. Por ejemplo, la podredumbre radical del olivo provocada por *Phytophthora* spp. presenta unos síntomas aéreos no específicos que se pueden confundir con el síndrome de "apoplejía" provocado por *V. dahliae* (Sánchez-Hernández et al., 1998). Por esta razón, es necesario el aislamiento e identificación del patógeno a partir del material vegetal infectado con el objetivo de realizar un diagnóstico adecuado (Trapero y Blanco-López, 2008), o bien emplear herramientas moleculares que lo permitan.

Entre diferentes aislados de esta especie, es frecuente una virulencia diferencial sobre algunos huéspedes (Bhat y Subbarao, 1999). De esta forma, en algodonero se han identificado los patotipos defoliante y altamente virulento (D) y no defoliante (ND), denominados de esta forma por el síndrome característico que producen en esta planta, y por la capacidad que posee el defoliante de destruir la planta (Jiménez-Díaz et al., 1998). En España, las investigaciones realizadas han puesto de manifiesto que estos aislados de *V. dahliae* se expresan en el olivo con el mismo nivel de virulencia que presentan en cultivares de algodón (Schnathorst y Sibbett, 1971; Rodríguez-Jurado, 1993). Las infecciones producidas por el patotipo D pueden dispersarse de una forma más rápida y a mayor distancia, a través de la dispersión por el viento de hojas infectadas que caen prematuramente de árboles enfermos (Navas-Cortés et al., 2008).

En la década de los 80, los aislados del patotipo D se encontraban localizados en la zona de las Marismas del Guadalquivir, sin embargo, prospecciones recientes han confirmado la presencia en otras áreas de Andalucía y suponen una grave amenaza para las plantaciones de olivar, sobre todo para las de nueva creación (López-Escudero y Blanco-López, 2001). Asimismo, el

patotipo defoliante o aislados de virulencia similar han sido detectados en América (Mathre et al., 1966; Schnathorst, 1969), China (Zhengjun et al., 1998), Israel (Korolev et al., 2000) y Túnez (Triki et al., 2011). Recientemente, los patotipos ND y D han sido caracterizados como raza 1 y 2, respectivamente (Hu et al., 2015). En una identificación realizada en la colección de aislados de *V. dahliae* del Departamento de Agronomía de la Universidad de Córdoba (UCO), el 100% de los aislados caracterizados como patotipo defoliante correspondieron a la raza 2. Sin embargo, respecto al patotipo no defoliante, el 71,43% y el 28,7% correspondieron a la raza 1 y 2, respectivamente (Raya et al., *datos no publicados*).

AGENTE CAUSAL

El género *Verticillium* fue establecido en 1816 por Nees von Esembek, de acuerdo con la disposición verticilada de sus conidióforos. Este género se ha englobado tradicionalmente dentro de la clase Hyphomycetes, orden Moniliales y familia Moniliaceae. Se trata de un hongo hemibiótrofo haploide de suelo, de reproducción sexual desconocida, que se reproduce asexualmente por medio de conidios, produciendo microesclerocios (MS) adaptados a soportar condiciones ambientales adversas (Trapero y Blanco-López, 2008; Klosterman et al., 2009). Sin embargo, en la clasificación actual de los hongos (Hibbett et al., 2007), se engloba dentro de los hongos mitospóricos, sin clasificación de taxa supragenéricos y se incluyen en la división Ascomycota, subdivisión Pezizomycotina, clase Sordariomycetes y familia Plectosphaerellaceae (Inderbitzin et al., 2011; Inderbitzin y Subbarao, 2014).

Verticillium dahliae está considerado un invasor del suelo, caracterizado por una fase parasítica extensa sobre el tejido del huésped vivo y una fase saprofita declinante después de la muerte de éste (Powelson y Rowe, 1993). Los MS son estructuras de resistencia multicelulares y melanizadas, que germinan en respuesta a estímulos proporcionados por los exudados de las raíces de plantas huéspedes y no huéspedes, o bien por el secado al aire del suelo durante al menos 5 semanas (Butterfield y DeVay, 1977; Mol y Scholte, 1995). Al germinar, se produce la penetración de las hifas infectivas en las raíces, que puede producirse

por los puntos de emergencia de raíces laterales, por raicillas intactas o a través de heridas (Schnathorst, 1981). Las hifas penetran en la raíz colonizando las células epidérmicas y el córtex (Klosterman et al., 2009). Dentro de los vasos, el patógeno produce conidióforos y conidios que pueden moverse por la corriente de transpiración en sentido ascendente. Se produce un desequilibrio en el balance hídrico debido a la reducción en el aporte de agua que se produce por la obstrucción mecánica de los vasos xilemáticos (Eynck et al., 2007). Los MS se forman en los tejidos necrosados de la planta enferma, especialmente en hojas, ramas y tallos durante las últimas etapas de la fase parasítica del ciclo de vida del patógeno y son incorporados al suelo tras la degradación de los restos vegetales (Mol y Scholte, 1995). Al final del ciclo, los MS son incorporados al suelo mediante los tejidos senescentes de las plantas infectadas (Wheeler y Johnson, 2016).

La gravedad de la enfermedad se ve acrecentada por la gran dificultad que presenta su control. Así, el contexto de la enfermedad queda conformado por la ineficacia de los tratamientos químicos para el control, unida a la actual concienciación ambiental respecto al uso de fungicidas químicos en el manejo de las enfermedades. Ante la ausencia de métodos completamente eficaces contra la enfermedad, un nuevo enfoque es requerido para el manejo del cultivo donde se integren todas las medidas de control disponibles y entre ellas, el control biológico, surge como una solución alternativa y eficaz para el control de la Verticilosis (Hiemstra y Harris, 1998; Pegg y Brady, 2002).

CONTROL BIOLÓGICO: UNA ALTERNATIVA POTENCIAL DE CONTROL FRENTE A LA VERTICILOSIS DEL OLIVO

El cultivo del olivo basado en monocultivo de variedades genéticamente uniformes y un alto aporte de insumos (como por ejemplo la irrigación), propio de la agricultura convencional y de las nuevas plantaciones intensivas, ha favorecido, como en otros cultivos, el desarrollo epidémico de muchas enfermedades causadas por hongos, bacterias, nematodos y virus (Van Bruggen, 1995). La aplicación de fungicidas químicos, en su mayoría de amplio espectro, provoca la

inhibición del metabolismo de los microorganismos patógenos, pero también actúa sobre los organismos presentes en el suelo, entre los cuales se presentan organismos beneficiosos para los cultivos (Tuzun y Kloepper, 1995), limitando así el beneficio que pueden aportar a los cultivos. Muchos de estos fungicidas son de efecto temporal y requiere la aplicación de nuevas dosis, facilitando la aparición de cepas resistentes a estos fungicidas y dificultando su control.

El empobrecimiento biológico de los ecosistemas que provoca vulnerabilidad ante la recolonización de patógenos de plantas, junto con el aporte de altas concentraciones de nitrógeno, la pérdida de materia orgánica del suelo y los desequilibrios en la nutrición de la planta, han provocado un incremento de la susceptibilidad de los cultivos agrarios, claro ejemplo se puede encontrar en gran parte de los paisajes olivareros (Garbeva et al., 2004; Datnoff et al., 2007).

Actualmente, no existen medidas de control eficaces para la Verticilosis debido a diversos factores tales como la capacidad del patógeno de sobrevivir prolongadamente en el suelo, la amplia gama de plantas susceptibles a la infección y la ineficacia de los tratamientos químicos hasta ahora explorados para combatir al hongo durante su fase parasítica en el xilema (Fradin y Thomma, 2006; Tsror, 2011).

Por estos motivos, el control biológico surge como un componente esencial para el control integrado de la Verticilosis en olivo, tanto en nuevas plantaciones como, sobre todo, en olivares establecidos donde la resistencia genética no es aplicable.

En numerosos cultivos se ha intensificado la búsqueda de métodos biológicos alternativos para el control biológico de la Verticilosis (Hiemstra y Harris, 1998; Pegg y Brady, 2002). Entre ellos, se ha investigado principalmente el uso de numerosos microorganismos antagonistas (Berg et al., 1994, 2005; Tjamos et al., 2004, 2005; Ownley et al., 2009; Erdogan y Benlioglu, 2010; El Hadrami et al., 2011; Yang et al., 2013, 2014; Bubici et al., 2013; Xue et al., 2013; Angelopoulou et al., 2014), extractos microbianos (Dayan et al., 2009; Kaewchai et al., 2009; Pane et al., 2014) extractos vegetales (Uppal et al., 2008; Arslan y Dervis, 2010; Yohalem y Passey, 2011), enmiendas orgánicas

(Lazarovits et al., 2000; Bonanomi et al., 2007; Goicoechea, 2009; Papasotiriou et al., 2013) y el enterrado de cubiertas vegetales o biofumigación (Sarwar et al., 1998; Tsror et al., 2007; França et al., 2013; Neubauer et al., 2014, 2015).

En esta búsqueda de medidas alternativas, la Verticilosis del olivo no ha sido una excepción, aunque la dificultad añadida por la naturaleza del huésped, especie leñosa longeva, con una anatomía compleja y gran sistema radicular, ha limitado notablemente este tipo de investigaciones (Trapero y Blanco, 2008; López-Escudero y Mercado-Blanco, 2011). No obstante, en las últimas décadas se observa un incremento del interés en esta materia.

Ante la carencia existente en el control de esta enfermedad en el olivar, desde el grupo de Patología Agroforestal de la UCO se ha desarrollado una línea de investigación sobre control biológico de la Verticilosis del olivo. En este sentido, la presente tesis doctoral, tiene como finalidad principal realizar una selección masiva de posibles tratamientos biológicos para el control de dicha enfermedad. Entre estos tratamientos se encuentran agentes de control biológico, enmiendas orgánicas y sustancias naturales.

Agentes de control biológico

Desde la perspectiva moderna de una agricultura sostenible, una de las medidas de control a utilizar para el establecimiento de una estrategia eficaz para el control de enfermedades, es el uso de antagonistas microbianos, con potencial como agentes de control biológico (ACBs). Estos presentan diferentes modos o mecanismos de acción que les permiten el control de hongos fitopatógenos. Hasta ahora, sólo un pequeño grupo de especies bacterianas y fúngicas han demostrado ser antagonistas efectivos de *V. dahliae*; y de ellas, sólo un reducido número ha mostrado capacidad de biocontrol eficiente de la enfermedad causada por este patógeno en olivo (Hiemstra et al., 1998; Pegg y Brady, 2002).

En cultivos distintos al olivo, son destacables cepas de *Bacillus subtilis*, *Pseudomonas fluorescens, Stenotrophomonas maltophilia*, por ser efectivas en el control de *V. dahliae* en *Brassica napus* (Berg et al., 1994). Al igual que, determinadas cepas de *Talaromyces flavus*, *Paenibacillus alvei*, y *B. amiloliquefaciens*, en solanáceas (Marois et al., 1982; Tjamos et al., 2004), y

distintas bacterias endofíticas en algodonero (Zhengjun et al., 1996), aunque son muy escasos los productos fitosanitarios de base biológica registrados para el control de la Verticilosis. Entre ellos es destacable “RhizoStar®”, formulado con una cepa de *Serratia plymuthica* (HRO-C48), con el que se obtuvieron buenos resultados en condiciones controladas y en campo para el control de la enfermedad en fresa (Kurze et al., 2001). Otra formulación comercial, “Mycostop®”, de la cepa K61 de *Streptomyces griseoviridis* (Minuto et al., 2006) registrada en Italia para tomate, demostró ser también efectiva para el control de la Verticilosis en tomate cuando se utilizaba en combinación con solarización. Dentro del género *Pseudomonas*, se ha demostrado que las cepas de *P. fluorescens* ejercen un efecto notable sobre la incidencia y severidad de la enfermedad en berenjena (Malandraki et al., 2008) y patata (Uppal et al., 2008).

En olivo, determinadas cepas de bacterias endofíticas como la cepa HRO-C48 de *S. plymuthica*, han sido las más estudiadas (Müller et al., 2008). Se ha demostrado que cuando se aplican en condiciones controladas y semicontroladas muestran una prolongada colonización de la rizosfera en plantaciones de cv. Arbequina y un control efectivo de la Verticilosis. Otro ejemplo, la cepa K165 de *Paenibacillus alvei* (Markakis et al., 2016; Tjamos et al., 2004), aislada de la rizosfera de plantas de tomate, también tiene la capacidad de controlar la enfermedad en olivo tanto en condiciones semicontroladas como en campo.

Además del efecto de biocontrol, en determinadas cepas de bacterias de los géneros *Bacillus* spp. y *Pseudomonas* spp. (Mercado-Blanco y Bakker, 2007; Höfte and Altier, 2010), se ha detectado un efecto promotor del crecimiento de la planta. A estas bacterias se las denomina PGPR (por sus siglas en inglés, que significan ‘Plant Growth Promoting Rhizobacteria’, o Rizobacterias Promotoras del Crecimiento Vegetal) (Kloepper y Schroth, 1978). En el caso particular del olivo, diversas cepas PGPR de *Pseudomonas* spp. han surgido como ACBs muy prometedores (Mercado-Blanco et al., 2004; Debode et al., 2007; Sanei y Razavi, 2011; Triki et al., 2012). La cepa endófita PICF7 de *P. fluorescens* se caracteriza por una prolongada colonización y es considerada como un efectivo ACB de la Verticilosis del olivo causada por el patotipo defoliante y altamente virulento (Mercado-Blanco et al., 2004; Prieto et al., 2009; Maldonado-González et al.,

2015). La efectividad de esta cepa ha sido demostrada en diferentes cultivares en vivero bajo condiciones controladas y semicontroladas (Prieto et al., 2009).

Por otro lado, diferentes géneros de hongos ejercen un papel muy importante dentro del control biológico de la Verticilosis en olivo, entre ellos, determinadas cepas de *Trichoderma* spp. (Lima et al., 2007; Otero et al., 2012). Este género está ampliamente distribuido y ha sido evaluado como ACB, aunque el único bioformulado que está actualmente comercializado en España para esta enfermedad en olivar es la mezcla de dos especies, *T. asperellum y T. gamsii*. La efectividad de esta mezcla ha sido demostrada en plantas de vivero (Jiménez Díaz et al., 2009). Otra combinación estudiada por Lima et al. (2007) demostró el potencial de *T. viride* con una enmienda compostada para la eliminación de microesclerocios en suelo artificialmente infestado. Sin embargo, ensayos realizados con plantones inoculados con cepas de *Glomus intraradices, G. mosseae y G. claroideum* no mostraron resistencia a la infección de *V. dahliae* (Porras-Soriano et al., 2006).

En los últimos años, cepas no patogénicas de *Fusarium* han sido identificadas como prometedores ACBs para la Verticilosis en varios cultivos (Fravel et al., 2003; Angelopoulou et al., 2014; Veloso et al., 2015). Solo un estudio hasta la fecha, que está comprendido en esta tesis (Capítulo 3), ha demostrado su eficacia en olivo (Varo et al., 2016). El modo de acción de estas cepas cubre un amplio rango de mecanismos. Cada cepa actúa de forma diferente. Así, *F. oxysporum* F2 es capaz de prevenir el ataque de *V. dahliae* mediante la interacción antagonista en cultivos de berenjena (Pantelides el al., 2009), mientras que la cepa Fo47 actúa por inducción de resistencia en cultivo de pimiento (Veloso y Díaz, 2011).

Sin embargo, en el diseño de estrategias que persiguen el uso de microorganismos como ACBs, se constata a menudo la ausencia de correlación entre los resultados obtenidos del antagonismo del ACB sobre el patógeno en condiciones controlados (*in vitro*) y en experiencias en planta y en campo (*in vivo*) (Hall et al., 1986; Fravel, 1988; Paulitz et al., 1992).

Enmiendas orgánicas

Otra categoría muy prometedora de tratamientos para el control biológico de *V. dahliae*, consiste en el uso de residuos de cultivos y enmiendas orgánicas compostadas (Bailey y Lazarovits, 2003). El compostaje es un proceso biológico controlado en el que la materia orgánica es degradada por diferentes grupos de microorganismos (Dees y Ghiorse, 2001), obteniendo como resultado una enmienda biológicamente estable (Adani et al., 1995), libre de patógenos y con una estructura ideal para albergar microorganismos antagonistas. Las enmiendas orgánicas poseen un efecto directo sobre el equilibrio nutritivo de los cultivos e influyen en el balance de microorganismos beneficiosos y patógenos establecidos en el suelo (Uppal et al., 2008), además su uso contribuye a reducir residuos originados por la agroindustria. Las enmiendas orgánicas compostadas pueden proteger frente a patógenos mediante la mejora del estado nutricional y/o toxicidad química o biológica directa. Al final de la etapa de maduración del compost, la temperatura del mismo decrece y es colonizado por microorganismos mesófilos, principalmente bacterias de los géneros *Bacillus* y *Pseudomonas*, además de especies fúngicas de *Alternaria*, *Aspergillus*, *Bipolaris*, *Fusarium*, *Mucor*, *Rhizopus*, *Peziza*, *Phoma* y *Trichoderma* (Mehta et al., 2014). A pesar del potencial que posee este tipo de medida de control para el olivar, son escasos los estudios de control biológico de la Verticilosis en olivo con enmiendas orgánicas (Avilés et al., 2011).

Al igual que las enmiendas sólidas, los extractos acuosos de compost o tés de compost han sido estudiados en cultivos como patata y fresa, donde han mostrado un efecto de inducción de resistencia, aunque este efecto es variable en función de las cepas de microorganismos que contenga y el efecto que éstas provoquen sobre la ruta del ácido salicílico en la planta (Yohalem et al., 1994; Van Wees et al., 1997).

En las enmiendas compostadas (sólidas o líquidas) se encuentran determinados microorganismos que actúan como ACBs y sustancias químicas indeterminadas (Cronin et al., 1996), las cuales son las responsables del efecto protector frente a patógenos. Una posible aproximación a este enfoque sería el

enriquecimiento de determinados compost con ACBs. Varios estudios han demostrado el efecto sinérgico de cepas de *Trichoderma* spp. y cepas no patogénicas de *F. oxysporum* al mezclarlas con compost, observándose mejores resultados en la supresividad de la enfermedad (Postma et al., 2003; Trillas et al., 2006).

El uso de esta estrategia aparece como una solución viable en campo, debido al incremento de la diversidad de los microorganismos aportado al suelo por las enmiendas compostadas, provocando un aumento de la resiliencia del suelo y de la planta a la invasión por el patógeno. Aunque parece ser una solución muy prometedora, es necesario un mayor conocimiento de las interacciones que se pueden dar entre microorganismos, de su efecto a largo plazo, así como la realización de estudios de toxicidad a suelos.

Sustancias naturales

Otra estrategia de control biológico está focalizada en la búsqueda de sustancias naturales como extractos de plantas y aceites esenciales, como alternativa al empleo de sustancias químicas. Esta medida de control presenta muchas ventajas en términos de sostenibilidad, modo de acción y toxicidad frente a patógenos, como por ejemplo *V. dahliae*, dentro de una estrategia de control integrado (Nega, 2014). Además, tienen una rápida degradación en el medio ambiente y generalmente tienen menos efecto tóxico sobre los microorganismos no diana (Thakore, 2006).

Los aceites esenciales son las fracciones líquidas volátiles, generalmente destilables por arrastre con vapor de agua y obtenidas a partir de material vegetal. En las últimas décadas, varios trabajos han centrado sus estudios en la obtención de metabolitos secundarios procedentes de extractos de plantas y aceites esenciales, como sustancias con un alto potencial frente a patógenos. Ejemplos como Carvacrol (5-isopropyl-2-methylphenol) y thymol (2-isopropyl-5-methylphenol) son producidos por determinadas plantas como un mecanismo de defensa de origen químico frente a organismos fitopatógenos (Vázquez et al., 2001; Falcone et al., 2005). Los metabolitos secundarios causan alteraciones en la morfología de las hifas de determinados patógenos como *Sclerotinia*

sclerotorium, resultando en una lisis de las paredes del hongo. Además, tienen un efecto inhibidor de la germinación de las estructuras de resistencia de *S. sclerotorium* (Soylu et al., 2007). Mathela et al. (2010) demostraron que la modificación química de esos componentes fenólicos en varios derivados procedentes de éteres y ésteres posee actividad biológica. De nuevo, son escasos los trabajos en los que se ha estudiado el efecto antifúngico de extractos de plantas y aceites esenciales frente a *V. dahliae* y más escasos aún, en olivo (López-Escudero y Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012). Por ello, el estudio de un conjunto amplio de aceites esenciales, extractos de plantas y metabolitos individuales para el control de *V. dahliae* en olivo, surge como necesidad, con el objetivo de llevar a cabo un 'screening' masivo de sustancias naturales para identificar las sustancias de mayor potencial capaces de controlar la enfermedad.

OBJETIVO DE LA TESIS DOCTORAL

Teniendo en cuenta lo anteriormente descrito, el objetivo general de esta Tesis Doctoral ha sido abordar, por primera vez, un estudio amplio que permita realizar una selección masiva de tratamientos potenciales para su aplicación en campo frente a la Verticilosis del olivo mediante el uso de compuestos pertenecientes a tres grandes grupos de compuestos aptos para el control biológico: i) Microorganismos antagonistas, ii) enmiendas orgánicas, y iii) sustancias naturales. El estudio para la selección de tratamientos eficaces se ha llevado a cabo hasta en cuatro tipos de condiciones: i) *in vitro*, ii) en suelo naturalmente infestado, iii) en condiciones controladas con plantones de olivo, y iv) en condiciones naturales en campo. Previamente se desarrollará una metodología para la inoculación artificial de plantones de olivo. Los trabajos realizados para alcanzar el objetivo propuesto, han conducido con la publicación de cinco artículos científicos que se recopilan y se presentan en esta Tesis Doctoral como capítulos 2, 3, 4, 5 y 6, además de un capítulo 7 adicional, todavía no publicado, donde se reúnen los experimentos de campo.

DESCRIPCIÓN DE LA TESIS DOCTORAL

En los capítulos 2 y 3 de esta Tesis se aborda la limitación ante una falta existente de un método eficaz de inoculación de plantas que reproduzcan las condiciones naturales de infección y que permita el estudio de aspectos epidemiológicos y de control biológico. En el capítulo 2 se describe la evaluación y optimización de medios de producción de MS de *Verticillium* de forma fácil y eficiente, incluso para aislados con nula capacidad de producción de estas estructuras en medios de cultivos habituales. En el capítulo 3 se han comparado cinco métodos de inoculación artificial diferentes, en aras de seleccionar el método más efectivo, en base a la respuesta severa, consistente y homogénea de las plantas.

La aplicación de esta metodología ha permitido evaluar un total de 162 tratamientos biológicos potenciales en diferentes formulaciones, de los cuales 47 ACBs han sido evaluados en el capítulo 4, 51 enmiendas orgánicas han sido evaluadas en el capítulo 5, y 44 extractos de plantas y 20 aceites esenciales han sido descritos en el capítulo 6.

El capítulo 7 aborda la evaluación en campo de los 14 tratamientos que han sido seleccionados por su eficacia en el control de la enfermedad en los capítulos anteriores. Se han realizado tres experimentos en tres localizaciones diferentes y en suelos naturalmente infestados con diferentes densidades de inóculo de *V. dahliae*.

Finalmente, se presentan la Discusión General y las Conclusiones finales de los trabajos abordados en esta Tesis Doctoral.

2

Microsclerotia mass production

2

Enhanced production of microsclerotia in recalcitrant *Verticillium dahliae* isolates and its use for inoculation of olive plants

ABSTRACT

Aims: The optimization of a simple protocol for the mass production of viable microsclerotia (MS) of *Verticillium* spp., even for recalcitrant isolates, to the inoculation of olive cuttings.

Method and Results: Four *Verticillium* spp. isolates were characterized by growth rate and morphology. Then, the production ability and the viability of MS over time were assessed in seven solid culture media and five aqueous media. The best culture medium, according to the quantity and the quality (size) of the MS produced, was the alkaline-modified sodium polipectate (AMSP) aqueous medium. The MS viability was higher in peat moss substrates. Finally, the MS obtained in this work were infective causing 100% incidence of Verticillium wilt (VW) disease in inoculated olive plants.

Conclusion: This study demonstrates that the modified sodium polipectate medium amended with 0·1% agar is the most suitable for the production of MS of *Verticillium dahliae* isolates that have lost the ability to produce MS in standard culture media.

Significance and Impact of the Study: Mass production of MS for artificial infestation of soil is critical to the study of epidemiological and control aspects of the VW. To overcome the failure in the production of MS in recalcitrant isolates, a culture media was optimized and a successful plant inoculation experiment was carried out with artificial MS.

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INTRODUCTION

Representative species of the genus *Verticillium* Nees 1816 are commonly found in agricultural soils (Domsch et al., 1980). This genus includes the plant pathogenic species *Verticillium dahliae* Kleb, *Verticillium albo-atrum* Reinke and Berthold, and *Verticillium longisporum* Stark (Karapapa et al., 1997), as well as other species with low, intermediate saprotrophic abilities such as *Verticillium tricorpus* which is known to cause significant losses in several host plants (Platt et al., 2000). Verticillium wilt (VW) caused by *V. dahliae* has the greatest economic impact, causing severe yield losses and plant death in many crops (Pegg and Brady, 2002). During recent years, this disease has become a major challenge for olive growing in the Mediterranean basin, due to the lack of an effective control method (López-Escudero and Mercado-Blanco, 2011).

Populations of *V. dahliae* infecting olive plants are formed by two distinctive virulence groups called defoliating (D) and nondefoliating (ND) pathotypes. The D pathotype is highly virulent and the ND pathotype is moderately severe in olive plants (Mercado-Blanco et al., 2003). Recently the D and ND have been characterized as race 2 and 1 respectively (Hu et al., 2015).

Verticillium dahliae produces long-lasting surviving structures called microsclerotia (MS), which constitute the main potential infective inoculum of the pathogen in soils. These MS are produced in senescent tissues of the affected plants (López-Escudero and Mercado-Blanco, 2011). Broad differences in MS size and shape can be found (Wilhelm, 1955), and this morphological criterion has been used to establish a suitable distinction among species and subspecific groups, which may display differential pathogenicity or virulence (Goud et al., 2003; López-Escudero and Blanco-López, 2005).

Differences among *Verticillium* species regarding the amount and morphology of the MS produced on potato dextrose agar (PDA) are pronounced. Isolates of *V. tricorpus* form large and irregularly shaped MS, usually with melanized hyphae growing from them, whereas *V. dahliae* isolates form smaller and from oval to elongated MS, which are sharply differentiated from the hyaline mycelium and conidiophores. Additionally, differences in

pathogenicity are found with *V. dahliae* causing serious wilt diseases of over 200 host species worldwide where *V. tricorpus* is generally harmless and it has a narrower host range (Hiemstra and Harris, 1998).

Diverse studies have indicated that the morphology of MS produced by *V. dahliae* in different culture media might be correlated with the virulence of the isolate. Large MS have a stronger inoculum potential because they germinate easily and show high levels of virulence (Hawke and Lazarovits, 1994). Butterfield and DeVay (1977) and López-Escudero and Blanco-López (2005) reported differences in the virulence and the average length/width ratio among MS produced by different isolates on modified sodium polipectate agar (MSPA) medium (Butterfield and DeVay, 1977; López-Escudero and Blanco-López, 2007).

A fundamental limitation to deepen the epidemiology and to develop control measures of the VW lies in the lack of an effective plant inoculation method that reproduces the natural infection conditions. Regarding this limitation, the mass production of MS for artificial infestation of the soil has a crucial importance. Several media have been used for MS production of *V. dahliae* (Lacy and Horner, 1966; Basu, 1987; Francl et al., 1988; Spink and Rowe, 1989; Hawke and Lazarovits, 1994; Nagtzaam et al., 1997; Xiao and Subbarao, 1998; Blok et al., 2000; López-Escudero and Blanco-López, 2007); however, according to our experience, some isolates with long-term storage or subsequent manipulation in experiments often change in viability and ability of MS production. These isolates are called recalcitrant due to the difficulty to produce MS using standard methods. To recover the success for MS production, we compared several published media and some modifications of them to assess the mass production of MS for normal and recalcitrant strains of *Verticillium* in these media.

The detection and estimation of *V. dahliae* in naturally infested soil is useful for gauging the disease risk associated with sites before planting susceptible crops. Researchers only familiar with *V. dahliae* on pectate-based agar media can easily be confused with *V. tricorpus* MS colonies

(Termorshuizen et al., 1998; Goud et al., 2003). The latter has not been reported as a pathogen in olive, but it has recommended for the biocontrol of *Rhizoctonia solani* in cotton seedlings (Paplomatas et al., 2000) and *V. dahliae* in potato (Davis et al., 2000).

This study provided improved methods for the production of MS of *V. dahliae*-recalcitrant isolates in a number suitable for the establishment of disease in olive plants.

MATERIALS AND METHODS

Fungal material

Four representative isolates belonging to the *Verticillium* collection of the Department of Agronomy at the University of Córdoba were selected for this study. They included two highly virulent defoliating (D) *V. dahliae* strains, V024 and V117, isolated from olive and cotton, respectively, and the mildly virulent ND strain, V004, isolated from cotton (Blanco-López et al., 1989; Rodríguez-Jurado, 1993). An isolate of *V. trichorpus* (V025), originally misidentified as *V. dahliae*, was selected because it produces a lot of MS in all culture media and does not lose the ability to produce MS after a routine laboratory use. The three *V. dahliae* isolates were collected in the Andalusia region, southern Spain, and the *V. tricorpus* V025 was collected from the soil in the Valencian region, east Spain.

The selected isolates were stored on PDA slants at 5°C. Conidial suspensions of each isolate were prepared from single-spore stock cultures maintained on PDA at 4°C. Mycelium was spread on the PDA plates and grown for 7 days at 24°C in the dark. The plates were flooded with sterile distilled water (SDW) and rubbed gently with a rubber-tipped glass rod. The resulting suspension was filtered through a double cheesecloth, counted with a haemocytometer and diluted to 10^6 conidia ml/1.

Effect of temperature on mycelial growth

To identify the optimum temperature for micelial growth of the four isolates of this study, an experiment under controlled conditions was conducted.

Thus, the daily rate of growth and the optimal temperature for development of each isolate were studied in detail. Mycelial plugs of 7-mm diameter were taken from the edge of 4-day-old colonies of each isolate and grown on PDA medium and were placed in the centre of each Petri dish containing the same medium. The inoculated Petri dishes were incubated at 3, 5, 7, 10, 15, 20, 25, 30 and 35°C in high precision (±0.2°C) incubators in the dark. The colony diameter was measured every 3 days throughout the 21 days. The growth rate at each temperature was converted to mm day^{-1}. Three dishes per isolate and each temperature combination were prepared. A factorial design was examined with the temperature and isolates as factors, and the Petri dishes as replications. The experiment was conducted twice.

For each isolate and replication, regression curves were fitted to the values of radial growth (mm/day) vs temperature. Three parameters were calculated in the fitted equation for each isolate and replication including optimum temperature (°C) for radial growth, maximum daily growth rate (mm/day) and area under the growth line (AUGL). The Analytis Beta model (Hau and Kranz, 1990) was selected among various linear and nonlinear regression models because it showed a good fit to describe the effect of temperature to the mycelial growth rate of the pathogen. The following equation of the Analytis Beta model described the influence of the temperature on growth rate:

$$[Y= K \times (T-T_{min})^a \times (T_{max}-T)^b].$$

in which Y = daily mycelial growth rate, T = temperature (ºC), and K, a, and b are unknown parameters. T_{min} and T_{max} depended on isolate according to minimal and maximal temperatures for growth, respectively. These parameters were obtaining using the Analytis Beta model and varying the values of Tmin (0–7°C) and Tmax (30–35°C) at intervals of 0.1°C. The adjusted optimum temperature was estimated by setting the first derivate of the equation to zero. The parameters used in this study were developed with an empirical approach, that is, the form of the model was determined by the collected data.

Culture media for microsclerotia production

To evaluate and compare the production of *Verticillium* spp., seven solid and four aqueous media were evaluated in two experiments (Table 1).

Experiment 1: Six solid and three aqueous media were used (Table 1). To enhance MS formation, the solid media were covered with a cellophane disc of 600 µm, according to López-Escudero et al. (2006). One of the three liquid media was modified from solid into aqueous culture by adding 1 g of agar per litre (0.1%) to optimize the recovery of individual MS. This media is called modified sodium polipectate (MSP).

Experiment 2: To compare the effect of the pH media on the capacity of MS production, a new experiment was conducted to compare the production of MS on the MSP medium with an unadjusted pH of 6.5, on the MSP adjusted to pH 11.5 (alkaline modified sodium polipectate (AMSP)) and on the basal modified agar (BMA) medium with pH 11.5 with a cellophane disc (Hu et al., 2013) (Table 1). The BMA medium was also modified into aqueous medium with 1 g of agar, which allows the recovery of individual MS. This media was renamed as BM. The aqueous media were prepared in Erlenmeyer flasks of 250-ml capacity each containing 100 ml of the medium.

Production of microsclerotia

In both experiments, 300 µl of a conidial suspension (10^6 CFU/ml) of each isolate was used to inoculate aqueous and solid media except in the case of cellophane media that was inoculated using 600 µl. The liquid cultures were shaken in an orbital incubator at 2.3 g. All liquid and solid media were incubated at 22°C (Soesanto and Termorshuizen, 2001) for 28 days in the dark.

The harvesting procedure to obtain individual MS was adapted from Tjamos and Fravel (1995). In the case of the solid media, the plates were flooded with SDW and MS were detached from the plates with a sterile scalpel. In the liquid cultures, the suspensions were centrifuged (21.8 g, 4°C, 20 min) to remove the supernatant growth medium and the pellet was resuspended in SDW. After the harvesting procedure to obtain individual MS, all suspensions

from both types of media were blended in a polytron homogenization (KINEMATICA, Lucerne, Switzerland) for 4 s, and they were then sifted through a 35-µm sieve, which is the size range reported for MS present in soils and usually considered in the quantification methods of the inoculum density of *V. dahliae* in soil (Butterfield and DeVay, 1977). The retained MS in the 35-µm sieve were recovered in a flask in SDW.

The obtained MS were counted using a haemocytometer and the length, width and length/width ratio of 30 MS were measured for each culture medium. The experiment was conducted three times. A factorial randomized complete block design was used with the three experiments as blocks, culture media and fungal isolates as the independent variables, and four plates or flasks as replications. Data analysis was applied to the total number and size of the recovered MS.

Viability of microsclerotia

To assess the viability of the artificially produced MS, two experiments were carried out. In Experiment 1, aliquots of 0.5 ml of each isolate from a MS suspension in SDW were spread onto MSPA plates. The suspensions were previously adjusted to obtain densities between 25 and 30 MS per plate with three replications per isolate and medium. The cultures were incubated at 24°C in the dark for 14 days, and the number of developed colonies was counted assuming that each colony came from a single MS.

Experiment 2 was designed to test the effect of different substrates on MS viability over time. Three different substrates were used (peat moss, sand and sterile sand) and they were inoculated with a MS suspension in SDW to obtain an initial inoculum density of 300 MS g/1 of substrate. The inoculum density of the pathogen was assessed in each substrate 1, 8 and 20 weeks after inoculation using the wet sieving technique (Butterfield and DeVay, 1977). To determine the viability of MS, samples from each substrate (25 g) were mixed and air-dried for 2 weeks at room temperature. Each sample was suspended in 100 ml of distilled water, shaken at 11.41 g for 1 h, and filtered through 150- and 35-µm sieves. The residue retained on the 35-µm sieve was recovered in

100 ml of distilled water, and then, 1 ml of the suspension from each treatment was plated on MSPA using 10 replicated plates per treatment. After 2 weeks of incubation at 22 ± 2°C in the dark, the soil residues were removed from the agar surface with tap water and the colonies of *V. dahliae* were counted under a stereoscopic microscope.

Inoculation of olive plants with microsclerotia

Substrate consisting of peat was inoculated with MS produced on AMSP from the *V. dahliae* D isolates (V024 and V117), the ND isolate (V004) and *V. tricorpus* (V025) that were suspended in SDW and adjusted to 350 UFC g/1. Five-month-old rooted olive cuttings of the susceptible cv. Picual were planted in plastic pots with 0.8 l of capacity with the mixture of MS and sterile substrate. After inoculation, all of the plants were transferred to a growth chamber (22 ± 2°C, L:D 12:12 (10.000 lux) and 60% RH) and were watered daily. The disease was weekly assessed by the severity of symptoms for 10 weeks using the following rating scale developed by Tjamos et al. (1991) that considers the percentage of the plant tissue affected by chlorosis, leaf and shoot necrosis or defoliation: 0 = absence of symptoms, 1 = light foliar symptoms in <33% of plant, 2 = moderate foliar symptoms and light defoliation (34–66%), 3 = severe foliar symptoms and moderate defoliation (67–99%), and 4 = total defoliation or plant death. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (López-Escudero et al., 2004). There were six replicate plants per fungal isolate, and the experiment was conducted twice. A control treatment was used in the absence of fungus. A randomized complete block design was used with the two experiments as blocks, fungal isolates as the independent variable, and plants as replications. A control treatment was planted only with sterile substrate.

Table 1. Culture media used to produce microsclerotia of *V. dahliae* and *V. tricorpus*

MEDIA	ACRONYMS	REFERENCE	DESCRIPTION	
			State	**pH**
Experiment 1				
Czapek Dox	CD	Hawke and Lazarovits, 1994	Aqueous	6.0
Minimal medium Agar	MMA	Puhalla and Mayfield, 1974; Markakis et al., 2009	Solid	6.0
Modified Sodium Polipectate Agar	MSPA	Butterfield and DeVay, 1977; Lopez-Escudero et al., 2007	Solid	6.5
Modified Sodium Polipectate	MSP	This study	Aqueous	6.5
Potato Dextrose Agar	PDA	Dhingra and Sinclair, 1995	Solid	6.5
Potato Dextrose Agar diluted to 10%	PDA10%	This study	Solid	6.0
Potato stems	Potato	Mol and Scholte, 1995	Solid	-
Sucrose sodium nitrate	SSN	Malandraki et al., 2008	Aqueous	6.5
Water-Agar	WA	Termorshuizen et al., 1998; Goud et al., 2003	Solid	6.0
Experiment 2				
Alkaline Polipectate Sodium Modified	AMSP	This study	Aqueous	11.5
Modified Sodium Polipectate	MSP	This study	Aqueous	6.5
Basal medium agar	BMA	Hu et al., 2013	Solid	11.5
Basal medium modified	BM	This study	Aqueous	11.5

AMSP, alkaline modified sodium polipectate; BMA, basal modified agar; MSP, modified sodium polipectate; MSPA, modified sodium polipectate agar; PDA, potato dextrose agar.

Data analysis

All statistical analyses were conducted using STATISTIX 10.0 (Analytical Software, Tallahassee, FL). The Analytis Beta model was adjusted to the mycelial growth data using the nonlinear procedure for each fungal isolate. A linear regression was applied to test the relationship between the estimated data by nonlinear regression and observed data. The regression model was chosen from many combinations of terms, based on the significance of the estimated parameters ($P \leq 0.05$), Mallow's Cp statistic, Akaike's information criterion modified for small data sets, the coefficient of determination (R^2), R^2 adjusted for degrees of freedom (R_a^2), centred R^2 for no-constant models (R^2), the predicted residual sum of squares and pattern of residuals over predicted and independent variables. Analysis of variance (ANOVA) were performed to compare the fungal isolates for the adjusted maximum rate, adjusted optimum temperature and the area under the adjusted growth lines (AUGL).

ANOVA was also performed for the counting, length, width and length/width of MS. Data on the number of produced MS were transformed to log10 prior to analysis in order to achieve homogeneity of variance. When ANOVA was significant, mean values were compared using the Fisher's protected Least Significant Difference test ($P = 0.05$). Statistical analysis of the final disease severity and RAUDPC in the inoculated olive plants experiment, were performed using the nonparametric Kruskal–Wallis and Dunn's tests. Both incidence and mortality were analysed by the multiple comparisons for proportions test ($P = 0.05$) (Zar, 2010), which considered the observed and expected frequencies of symptomatic and dead plants respectively.

RESULTS

Effect of temperature on mycelial growth

The mycelial growth rate (mm/day) of the isolates of *V. dahliae* and *V. tricorpus* in PDA varied significantly among the isolates, temperature and the interaction between both factors. The growth of the isolates was well fitted to the Analytis Beta model, all of the estimated parameters of the equation were

significant ($P < 0.001$) and the coefficients of determination (R^2) ranged from 0.983 to 0.998 (Table 2).

The isolates showed significant differences ($P < 0.001$), forming two homogeneous groups, according to the parameters T_{opt} and Y_{max}, however, no significant differences were observed to AUGL ($P = 0.820$). The first group (T_{opt} and Y_{max}) was formed by the *V. dahliae* isolates whose T_{opt} were 21.7, 23.2 and 23.4°C and Y_{max} were 3.35, 3.55 and 3.65 mm/day for V004, V024 and V117 isolates respectively. The second group was formed by the *V. tricorpus* V025 (T_{opt} = 20.1°C and Y_{max} = 2.56 mm/day). This isolate grew faster than the remaining of isolates at low temperatures (<15°C) and slower at high temperatures (≥ 15°C). The D isolates of *V. dahliae* (V024 and V117) grew better than the ND isolate (V004) at high temperatures (≥15°C). In addition, the isolate V117 grew worse than the other three at 7°C, and this isolate did not grow at 5°C (Table 2 and Fig. 1).

Table 2. Effect of temperature on the mycelial growth rate of *Verticillium* isolates growing on the PDA medium.

Isolate		Adjusted model*				Optimal T[a] (°C)[†¶]	Max. tax[‡¶] (mm d^{-1})	AUGL[§¶]
		R^2	a	b	k			
V. dahliae	V004	0.997	1.679	0.774	$4{,}5*10^{-03}$	21.1 ± 0.2ab	3.35 ± 0.6b	344.9 ± 54.4a
V. dahliae	V024	0.998	1.561	0.612	$9{,}8*10^{-03}$	23.2 ± 0.4a	3.56 ± 0.7ab	358.2 ± 56.2a
V. tricorpus	V025	0.983	1.669	0.900	$2{,}4*10^{-03}$	20.1 ± 0.3b	2.56 ± 0.7c	298.5 ± 36.4a
V. dahliae	V117	0.996	1.222	0.448	0.0424	23.4 ± 1.4a	3.66 ± 1.5a	358.0 ± 62.6a

*Mycelial growth rate on potato dextrose agar (PDA) at 3 to 35°C was adjusted to the Analytis Beta model: $Y = K \times (T-T_{min})^a \times (T_{max}-T)^b$, in which Y = mycelial growth rate (mm d^{-1}); a, b, and c are the regression coefficients; and R^2 = coefficient of determination. †Optimal temperature ± SE estimated by the adjusted model. ‡Maximum growth rate estimated by the adjusted model. §¶Area ± SE under the growth lines of the mycelial growth rate over time. ¶In each column, mean values followed by different letters are statistically significant according to Fisher's protected least significant difference test at $P = 0.05$.

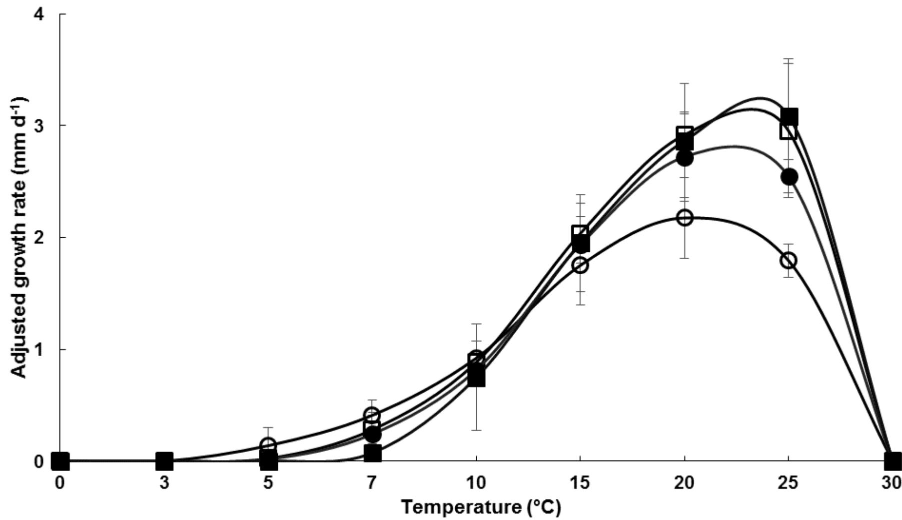

Figure 1. Adjusted growth curves according to the Analytis Beta model for three *V. dahliae* isolates: V004 (●), V024 (□) and V117 (■) and one *V. tricorpus* isolate, V025 (○) growing on PDA at different temperatures.

Production of microsclerotia

The first Verticillium MS were detected after 10–12 days of incubation. No global differences in the production of MS were found among *Verticillium* isolates (P = 0.3013). However, the production of MS depended on the interaction between the isolate and culture medium. The isolate, V117, with no previously consistent production of MS, only produced MS in MSP (33,620 MS/ml), CD (2.203 MS/ml) and WA (69 MS/ml) while the three remaining isolates produced MS in all of the tested media. Different media were suitable for different isolates to produce the highest number of MS: MM for isolates V004 and V025, MSP for isolate V117, and PDA 10% for isolate V024 (Table 3).

The size of MS greatly varied among culture media, highlighting the MSP medium, which produced the largest MS for all isolates tested. Regarding the morphology, MS produced by *V. tricorpus* V025 isolates were elongated and showed a greater average length/width ratio of 2.26 than the *V. dahliae* isolates. Among the MS produced by the *V. dahliae* isolates, the highly virulent D isolates (V024 and V117) were more elongated than the midly virulent ND

isolate (V004), which were more rounded and showed an average ratio of 1.36 (Table 3).

In the experiment 2, the *V. dahliae* D isolates (V024 and V117) produced greater amount of MS in the AMSP medium than in the other two selected media. Remarkably, the recalcitrant V117 isolate had the highest MS production (54,161 MS/ml) in the MSP medium alkalinized to a pH of 11.5 (AMSP medium). Regarding the other two isolates (V004 and V025), the most suitable media for MS production were BM or BMA (Table 4). Both liquid media (AMSP and BM) produced MS larger than the solid BMA medium, although the MS had the highest length/width ratio in the AMSP medium (Table 4).

Viability of microsclerotia

In the first survival experiment, the germination of individual MS through time in AMSP and BM aqueous media, showed significant differences between the *V. dahliae* and *V. tricorpus* isolates ($P = 0.0006$). MS germination in the AMSP plates started after 72 h of incubation. The final percentages after 14 days of incubation varied from 80 to 90% for the V004, V024 and V117 isolates and it was only 30% for V025. The survival of MS in different substrates showed a significant interaction between factors (isolate and substrate) from 1 to 20 weeks of evaluation. Broadly, at the beginning of the experiment (1 week after inoculation), MS production clearly depended on the isolate and substrate, being the most suitable for all isolates, the sterile peat ($P < 0.001$). The results showed percentages of viability >100% due to the breakage and posterior germination of MS during their manipulation. Despite this fact, the remaining MS were appropriate for germination. However, the percentage of germination decreased through time. In the second and third evaluations, the best MS production medium was MSP ($P = 0.0017$), with the most viable isolate through time being V024. In the third evaluation, at 20 weeks, the trend changed, and the most suitable substrate was the sand ($P < 0.001$) (Fig. 2).

Table 3. Amount and morphological parameters of microsclerotia formed by four *Verticillium* isolates growing in several cultures media*.

Isolate, Culture media	Log MS ml^{-1} †‡	Length (μm) ‡	Width (μm) ‡	Length/Width (μm) ‡
V004				
MMA	4.2 ± 0.0	55.9 ± 3.7	33.5 ± 1.6	1.3 ± 0.1
PDA	3.7 ± 0.2	71.8 ± 5.9	50.9 ± 0.0	1.3 ± 0.1
PDA 10%	3.6 ± 0.2	68.3 ± 5.8	48.9 ± 6.9	1.3 ± 0.1
MSPA	3.4 ± 0.1	71.8 ± 2.4	50.9 ± 1.7	1.4 ± 0.1
SSN	3.1 ± 0.1	69.31 ± 0.1	48.9 ± 6.0	1.5 ± 0.3
MSP	2.7 ± 0.1	214.5 ± 15.0	137.5 ± 12.3	1.5 ± 0.1
CD	2.5 ± 0.1	66.0 ± 6.7	56.4 ± 6.9	1.2 ± 0.0
WA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Potato	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LSD(20 df)$_{0.05}$	**0.24**	**18.53**	**15.92**	**0.32**
V024				
PDA 10%	3.4 ± 0.1	156.3 ± 13.7	96.1 ± 1.8	1.4 ± 0.2
MSP	3.3 ± 0.1	215.1 ± 11.5	72.1 ± 8.5	1.6 ± 0.4
CD	3.3 ± 0.1	113.6 ± 9.8	68.2 ± 3.6	1.7 ± 0.3
WA	3.1 ± 0.1	48.3 ± 7.3	28.3 ± 4.4	1.7 ± 0.2
MSPA	3.0 ± 0.1	74.8 ± 8.6	48.6 ± 7.3	1.6 ± 0.3
MMA	2.2 ± 1.1	30.6 ± 16.4	25.6 ± 13.4	1.3 ± 0.4
PDA	1.9 ± 1.0	70.8 ± 16.6	42.2 ± 5.0	3.3 ± 1.1
Potato	1.8 ± 1.0	63.7 ± 3.8	34.9 ± 3.7	1.9 ± 0.3
SSN	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LSD (20df)$_{0.05}$	**1.78**	**46.25**	**27.31**	**1.37**
V025				
MMA	4.9 ± 0.1	82.5 ± 15.1	66.7 ± 13.3	2.5 ± 0.6
PDA 10%	4.2 ± 0.2	106.7 ± 14.5	65.7 ± 19.6	3.3 ± 1.0
SSN	3.6 ± 0.3	173.7 ± 41.4	70.8 ± 9.2	2.6 ± 0.7
PDA	3.3 ± 0.1	93.2 ± 9.7	68.4± 12.7	2.2 ± 0.0
MSP	3.1 ± 0.1	277.1 ± 10.2	87.2 ± 4.6	2.3 ± 0.3
WA	3.0 ± 0.1	53.3 ± 3.3	45.0 ± 2.8	3.4 ± 0.3
CD	2.9 ± 0.1	146.2 ± 49.1	67.9 ± 27.8	3.3 ± 0.7
MSPA	2.8 ± 0.1	173.7 ± 4.9	51.3 ± 4.9	4.1 ± 0.7
Potato	1.9 ± 1.0	45.2 ± 23.4	44.5 ± 22.8	0.9 ± 0.1
LSD(20 df)$_{0.05}$	**1.00**	**68.33**	**43.42**	**1.99**
V117				
MSP	4.5 ± 0.1	213.3 ± 5.6	84.9 ± 5.7	2.6 ± 0.1
CD	3.3 ± 0.3	81.7 ± 7.7	58.1 ± 2.9	1.8 ± 0.1
WA	1.2 ± 1.3	18.3 ± 9.2	6.7 ± 3.3	1.8 ± 1.0
PDA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
PDA 10%	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MSPA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
MMA	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
SSN	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Potato stems	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
LSD(20 df)$_{0.05}$	**1.13**	**21.75**	**11.79**	**0.87**

*Data are the means of three experiments with four replicates for each culture medium and isolate. †The number of MS ml^{-1} was log-transformed for the statistical analysis. ‡Significant differences between any treatment means given by the least significant difference (LSD) critical value at P = 0.05, for 20 degrees of freedom (df).

Table 4. Amount and morphological parameters of microsclerotia formed by four *Verticillium* isolates growing in three selected culture media*.

Isolate, Culture media	LogMS ml^{-1} †‡	Length (µm)‡	Width (µm) ‡	Length/Width (µm)
V004				
BM	4.8 ± 0.1	273.6 ± 50.1	166.7 ± 32.1	1.0 ± 0.1
BMA	4.2 ± 0.1	55.9 ± 1.4	55.0 ± 3.0	1.3 ± 0.1
AMSP	3.7 ± 0.1	227.2 ± 5.0	153.3 ± 1.4	1.9 ± 0.1
LSD(6 df)$_{0.05}$	**0.21**	**116.02**	**86.91**	**0.31**
V024				
AMSP	4.7 ± 0.0	228.3 ± 11.0	147.5 ± 13.3	1.6 ± 0.2
BM	4.3 ± 0.1	263.9 ± 30.0	175.8 ± 7.6	1.5 ± 0.2
BMA	3.7 ± 0.1	60.0 ± 5.0	25.0 ± 3.0	2.5 ± 0.4
LSD(6 df)$_{0.05}$	**0.18**	**71.88**	**35.70**	**1.02**
V025				
BMA	4.1 ± 0.1	73.3 ± 4.0	63.3 ± 1.5	1.2 ± 0.1
BM	3.9 ± 0.1	235.8 ± 15.0	167.2 ± 22.7	1.5 ± 0.3
AMSP	3.2 ± 0.1	188.1 ± 40.1	95.0 ± 10.2	2.3 ± 0.3
LSD(6 df)$_{0.05}$	**0.30**	**93.90**	**54.64**	**0.82**
V117				
AMSP	4.7 ± 0.1	205.3 ± 17.1	132.8 ± 16.3	1.6 ± 0.1
BM	4.0 ± 0.0	207.2 ± 36.5	162.8 ± 28.6	1.3 ± 0.2
BMA	1.9 ± 1.7	76.7 ± 66.6	58.3 ± 50.6	1.3 ± 0.6
LSD(6 df)$_{0.05}$	**2.45**	**89.75**	**69.60**	**0.91**

* Data are the means of three experiments with four replicates for each culture media and isolate. †The number of MS ml^{-1} was log-transformed for statistical analysis. ‡Significant differences between any treatment means given by the least significant difference (LSD) critical value at P = 0.05 for 6 degrees of freedom (df).

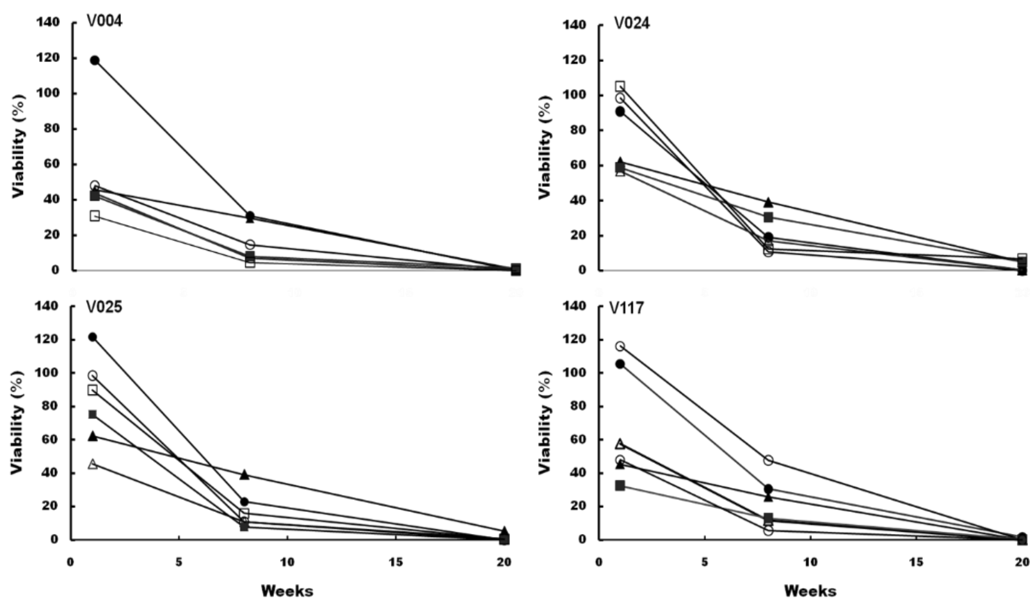

Figure 2. Viability over time of microsclerotia of *Verticillium dahliae* isolates (V004, V024 and V117) and *V. tricorpus* isolate (V025) obtained from peat AMSP (●), peat BM (○), sand AMSP (■), sand BM (□), sterile sand AMSP (▲), and sterile sand BM (Δ).

Inoculation of olive plants with microsclerotia

This experiment was carried out with the MS of the four selected Verticillium isolates. All of the plants inoculated with the isolates D (V024 and V117) of *V. dahliae* showed VW symptoms. The affected plants exhibited typical disease symptoms, including wilting, dieback and defoliation. No symptoms were observed in the plant inoculated with V025 and V004 isolates and control plants (Fig. 3). The pathogen was isolated from all the affected plants which were inoculated with V024 and V117, but it was not isolated from the asymptomatic plants inoculated with V004 and V025.

The onset and severity of the symptoms did not show differences between the D isolates. The mean values of RAUDPC and final severity of symptoms were higher in the plants infected with the V117 isolate, where 100% of mortality at

the end of experiment was observed despite the symptoms appearing 3 weeks after sowing, and the plants were severely affected by both isolates (Table 5).

Figure 3. Differential response of susceptible olive plants of cv. Picual at 14 weeks after inoculation with microsclerotia of *Verticillium* isolates (V004, V024, V025 and V117) and a sterile control. Typical defoliation symptoms were apparent in olive plants inoculated with isolates V024 and V117. No symptoms were observed in the control plants growth in sterile soil and in plants inoculated with isolates V004 and V025.

Table 5. Disease parameters of olive plants inoculated with microsclerotia of four isolates of *Verticillium dahliae* and *V. tricorpus*

Isolate		Incidence (%)*	Mortality (%)*	Disease severity†	RAUDPC†
Sterile Control		0 b	0 b	0.0 ± 0.0b	0.0 ± 0.0b
V. dahliae	MS V004	0 b	0 b	0.0 ± 0.0b	0.0 ± 0.0b
V. dahliae	MS V024	100 a	57 a	3.7 ± 0.2a	62.8 ± 7.0a
V. tricorpus	MS V025	0 b	0 b	0.0 ± 0.0b	0.0 ± 0.0b
V. dahliae	MS V117	100 a	50 a	3.8 ± 0.3a	72.4 ± 1.5a

*Percentage of plants showing Verticillium wilt symptoms or killed 14 weeks after inoculation. Mean values in the same column followed by the same letter are not significantly different according to the multiple comparisons for proportions test at P = 0.05 (Zar, 2010). †Final disease severity 14 weeks after inoculation based on a 0-4 rating scale and relative area under the disease progress curve (RAUDPC) developed over the assessment period. In each column, mean values followed by a common letter are not significantly different according to the non-parametric Kruskal-Wallis – Dunn's test at P = 0.05.

DISCUSSION

Successful implementation of an effective control of VW of olive plants requires a greater understanding of the role of microsclerotia on pathogen survival and plant infection under field conditions. During recent years, a remarkable number of studies about pathogen survival and control of the disease have been carried out (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012), even though the variability between experiments due to the erratic artificial production of MS in the laboratory has restricted the results. The procedure outlined here can be used to produce a large number of MS, even for recalcitrant isolates. The inoculation of olive cuttings with artificial MS may have enormous potential because the natural conditions of inoculation are reproduced. The MS produced by this method maintained high and uniform viability for the first weeks after inoculation when the infection is performed.

The selected isolates of *Verticillium* spp. used in this study were characterized through the growth rate and morphology. According to our results, the D isolates of *V. dahliae* grew better at higher temperatures (22.9–23.4°C) than the ND *V. dahliae* (21°C) and the *V. tricorpus* isolate (20.1°C). As previously found by Jiménez-Díaz et al. (2012), this fact could explain the predominance of the D isolate of *V. dahliae* in the most important olive growing areas, where the increase in the virulence of the disease and warmer temperatures cause severe yield losses and plant death.

Soesanto and Termorshuizen (2001) demonstrated that temperatures between 15 and 20°C scarcely influence the microsclerotia production. Here, we focused on the effect of several media in MS at these temperatures, and the results showed that the MSP was the most suitable medium. At the same time that this study was carried out, Hu et al. 2013 demonstrated that the modified basal agar (MBA) was appropriate for selected Chinese isolates.

After comparing equal terms for both media (pH 11.5, aqueous and shaking conditions), an excellent production of MS was obtained by growing the cultures in the aqueous alkalinized media AMSP. Remarkably, the modified medium, MB, in an aqueous state and agitated can reach better results than the unmodified solid MBA. The use of only 1 g agar/l medium allowed for individual MS from the mixture with conidia and mycelia. In further studies in which mass production of recalcitrant isolates is required, the MSP medium is the most suitable, producing significantly better results in the production of these resting structures, although a significant interaction was found between the culture media and isolate. This fact indicates that the effectiveness of a culture medium to produce MS depends on the isolate of *Verticillium* spp. used.

MS of *V. dahliae* in naturally infested soil vary greatly in their size (diameter), ranging between 11 and 125 µm, and larger aggregates may occur (Ashworth et al., 1974; DeVay et al., 1974). Large microsclerotia (>75 µm), and fully melanized, probably contain more layers of cells and are likely to be more tolerant to harsh external conditions (Hawke and Lazarovits, 1994) and consequently, these structures are more suitable for biological and epidemiological studies. In this study, the MSP or AMSP media produced the larger MS, ensuring the pathogenic ability of the isolates for inoculation experiments. Both media, but especially the AMSP medium, were the most suitable according to the quantity and quality of MS produced, even for recalcitrant strain, V117, that had lost the ability to produce MS by the standard methods.

The rates of germination and viability of MS produced in two culture media and inoculated in three substrates were affected by the *Verticillium* isolate, inoculated substrate and culture medium source of MS. In general, *V. dahliae* isolates had a better germination rate than *V. tricorpus*. The viability of MS depended on the type of substrate (peat, sand and sterile sand). In general the peat was the most suitable substrate because it maintained MS viability higher than 24% up to 8 weeks after inoculation; however, the V024 isolate showed the best results in the sand substrate.

Although there was a loss of the viability of MS over time, however, during the first 8 weeks, MS viability remained above 24% in the peat, which shows that MS are an effective inoculum for plant infection under controlled conditions, because in these conditions infections occur during the first weeks after inoculation (López-Escudero et al., 2004). Under field conditions, MS may germinate and sporulate several times and still have the capacity for growth and infection when contacted by host roots. Nevertheless, in potted plants the amount of exudates decrease (Farley et al., 1971), and this fact may explain the drastic viability decrease of MS in the selected substrates.

The inoculation of olive plants with MS confirmed the suitability of MS produced in artificial media to infect and cause VW disease under controlled conditions. In this experiment, sterile peat was the substrate used, based on previous results. Disease development in plants growing in the substrate artificially infested with MS of defoliant isolates of *V. dahliae* reached 100% incidence and 50–57% of mortality. However, olive plants inoculated with MS of ND V004 and V025 did not show symptoms.

V. tricorpus has not been described as an olive pathogen; however, this species is causing a devastating disease in potato (Moukhamedov et al., 1994). In a previous study, López-Escudero and Blanco-López (2005), demonstrated that the D isolate, V117, produced 100% mortality in cotton plants, while

values for the isolated ND V004 were much lower, confirming the results shown here. In our study, the same isolate was not pathogenic on olive plants inoculated with MS; however, this fact can be due to the inoculation conditions which are less aggressive than the root-dip inoculation used by López-Escudero et al. (2005).

The pathogen was reisolated from the aerial parts in symptomatic plants (inoculated with V024 and V117 isolates), however, this reisolation was not possible in asymptomatic plants (inoculated with V004 and V025 isolates), confirming that the pathogen did not colonize aerial tissues. These results agreed with Rodríguez-Jurado (1993) and Mercado-Blanco et al. (2003), where D isolates showed higher colonization ability in comparison with ND isolates. The viability of the artificial MS had not influenced in the virulence of the isolates, while the nature of the isolate was a determining factor.

The media and method presented here for the production and manipulation of MS, even for recalcitrant isolates of *V. dahliae*, could provide a tool to deepen the study of VW diseases and to develop new methods for disease management.

ACKNOWLEDGEMENTS

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3

Inoculation method to asses biological treatments

3

Development and validation of an inoculation method to assess the efficacy of biological treatments against Verticillium wilt in olive trees

ABSTRACT

Verticillium wilt in olive trees, caused by the soil-borne fungus *Verticillium dahliae*, is one of the most serious diseases of this crop due to high tree mortality and the difficulty of control. One of the major constraints to developing control measures against this disease is the lack of inoculation methods to evaluate the effectiveness of treatments. Here, we compared five inoculation methods for screening biological control agents (BCAs). The soil infested with Cornmeal Sand medium (CMS) at 20% w/w performed the best, and its effectiveness was further tested in olive plants treated with six BCAs, four strains of the fungal species *Fusarium moniliforme*, *F. oxysporum*, *Gliocladium roseum* and *Phoma* sp., and two fermented mixtures of several yeast and bacteria. Strains of *F. oxysporum*, *Phoma* sp. and the two mixtures of microorganisms significantly decreased the severity of the disease in potted plants of the susceptible cv. Picual.

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INTRODUCTION

Verticillium wilt of olive trees (*Olea europaea* L.) (VWO), caused by the soil-borne fungus *Verticillium dahliae* Kleb., is one of the most serious diseases for this crop (López-Escudero and Mercado-Blanco, 2011). This fungus produces resting and infecting structures, microsclerotia (MS), which are stimulated to germinate by root exudates. The hyphae penetrate and invade the xylem vessels where conidia are formed. The fungus produces MS in symptomatic plants that allow the fungi to survive in the soil for up to 13 years, even though the conidia may live for only a few days in the soil (Pegg and Brady, 2002).

One concerning observation about this disease in several important Mediterranean olive-growing regions is the rapid spread of *V. dahliae* isolates belonging to a defoliating (D) pathotype, which is more virulent than the dominant non-defoliating (ND) pathotype (López-Escudero et al., 2004). This fact, together with the severity of the infections, the prolonged survival of *V. dahliae* in the soil, the wide host range, and the ineffective control by chemical compounds, form the context of this devastating disease (Tjamos, 2000). Because there is no single method that is sufficiently effective when individually applied, management strategies should be focused on integrated control measures (López-Escudero and Mercado-Blanco, 2011). Among these measures, the use of resistant cultivars and biological control practices appear to be effective and sustainable strategies in olive trees. However, the biological control of VWO has been investigated to a lesser extent compared with herbaceous species due to the longevity and inherent particularities of crop management of perennial hosts (López-Escudero and Mercado-Blanco, 2011).

Over the last few years, different studies have been conducted to evaluate the effect of BCAs in controlling VWO under controlled conditions. The most prominent examples are the use of bacterial strains, such as *Paenibacillus alvei* (Markakis et al., 2015), *Pseudomonas fluorescens* (Mercado-Blanco et al., 2004; Sanei and Razavi, 2011), *P. putida* (Mercado-Blanco et al., 2004), or *Serratia plymuthica* (Müller, 2006). Remarkably, only one biological treatment (Bioten®), which is composed by *Trichoderma asperellum* and *T. gamsii*, is currently available to farmers in Spain (Jiménez Díaz et al., 2009), but its application is

limited to the treatment of olive trees before planting. Nevertheless, an optimum inoculation method that provides a mass screening of potential new BCAs is needed. One of the limiting factors to the development of an optimum inoculation method is the difficulty of producing large amounts of MS. These can be obtained from naturally infested soil or produced artificially using different culture media. Unfortunately, when potted olive plants are grown in naturally infested soil, the occurrence of wilt disease is very erratic due to the slow progression of symptoms and the lack of infection in some plants, which limits data analysis (M. Blanco-López, *personal communication*). In addition, it is difficult to find a homogeneous soil highly infested with MS, at least in Spain. Conversely, naturally infested soil has been used to inoculate herbaceous plants with good results (Termorshuizen and Mol 1995; Xiao and Subbarao, 1998).

In most experiments, olive plants are infected with *V. dahliae* using a root-dip inoculation that exposes roots to a suspension of pathogenic conidia, after which the inoculated plant is established in soil or a potting mixture (Colella et al., 2008; Trapero et al., 2013a). Conversely, under natural conditions, MS infect the olive roots and subsequently invade the xylem (Beckman and Roberts 1995). In addition, the root-dip inoculation method makes it difficult to evaluate the potential effect of BCAs due to the high inoculum pressure of the pathogen and the fact that, once the pathogen has vascularly colonized the plants, it is highly inaccessible to BCAs.

An alternative to both conidial suspension and naturally infested soil inoculation methods is the use of a culture media that can produce a high amount of quality inoculum. Among these media, specific mention can be made of the cornmeal sand Mixture (CMS) medium that has been used for other soil-borne diseases, such as Fusarium wilts (Trapero-Casas and Jiménez-Díaz 1985), although it has not been optimized for *V. dahliae*. Thus, the main goal of this study was developed an effective and standardized inoculation method for a better comparison of biological control treatments against *V. dahliae*.

Material and methods

Plant material

Five-month-old olive rooted cuttings of susceptible cv. Picual were used in the five experiments of the current study. The plants were maintained in a growth chamber for one month before the inoculations to force active growth. They were then planted in 0.8 l plastic pots in different substrates, depending on each experiment. In the experiment 1, cotton seedlings of the susceptible cv. Coco growing in 0.5 l plastic pots. All plants were incubated at 60% relative humidity and a 14 h photoperiod of fluorescent light adjusted to 216 µmol/m^2 s and 22ºC.

Fungal isolates and inoculum production

Two defoliating isolates (V024 and V117) belonging to the fungal collection of the Department of Agronomy at the University of Córdoba were used in the different experiments. The cotton isolate V117 is considered highly virulent in olive plants (López-Escudero et al., 2004; López-Escudero and Blanco-López, 2007), even though there were difficulties developing MS *in vitro*. For this reason, we included the olive isolate V024 that showed a high production of MS in *in vitro* conditions.

Naturally infested soils (Experiment 1)

To test the effect of naturally infested soil as an inoculation method for potted plants, rooted olive cuttings and cotton seddlings were planted in 0.7 l plastic pots filled with natural soils with different inoculum densities. These soils were collected from four Andalusian orchards: orchard 1, located in the municipality of Villanueva de la Reina (UTM coordinates X: 38.012845; Y: 3.909219) with a vertisol soil containing 110 MS/g; orchard 2, located in the municipality of Utrera (UTM coordinates X: 37.067898, Y: 5.911201) with a alfisol soil containing 26 MS/g; orchard 3, located in the municipality of Andújar (UTM coordinates X: 409.007,90763; Y: 4.204.618,5853) with a inceptisol soil containing 23 MS/g; and orchard 4, located in Guadalcázar (UTM coordinates X: 37.779982, Y: 4.965521) with a vertisol soil containing 2 MS/g.

The inoculum density of the pathogen in the four soils was determined at the beginning and end of each experiment, i.e. at 4 weeks and 12 months after inoculation in cotton and olive, respectively, using the wet sieving technique and the modified sodium polipectate agar (MSPA) medium (Butterfield and DeVay 1977); López-Escudero and Blanco-López, 2007). Ten replicated plates per treatment were used, and colonies of *V. dahliae* were counted under a stereoscopic microscope.

Soil infestation with cornmeal sand inoculum (Experiment 2)

The following experiment was conducted to optimize the CMS as the inoculum source of *V. dahliae*. This experiment was carried out with the V117 isolate. The CMS consisted of a sterilized mixture of 9:1:2-weight proportion of dry sand, cornmeal, and distilled water, respectively. Afterward, 1 kg of the CMS was distributed in Erlenmeyer flasks of 2 l and inoculated using fifty 5-mm PDA disks with pathogen mycelia. The flasks were agitated every 4 days to promote the homogenous colonization of the substrate by the fungus. After 28 days, the CMS colonized by the pathogen was mixed into sterile peat moss at the following proportions by weight: 5, 7.5, 10, 20, 22.5, 35, and 50% (CMS / peat moss). Later in the inoculation, all plants were transferred to controlled environmental conditions and watered daily.

Comparison of inoculation methods (Experiment 3)

We conducted a third experiment to compare the CMS method at a low and middle proportion (5 and 20%, respectively) with other three inoculation methods using the two isolates of *V. dahliae*. The new inoculation methods were: 1) **soil infestation with MS,** MS developed in MSPA medium were mixed with sterile soil and adjusted to 200 MS/g of sterile soil; 2) **root dipping in a conidial suspension,** olive plants were inoculated by dipping their bare root systems in each *V. dahliae* isolate conidial suspension at 10^7 conidia ml^{-1} for 30 min (Colella et al., 2008; Trapero et al., 2013a) and transplanted to pots with sterile peat moss; 3) **root impregnation with a paste of PDA,** plant root systems were drenched for 5 min with a blended PDA paste obtained from 1-week-old cultures of *V. dahliae* (Hiemstra and Harris 1998).

Validation of selected inoculation methods for biocontrol assays (Experiment 4 and 5)

To evaluate the effectiveness of the previous inoculation methods for screening BCAs, we conducted two bioassays using olive plants. In the first bioassay (Experiment 4), plants were inoculated with the pathogen using CMS medium or PDA paste and were then planted in 0.8 l plastic pots. The inoculated plants were treated separately with two biological control treatments consisting of two different conditions of fermented mixtures of microorganisms (*Rhodopseudomonas palustris, Rhodobacter sphacrodes, Lactobacillus plantarum, L. casei,* and *Streptococcus lactis, Saccharomyces* spp., and *Streptomyces* spp.), which are based on the trademarked product EM-1 Microbial Inoculant (Higa and Ke 2001). Both biocontrol treatments were applied directly by watering the pots until field capacity. A second bioassay (experiment 5) was conducted using 20% CMS as the inoculum and the following BCAs were evaluated: *Fusarium moniliforme* (FM01)*, F. oxysporum* (FO12), *Gliocladium roseum* (GR01) and *Phoma* sp. (Ph02). All the isolates were previously selected according to their biological control capacity and belong to the Department of Agronomy at the University of Córdoba (Varo and Trapero*, data not published*).

The four BCAs were grown in PDA, and conidia of each isolate were collected from 4-day-old cultures and adjusted to 10^4 conidia ml^{-1}. Liquid inoculum was grown in potato dextrose broth (PDB) in shaken culture. Pots with transplanted plants in sterile peat moss with 20% of the CMS inoculum were treated by watering with the BCA inoculum until field capacity. All plants were maintained in a high humidity, dark chamber 3 days after inoculation and were then removed to a growth chamber.

Disease assessments

Disease severity was periodically assessed for 14 weeks using a rating scale developed by Tjamos et al. (1991) that considers the percentage of plant tissue affected by chlorosis, leaf and shoot necrosis or defoliation: 0 = absence of symptoms, 1 = light foliar symptoms in <33% of plant, 2 = moderate foliar symptoms and light defoliation (34-66%), 3 = severe foliar symptoms and

moderate defoliation (67–99%) and 4 = total defoliation or plant death. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (Wilhelm and Taylor 1965; López-Escudero et al., 2004).

Data analysis

In all experiments, the plants were arranged in a completely randomized design. Up to three control treatments were used depending on the experiment; one control dipped the roots in sterile water, the second impregnated the roots with a PDA paste and the third transplanted the plants into sterile peat moss. An analysis of variance (ANOVA) of the RAUDPC was performed for each experiment because the RAUDPC values met the assumptions of normality and homogeneity of variances for this analysis. When ANOVA showed significant differences ($P < 0.05$) among treatments, mean values were compared using Fisher's protected Least Significant Difference (LSD) test at $P = 0.05$. Both incidence and mortality were analyzed by multiple comparisons for proportions test ($P = 0.05$) (Zar, 1999), which considered the observed and expected frequencies of symptomatic and dead plants, respectively. In experiment 2, linear regression was used to study the effect of CMS doses on disease. The RAUDPC data were transformed by the monomolecular model as $Y = ln\ [(100/(100-RAUDPC))]$ (Campbell and Madden 1990) and linear regression was fitted as $Y = aX + b$ (X = CMS dose). Statistical analysis of the data was conducted using Statistix 10.0 (Analytical Software, Tallahassee, USA).

RESULTS

Disease development in naturally infested soils (experiment 1)

Most cotton plants growing in infested soils showed similar symptoms, including defoliation and sudden wilt, although plants sowed in soil from orchard 1 (111.9 MS/g) were also stunted. The pathogen affected all plants sowed in soils containing >20 MS/g (orchards 1, 2, and 3). Conversely, the pathogen only

affected 80% of the plants sowed in soil with lower inoculum density (2.2 MS/g). Significant differences were observed between the four soils in the disease severity ($F_{(3,16)} = 7.31$ and $P = 0.0026$) and RAUDPC ($F_{(3,16)} = 19.09$ and $P < 0.0001$) (Table 1). The first symptoms were observed 1 week after inoculation of cotton plants sowed in soil from orchard 1. In the rest of the treatments, the first symptoms were observed 2-3 weeks after the plants were sowed. The increase in disease lasted for 3 weeks, although disease onset became earlier as the inoculum density increased.

Table 1. Disease parameters of olive plants growing in four soils naturally infested by *Verticillium dahliae*

Host	**Soil**	**UFC/g**[a]	**Incidence (%)**[b]	**Mortality (%)**[b]	**Disease Severity**[c]	**RAUDPC**[d]
Cotton	1	111.9 ± 20.4a[e]	100 ± 0.0a	100 ± 0.0a	4.0 ± 0.00a[e]	100.0 ± 0.00a[e]
(*n*=5)	2	27.2 ± 2.6b	100 ± 0.0a	100 ± 0.0a	4.0 ± 0.00a	88.6 ± 1.75a
	3	22.6 ± 4.8b	100 ± 0.0a	100 ± 0.0a	4.0 ± 0.00a	81.1 ± 5.60a
	4	2.2 ± 0.1b	80 ± 17.8b	80 ± 17.8b	2.3 ± 0.61b	11.6 ± 3.26b
Olive	1	111.9 ± 20.4a	100.0 ± 0.0a	10.0 ± 9.5a	1.3 ± 0.32a	16.5 ± 4.92a
(*n*=10)	2	27.2 ± 2.6b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.00b	0.0 ± 0.00b
	3	22.6 ± 4.8b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.00b	0.0 ± 0.00b
	4	2.2 ± 0.1b	0.0 ± 0.0b	0.0 ± 0.0b	0.0 ± 0.00b	0.0 ± 0.00b

[a]Mean initial values ± standard error (SE) of soil inoculum density of the inoculated plants maintained in controlled conditions.

[b]Percentage of plants ± SE showing symptoms or killed by *V. dahliae* 14 weeks after inoculation. For each host and in each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at $P = 0.05$.

[c]Final disease severity 14 weeks after inoculation ± SE.

[d]Mean value for the relative area under the disease progress curve (RAUDPC) developed over the assessment period ± SE.

[e]For each column and host, mean values followed by the same letter were not significantly different according to Fisher protected LSD test ($P = 0.05$).

In olive plants, the soils formed two homogenous groups according to the disease parameters. The soil from orchard 1 formed the first group with the following disease parameters: 16.5% RAUDPC ($F_{(3, 35)} = 10.85$, $P < 0.0001$), 1.3 final disease severity ($F_{(3,35)} = 15.15$, $P < 0.0001$), and 10% mortality. The remainder of the soils formed another homogeneous group because no disease symptoms were observed (Table 1). Even for the soil from orchard 1, the disease

data were not robust or homogeneous due to the appearance of completely affected and non-symptomatic plants growing in the same soil. In this case, the first Verticillium wilt symptoms were observed 11 weeks after planting.

The inoculum density of the four soils decreased steeply during the 12 months of the experiment. Thus, the inoculum density varied from 111.9, 27.2, 22.6, and 2.2 MS/g to 61.9, 13.7, 7.2, and 1.7 MS/g (i.e., decreasing 55, 50, 50 and 20%, respectively). At the end of the experiment, there were significant differences in the inoculum densities among all treatments ($F_{(3,8)}$ = 21.04, P = 0.0004).

Influence of CMS doses on the development of Verticillium wilt (experiment 2)

More than 80% of the plants in the experiment with different CMS doses showed Verticillium wilt symptoms during the experiment. The affected plants exhibited typical disease symptoms, including wilting, dieback and defoliation. No symptoms were observed in the control plants.

The disease progressed faster at higher doses of CMS, and the first symptoms appeared 3-4 weeks after planting. All the plants growing at CMS doses greater than 5% showed Verticillium symptoms, meanwhile disease incidence at CMS 5% was 78%. The disease severity and RAUDPC increased with increasing CMS doses with a tendency toward saturation at higher doses. This relationship was well explained by the monomolecular model (R^2 = 0.9061, P = 0.0003) (Fig. 1).

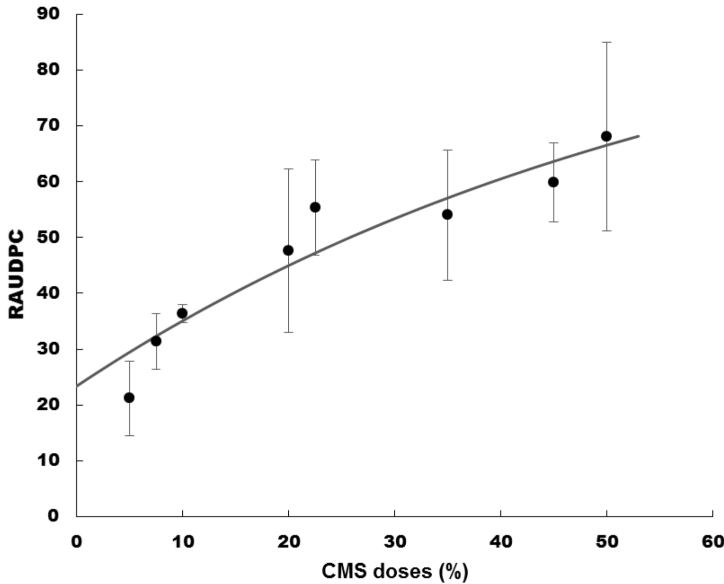

Figure 1. Relative area under the disease progress curve (RAUDPC) in rooted cuttings of susceptible cv. Picual at different CMS doses. Black dots and vertical bars refer to the mean and standard error of the mean (SE) of the observed data, and the line represent the adjusted monomolecular model (R2 = 0.9061; P = 0.0003)

Comparison of inoculation methods (experiment 3).

Verticillium wilt symptoms were observed in all olive plants inoculated by the different inoculation methods. No symptoms were detected in any of the control plants. The olive plants exhibited a high susceptibility to both pathogen isolates, even at very low inoculum levels, and there were no differences between isolates ($F_{(1,25)} = 2.57$, $P = 0.1215$). The PDA paste was the inoculation method that caused the highest mortality and RAUDPC, although disease incidence was 100% regardless of the method of inoculation. The final severity recorded at 90 days and the RAUDPC significantly differed among the inoculation methods (RAUDPC: $F_{(7,48)} = 11.82$ and $P < 0.0001$ and final disease: $F_{(7,48)} = 3.02$ and $P = 0.0103$) (Table 2). When the olive plants were inoculated by root dipping inoculations, the first symptoms appeared 21 days after inoculation, and increased disease severity lasted 67 days, while the first symptoms appeared at 33 days with the soil infestation methods, and the progress of the disease lasted 90±10 days.

Comparison and efficiency of the CMS and PDA paste methods for assessing biological control agents (experiment 4).

Table 2. Disease parameters of olive plants inoculated with two highly virulent isolates (V117 and V024) of *Verticillium dahliae* using different inoculation methods

Inoculation method	Incidence (%)[a]	Mortality (%)[a]	Disease severity[b]	RAUDPC[c]
CMS 5% V117	100 ± 0.0a	43 ± 18.7bc	3.2 ± 0.33ab[d]	45.6 ± 7.50b[d]
CMS 20% V117	100 ± 0.0a	57 ± 18.7b	3.3 ± 0.38ab	50.1 ± 6.55b
CMS 5% V024	100 ± 0.0a	29 ± 17.1c	2.0 ± 0.33b	37.5 ± 6.79b
CMS 20% V024	100 ± 0.0a	57 ± 18.7b	2.6 ± 0.52ab	42.0 ± 10.44b
MS V024	100 ± 0.0a	57 ± 18.7b	3.7 ± 0.58a	57.8 ± 6.87b
Root dipping V117	100 ± 0.0a	57 ± 18.7b	2.9 ± 0.27ab	42.1 ± 8.35b
Root dipping V024	100 ± 0.0a	57 ± 18.7b	3.4 ± 0.20ab	51.0 ± 6.85b
Paste PDA V024	100 ± 0.0a	86 ± 13.2a	3.9 ± 0.11a	100.0 ± 4.47a

[a]Percentage of plants ± standard error (SE) showing symptoms or killed by *V. dahliae* 14 weeks after inoculation (n = 7). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at P = 0.05.

[b]Final disease severity 14 weeks after inoculation ± SE.

[c]Mean value for the relative area under the disease progress curve (RAUDPC) developed over the assessment period ± SE.

[d]In each column, mean values followed by the same letter were not significantly different according to Fisher protected LSD test (P = 0.05).

Olive plants inoculated with 20% CMS showed lower disease severity than those inoculated using the PDA paste method. In the latter case, the first symptoms appeared 1 week earlier, and plants died 5 weeks before the olive plants growing in peat moss infested with CMS. There was no effect ($F_{(2,24)} = 1.00$ and $P = 0.3827$ for final severity and $F_{(2,24)} = 0.08$ and $P = 0.9220$ for RAUDPC) observed from biological control mixtures in the final disease severity of olive plants inoculated with the PDA paste (Fig. 2A). The increase in disease severity lasted 4 weeks in the control treatment and 3 weeks in the treatment with the two different mixtures of microorganisms.

The two mixtures of microorganisms had a significant effect ($F_{(2,26)} = 15.77$ for RAUDPC and $F_{(2,26)} = 16.93$ for final severity, both at $P < 0.0001$) on disease development in olive plants that were inoculated using 20% CMS, and there was a significant difference between them. MOM2 treatment reduced disease severity by 82.08%, while the MOM1 mixture reduced disease severity by 25.72% with respect to the control (Fig. 2B). Therefore, the CMS method allowed better discrimination of the effects of the biocontrol treatments.

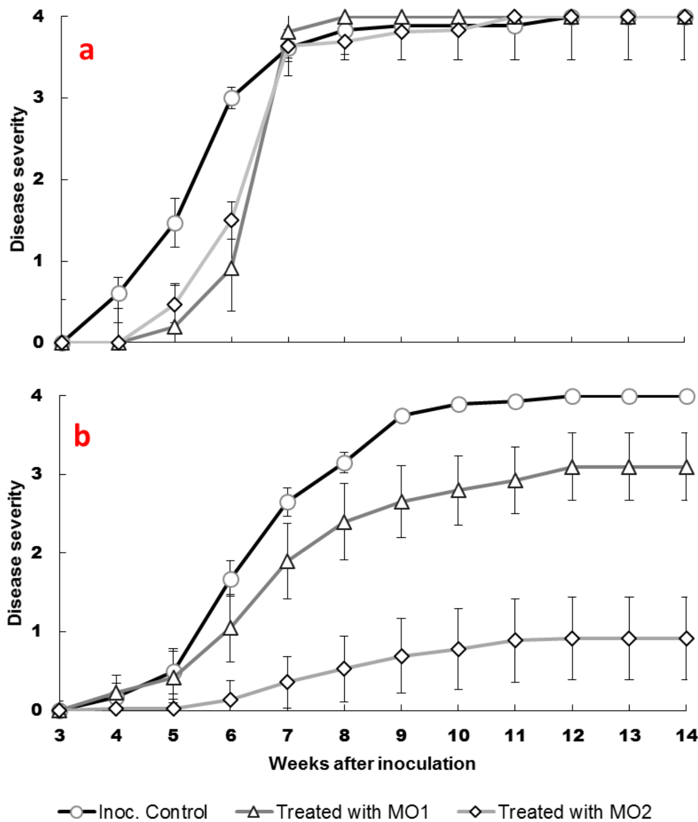

Figure 2. Disease severity progress curves for olive plants inoculated with the PDA paste (A) or 20% CMS methods (B). Plants were inoculated with a virulent isolate (V024) of *Verticillium dahliae* and treated with two different mixtures of microorganisms (MOM1 and MOM2). Disease severity was rated weekly using a 0–4 scale. Dots and vertical bars along the curve are the mean and standard error of the mean for each evaluation

Efficiency of the CMS method for assessing antagonist microorganisms (experiment 5)

The 20% CMS allowed us to evaluate the efficacy of different antagonistic fungi used against *V. dahliae*. The effect of different treatments on disease parameters ranged from a total reduction of the disease, which occurred in plants treated with *F. oxysporum* (FO12), to very low effects that did not differ significantly ($F_{(4,39)}$ = 6.99 and P = 0.0002) from the inoculated control plants, which was observed with plants treated with *Gliocladium roseum* (GR01) and *F. moniliforme* (FM01). Plants treated with *Phoma* sp. (PH02) showed an intermediate response with a significant reduction in the disease severity ($F_{(1,17)}$ = 10.12 and P = 0.0055) and the RAUDPC ($F_{(1,17)}$ = 5.62 and P = 0.0299) of approximately 50% respect to the control treatment (Fig. 3; Table 3).

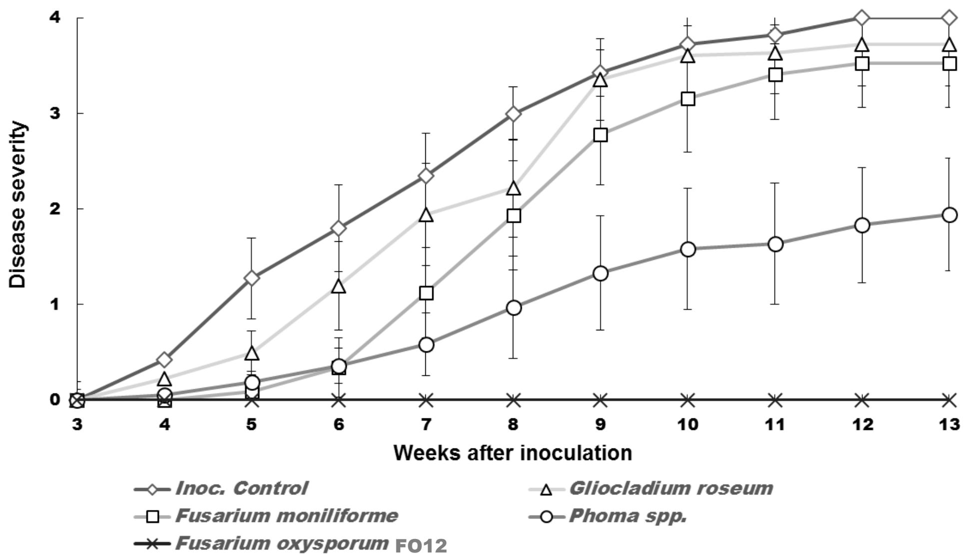

Figure 3. Disease severity progress curves for olive plants inoculated by CMS (20%) of the virulent isolate (V024) of *Verticillium dahliae* and treated with four biological control agents. The disease severity was rated weekly using a 0–4 scale. Dots and vertical bars along the curve are the mean and standard error of the mean for each evaluation

Table 3. Disease parameters of olive plants inoculated with a highly virulent isolate of *Verticillium dahlia*e (V024) by the CMS method and treated with different antagonistic fungi

Treatment	Incidence (%)[a]	Mortality (%)[a]	Disease severity[b]	RAUDPC[c]
Inoculated control	100 ± 0.0a	100 ± 0.0a	4.0 ± 0.00a[d]	93.8 ± 11.11a[d]
Sterile control	0 ± 0.0b	0 ± 0.0d	0.0 ± 0.00c	0.0 ± 0.00c
Gliocladium roseum	100 ± 0.0a	80 ± 12.6b	3.4 ± 0.43a	96.4 ± 17.15a
Fusarium moniliforme	100 ± 0.0a	90 ± 9.5b	3.5 ± 0.46a	82.0 ± 15.22ab
Phoma sp.	70 ± 14.5b	30 ± 14.5c	2.1 ± 0.62b	44.1 ± 18.39bc
Fusarium oxysporum	0 ± 0.0c	0 ± 0.0d	0.0 ± 0.00c	0.0 ± 0.00c

[a]Percentage of plants ± standard error (SE) showing symptoms or killed by *V. dahliae* 12 weeks after inoculation (n = 10). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at P = 0.05.

[b]Final disease severity 12 weeks after inoculation ± SE.

[c]Mean value for the relative area under the disease progress curve developed over the assessment period ± SE.

[d]In each column, mean values followed by the same letter were not significantly different according to Fisher protected LSD test (P = 0.05).

DISCUSSION

Verticillium wilt is the most important olive disease in the Mediterranean region. Control of VW in olive trees must be based on the integration of different control measures due to the inefficiency of these measures when individually applied (Klosterman et al., 2009; López-Escudero and Mercado-Blanco, 2011). The use of biological agents or treatments is the control measure that has aroused the most interest for VW of olives (Mercado-Blanco et al., 2004; Martos-Moreno et al., 2006; Porras-Soriano et al., 2006; Mercado-Blanco and Bakker, 2007; Müller and Berg, 2008; Jiménez Díaz et al., 2009; Markakis et al., 2015), although a general evaluation of potential BCAs has not been made. During the selection process of potential BCAs, it is necessary to conduct a massive screening of different microorganisms or other biological treatments. For this reason, it is essential to develop a fast and efficient inoculation method for olive plants that allows the evaluation of these BCAs against *V. dahliae*. Here, we compared different inoculation methods (naturally infested soil, soil infested with MS, soil infested with CMS medium colonized by the fungus, root dipping in a

conidial suspension, root impregnation with a paste of PDA+fungus) to evaluate the efficiency of six potential BCAs, and the most effective method used CMS at 20% as the inoculum source.

In experiments conducted for our group, 100% of the final incidence of VW in the susceptible cv. Picual were reached using 1.000 l microplots and 10 MS/g of inoculum (Lopez-Escudero and Blanco-Lopez, 2007; Pérez-Rodríguez et al., 2015). Conversely, we have never been successful using plastic or clay pots from 0.7 to 20 l. In our experiment using 0.7 l pot, all olive plants that were planted in the soil infested with 111.9 MS/g showed slight symptoms of VW, while none of the plants planted in the remaining soils (< 28 MS/g) showed disease symptoms. These soils, however, can be considered conductive because they were collected from olive orchards and cotton fields with a high incidence of disease. Our results using potted plants contrast with the cv. Picual responses to the pathogen under field conditions, where 0.8-4 MS/g is enough to cause a severe epidemic during the first years after planting (Trapero et al., 2013b; Roca et al., 2015). The great variability in the response of potted olive plants to the pathogen using naturally infested soil may be due to the architecture or development of confined root systems or changes in the chemical stimulants of the root exudates of olive plants. Conversely, the cotton plants showed homogeneous severity of Verticillium wilt symptoms in the four soils used. Similar results were obtained by other authors working with herbaceous crops (Xiao and Subbarao, 1998).

Unlike the case of naturally infested soil, most olive plants planted on mixtures of infested CMS and sterile soil showed symptoms of disease when CMS medium was used as the inoculum source of *V. dahliae*, even at the lowest CMS dose (5%). Moreover, the severity of symptoms in olive plants increased according to monomolecular model with increasing the CMS dose. Similar results have been described in other host plants of *V. dahliae* (Pullman and DeVay, 1982; Paplomatas et al., 1992). When we compared different inoculation methods, olive plants that were inoculated using the PDA paste method were more severely

affected than those inoculated using the root dip. Overall, the root dip method is considered a successful method to evaluate the resistance/susceptibility of olive cultivars (López-Escudero et al., 2004; Gordon et al., 2005; Trapero et al., 2013a), but there are some limitations to the screening for BCAs. For example, olive plants are frequently treated with BCAs by root dip, after which they are also inoculated with *V. dahliae* by root dip; this double inoculation is very aggressive for the plants and it can cause moderate plant mortality. Furthermore, in the root dip method, the root system is exposed to the pathogen at the time of inoculation, and the plant is rapidly colonized by the pathogen. This fact limits the efficacy of the BCAs due to the lack of access to the vascular system of the plant. In addition, the root dip method is far from the natural conditions in which the pathogen and the BCAs interact in the soil and rhizosphere (Hiemstra and Harris, 1998; Pegg and Brady, 2002). In addition, inoculation methods, in which *V. dahliae* MS are artificially produced in culture media have an important limitation because frequently the reference strains of the pathogen reduce their capacity to produce MS in culture after prolonged storage (Hu et al., 2013).

The above constraints are overcome by the use of the CMS method at 5 and 20%, which provides highly consistent results in inoculated olive plants that use the two isolates of the pathogen. In addition to olive plants, the CMS method has been successful with cotton, eggplant and watermelon plants, even with low doses of CMS (Varo and Trapero, *unpublished data*). The main limitation of this method was that the inoculum density of *V. dahliae* decreased over time. This observation is consistent with previous reports using other inoculum sources of the pathogen (Blok et al., 2000; López-Escudero and Blanco-López, 2007). Even so, 20% CMS was more effective than the PDA paste method in testing the effect of the two mixtures of beneficial soil microorganisms, mainly bacteria and yeast (Higa and Ke, 2001). For this reason, we used the CMS method to evaluate the biological control capacity of four antagonistic fungal species. Among them, the non-pathogenic *F. oxysporum* strain F012 showed total control of the disease in our conditions. The biological control capacity of species of the *Fusarium* genus has been well known since the 1980s (Alabouvette and Couteaudier, 1992;

Malandraki et al., 2008; Veloso and Díaz, 2012). The *Phoma* sp. strain 02 also displayed effectiveness against the pathogen but on a more limited scale; this genus had not been reported as a non-pathogenic biological control agent in previous studies.

Based on these results, the method of inoculation of CMS at 20% is of great interest to the assessment of BCAs potentially effective against VW in olive plants. Currently, this method is being used to evaluate more than 200 biological treatments for their efficacy against isolates of *V. dahliae* prevalent in southern Spain before their final evaluation under field conditions (Varo et al., 2015).

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4

Main study:
Microorganism screening

4

Selection and evaluation of microorganisms for biocontrol of *Verticillium dahliae* in olive

ABSTRACT

Aims: Identify potential biological control agents against Verticillium wilt in olive through a mass screening approach.

Method and Results: A total of 47 strains and nine mixtures of microorganisms were evaluated against *Verticillium dahliae* in a three stage screening: i) *in vitro*, by the effect on the mycelial growth and spore germination of the pathogen; ii) in natural infested soil, by the effect on the reduction of microsclerotia of the pathogen; iii) *in planta*, by the effect on the infection of olive plants under controlled conditions. Various fungal and bacterial strains and mixtures inhibited the pathogen and showed consistent biocontrol activity against Verticillium wilt of olive.

Conclusion: The screening has resulted in promising fungi and bacteria strains with antagonistic activity against Verticillium, such as two non-pathogenic *Fusarium oxysporum*, one *Phoma* sp., one *Pseudomonas fluorescens* and two mixtures of microorganisms that may possess multiple modes of action.

Significance and Impact of the Study: This study provides a practical basis for the potential use of selected strains as biocontrol agents for the protection of olive plants against *V. dahliae* infection. In addition, our study presented an effective method to evaluate antagonistic microorganisms of *V. dahliae* in olive.

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INTRODUCTION

Verticillium wilt of olive (*Olea europaea* L.) (VWO), caused by the soil-borne fungus *Verticillium dahliae* Kleb., is a destructive disease widely distributed throughout all regions of the world where olive trees are grown. The formation of resting structures known as microsclerotia (MS) is a critical factor in the survival, dissemination, and epidemiology of the Verticillium wilt pathogen. This disease is more difficult to control with the presence of two groups of *V. dahliae* isolates that have been identified on cotton and olive: highly virulent defoliating (D) and moderately virulent non-defoliating (ND) pathotypes (Rodríguez-Jurado, 1993). Recently the D- and ND-pathotypes have been characterized as race 2 and 1, respectively (Hu et al., 2015).

The lack of efficacy of chemical compounds to control soil-borne vascular pathogens, together with the current environmental awareness in managing diseases, have intensified the search for alternative methods to control Verticillium wilt diseases (Pegg and Brady, 2002). An interesting approach to control VWO within an integrated control framework (López-Escudero and Mercado-Blanco, 2011) is the use of biological control agents (BCAs). These microorganisms directly or indirectly interact with plants by protecting them from the deleterious effect of the pathogens, competing for nutrients and colonization space, inducing systemic resistance (ISR) or promoting plant growth through the production of phytohormones and the delivery of nutrients (Whipps, 2001). However, the added difficulty of the nature of woody species host confined to areas with a Mediterranean climate, significantly limits such research.

Nevertheless, a few promising examples have been reported as effective BCAs against VWO. To date, endophytic bacteria remain the most studied in olive, such as *Serratia plymuthica* strain HRO-C48 (Müller et al., 2008), and the genera *Bacillus*, *Paenibacillus*, and *Pseudomonas* (Mercado-Blanco et al., 2004). Different *Pseudomonas* spp. strains have been identified and evaluated in olive plants (Triki et al., 2012); one of the most promising isolates is *P. fluorescens* PICF7 (Gómez-Lama-Cabanás et al., 2014). This strain displayed effective

control of VWO in different nursery-produced olive cultivars under controlled (growth-chamber) or semi-controlled (greenhouse) growth conditions (Prieto et al., 2009). Among the fungi, *Trichoderma* spp. are widely distributed in many ecological niches and have also been studied as BCAs for the control of VWO. Currently, the only commercial bioformulation available for the control of VWO in Spain is a mixture of two species of *Trichoderma*, *T. asperellum* and *T. gamsii,* but this mixture is only approved for the treatment of olive plants in the nursery, before final planting in the field (Jiménez Díaz et al., 2009). Other studies have shown the potential of combining *Trichoderma* strains with soil solarization (Lima et al., 2007). However, inoculation with *Glomus intraradices, G. mosseae* and *G. claroideum* was not able to suppress VWO (Porras-Soriano et al., 2006).

In recent years, non-pathogenic strains of *Fusarium oxysporum* have also been identified as potential BCAs for Verticillium wilt diseases (Angelopoulou et al., 2014; Veloso et al., 2015), but there were no previous reports about the suppressive effect of non-pathogenic *F. oxysporum* strains on VWO. Previous infection and vascular colonization of olive plants by a ND isolate of *V. dahliae* (artificial inoculation by root dipping) was shown to protect the plants to some extent against further infections by a D isolate (Martos-Moreno, 2003). Most of these previous studies have specifically targeted the disease suppression by one determinate strain or broadly by determinate genera. Therefore, it has also been noted that combinations of BCAs have to be compatible to establish better and more consistent disease suppression (Raupach and Kloepper, 1998).

Because there is very limited knowledge regarding the biological control of VWO, a mass screening of microorganisms to find potential biological control agents is necessary. However, this is a typical time- and labour-consuming process and has not been done. The aim of this study was to compare the effects on the mycelial growth of the pathogen, the viability of individual MS and the infection in olive plants of many fungal and bacterial strains or mixtures of them to select potential BCAs against the VWO. Our screening was based on the biological activity of the selected strains *in vivo* rather than *in vitro* because there

is no clear relationship between the antagonism *in vitro* and *in vivo* (Lemanceau and Alabouvette, 1991).

MATERIALS AND METHODS

Pathogen isolates

Two *V. dahliae* isolates were used in this study, the mild-virulent strain of the ND pathotype namely V004 (Rodríguez-Jurado, 1993) and the D pathotype V024, characterized as highly virulent (Varo et al., 2015). Both isolates were collected from the Andalusia region in southern Spain and belonging to the fungal collection of the Department of Agronomy at the University of Córdoba. The isolates were maintained on potato dextrose agar (PDA) slants at 5ºC. Plates of 6-d-old single spore culture incubated on PDA at 24ºC in the dark were used as the pathogen inoculum source.

Potential biocontrol agents

The selected fungi and bacteria used in this study are listed in Table 1. All potential BCAs were isolated from plant tissue or soil based on the methods described by Dhingra and Sinclair (1995). The identification of the selected fungal and bacterial strains was made by PCR and morphological characteristics.

All bacterial cultures were cryopreserved with 30% glycerol at 80ºC. The bacterial inoculum was prepared from the colonies grown on King's B agar (KBA) (Dhingra and Sinclair, 1995) plates at 25ºC for 48 h and scraped from the medium with a sterile glass rod. The densities of each strain was adjusted to 10^8 cel ml^{-1} (Mercado-Blanco et al., 2004). When an aqueous inoculum was required, crude bacterial culture filtrates of each strain were used to inoculate a 250 ml flask containing 100 ml of sterile KBA before incubation in an orbital shaker (2.5 *g*, 26ºC and 12 h light:dark) for three days.

The potential biocontrol fungal strains were prepared from single-spore stock cultures maintained on PDA slants at 4°C. The fungal isolates were grown in PDA, and conidia of each isolate were collected from four-d-old cultures and adjusted to 10^5 conidia/ml. When an aqueous inoculum was required, crude fungal

cultures of each strain were used to inoculate a 250 ml flask containing 100 ml of sterile potato dextrose broth (PDB) before incubation in an orbital shaker (2.5 *g*, 25ºC and 12 h light:dark) for five days.

Plant material

Five-month-old olive rooted cuttings of susceptible cv. Picual were used in the five experiments of the current study. Olive plants cv. Picual were shown to be very susceptible to the D *V. dahliae* pathotype and moderately susceptible to the ND pathotype in previous studies (López-Escudero et al., 2004). The plants were maintained in a growth chamber for one month before inoculation to force active growth.

Dual culture assay

The potential BCAs were tested for antagonism against V117 isolate of *V. dahliae*, using the dual culture technique in PDA petri dishes. Pathogen mycelial plugs of seven-mm-diameter were taken from the edge of four-d-old colonies of each isolate grown on PDA medium and were placed at the periphery of culture plates. A fungal mycelial plug or a five µl bacterial suspension drop were placed at four cm to the *V. dahliae* plug and were incubated for seven days at 25ºC in the dark. As negative controls, three plates were inoculated only with the *V. dahliae* isolate. Each combination of pathogen/antagonist was replicated three times in a randomized complete block design. The experiment was conducted twice. The radius of the *V. dahliae* colony was recorded every two days for 10 days. The percentage inhibition of mycelial growth was calculated using the following formula:

$$Inhibition\ (\%) = \left[\frac{(R-r)}{R} x\ 100\right]$$

where, r is the radius of the *V. dahliae* opposite the BCA colony and, R is the maximum radius of the *V. dahliae* colony away from the BCA colony.

Effect of BCAs on microsclerotia viability

This assay was carried out to determine the effect of the potential BCAs to suppress MS of *V. dahliae* in a naturally infested soil. The soil contained 110 CFU g^{-1} of the pathogen and was collected from a cotton field in southern Spain. The soil was air-dried at room temperature and sifted through a 0.8 mm sieve to remove organic debris and large particles. Sterile 100 ml containers were drilled to facilitate the percolation of the liquids, and filled with 60 g of the infested soil. The aqueous inoculum of each BCA was added to the containers by watering until field capacity of the soil. A total of 47 strains of BCAs and nine mixtures of microorganisms were evaluated. In addition, a naturally infested soil treated with water was included as control treatment. The containers (three per treatment) were covered and incubated for 72 h at 25ºC in the dark.

After 72 h, the soil from each container was air-dried and the *V. dahliae* inoculum density was estimated by wet sieving (Huisman and Ashworth, 1974). Each sample was suspended in 100 ml of distilled water, shaken at 11.4 *g* for one h, and filtered through 150- and 35-µm sieves. The residue retained on the 35-µm sieve was recovered in 100 ml of distilled water, and then, one ml of the suspension from each treatment was plated on modified sodium polypectate agar medium (MSPA) (López-Escudero and Blanco-López, 2007) using 10 replicated plates per each soil sample. Plates were incubated at 24ºC in the dark for 14 d, thereafter soil residues were removed with tap water and *V. dahliae* colonies were counted. The inoculum density in each soil sample was estimated from the number of *V. dahliae* colonies and expressed as the number of MS or propagules per gram of air-dried soil (ppg) (López-Escudero et al., 2003). A randomized complete block design was used and the experiment was conducted twice.

Suppression of Verticillium wilt of olive by BCAs

All the BCA isolates that produced more than 75% inhibition of *V. dahliae* mycelial growth in the *in vitro* assay or showed effective inhibition of the pathogen MS (beyond 90% inhibition) were selected for the *in planta* evaluation in a growth chamber under controlled conditions. In addition, selected BCAs that

previously showed biological control activity towards several soil-borne pathogens by inducing systemic resistance were tested in these experiments. Olive plants of cv. Picual were planted into 0.8 L pots (one plant per pot), containing sterile peat moss with 20% (weight / weight) of the cornmeal sand mixture (CMS) infested with the pathogen (Varo et al., 2016a). Inoculated and control plants were incubated at 60% RH with a 14 h photoperiod of fluorescent light adjusted to 216 µmol $m^{-2}s^{-1}$ and 22ºC.

Two experiments (I and II) were conducted to determine the ability of the selected BCAs isolates to suppress Verticillium wilt of olive caused by the D *V. dahliae* pathotype. The inoculum density of the pathogen was determined at the beginning of both experiments using the wet sieving technique and the MSPA medium.

For experiment I, the olive plants were treated by watering with the BCA inoculum (12 strains and six mixtures of microorganisms), at a 20%-dose until field capacity of soil. Two control treatments were used: transplanted plants in sterile peat moss with the pathogen but in absence of the BCAs and transplanted plants in absence of the pathogen and BCAs. The experiment was carried out twice in a randomized complete block design, each block comprising ten pots.

Experiment II was conducted to identify the potential systemic inducted resistance of four aerial fungal strains. Plants were treated twice (14 days before and the same day of the inoculation with *V. dahliae*) by spraying over the aerial part of the plants with an aqueous inoculum suspension of the BCAs. Two control treatments were used: transplanted plants in sterile peat moss with the pathogen but in absence of the BCAs and transplanted plants in absence of the pathogen and BCAs. The experiment was conducted twice in a randomized complete block design, each block comprising ten pots.

Disease assessment

Disease severity on inoculated plants was weakly determinate by assessing visible symptoms for 14 weeks. Each olive tree was assessed for disease severity

with a 0 to 16 rating scale. The scale estimated percentage of affected tissue using four main categories or quarters (≤25, 26-50, 51-75, and 76-100%) with four values per each 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: $Y = 6.25X - 3.125$. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (López-Escudero et al., 2004).

Data analyses

An analysis of variance (ANOVA) of the inoculum density, the final disease severity and the RAUDPC were performed for experiments because the mean values to each parameter met the assumptions of normality and homogeneity of variances for this analysis. When the ANOVA showed significant differences ($P < 0.05$) among BCAs treatments, mean values were compared using the Fisher's protected LSD test at $P = 0.05$. Both incidence and mortality were analysed by multiple comparisons for proportions test ($P = 0.05$) (Zar, 1999), which considered the observed and expected frequencies of symptomatic and dead plants, respectively. Statistical analysis of the data was conducted using Statistix 10.0 (Analytical Software, Tallahassee, USA).

RESULTS

Effect of the BCA on mycelial growth

The different BCA isolates varied in their ability to inhibit the mycelial growth of *V. dahliae*. Antagonism could be observed in two ways: (i) by the detection of clear inhibition zones up to 20 mm without Verticillium mycelium or (ii) by hyperparasitism resulting in the destruction and discoloration of the Verticillium mycelium and MS. Both phenomena were found for a large proportion of the BCA isolates. The effect (i) was detected by *F. oxysporum* strains FO02, FO03, FO04,

FO12, *Mucor* sp strain MU01, *Rhizopus* sp. strains RZ01, RZ02, and *Trichoderma* sp. strains TV01, Bioten® and THS. These strains overgrew the *V. dahliae* colony and covered the plate completely, preventing the growth of the pathogen. In contrast, VIN01, VIN02 (mixtures of microorganism from vineyard), and MO1 (fermented mixture of microorganisms) showed clear inhibition zones showed the effect (ii). The remainder of the isolates (14 strains) did not exhibit inhibitory ability over the growth of the pathogen (Table 1).

Effect of the BCAs on microsclerotia viability.

The effect of the BCA extracts (47 strains and nine mixtures of microorganisms) on the survival of MS of *V. dahliae* showed significant differences among treatments ($P < 0.0001$) (Fig. 1). The *F. oxysporum* strains FO02, FO03, FO04, FO12, FO13 and the *G. roseum* strain GR02 isolates, the MO1 and MO2 (two different conditions of fermented mixtures of microorganisms), and commercial product Bioten® completely inhibited the *V. dahliae* propagules. The *Phoma* sp. isolate PH01 reduced the viability by 92%. However, between 60% and 30% of MS of *V. dahliae* remained viable after treated with VIN01 and VIN02, the TA01, BT345, BT463, selected *B. subtilis* and *Mucor* sp. isolates. Among the remaining isolates (22 strains and 2 mixture of microorganisms), which failed to suppress MS viability with less than 40%, the selected strains of *Aureobasidium* sp. and *Pseudomonas* sp. were found.

Table 1. Fungi and bacteria strains or mixtures selected for evaluation against *Verticillium dahliae* and their effect on the mycelial growth of the pathogen in dual cultures.

ISOLATE* NO.	SPECIES	ORIGIN	INHIBITION (%)†	ISOLATE* NO.	SPECIES	ORIGIN	INHIBITION (%)†
AF04	*Aureobasidium foliicola*	*Quercus suber* (Leaf)	75.1	**PH02**	*Phoma* sp.	*Olea europaea* cv. Picual (Leaf)	45.0
AP06	*Aureobasidium pullulans*	*Olea europaea* cv. Picual (Leaf)	57.8	**PS01**	*Pseudomonas* sp.	Fungikiller® Bio-Iliberis	7.5
AP07	*Aureobasidium pullulans*	*Olea europaea* cv. Arbequina (Leaf)	56.0	**PS02**	*Pseudomonas* sp.	*Olea europaea* cv. Picual (Rhizosphere)	8.9
AP08	*Aureobasidium pullulans*	*Olea europaea* cv. Picual (Leaf)	55.9	**PFF**	*Pseudomonas fluorescens*	*O. europaea* cv. Picual (Rhizosp)	54.8
AP09	*Aureobasidium pullulans*	*Olea europaea* cv. Picual (Leaf)	58.3	**PICF4**‡	*Pseudomonas fluorescens*	*O. europaea* cv. Picual (Rhizosp)	53.5
BA01	*Bacillus amyloliquefaciens*	*Olea europaea* cv. Picual (Rhizosphere)	18.1	**PICP2**‡	*Pseudomonas putida*	*O. europaea* cv. Picual (Rhizosp)	62.0
BPS	*Bacillus pumillus*	*O. europaea* cv. Picual (Rhizosp)	34.0	**PO1B**	*Pythium oligandrum*	*O. europaea* cv. Picual (Rhizosp)	71.0
BSS	*Bacillus subtilis*	Serenade ® BASF	31.0	**PO06**	*Pythium oligandrum*	*O. europaea* cv. Picual (Rhizosp)	75.3
BS10	*Bacillus subtilis*	*O. europaea* cv. Picual (Rhizosp)	27.8	**RZ01**	*Rhizopus* sp.	Soil	96.6
BS165	*Bacillus subtilis*	*O. europaea* cv. Picual (Rhizosp)	22.6	**RZ02**	*Rhizopus* sp.	Composted grape marc	96.2
BT345	*Bacillus thurigiensis*	*O. europaea* cv. Picual (Rhizosp)	31.2	**MOST01**	Must: *Saccharomyces cerevisiae, Kluyveromyces fragilis, Torulaspora* sp. and	Grape must intended for wine elaboration	96.4

					Zymomonas mobilis		
BT463	*Bacillus thurigiensis*	*O. europaea* cv. Picual (Rhizosp)	31.6	**MOST02**	Fermented must: *Saccharomyces cerevisiae, Kluyveromyces fragilis, Torulaspora* sp. and *Zymomonas mobilis*	Fermented grape must intended for wine elaboration	96.0
CH04	*Coniothyrium minitans.*	*O. europaea* cv. Picual (Rhizosp)	53.5	**TOE1**	*Trichoderma* sp.	*O. europaea* cv. Picual (Rhizosp)	96.2
CMC	*Coniothyrium minitans*	Constans® Bayer	53.8	**Bioten®**	*Trichoderma asperellum + T. gamsii*	Bioten® Isagro	94.3
FM02	*Fusarium moniliforme*	*Olea europaea* cv. Picual (Leaf)	34.1	**TA01**	*Trichoderma atroviride*	*O. europaea* cv. Picual (Rhizosp)	76.5
FO02	*Fusarium oxysporum*	*O. europaea* cv. Picual (Rhizosp)	82.3	**THS**	*Trichoderma harzianum + T. asperellum*	Tusal® Koppert	92.3
FO03	*Fusarium oxysporum*	*O. europaea* cv. Picual (Rhizosp)	91.0	**TV01**	*Trichoderma virens*	Soil	92.0
FO04	*Fusarium oxysporum*	Olives fermentation liquid	90.2	**MO1**	Microorganism suspension (*Rhodopseudomonas palustris, Rhodobacter sphacrodes, Lactobacill-us plantarum, L. casei, Saccharomyces* sp., and *Streptomyces* sp.)	EM®	95.2
FO12	*Fusarium oxysporum*	*Quercus suber* (Cork)	90.5	**MO2**	Microorganism suspension (*Rhodopseudomonas palustris, Rhodobacter*	EM® under continuous fermentation	36.4

					sphacrodes, Lactobacillus plantarum, L. casei, Streptococcus lactis, Saccharomyces sp. and *Streptomyces* sp.)		
FO13	*Fusarium oxysporum*	*Olea europaea* cv. Arbequina (Leaf)	51.6	**MO21**	Unidentified bacteria 1	Unknow	7.8
GR02	*Gliocladium roseum*	*O. europaea* cv. Picual (Rhizosp)	48.9	**MO22**	Unidentified bacteria 2	Unknow	7.3
GIM	*Glomus intraradices*	*O. europaea* cv. Picual (Rhizosp)	52.5	**MO23**	Unidentified bacteria 3	Unknow	6.5
LBF	*Lactobacillus* sp.	Fuego® Biagro	26.1	**MO24**	Unidentified bacteria 4	Unknow	6.4
MU01	*Mucor* sp.	Soil	93.0	**MO25**	Unidentified bacteria 6	Unknow	10.3
MU02	*Mucor* sp.	Soil	90.1	**V025**	*Verticillium tricorpus*	Soil	49.7
PEG01	*Paenibacillus* sp.	*Olea europaea* cv. Wardan (Rhizosphere)	95.9	**VIN01**	Bacterial suspension (species of *Acetobacter, Gluconacetobacter, Acidomonas, Swaminathania, Neoasia, Granulibacter* and *Saccharibacter*)	Vinegar	60.3
PS02	*Paenibacillus* sp.	Soil	18.1	**VIN02**	*Acetobacter aceti*	Vinegar	63.4
PH01	*Phoma* sp.	*Olea europaea* cv. Picual (Leaf)	49.6				

*Fungal Culture Collection of the Department of Agronomy, University of Córdoba, Spain.

†Significant differences between any treatment means are given by a critical value for means comparison of 13.5X, according to the Tukey's HSD test at P = 0.05

‡These strains were characterized and supplied by Dr. Jesús Mercado-Blanco from CSIC-IAS.

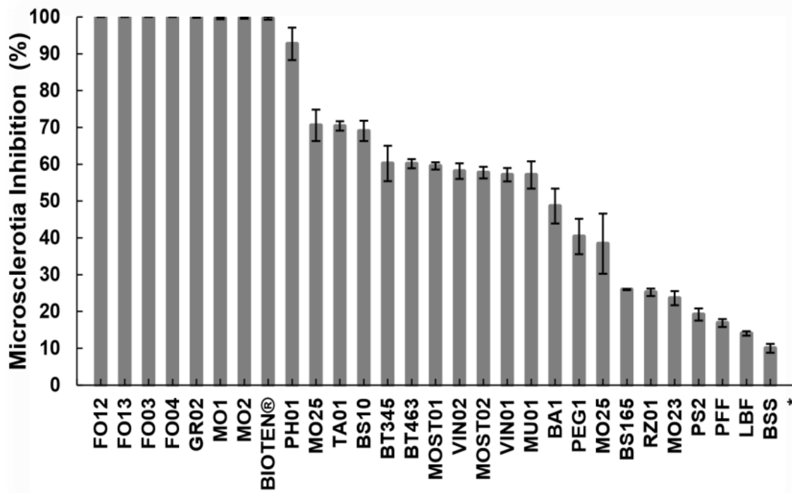

Figure 1. Inhibitory effect (%) and standard error of the potential BCAs on the viability of microsclerotia of *Verticillium dahliae* in a naturally infested soil (*the 29 remainder strains showed no effect on *V. dahliae* microsclerotia). Error bars represent standard error.

Effect of the BCAs on Verticillium wilt development in olive plants

The initial artificial infested substrate had an inoculum density of 750 CFU g-1 soil. Inoculated plants grown in the V024 *V. dahliae* infested soil showed symptoms characteristic of those caused by the D pathotype in olive cv. Picual (Figure 2). In those plants, the first symptoms developed by 28 days after inoculation reaching a disease incidence of 100% (Table 2). FO12 had the best protection effect (0% disease incidence), and treatment with FO04, MO1, PF04, FO12+PF04, PH01 and VIN02 reduced significantly the final disease incidence (1.9, 19.4, 26.9, 56.9, 11.9 and 0.95%, respectively) and the RAUDPC (4.1, 19.1, 24.2, 16.1, 47.0 and 18.6%, respectively) compared to the control ($P< 0.0001$), though to a lesser extent than reduction by isolate FO12. Regarding the final incidence and mortality of plants Bo165, FO47, GR02, MO2 and PF07 did not show significant differences compared with the control; the remainder of the BCAs showed intermediate results.

Table 2. Disease related parameters for olive plants growing in soil artificially infested with the defoliating isolate of *Verticillium dahliae* and treated by irrigation with different BCAs.

BCA TREATMENT	**INCIDENCE (%)***	**MORTALITY (%)***	**DISEASE SEVERITY†**	**RAUDPC†**
Sterile control	0 E	0 E	0 E	0 F
Inoc. control	100 A	100 A	100 A	100 ± 0.0 ABC
AP06	78 B	56 C	89.4 ± 8.8 AB	97.8 ± 2.2 ABC
BIOTEN®	78 B	56 C	56.9 ± 15.0 BC	59.9 ± 15.6 CDE
BS165	100 A	80 B	79.4 ± 17.5 ABC	118.7 ± 1.48 AB
BT345	89 B	67 BC	69.4 ± 14.0 ABC	92.3 ± 7.7 ABCD
FM02	80 B	70 BC	84.4 ± 11.7 AB	87.4 ± 12.2 ABCD
FO03	60 C	40C	51.9 ± 22.1 BCD	66.2 ± 11.7 BCDE
FO04	14 D	0 E	1.9 ± 4.9 E	4.1 ± 3.8 F
FO12	0 E	0 E	0 E	0 F
FO47	100 A	60 BC	61.9 ± 14.4 BC	79.1±17.7 BCD
GR02	100 A	89 B	81.9 ± 10.9 ABC	102.7 ± 17.2 ABC
MO1	30 D	10 D	19.4 ± 12.7 DE	19.1 ± 10.9 EF
MO2	100 A	40 C	81.9 ± 8.7 ABC	86.4 ± 13.3 ABCD
PICF04	30 D	20 D	26.9 ± 14.5 DE	24.2 ± 11.7 EF
PICP02	90 B	70 BC	81.9 ± 9.8 ABC	86.2 ± 14.5 ABCD
FO12 + PIC04	22 D	11 D	11.9 ± 12.7 DE	16.1 ± 13.3 EF
PH01	67 BC	44 C	49.4 ± 15.3 CD	47.0 ± 18.4 DEF
VIN01	88 B	62 BC	71.9 ± 14.4 ABC	125.3 ± 23.9 A
VIN02	11 D	0 E	0.95 ± 1.7 E	18.6 ± 10.9 EF

*Percentage of plants ± standard error (SE) showing symptoms or killed by *V. dahliae* 12 weeks after inoculation (n = 20). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at P = 0.05.

†Final disease severity ± SE 14 weeks after inoculation based on scale of 0 to 16 (0 = no lesions, 16 = 94-100% of affected tissue) and relative area under the disease progress curve (RAUDPC) ± SE developed over the assessment period. In each column, mean values followed by a common letter are not significantly different according to Fisher's protected LSD test at P = 0.05.

In experiment II, prior treatments with the BCAs showed significant differences (*P*< 0.001). In control plants grown in soil infested with the pathogen, the first symptoms developed by 32 d after inoculation with *V. dahliae*, and the disease developed to reach a final incidence of 100% (Table 3). In plants treated with aerial fungi the first symptoms appeared 39 d after the inoculation in the AP06 treatment and 46 d to the rest of treatments. The treatment with PH01 reduced the disease severity compared to with the control (88.1% of final disease severity and 85.6% of RAUDPC). Also, The FO12 and FM02 showed an effective control reaching the reduction of disease severity by 65.6 and 80.6%, respectively.

Table 3. Disease related parameters for olive plants growing in soil artificially infested with the defoliating isolate of *Verticillium dahliae* and sprayed with different BCAs to induce resistance.

BCA TREATMENT	**INCIDENCE (%)***	**MORTALITY (%)***	**DISEASE SEVERITY**†	**RAUDPC**†
Sterile control	0 E	0 D	0 C	0 B
Inoc. control	100 A	100 A	100 A	93.8 ± 11.1 A
AP06	100 A	60 B	76.9 ± 9.6 A	88.2 ± 17.4 A
FM02	50 C	0 D	19.4 ± 9.7 B	26.4 ± 13.0 B
FO12	50 C	20 C	34.4 ± 13.1 B	33.7 ± 14.5 B
PH01	40 CD	0 D	11.9 ± 6.4 B	8.2 ± 3.9 B

*Percentage of plants ± standard error (SE) showing symptoms or killed by *V. dahliae* 12 weeks after inoculation (*n* = 20). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at *P* = 0.05.

†Final disease severity ± SE 14 weeks after inoculation based on a scale of 0 to 16 (0 = no lesions, 16 = 94-100% of affected tissue) and relative area under the disease progress curve (RAUDPC) ± SE developed over the assessment period. In each column, mean values followed by a common letter are not significantly different according to Fisher's protected LSD test at *P* = 0.05.

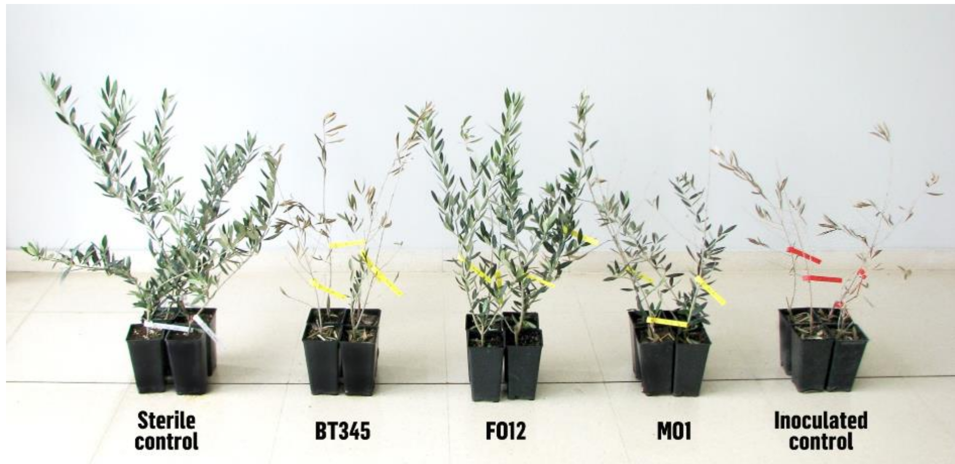

Figure 2. Differential response of susceptible olive plants of cv. Picual at 14 weeks after inoculation with *Verticillium dahliae* and different biological control agents. From left to right: Sterile control, plants treated with water; plants treated with *Bacillus thuringiensis* BT345 strain; FO12 plants treated with non-pathogenic *Fusarium oxysporum* FO12 strain; MO1 plants treated with mixture of microorganisms MO1 and Inoculated control in absence of BCAs.

DISCUSSION

The first step to achieve effective biological control of Verticillium wilt of olive is to find an appropriate source of potential BCAs. This study presents the results of a mass screening designed to detect strains of bacteria, fungi, and mixtures of bacterial and fungal species with significant antagonistic activity to *V. dahliae* and to evaluate their potential as biocontrol agents by studying their effect on mycelial growth, MS viability and infection of olive plants in controlled conditions. The approach followed was to carry out the experiments using a woody plant such as the olive instead of a model plant. This is the first report that identified a broad spectrum of antagonistic microorganisms against the VWO and used a comparison of the suppression capacity of several BCAs to control Verticillium wilt in olive.

Based on dual culture assay, our results suggested that 33 (60%) of the BCAs reached an inhibition greater than 50% of the mycelial growth of *V.*

dahliae and the that the effect of BCAs on the viability of MS of *V. dahliae* showed significant differences with the inoculated control in 29 (52%) treatments. As a result, 12 strains and six mixtures of microorganisms were selected for experiments with olive plants.

The high proportion of effective *in vitro* BCA treatments is not uncommon (Zheng et al., 2011), however the tested BCAs were also selected based on their effectiveness against other pathogens. In this study, the selection criterion chosen was BCA efficacy against VWO, regardless of the mechanism of action. Our findings have shown that several isolates have antagonistic activity against *V. dahliae* when tested *in vitro*, on MS and *in planta*. These BCAs were identified as FO12, FO04, M01 and VIN02 and they significantly reduced symptom expression in olive plants, indicating that this effect is most likely due to a direct effect against the pathogen that an indirect effect of induced resistance in olive plants.

The inhibition rate on mycelial growth of the pathogen varied significantly according to the type of antagonist. The highest inhibition rate was recorded using four *F. oxysporum*, two *Mucor* spp., two *Rhizopus* spp., and four *Trichoderma* spp. strains. This experiment also showed that there are two types of mechanisms used by antagonistic fungal isolates, one in which the entire plate on PDA media is invaded, such as with the fungi mentioned above, and the other in which *V. dahliae* growth is weakly inhibited (e.g. microorganism mixtures VIN 01, VIN02 and MO1).

Reduction in *Verticillium* MS germination and formation of secondary MS may result in a lower Verticillium infection pressure in the field and in a reduced survival of *Verticillium* MS in soil. As a result, 39 strains or mixture of strains has not been selected to plant experiments. Among these strains, it can be found strains with proved potential biocontrol, as *Coniothyrium minitans* (Fiume and Fiume, 2005), *Paenobacillus* spp. (Antonopoulos et al. 2008) and *Bacillus* spp. (Li et al., 2008), in other diseases although no control was showed in our study.

However, other possible effect of these BCAs, such as the induction of resistance (Tjamos *et al.*, 2005; Veloso and Díaz, 2012), have not been evaluated in our study.

When BCAs were applied by irrigation, the most effective treatments were two *F. oxysporum* strains (FO04 and FO12) and an unidentified mixture of vinegar yeasts (VIN01), specially highlighting the FO12 strain of *F. oxysporum*. Non-pathogenic *F. oxysporum* strains are well known to protect against pathogenic *F. oxysporum* (Alabouvette *et al.*, 2009), although only a few papers have reported efficacy against other pathogens, as *Pythium ultimum* (Benhamou et al., 2002), *Phytophthora capsici* (Silvar *et al.*, 2009) and *Verticillium dahliae* in eggplant (Pantelides et al., 2009). This is the first report showing the effective control of VWO. The protective strains are usually more effective when they are applied a few days before the inoculation of the pathogen; and the protection is often improved when the strains are associated with rhizobacteria, especially *P. fluorescents* (Lemanceau and Alabouvette, 1991; Saman, 2009). In this report the best protective strain was applied at the same time as the pathogen in an artificial inoculation and showed reduction of the disease close to 100%. It should be noted that we infested the substrates with high inoculum densities of *V. dahliae*, while in the field the pathogen is in low densities (López-Escudero and Blanco-López, 2007). For this reason, all treatments those were effective to protect olive plants against *V. dahliae* in our study, have a great interest to be evaluated in field conditions for the control of VWO.

Although the action mechanisms of BCAs have not been studied, the reduction of Verticillium wilt when leaves were sprayed with several BCAs suggests a possible indirect effect of induction of resistance in olive plants. Of the four BCAs that were sprayed on olive plants, three were effective in reducing Verticillium wilt, highlighting especially a *Phoma* sp. strain (PH01). The FO12 strain showed a resistance-inducing effect in addition to its direct effect against the pathogen already noted.

Despite the number of scientific papers investigating the efficacy of protective strains of *F. oxysporum*, the modes of action of theses strains have not been fully elucidated. Competition is a well-studied phenomenon in the interaction of non-pathogenic *F. oxysporum* isolates with pathogenic Fusaria, but it is unexplored with *V. dahliae*. Previous studies have reported the ability of several non-pathogenic *F. oxysporum* strains to trigger ISR in plants against different pathogenic *formae speciales* of *F. oxysporum* (Fuchs et al., 1997; Mandeel and Baker, 1991). A non-published study with a confocal microscope had showed that the FO12 strain grows as epiphyte, remaining in the root surface of various herbaceous plants (A. Varo, D. Rybakova and G. Berg 2014, *personal communication*).

One of the potential 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 another species of *Fusarium* (Lemanceau and Alabouvette, 1991). Similarly, the selected strain of *Phoma* sp. (PH01) represents a promising biocontrol agent exhibiting potential biocontrol mechanisms because it is applied in the aerial part of olive plant that is an easy and economic method of application.

Contrary to the study of Debode et al. (2007), and according to the common assumption that melanised structures are resistant to microbial attacks (Bell and Wheeler, 1986), the present studies showed that *Pseudomonas* spp. were unable to suppress the viability of *Verticillium* MS *in vitro*. However, the results *in planta* with the PICF4 are promising.

It is likely that most cases of naturally occurring biological control result from mixtures of antagonists, rather than from high populations of a single antagonist. In this study, we demonstrate this fact with the mixture of microorganisms MO1. For example, mixtures of antagonists are considered to account for protection with disease-suppressive soils (Schippers, 1992). Consequently, the application of a mixture of introduced biocontrol agents would

more closely mimic the natural situation and might broaden the spectrum of biocontrol activity and enhance the efficacy and reliability of the control (Duffy and Weller, 1995). A promising treatment using the association of non-pathogenic *F. oxysporum* FO12 with the PICF4 isolate (Mercado-Blanco et al., 2004) was developed as mentioned Lemanceau and Alabouvette (1991).

The *in vivo* trials of this study showed a consistent and significant antagonistic activity against *V. dahliae*. Furthermore, a significant positive correlation was observed between the natural MS *in vitro* and the *in planta* assays. However, application of these microorganisms under field conditions warrants more investigations on their mass production, their formulation, and their delivery methods.

In conclusion, we report for the first time a high diversity of BCAs against VWO. This represents a first step to develop an effective and environmentally friendly biological treatment against VWO in the field.

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5

Main study: Organic amendment screening

5

Identifying potential organic amendments to suppress the Verticillium wilt disease of olive

ABSTRACT

Biological control of plant diseases using soil amendments such us animal manure and composted materials minimize organic waste and has been proposed as an effective strategy in plant disease. In this study, thirty-five organic amendments and sixteen compost mixtures have been assessed against *Verticillium dahliae* by the antagonistic effect on the mycelial growth and spore germination; and in natural infested soil, by the effect on the reduction of microsclerotia of the pathogen. Nine OAs and fifteen compost mixtures produced a consistent *in vitro* inhibition effect against *Verticillium dahliae.* Therefore, their biocontrol potential was assessed by the effect on the infection of olive plants under controlled conditions. The significant reduction in the severity of the symptoms of *Verticillium dahliae* provides a practical basis for the potential use of grape marc compost (100% reduction of the disease) and solid olive oil waste (alperujo) combined with other OAs, such as microorganism mixtures (73% reduction of the disease) or dairy waste (63% reduction of the disease). In addition, we conclude that the mixture of agro-industrial waste with other biological control agents is a promising strategy against Verticillium wilt of olive trees in the Mediterranean basin.

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INTRODUCTION

Agroindustrial processing in olive, grape (*Vitis vinifera* L.), cork (*Quercus suber* L.), and dairy production give rise to large amounts of agro-industrial by-products, such as a semi-solid residue from the extraction of olive oil by the two-phase system which is called "Alperujo" in Spanish, grape marc, cork waste and lactic acid. Spain generates approximately 6 million tonnes of alperujo annually (FAOSTAT, 2015), which means large quantities are produced during a short period of time. These by-products cause serious management problems due to phytotoxicity and the semisolid texture. Consequently, finding appropriate methods for their disposal is an urgent need in some Mediterranean countries (Papasotiriou et al., 2013).

Organic amendments (OAs) can include solid and liquid materials or mixtures of them with a highly diverse composition that are from a wide range of animal and plant origins in agroindustry. They are applied as fertilizers, contributing to reduced agrochemical inputs, thereby minimizing residues originating from farming activity. In addition, their use also contributes to reduce foliar and soil-borne diseases (Trillas et al., 2006).

Verticillium wilt (VW) disease, caused by the widespread soil-borne fungus *Verticillium dahliae,* is one of the most serious worldwide diseases in olive (*Olea europaea* L.), causing severe losses and plant death (López-Escudero & Mercado Blanco, 2011). The pathogen can survive over long periods in soil by producing microsclerotia (MS), which constitute the infective and spreading structures of the fungus. In addition, the severity of the infections, the wide host range, and the ineffective control by chemical compounds constitute the context of this devastating disease. In this respect, difficulty in controlling *V. dahliae* in woody plants, and particularly in olive, is added due to the localization of the pathogen within the vascular system, a site always difficult to reach by chemical or biological treatments (López-Escudero & Mercado-Blanco, 2011).

Nevertheless, biocontrol measures are feasible for olive by enhancing soil suppressiveness to VW, using OAs as an alternative biocontrol approach to the conventional paradigm of plant disease control. This alternative is perceived to be safer and to have a minimal environmental impact. Usually OAs are rich in microorganisms and the use of several microorganisms at the same time to control one or many pathogens is possible rather than the conventional use of one active ingredient or microbial agent (Mehta et al., 2014). An increase in the diversity of the microbial community in the soil should add more competition and more resilience to a pathogen invasion.

Biological control of VW with OAs has been investigated to a lesser extent compared with other vascular wilt diseases (Avilés et al., 2011; Goicoechea, 2009). Most research has been conducted on vegetable crops (Termorshuizen et al., 2006), but the mechanisms of OAs that drive suppression to *V. dahliae* currently are not clear or well-studied (Avilés et al., 2011). In olive, despite the increase in interest in biocontrol of VW diseases with OAs, only two studies have been published. The first of these studies was the first report worldwide for controlling a plant disease caused by *V. dahliae* using AOs, highlighting the importance of this olive disease and the need for control measures. In this study, performed on olive trees under field conditions in California, a remarkable control of VW of olive was obtained by applying dry wood shaving (Wilhelm et al., 1962). The other study showed the effects of olive mill liquid wastes on nursery-grown olive plants (Vitullo et al., 2013).

Several reports have suggested that compost water extracts reduce the severity of foliar diseases, such as the grey mould of strawberries and late blight of the potato, however this occurs with variable efficacy (Yohalem et al., 1994). Compost extracts contain biocontrol agents and unidentified chemical factors, which appear to play a role in their efficacy, although no studies have been published in VW or indeed, in olive. For that reason, evaluation of disease control achieved by compost extracts may be an interesting strategy in olive, because the

protective effects of compost extracts appear to be due, at least in part, by the induction of systemic resistance in the treated plants (Zhang et al., 1998).

The ability of biological control agents (BCAs) isolated from compost to control *V. dahliae* in several crops has been reported (Castaño et al., 2013; Markakis et al., 2016). Introduction of BCAs into soils, either directly by application of microbial antagonist formulations or indirectly by combination with OAs, also has a potential impact on indigenous soil microbial communities (Ruano-Rosa & Mercado-Blanco, 2015). Vitullo et al. (2013) demonstrated effective control of MS and development of VW of olive with olive mill wastes in combination with *Bacillus amyloliquefaciens* and *Burkholderia cepacia*. Hence, OAs must also be applied in combination with disease control strategies to define an integrated research strategy (Melero-Vara et al., 2011).

Effective management strategies to control VW, including biocontrol, should aim to eradicate MS or avoid their germination (Antonopoulos et al., 2008). For this reason, we set-up different sequential assays to evaluate its effects on conidia and mycelium, MS from naturally infested soil and then, on the infection of olive rooted cuttings in controlled conditions.

The aim of this study was to determine the ability of a batch of OAs from agroindustry waste and their water extracts to protect planting material of the highly susceptible olive cv. Picual against *V. dahliae* in olive. This was accomplished through comparing the (i) *in vitro* evaluation, (ii) *in vivo* evaluation of the OAs' disease-suppressive effects on VW in the olive plant cv. Picual, and (iii) the different combinations of olive waste compost with BCAs and other OAs with regard to Verticillium wilt reduction under the same *in vitro* and *in vivo* conditions.

MATERIALS AND METHODS

Pathogen isolates

Two *V. dahliae* isolates were used in this study, the mild-virulent strain namely V004 and the highly virulent pathotype V024 (Varo et al., 2016b). Both isolates were collected from the Andalusia region of southern Spain and they are belonging to the fungal collection of the Department of Agronomy at the University of Córdoba. The isolates were maintained on potato dextrose agar (PDA; Difco, MD, USA) slants at 4ºC. Plates of a six-day-old single spore culture incubated on PDA at 24ºC in the dark were used as a pathogen inoculum source.

Compost material

The bioassays were carried out using OAs from agroindustry waste of different natures and from different areas of Spain (Table 1). Composts were collected from commercial and experimental composting plants and were proved to be mature and stable in terms of chemical and microbiological characteristics to avoid phytotoxicity. Then, a grinding process was applied to achieve a suitable volatile compound release. The commercial copper product Folicupro® (47% copper oxychloride, Nufol, Spain), authorized for organic farming, was employed as a control treatment. The same compost batch was used in all *in vitro* experiments.

Compost tea and extracts

To evaluate the suppressive effect of compost water extracts, we evaluated five crude extracts and two commercial compost teas that consisted of fermented aqueous extracts of composted materials. Regarding the crude extracts, compost samples were suspended in sterile distilled water (1:4, v:v). The flasks were incubated at 25ºC and 150 rpm for 7 days and then the aqueous solution was filtered, then all treatments were stored at 4ºC until further use.

Compost mixtures

To evaluate the combined use of compost with other OAs or BCAs, two preselected composting mixtures were used, MB14 (alperujo compost plus sheep manure as nitrogen source) and MC14 (alperujo compost plus urea as nitrogen source), characterized and studied by Fernández-Hernández et al. (2014). Both compost mixtures were treated with four different aqueous treatments applied by direct watering (1:5, v:v) before mixing. The four aqueous treatments were: a fermented mixture of microorganisms (MO1) containing *Rhodopseudomonas palustris, Rhodobacter sphaeroides, Lactobacillus plantarum, L. casei, and Streptococcus lactis, Saccharomyces* sp., and *Streptomyces* sp., which is based on the trademarked product EM-1 Microbial Inoculant (Varo et al., 2016a); a non-pathogenic *Fusarium oxysporum* strain (FO12) (Varo et al., 2016a); a compound based on lactic acid (LAC) from the dairy industry, and a compost tea (TEA01) obtained from the alperujo compost CAL03 (Table 1). Each compost mixture was placed in a container during the maturation phase of the compost. During that time the compost was recolonized with mesophilic bacteria and fungi (Mehta et al., 2014).

Plant material

Five-month-old olive rooted cuttings of susceptible cv. Picual were used in the current study. This cultivar was shown to be very susceptible to the highly virulent *V. dahliae* pathotype in previous studies (López-Escudero et al., 2004). The plants were maintained in a growth chamber for one month before the inoculations to force active growth.

Biofumigation effect of organic amendments on mycelial growth

The experiment was set up to test the biofumigant effect of the OAs against *V. dahliae* isolates V004 and V024. Seven-mm-diameter agar plugs taken from the edge of *V. dahliae* colonies actively growing in PDA medium for 7 days at 25ºC were transferred to the centre of Petri dishes containing fresh PDA medium and

were then immediately placed as lids of plastic beakers (internal upper diameter 9 cm; high 12 cm; 0.4 l in volume) containing the OAs at the bottom. Dry ground matter plus the needed weight of sterile deionized water to achieve 90% of moisture content of fresh matter was prepared, including treatment with sterile water with the absence of OA as a control. The beakers were hermetically sealed with Parafilm® (Pechiney, Chicago, USA) to avoid the occasional loss of volatiles and then were released and placed in a controlled environment room at 25°C with white fluorescent lighting with a photoperiod of 14:10 L:D. The experiment was conducted twice as a complete block design with two blocks (experiments) and four replicated plates per OA and *V. dahliae* strain.

The radial growth of the colonies was recorded every two days until the control colonies covered the entire surface of the plates. The percentage inhibition of mycelial growth was calculated using the following formula: Inhibition (%) = ([R-r]/R x 100), where r is the radius of the *V. dahliae* in the presence of the OA, and R is the maximum radius of the *V. dahliae* colony away from the OA.

At the end of the experiment, plates were removed from the beakers, covered with a new sterile Petri dish lid and incubated for one week at 25ºC in the dark to check the ability of the colonies to continue growing after exposition to the volatiles released by the OA.

Effect of the OA on microsclerotia viability

To determine the effect of the potential OA to suppress MS of *V. dahliae* from naturally infested soil, two experiments (I and II) were conducted. A naturally infested soil, containing 106 CFU/g of the pathogen, was collected from a cotton field grown continuously for 50 years in the municipality of Villanueva de la Reina (UTM coordinates X: 38.012845; Y: 3.909219) in southern Spain. It is a vertisol soil with the following characteristics: pH in ClK= 7.66, organic matter = 1.80%, total CaCO3 equivalent = 21.28%, active CaCO3 = 9.89%, available K = 222 ppm, available P = 12.6 ppm, and organic N = 0.10%. The soil was air-dried at room temperature and sifted through a 0.8 mm sieve to remove organic debris and large

particles. Sterile 100 ml containers were drilled to facilitate the percolation of the liquids and were then filled with the infested soil. The experiments were conducted twice, and four replications of each treatment were considered in a completely randomized experimental design. Infested soil in the absence of OAs was included as a control treatment.

Experiment I was carried out with all OAs. Regarding the solid OAs, amended mixed samples, consisted of the naturally infested soil (40 g) and compost or a mixture of the amended compost (20 g), were used to fill the containers and they were watered with sterile water until the field capacity of the soil was reached. In the case of aqueous OAs, a 60 g sample of soil was watered with an OA suspension in water (1:2, v:v) until the field capacity of the soil was reached. The containers were covered and incubated for 72 h at 25ºC in the dark.

Experiment II was carried out using the composted poultry manure MAN01 and the same protocol as that of Experiment I, although the effect on *V. dahliae* MS was determined after two, eight and 16 days of incubation, and the dose of manure was reduced to 10%.

Subsequently, the soil from each container in both experiments was air-dried. To determine the effect of the OAs, the *V. dahliae* inoculum density was estimated by wet sieving using 10 replications of modified sodium polypectate agar medium (MSPA) (López-Escudero & Blanco-López, 2007). The plates were incubated at 24ºC in the dark for 14 days; after that, the soil residues were removed with tap water, and the *V. dahliae* colonies were counted. The inoculum density in each soil sample was estimated from the number of *V. dahliae* colonies and expressed as the number of MS or propagules per gram of air-dried soil (ppg). The experiment was conducted twice and arranged in a completely randomized experimental design.

Table 1. Individual organic amendments and mixtures evaluated for their effectiveness against *Verticillium dahliae*.

CODE	COMPOSTED MATERIAL[a]	COMPANY/ ORIGIN	CODE	COMPOSTED MATERIAL[a]	COMPANY/ ORIGIN
MAN01	Poultry manure	I/Córdoba	**CGR01**	Grape compost	XIII/Córdoba
MAN02	Pelleting poultry manure	XXI/Cádiz	**CGR02[b]**	Grape compost	XIV/Córdoba
MAN03	Sheep manure	II/Córdoba	**CGR03[b]**	Grape compost	XIV/Córdoba
MAN04	Piggery slurry	II/Córdoba	**CGR04**	Grape waste	XIV/Córdoba
MAN05	Cow manure	II/Córdoba	**CGR05**	Grape waste	XIV/C. Real
CAL01	Olive waste compost	III/Almería	**RSU01**	Municipal sewage sludge	XV/Córdoba
CAL02	Olive waste compost	IV/Córdoba	**RSU02**	Compost municipal sewage sludge	XV/Córdoba
CAL03	Olive waste compost	V/Córdoba	**RSU03**	Compost municipal sewage sludge	XV/Córdoba
CAL04	Olive waste compost	VI/Jaén	**RSU04**	Compost municipal sewage sludge	XV/Córdoba
CAL05	Olive waste compost	VII/Jaén	**HUM01**	Vermicompost	XVI Lombricor® /Córdoba
CAL06	Olive waste compost	VIII/Jaén	**HUM02**	Vermicompost	XVII Fertil®/ Albacete
CAL07	Olive waste compost	IX/Málaga	**HUM03**	Humic acids	XVIII Fulvisil® /Mexico
CAL08	Olive waste compost	III/Almería	**HUM04**	Leonardite[c] (60%), Compost (40%)	XIX/Sevilla
CAL09	Olive waste compost	IV/Córdoba	**HUM05**	Fulvic and humic acids	XIX/Sevilla
CAL10	Olive waste compost	X/Córdoba	**LAC02**	Dairy waste (Lactic acid)	XX Plantiforte®/Jaén
CAL15	Olive waste compost	VIII/Jaén	**TEA01**	Compost tea	V/Córdoba
COR01	Cork compost (0.5-2 µm particle size)	XI/Córdoba	**TEA02**	Compost tea	X/Córdoba
COR02	Cork compost (2-3 µm particle size)	XI/Córdoba	**COPP**	47% copper oxychloride + 4% nitrogen	XXI/Jaén

COMPOST MIXTURES		
CODE	**COMPOSTED MATERIAL[a]**	**COMPANY/ORIGIN**
MB14	CAL15 (70%) + sheep manure (30%)	VIII/Jaén
MBFO12	MB14 + FO12 (2.5% volume 108 con/ml)	VIII/Jaén
MBLAC	MB14 + Plantiforte (2.5%)	VIII/Jaén
MBMO1	MB14 + MO1 (2.5%)	VIII/Jaén
MBTEA01	MB14 + TEA01 (2.5%)	VIII/Jaén
MB15	CAL15 (58%) sheep manure (42%)	VIII/Jaén
MB152	CAL15 (62%) + sheep manure (35%) + olive leaves (3%)	VIII/Jaén
MB153	CAL15 (50%) + cow manure (50%)	VIII/Jaén
MC14	CAL15 (98%) + urea (2%)	VIII/Jaén
MCFO12	MC14 + FO12 (2.5% volume 108 con/ml)	VIII/Jaén
MCLAC	MC14 + Plantiforte (2.5%)	VIII/Jaén
MCMO1	MC14 + MO1 (2.5%)	VIII/Jaén
MCTEA01	MC14+ TEA01 (2.5%)	VIII/Jaén
MC15	CAL15 (87%) + olive leaves (13%) + urea (2%)	VIII/Jaén
MC152	CAL15 (65%) + horse manure (35%) + urea (4%)	VIII/Jaén

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

[b]CGR02 and CGR03 composts were taken from the same place and for two consecutive years; compost CGR03 was sampled and tested for disease suppressiveness one year after compost CGR02.

[c]Leonardite is a soft brown coal-like mineral deposit usually found in conjunction with lignite deposits.

Suppression of Verticillium wilt of olive by OAs

A bioassay was carried out to evaluate the Verticillium wilt suppressive effect of the OAs in five-month-old olive rooted cuttings. Inoculum of V024 *V. dahliae* was produced in a cornmeal sand mixture (CMS) inoculated by the pathogen (Varo et al., 2016a). Regarding the solid OAs, the amended-compost potting mixes consisted of sterile peat, compost and CMS *V. dahliae* inoculum (6:3:1 w:w:w, respectively). Regarding the aqueous OAs, the potting mixes consisted of sterile peat and CMS *V. dahliae* inoculum (9:1, w:w) watered with the

OA solution (1:4 v:v) until the field capacity of the substrate was achieved. The non-amended mixes consisted of sterile peat (sterile control) and sterile peat with *V. dahliae* (inoculated control).

Thereafter, the olive plants were planted in 0.8 L pots filled with potting mix. Then, inoculated and control plants were incubated at 60% of relative humid with a photoperiod of 14:10 (L:D; 10,000 lux) at 22ºC. The inoculum density of the pathogen was determined at the beginning of the experiments using the wet sieving technique as outlined in section 2.7. The experiment was carried out in a randomized complete block design with ten replicated pots per treatment.

Disease assessment

To evaluate the progress of Verticillium wilt in the olive plants, disease severity was weekly assessed by the severity of symptoms for 14 weeks. Each olive tree was assessed for disease severity with a 0 to 16 rating scale. The scale estimated percentage of affected tissue using four main categories or quarters (≤25, 26-50, 51-75, and 76-100%) with four values per each 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: $Y = 6.25X - 3.125$. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (López-Escudero et al., 2004).

Data analyses

An analysis of variance (ANOVA) of the inoculum density and the RAUDPC were performed for experiments because the mean values to each parameter met the assumptions of normality and homogeneity of variances for this analysis. The final severity was analysed using the nonparametric Kruskal-Wallis-Dunn test. When the ANOVA showed significant differences ($P < 0.05$) among

OAs treatments, mean values were compared using the Fisher's protected LSD test at P = 0.05. Both incidence and mortality were analysed by multiple comparisons for a proportions test ($P < 0.05$) (Zar, 1999), which considered the observed and expected frequencies of symptomatic and dead plants, respectively. In addition, a factorial design was performed to identify the interaction between the two compost mixtures MB14 and MC14 and the four aqueous treatments. Individual statistical analyses of the data were conducted using Statistix 10.0 (Analytical Software, Tallahassee, USA).

RESULTS

Biofumigation effect of organic amendments on mycelial growth

The effect of vapours from OAs on the mycelial growth of *V. dahliae* showed significant differences with the untreated control ($P < 0.001$) in 9 treatments (25.7%) and the remaining 26 treatments (74.3%) had no effect (Fig. 1). Our results suggested that poultry manure revealed the highest inhibitory activity. The mycelial growth was reduced by 100% and there was a fungicidal effect because the two pathogen isolates did not grow when they were maintained on PDA without exposure to the amendment. LAC01 and CGRAPE02 resulted in moderate inhibition, ranging from 60% for the V024 pathotype and 43% for the V004 pathotype. Nevertheless, CGRAPE01 reached a significantly lesser degree of inhibition (approximately 30%). The olive waste composts CALP01, -02 and -04, and the bovine and sheep manure resulted in a lower inhibition of *V. dahliae* ranging from 3% to 16%. Overall, no significant differences were found between *V. dahliae* isolates (P = 0.0632) or for the interaction between isolate and OA treatment (P = 0.3250).

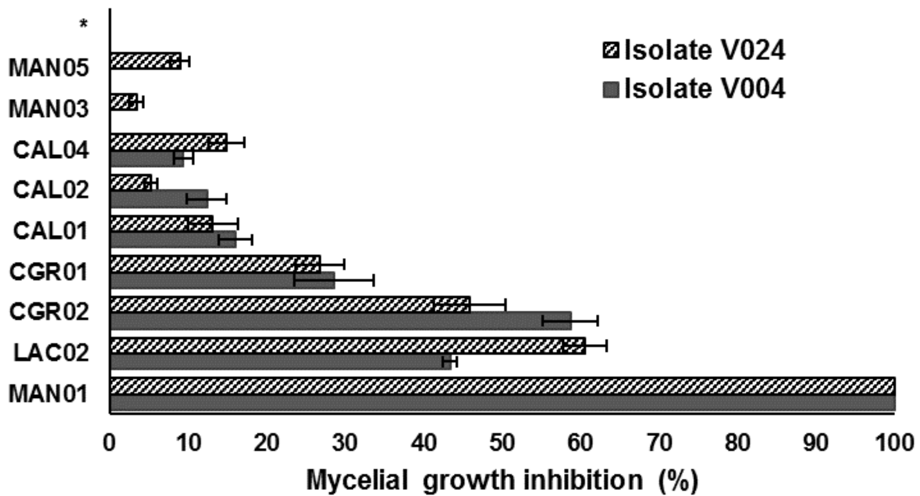

Figure 1. Inhibition (%) of mycelial growth of two isolates of *Verticillium dahliae* grown on PDA and exposed to vapours from OAs. Horizontal lines in bars are the standard error of the mean. *The remaining 26 OAs had no effect on mycelial growth.

Effect of OAs on microsclerotia viability

The effect of OAs on the viability of MS of *V. dahliae* showed significant differences with the inoculated control ($P < 0.001$) in 35 treatments (73%) (Fig. 2), and even showed a MS stimulation effect in 8 composts (17%). The highest and most consistent MS viability suppression (100%) was found for the individual treatments CGR01, CGR02, CGR03, MAN01 and LAC02, and for MBLAC, MCLAC, MCTEA01 and MB153 mixtures. Furthermore, OAs TEA01, RSU03 and MC152 showed a reduction in MS viability in the range 91.7–84.2%. The alperujo composts, which have the CAL code, diminished MS viability in the range 52.3–75.9%, being CAL01 the most effective of them. In contrast to grape composts, which completely inhibited mycelial growth, the uncomposted grape wastes CGR04 and the CGR05 showed a lower inhibitory effect that was close to 60%. The municipal sewage sludge showed different effects ranging from 60.7 to 83.4% (RSU01 and RSU02, respectively). With the exception of sheep manure MAN03 that showed 100% inhibition of MS viability, the remaining animal manures

reached a low MS inhibition effect of 20 to 44%. The cork compost COR01 showed a diminished MS viability of 53.7%, but COR02 had no effect on MS (Fig. 2).

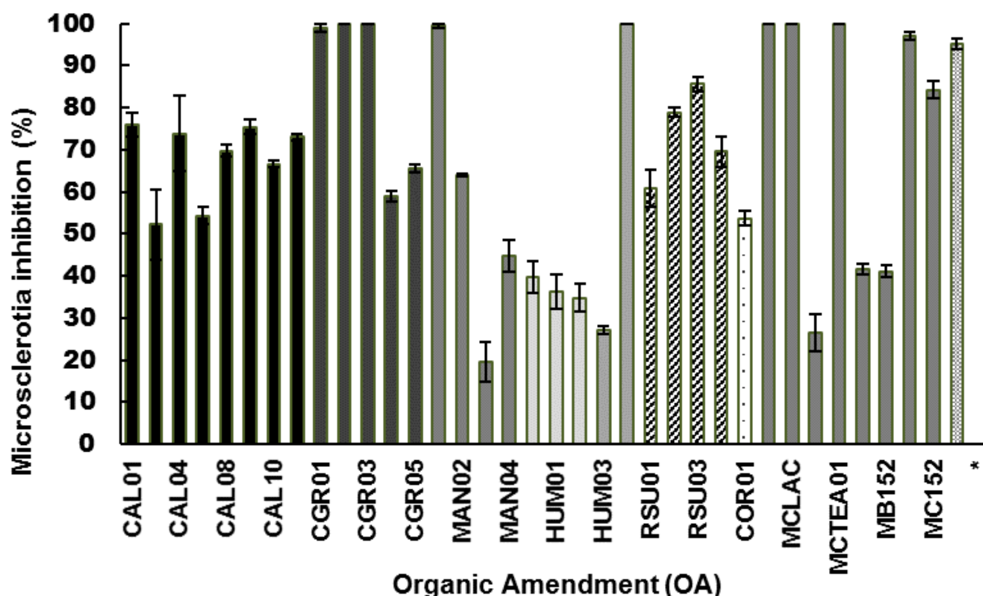

Figure 2 Inhibition (%) of *Verticillium dahliae* microsclerotia viability in a naturally infested soil amended with various OAs. Vertical lines in bars are the standard error of the mean. *The remaining 15 OAs were not significantly different from the control treatment.

Several alperujo composts, such as CAL03, CAL06 and CAL07, completely failed to suppress MS viability in the natural soil. On the other hand, the MC15, COR02, MB14, MC14, MBMO1, MCMO1, MBFO12, and MCFO12 individual composts or mixtures showed a stimulating effect on MS *V. dahliae*, inducing the germination of all potential MS (Fig. 2).

In addition to solid OAs, the effect of OA extracts and compost teas was evaluated on the survival of *V. dahliae* MS (Fig. 3). The aqueous OAs showed significant differences among treatments ($P < 0.0001$). The TEA01 inhibited the *V. dahliae* propagules at 91.7%, which was equal to the EXTCAL09, reducing the viability of the pathogen better than the original solid composts. However, the

CAL04, MAN05 and HUM01 extracts showed a lower effect than composts of origin.

With regard to mixtures, the alperujo composts combined with other OAs provided better reduction of MS viability in comparison with the unmixed alperujo composts. The alperujo compost mixed with LAC02 provided the most consistent reduction in MS viability (100%). The water extract from the compost mixture MC14 + TEA01 (MCTEA01) reached 100% of MS inhibition (Fig. 3), although the water extract from the compost mixture MB14 (MBTEA01) only reached 26% of inhibition. The alperujo compost mixed with animal manure showed a higher MS inhibition than other unmixed animal manures. The MB153 alperujo compost with cow manure affected MS viability more markedly (100%), the MC152 alperujo compost with sheep manure and olive leaves suppressed the MS in 84%, and MB15 and MB152 reduced viability to a lower effect (close to 30%).

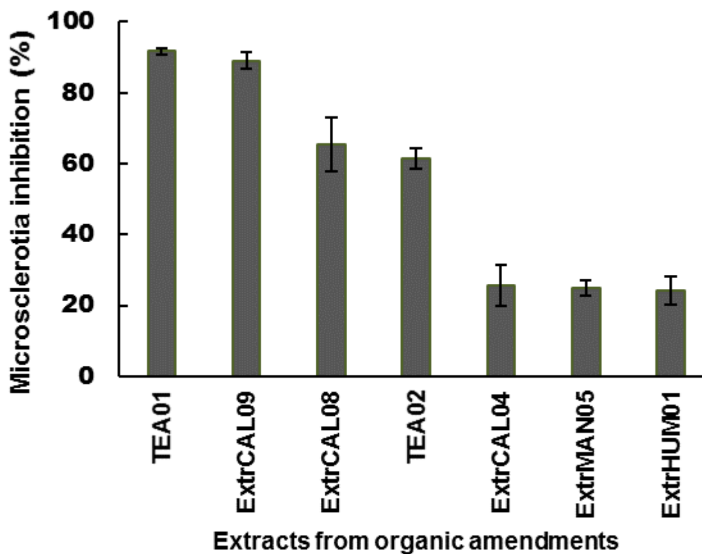

Figure 3. Inhibition (%) of *Verticillium dahliae* microsclerotia viability in a naturally infested soil amended with various crude extracts and compost teas from OAs. Vertical lines in bars are the standard error of the mean.

In Experiment II, the results of pathogen inoculum density at two, eight, and 16 days after application of a low dose of the MAN01 amendment, revealed a general trend of increased MS viability over the incubation time (Fig. 4). These results showed that this manure had a fungistatic effect on MS, due to the progressive loss of the inhibition effect.

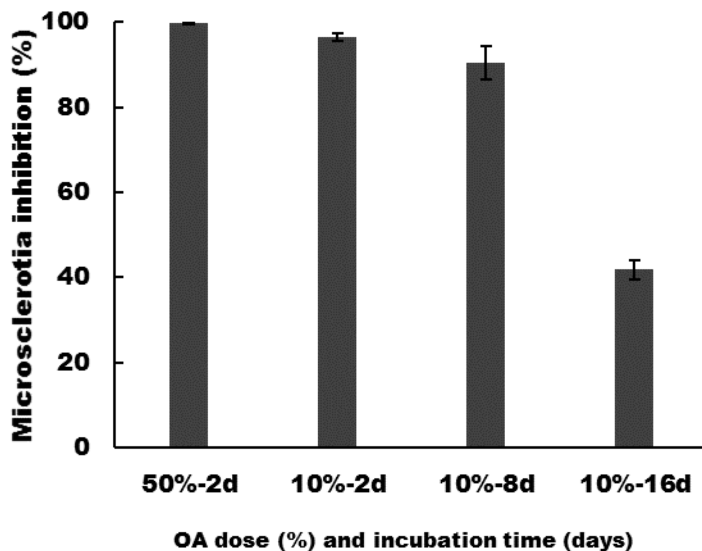

Figure 4. Inhibition (%) of *Verticillium dahliae* microsclerotia viability in a naturally infested soil amended with two doses (50% and 10%) of the poultry manure MAN01 and evaluated at two, eight and 16 days after the amendment. Vertical lines in bars are the standard error of the mean.

Effect of organic amendments on the progress of Verticillium wilt of olive

Because several studies have shown discrepancies between the antagonistic effects under *in vitro* and *in planta* efficacy (Reddy et al., 1994), the OAs with significant effects on MS were assessed in olive plants.

The initial inoculum density in the soil infested by the CMS method using the *V. dahliae* V024 isolate was 750 CFU/g soil. Olive plants grown in the infested

soil showed Verticillium wilt symptoms that are characteristics of the highly virulent pathotype. In those plants, the first symptoms appeared 33 days after inoculation and the increasing on disease severity lasted 70 days. Significant disease suppression ($P < 0.01$) was found in 12 (52%) cases. A slight disease aggravation was found in 5 (22%) cases, although this disease increase was not significant and could be caused by the high percentage of animal manure in the mixture compost (almost 50%). The effect of different treatments on disease parameters ranged from a total disease reduction (100% inhibition), which occurred in plants treated with grape composts CGR02 and CGR03, to little or no reduction that did not differ significantly from the inoculated control plants.

Intermediate responses with a significant reduction, up to 40% in the disease parameters were observed in plants treated with CAL15 and MAN01, and treatments containing dairy wastes (LAC02, MBLAC and MCLAC) or compost tea (TEA01, MBTEA01 and MCTEA01). The MCMO1 reduced the disease incidence to 80%. The fungicide COPP showed no effect on RAUDPC and the final incidence (Table 2).

The factorial ANOVA for the two compost types amended with four aqueous treatments showed a significant effect of compost type ($P = 0.0285$), treatment ($P = 0.0015$), but the interaction between compost type and treatment was no significant ($P = 0.3741$). The compost with urea was more suppressive for VW than the compost with sheep manure, being the average of RAUDPC 40.2 and 62.7%, respectively. Also, for both compost types, the treatments with compost tea (TEA01, RAUDPC = 32.1%), lactic acid (LAC, RAUDPC = 37.8%) and microorganism mixture (MO1, RAUDPC = 47.7%) were more effective than the treatments with the *F. oxysporun* strain (FO12, 88.2%).

Table 2. Disease related parameters for olive plants grown in artificially infested soil with the defoliating isolate of *Verticillium dahliae* and treated with different OAs.

OA treatment	INCIDENCE (%)[a]	MORTALITY (%)[a]	DISEASE SEVERITY[b]	RAUDPC[c]
Inoc. Control	100 [A]	100 [A]	96.9 ± 0 [A]	100 ± 5.5 [ABC]
CAL15	100 [A]	44 [B]	51.9 ± 12.8 [ABCD]	60.4 ± 14.3 [DEFG]
CGR02	0 [D]	0 [D]	0 [D]	0 [I]
CGR03	0 [D]	0 [D]	0 [D]	0 [I]
LAC02	50 [C]	30 [BC]	44.1 ± 15.6 [ABCD]	40.3 ± 16.7 [EFGH]
MAN01	90 [B]	40 [BC]	66.9 ± 11.5 [ABCD]	58.0 ± 12.0 [DEFGH]
MAN02	100 [A]	100 [A]	96.9 ± 0 [A]	100 ± 2.3 [ABC]
MB14	100 [A]	100 [A]	96.9 ± 0 [A]	100 ± 6.5 [ABC]
MB15	100 [A]	100 [A]	96.9 ± 0 [A]	125.6 ± 4.6 [A]
MB152	100 [A]	100 [A]	96.9 ± 0 [A]	108.1± 4.6 [AB]
MB153	100 [A]	89 [B]	91.9 ± 5.0 [AB]	128.0 ± 12.7 [A]
MBFO12	90 [B]	60 [B]	61.3 ± 9.2 [ABC]	75.8 ± 9.9 [BCD]
MBLAC	70 [C]	40 [BC]	54.4 ± 14.3 [ABCD]	38.9 ± 13.4 [EFGH]
MBMO1	100 [A]	70 [B]	86.9 ± 8.0 [AB]	72.8 ± 11.1 [BCDE]
MBTEA01	90 [B]	30 [BC]	68.4 ± 9.5 [ABCD]	40.2 ± 9.6 [EFGH]
MC14	50 [C]	0 [D]	27.1 ± 9.1 [BCD]	68.2 ± 24.1 [CDEF]
MC15	100 [A]	60 [B]	76.9 ± 10.2 [ABC]	86.4 ± 11.7 [BCD]
MC152	90 [B]	90 [B]	86.9 ± 0 [AB]	100.8 ± 5.2 [ABC]
MCFO12	100 [A]	70 [B]	89.4 ± 4.9 [AB]	101.1 ± 9.8 [ABC]
MCLAC	30 [C]	0 [D]	14.7 ± 8.5 [CD]	36.6 ± 20.6 [FGH]
MCMO1	50 [C]	0 [D]	9.4 ± 4.8 [CD]	22.6 ± 10.2 [H]
MCTEA01	40 [C]	0 [D]	8.8 ± 5.1 [CD]	24.0 ± 11.4 [GH]
TEA01	100 [A]	40 [BC]	61.2 ± 10.5 [ABCD]	55.5 ± 14.6 [DEFGH]
TEA02	56 [C]	22 [BC]	35.8 ± 14.1 [ABCD]	34.9 ± 12.7 [FGH]
COPP	90 [B]	89 [B]	69.4 ± 14.2 [ABCD]	86.6 ± 17.9 [BCD]

[a]Percentage of plants showing symptoms or killed by *V. dahliae* 14 weeks after inoculation (n = 20). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar 1999) at $P = 0.05$. [b]Final disease severity ± SE 14 weeks after inoculation based on a scale of 0 to 16 (0 = no lesions, 16 = 94-100% of affected tissue). Mean values followed by a common letter are not significantly different according to the non-parametric Kruskal-Wallis – Dunn's test at $P = 0.05$. [c]Relative area under the disease progress curve (RAUDPC) ± SE developed over the assessment period. Mean values followed by a common letter are not significantly different according to least significant difference (LSD) test at $P = 0.05$.

DISCUSSION

The application of organic amendments has been successfully used for reducing the *V. dahliae* microsclerotia in soil and disease incidence in olive plants. Compost suppressiveness to plant pathogens has been attributed to abiotic and/or biotic factors (Noble and Coventry, 2005).

Biological mechanisms of microbial communities such as antibiosis are probably responsible for the prevention of mycelial growth (Bailey & Lazarovits, 2003). Antibiosis due to biofumigant compounds produced by OAs might explain the inhibition observed in our experiments. Our results are in agreement with other studies in which the effects of vapours produced by organic amendments were also evaluated (Vitullo et al., 2013). The effect on mycelial growth is useful to assay the inhibition of the pathogen installation in the substrate because of the antagonistic characteristics of the OAs.

Effective management strategies to control VW diseases, including biocontrol, should aim to eradicate MS or avoid their germination (Antonopoulos et al., 2008). The potential use and efficacy of soil amendments to control VW, including their effects on MS viability, were reviewed by Goicoechea (2009) and concluded that the efficacy on these structures will increase with amendments with high lignin contents and soils that are able to accumulate ammonia from amendments. Most of the OAs included in this study had not been previously evaluated against *V. dahliae*, so there is no information about their effect on MS viability (Borrero et al., 2004). However, some OAs have actually been assessed, as several alperujo composts or animal manures, and the results obtained in this study agree with those of Termorshuizen et al. (2006), which demonstrated the efficacy of grape compost against *V. dahliae*.

Pathogen inhibition by compost teas are at least partially attributed to the presence of live microorganisms (Gea et al., 2009). However, to our knowledge, this is the first report on the effectiveness of inhibiting natural MS of *V. dahliae* with compost teas.

The remarkable effect on the viability of MS observed in our study with OAs including lactic acid from dairy agroindustry waste, could be due to the incorporation of a population of lactic acid microorganisms which may have antagonist activity against *V. dahliae*, as has been shown by Fhoula et al. (2013).

A very important aspect of OAs is the need for the amendments are not fresh but come from a previous composting process. Fresh OAs may have phytotoxicity problems and may even be carriers of *V. dahliae* inoculum. This last point was proven when a fresh sheep manure was applied as OA in a olive grove (López-Escudero and Blanco-López, 1999). The sheep had been previously fed in a cotton field affected with *Verticillium dahliae*, so their manure contained and transmitted pathogen propagules (MS), thereby contributing to the increase in the pathogen population in soil. Similar effects were observed by Termorshuizen et al. (2006) with municipal sewage sludge and yard waste composts.

Different effects were showed by the manure MAN01, which had a fungistatic effect on MS and a fungicidal effect on mycelia and conidia due to the susceptibility of these structures. Conversely, the effect was lower on MS, probably due to increased resistance of such structures being multicellular and compacted. Regular manure applications should be considered in this case because the effect decreased over time.

Overall, the results from this study showed that suppressive grape compost completely inhibited pathogen growth *in vitro* and disease development. Ideally, the biological control candidate should be screened in the plants rather than *in vitro*, and this study proved this statement. The lethal effect of grape compost on *V. dahliae* may be due to phenols and volatile organic acids. In general, the concentration of phenols in compost exhibits a slowly decreasing trend over time. Therefore, phenols and volatile organic acids, among other components, are considered as the most reliable indicators of the degree of compost maturity, reflecting temporal microbiological properties of active and cured compost. Indeed, phenols also exhibit antimicrobial properties (Obied et al., 2005), and potentially

induced plant resistance against soil-borne pathogens. These compounds and some of their degradation products have been found to exhibit fungicidal activity on various soil-borne pathogens (Yangui et al., 2010)

In our study, we infested the substrates with high inoculum densities of the pathogen, while in practice, the disease started at low pathogen inoculum densities, allowing slow disease development. This fact showed that some compost in field conditions have effectiveness against pathogens. On the other hand, the large standard errors in Table 2 are indicative of considerable variation among replicates within some treatments. There may be several explanations for this fact. It may be caused by the effect of volatile organic acids released by the OAs with phytotoxic results and also may be due to an error in the mixing technique used. Chemical and physical attributes of soil, including pH, organic matter and clay content, can contribute to the suppression of plant diseases, either directly or by activating living soil microorganisms (Mazzola, 2002). This fact could explain why the application of isolate FO12 and the amendment MBMO1 to the alperujo compost did not lead to any significant advantage. Probably, the high microbial diversity delayed the establishment of another microorganism or mixture of microorganisms in this case (Castaño et al., 2013).

The loss of efficacy in the treatment FO12 when mixed with alperujo composts could be due to inhibition by saprophytic microorganisms or chemicals in the compost itself, so more research is needed in this and other ways for the implementation of FO12 treatment.

Termorshuizen *et al*. (2006) demonstrated that the effectiveness and consistency of OA in disease suppression were influenced, among other factors, by the target pathosystem and by the variability due to the original source, chemical characteristics, and other factors of the OA. We therefore only studied the olive-*V. dahliae* pathosystem, although the use of olive woody plants posed a difficulty added to the study.

Scientific opinion about recycling of agroindustry waste in agriculture is controversial and some consider only the negative side, such as phytotoxicity and antimicrobial effects over beneficial microorganisms in this waste, and others only consider the positive side, such as soil fertilization and reuse of waste (Greco et al., 2006)). In our opinion, and according to Bailey & Lazarovits (2003), the aerated composting process is essential to avoid risks to human health and to reach accurate results. Also, uncomposted amendment can support high populations of microorganisms, but with a saprophytic activity that is not effective to disease suppression (Scheuerell et al., 2005). In agree with our results, some studies have demonstrated interesting bactericidal and fungicidal activities of olive-oil agroindustry waste and especially of its phenolic monomers such as hydroxytyrosol and tyrosol (Yangui et al., 2010).

The results of the present study demonstrated that grape compost and alperujo compost combined with dairy industry waste may exert a suppressive effect against *V. dahliae* in the field. This is the first report on disease suppression in olive with alperujo (waste from modern olive-oil industries). Therefore, future research should focus on the identification of the compounds or microorganisms present in composts that are responsible for disease suppressiveness to elucidate and explore their use. This would be a substantial advance in the way to control *V. dahliae*, where no chemical control treatments are available.

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6

Main study:

Essential oil and Plant extract screening

6

Screening water extracts and essential oils from Mediterranean plants against *Verticillium dahliae* in olive

ABSTRACT

Verticillium wilt in olive is considered the most serious biotic threat to this crop. Effective control of this disease relies on an integrated disease management strategy in which the biological control has an important role nowadays. This work describes the potential effect of 44 plant extracts and 20 essential oils against *Verticillium dahliae*. The results demonstrate the in vitro and in planta effectiveness of essential oil from *Thymus*, in particular *Thymus* sp. 04 (prepared in the laboratory), and the commercial product Thymus sp. 01, against *Verticillium dahliae*. The inhibition of mycelial growth and microsclerotia reached 100% in both treatments and achieved a disease reduction in olive plants by 65% and 42% for *Thymus* sp. 04 and sp. 01, respectively. These treatments showed the potential for essential oils use in the control of this pathogen in the frame of an integrated disease management strategy. This is the first report of the use of essential oils to control Verticillium wilt in olive plants. Further studies are warranted to identify the bioactive compounds in the essential oil that control *V. dahliae* and evaluate their potential use as natural fungicides.

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INTRODUCTION

The extensive and intensive cultivation of olives in the Mediterranean and other regions throughout the world is threatened by the soilborne fungus *Verticillium dahliae*, which causes Verticillium wilt (VW) and limits production in these areas (Blanco López et al., 1984; López-Escudero and Mercado-Blanco, 2011). The incidence of this disease has increased over the past 30 years because of the establishment of orchards in fields previously cropped with susceptible hosts of the pathogen, the use of infected planting material (Blanco-López et al., 1984; Jimenez-Díaz et al., 2012) and the expansion of irrigation in the olive groves (Pérez-Rodríguez et al., 2015).

The pathogen can survive in soil for several years as microsclerotia (MS). The parasitic phase of the *V. dahliae* life cycle begins with the germination of MS in soil in response to root exudates (Schreiber and Green, 1963) and favorable soil environmental conditions. Germination gives rise to the formation of infective hyphae, which penetrate the plant roots and grow within the xylem vessels, producing mycelium and spores (Talboys, 1962). As a result of xylem colonization by the pathogen, water flow decreases, leading to water stress (Ayres, 1978). Populations of *V. dahliae* infecting olive plants are formed by two distinctive virulence groups called defoliating (D) and non-defoliating (ND) pathotypes. The D pathotype is highly virulent and the ND pathotype is moderately severe in olive plants (López-Escudero and Mercado-Blanco, 2011). Recently the ND and D have been characterized as race 1 and 2, respectively (Hu et al., 2015). Strategies for the management of VW should be focused on reducing the survival of these resting fungal structures or preventing their germination (Antonopoulos et al., 2008).

Due to the ineffectiveness of chemical controls, natural products including plant extracts (PEs) and essential oils (EOs) present many advantages in terms of sustainability, mode of action and toxicity within an integrated management strategy for the disease (Nega, 2014), where biological control arises as an alternative challenge. Moreover, interest in secondary metabolites from PEs and

EOs, as potential antimicrobial agents for use in crop protection, has increased during recent decades (Isman 2000; Burt 2004).

Studies on the antifungal activity of PEs and EOs against plant pathogens have been conducted in *in vitro* conditions (Baruah et al., 1996; Carta et al., 1996; Bianchi et al., 1997; Wilson et al., 1997; Pina-Vaz et al., 2004). However, very few studies have focused on the antifungal activity of PEs and EOs against *V. dahliae* under *in vivo* conditions (Uppal et al., 2008).

The broad aims of this study were to investigate the antifungal effects of PEs and EOs obtained from Mediterranean plants against *V. dahliae* mycelial growth on Petri plates and on the viability of its MS in naturally infested soil. Additionally, research was extended to evaluate *in vivo* the potential biocontrol effect of PEs and EOs on VW disease in the susceptible olive cv. Picual.

MATERIALS AND METHODS

Pathogen isolates

Two *V. dahliae* isolates from the fungal collection of the Department of Agronomy at the University of Córdoba were used in this study: a mildly virulent strain ND pathotype V004 and a highly virulent D pathotype V117 (Blanco-López et al., 1989). The isolates were maintained on potato dextrose agar (PDA) slants at 4ºC. Plates of a 6-day-old single spore culture incubated on PDA at 24ºC in the dark were used as the pathogen inoculum source.

Plant material used for extracts and essential oils

Based on a literature survey, 29 commercial products and material from 35 botanical species (Table 1) were chosen for this study. Collection of wild species was assisted by Semillas Cantueso S.L. (Córdoba, Spain http://www.semillascantueso.com) that had previously identified the geographic place where different botanic populations grew in Andalucía (southern Spain). Surveys were conducted in diverse zones in the Sierra Morena and the Campus de Rabanales of the University of Cordoba. The plant material was processed in the

laboratory. The leaves of *Oleae europaea* cultivars were collected from the World Olive Germplasm Bank of Córdoba (WOGB), and the *Brassicaceae* species were characterized and supplied by Dr. de Haro from CSIC-IAS at the growing stage. The freshly cut plant materials were sorted, dried with active ventilation at room temperature, ground to a fine powder in a hammer mill (Retsch GmbH and Co. KG, Haan, Germany), packed in paper bags and stored at 5ºC until use.

Plant extracts

The plant extracts (PEs) were obtained from several sources by steam distillation. A first group was purchased from the same companies mentioned below, and the purity was available for some of them. Another group consisted of PE obtained in the lab from different botanical species. Ground plant material samples (25 g of leaves and stems) of each plant were extracted with 100 ml of organic solvent (acetone) in a Soxhlet extractor. The mixture was boiled for 3 hours, and the extract was concentrated by a distillation process to evaporate the acetone. The crude extracts obtained were then stored at -18°C until further use. The extraction for each plant extract was run in duplicate.

Particularly, for *Allium* and *Melia*, the juices of both species were obtained according to the methodology of Curtis et al. (2004). Samples (100 g of leaves and stems) were chopped into small pieces and homogenized using a household blender (Braun, Aschaffenburg, Germany). The homogenates were then centrifuged (5000 rpm, 20ºC, and 10 minutes) and filtered to separate the juices, which were stored at -18ºC until further use.

Essential oils

Almost all essential oils used in this study (Table 1) were experimental products from several Spanish companies: Agromed S.L. (Granada, Spain), Fagron S.A. (Barcelona, Spain), Trabe S.A. (Murcia, Spain) or Zoberbac S.L. (Barcelona, Spain), except one from *Thymus* sp. that was extracted in our lab according to the methodology of Benkeblia (2004).

Table 1. Plant extracts and essential oils evaluated for their effectiveness against *Verticillium dahliae.*

Extract from	Origin (Purity)	Extract from	Origin (Purity)
Allium sativum	Lab preparation	*Lantana camara*	Lab preparation
Allium sp.	Bio 125®, Agromed SL	*Laurus nobilis*	Lab preparation
Atropa belladona	Fagron SA (3.45)	*Lepidium* sp.	Lab preparation
Azadirachta indica	Neem® Trabe S.A.	*Marrubium vulgare*	Fagron SA
Brassica napus	Lab preparation	*Melia azedarach*	Lab preparation
Brassica rapa	Lab preparation	*Mentha sativa*	Lab preparation
Castanea sativa	Fagron SA	*Nerium oleander*	Lab preparation
Cistus albidus	Lab preparation	*Olea europaea cv.* Lechín SE	Lab preparation(5.6)
Cistus ladanifer	Lab preparation	*Olea europea* cv. Arbequina	Lab preparation
Cistus laurifolius	Lab preparation	*Olea europea* cv. Cornicabra	Lab preparation
Citrus aurantium	Fagron SA (2.75)	*Olea europea* cv. Empeltre	Lab preparation
Citrus sp.	Bio 150, Agromed SL	*Olea europea* cv. Frantoio	Lab preparation(5.3)
Diplotaxis erucoides	Lab preparation	*Olea europea* cv. Picual	Lab preparation(7.3)
Diplotaxis virgata	Lab preparation	*Origanum vulgare*	Lab preparation
Eucalyptus camaldulensis	Lab preparation	*Papaver rhoeas*	Fagron SA
Ginkgo biloba	Fagron SA (11.00)	*Pinus pinea*	Lab preparation
Hammamelis virginiana	Fagron SA	*Pistacia lentiscus*	Lab preparation(2.9)
Hedera helix	Fagron SA (1.06)	*Rosmarinus officinalis*	Fagron S.A. (2.00)
Hirschfeldia incana	Lab preparation	*Salvia officinalis*	Fagron S.A. (1.25)
Inula viscosa	Lab preparation (6.00)	*Sambucus nigra*	Fagron S.A. (3.03)
Juglans regia	Fagron SA (3.73)	*Thymus vulgaris*	Lab preparation
Juniperus communis	Fagron SA	*Urtica* sp.	Lab preparation
Essential oil from	**Origin**	**Essential oil from**	**Origin**
Citric acid 01	Fagron SA	*Origanum vulgare*	Fagron SA
Citric acid 02	Fruitcare, Zoberbac SL	*Pinus sp.*	Fagron SA

Cymbopogon sp.	Fagron SA	*Rosmarinus officinalis*	Fagron SA
Eucaliptus sp.	Fagron SA	*Salvia officinalis*	Fagron SA
Illicium verum	Fagron SA	*Satureja sp.*	Fagron SA
Laurus nobilis	Fagron SA	*Thymus* sp. 01	Oleatbio, Trabe SA
Melaleuca alternifolia	Fagron SA	*Thymus sp.* 02	Biofungi, Fagron SA
Melaleuca cajeputi	Fagron SA	*Thymus* sp. 03	Bio 75, Agromed SL
Mentha sp.	Fagron SA	*Thymus* sp. 04	Lab preparation
Mirtus communis	Fagron SA	*Verbena officinalis*	Fagron S.A.

[1]The purity, where available, of plant extracts indicates the percentage of dry matter, which was estimated by evaporating 1 ml of extract at 70°C and weighing the dried residue.

Effect of plant extracts and essential oils on mycelial growth

The PEs and EOs from each plant sample were added to a molten (45°C) PDA medium at different doses: 5, 50, 500 and 5000 mg/L. The controls were non-amended dishes containing only PDA. The PDA Petri dishes were then inoculated in the center by placing onto the medium 7 mm diameter agar plugs taken from the edge of a 6-day-old culture of *V. dahliae* grown on PDA medium. After being incubated for 7 days at 25ºC, radial growth was determined by measuring two perpendicular diameters of fungal colony and calculating the average value after subtracting the diameter of the fungal plug. The radial growth was measured every two days for ten days (by this time control colonies had reached approximately 9 cm of diameter). Growth inhibition was calculated using the following formula, expressed as a percentage: % Inhibition = ((R-r)/R x 100), where r is the radius of the *V. dahliae* colony in the presence of the plant extract or essential oil, and R is the maximum radius of the *V. dahliae* colony of the controls.

The experiment was conducted twice, and five replications were used for each dose. A factorial design with three non-subordinated factors (two fungal isolates, 64 treatments and four doses of each treatment) was used.

Effect of plant extracts and essential oils on microsclerotia viability

This experiment was conducted to determine the effect of the 28 PEs and 20 EOs on MS of *V. dahliae*. A naturally infested soil, containing 10^6 CFU/g of the pathogen, was collected from a cotton field grown continuously for 50 years in the municipality of Villanueva de la Reina (UTM coordinates X: 38.012845; Y: 3.909219) in southern Spain. The soil was air-dried at room temperature and sifted through a 0.8 mm sieve to remove organic debris and large particles. The *V. dahliae* inoculum density was estimated by wet sieving (Huisman and Ashworth, 1974) using 10 replications of modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977). Plates were incubated at 24ºC in the dark for 14 days, after which soil residues were removed with tap water and the *V. dahliae* colonies were counted. The inoculum density of each soil sample was calculated from the number of *V. dahliae* colonies and expressed as the number of MS or propagules per gram of air-dried soil (ppg) (López-Escudero and Blanco-López, 2005). The experiment was arranged in a completely randomized design using three replications per treatment. The initial inoculum density was 110 MS/g of soil, in which the proportions of the pathotypes D and ND were 57.7% and 42.3%, respectively (Ostos et al., *unpublished results*).

After inoculum density estimation, 100 mL sterile containers were filled with 60 g of the infested soil. The containers were previously pierced at the bottom to facilitate the percolation of liquids. Treatments that produced more than 90% inhibition of *V. dahliae* mycelial growth in the *in vitro* assay were tested in the natural infested soil experiment. The PEs and EOs treatments were added at doses of 500, 2500 or 5000 mg/L to the containers until the field capacity of the soil was reached. The containers (three per treatment) were covered and incubated for 72 h at 25ºC in the dark. Infested soil watered with sterile water was included as a control treatment.

After incubation, the soil from each container was air-dried and the inoculum density of each treatment was calculated as mentioned above. The experiment was conducted twice.

Effect of plant extracts and essential oils on Verticillium wilt in olive plants

The treatments that showed an effective inhibition of the pathogen MS (greater than 90% inhibition) were selected for the *in vivo* evaluation under controlled conditions in a new experiment of inoculation of olive plants. This bioassay evaluated the development of disease incidence and severity of symptoms and was conducted to determine the ability of the selected PEs and EOs to suppress VW in olives caused by the D pathotype of *V. dahliae*. Five-month-old rooted olive cuttings of the susceptible cv. Picual were used. The plants were maintained in a growth chamber for one month prior to inoculation to force active growth.

Olive plants were planted into 0.8 L pots (one plant per pot), containing sterile peat moss with a 20% (weight/weight) of a cornmeal sand mixture (CMS) infested with the D pathotype isolate according to Varo *et al.* (2016). Non-inoculated plants were used as a control treatment. Inoculated and control plants were incubated at 22ºC and 60% RH with a 14 h photoperiod under fluorescent light adjusted to 216 $\mu mol/m^2s^1$.

The inoculum density of the pathogen in the substrate was determined at the beginning of the experiments as explained above. The experiment was carried out in a randomized complete block design with ten replicates (pots). The olive plants were treated by watering with a 2% dose of the PEs or EOs until the field capacity of the soil was reached. The experiment was conducted twice.

Disease assessment

To evaluate the progress of wilt in the olive plants, each olive tree was weakly assessed over 14 weeks for disease severity with a 0 to 16 rating scale. The scale estimated percentage of affected tissue using four main categories or quarters (≤25, 26-50, 51-75, and 76-100%) with four values per each 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: $Y = 6.25X - 3.125$. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (López-Escudero et al., 2004).

Data analysis

Statistical analysis of the mycelial growth inhibition, inoculum density, RAUDPC and final severity were conducted according to Fisher's protected LSD test at $P = 0.05$. Both incidence and mortality were analysed by multiple comparisons for proportions test ($P = 0.05$) (Zar, 1999), which considered the observed and expected frequencies of symptomatic and dead plants, respectively. Statistical analysis of the data was conducted using Statistix 10.0 (Analytical Software, Tallahassee, USA). Individual factors and the interactions between them were analyzed with an analysis of variance using Statistix 10.0 for Windows (Analytical Software, Tallahassee, FL). The mean values of each parameter were compared via the LSD test at $P = 0.05$.

RESULTS

Effect of plant extracts and essential oils on mycelial growth

In general, the mycelial growth inhibition greatly varied among treatments and increased with increasing dose of the treatments, although the EOs exhibited greater inhibition activity against the pathogen than the PEs. Since the general ANOVA showed a significant effect of treatments, doses, fungal isolates and all their interactions, a one-way ANOVA was conducted for each treatment type (plant extract and essential oil) and each isolate (V004 and V117), using the four doses as blocks. The results of treatments for each isolate and dose that showed more than

5% inhibition of mycelial growth in any of the two fungal isolates are shown in Figs. 1 and 2.

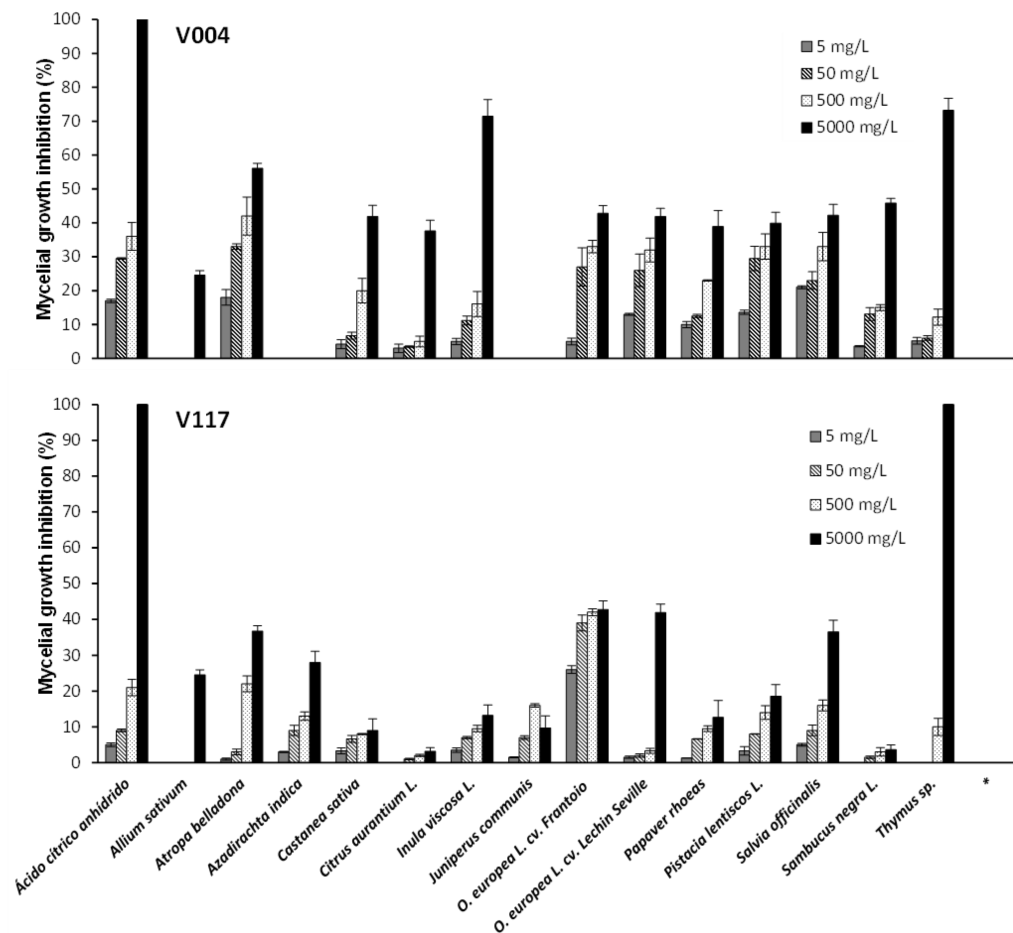

Figure 1. Mean inhibition (%) of mycelial growth and standard error of the mean for V004 and V117 pathotypes of *V. dahliae* grown on PDA amended with plant extracts at four doses (5, 50, 500 and 5000 mg/l). *Not shown treatments (28) did not inhibit mycelial growth of both pathotypes.

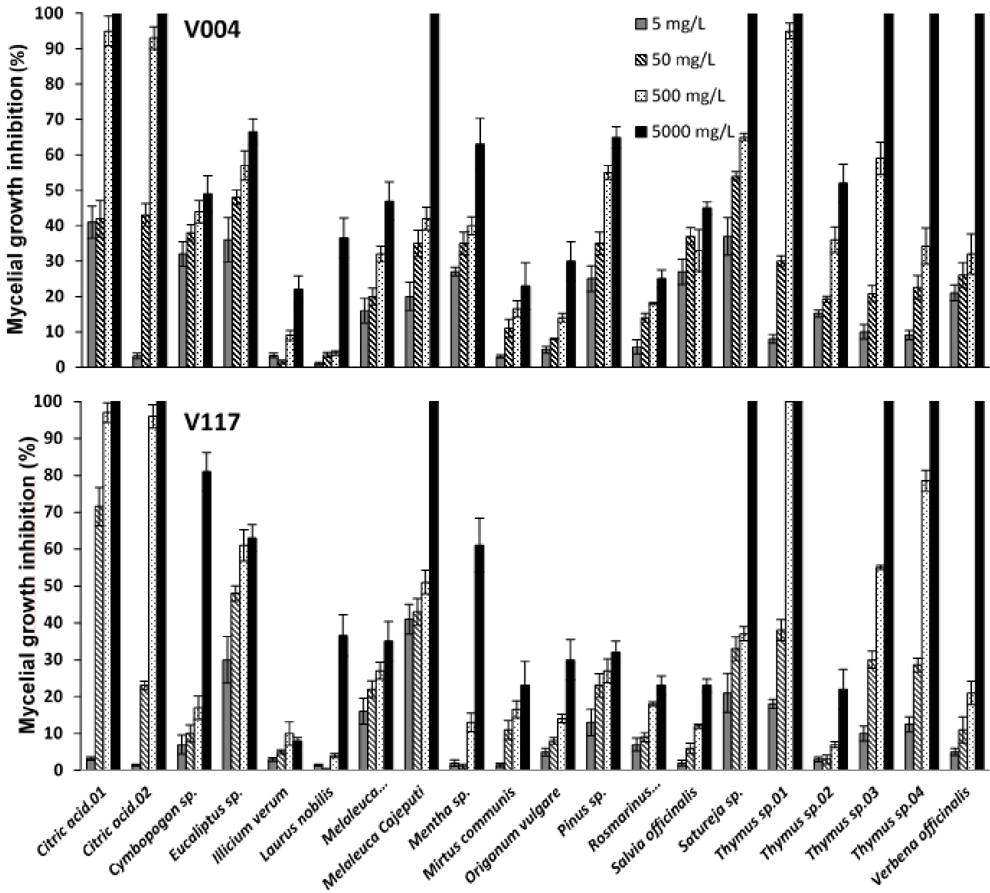

Figure 2. Mean inhibition (%) of mycelial growth and standard error of the mean for V004 and V117 pathotypes of *Verticillium dahliae* grown on PDA amended with essential oils at four doses (5, 50, 500 and 5000 mg/l).

With regard to the 44 PEs, lower doses (5, 50 and 500 mg/L) weakly inhibited the development of both pathotypes (Fig. 1); in Table 2 the mean value of the four doses are shown for each PE. At this mean dose, citric acid and *Thymus* sp. extract were the most effective treatments (100% for isolate V004 and 100% and 75% for isolate V117, respectively) and the extracts from *Allium* sp., *Atropa belladona, O. europaea* cv. 'Frantoio' and *Salvia officinalis* achieved an intermediate level of inhibition (36.4, 56.0, 42.7 and 42.2%, respectively, for V004

and 53.2, 36.1, 44.7 and 35.5 %, respectively, for V117). The remaining PEs treatments achieved a level of inhibition lower than 30% (Fig. 1). Overall, V117 was less sensitive to inhibitory activity than V004, although there was a reversal of these results for some treatments. When the inhibitory effect was analyzed for the overall of the four treatment doses, nine PEs treatments showed a growth inhibition higher than 25% for V004 isolate, while only four PEs (*Allium* sp., *Citrus* sp., *O. europaea* cv. 'Frantoio' and *Thymus* sp.) had the same effect for V117 isolate. The treatments not shown in Fig. 1 and Table 2 had no effect on mycelial growth.

Table 2. Mean inhibition (%) of mycelial growth for V004 and V117 pathotypes of *V. dahliae* grown on PDA amended with four doses of plant extracts.

Plant extract from	Fungal isolate	
	V004	V117
Allium sativum	6.1*	6.1*
Allium sp.	27.1	46.4
Atropa belladona	37.3	15.7
Azadirachta indica	0	13.3
Castanea sativa	18.2	6.7
Citrus aurantium	12.3	1.6
Citrus sp.	45.5	33.3
Inula viscosa	25.9	8.3
Juniperus communis	0	12.9
O. europea cv. Frantoio	26.9	37.4
O. europea. cv. Lechín SE	28.2	12.2
Papaver rhoeas	21.1	7.5
Pistacia lentiscus	29.0	10.9
Salvia officinalis	29.8	16.6
Sambucus nigra	31.7	8.0
Thymus sp.	24.2	27.5
$LSD_{0.05}$	**22.6**	**28.0**

*Mean values are the average of four plant extract doses (5, 50, 500 and 5000 mg/l), five replicated Petri dishes and two experiments. Significant differences between any treatment means are given by the least significant difference (LSD) test critical value at $P = 0.05$.

With regard to the twenty EOs, the highest dose (5000 mg/L) of citric acid 01 and 02, *Melaleuca cajeputi*, *Satureja* sp., *Thymus* sp. 01 and 03 and *Verbena officinalis* exhibited 100% inhibition activity against both *V. dahliae* pathotypes. In

addition, the inhibition by extracts from *Eucaliptus* sp. *Mentha* sp., *Pinus* sp., *Laurus nobilis*, and *Origanum vulgare* were significantly effective for the ND pathotype. The remaining EOs showed an intermediate effect that ranged from 64.9% to 18.4% inhibition for both pathotypes of *V. dahliae*. Even low doses (50 to 500 mg/L) of citric acid 01 and 03, *Melaleuca cajeputi* and *Thymus* sp. 01 and 03 led to a 40-95% inhibition of fungal growth for both isolates (Fig. 2). When the inhibitory effect was analyzed for the overall of the four treatment doses, the most effective treatments for both *V. dahliae* isolates were citric acid 01 and 02, *Eucalyptus* sp., *Melaleuca cajeputi*, *Satureja* sp. *Thymus* sp. 01, 03 and 04 (Table 3).

Effect of plant extracts and essential oils on microsclerotia viability

This experiment was conducted to determine the effect of PEs and EOs on the viability of *V. dahliae* MS in a naturally infested soil. There were no significant differences in the two repetitions of the experiment. The tested doses were 500, 2500 and 5000 mg/L. Although some PEs showed an inhibitory effect on the mycelial growth of the pathogen, none of them showed any effect on the *V. dahliae* MS viability. However, all EOs tested showed a significant reduction of MS viability although at different doses ($P < 0.001$). The extracts from *Thymus* sp. 04 showed a strong inhibitory effect on the viability of MS that reached the 77.3, 99.3 and 100% at doses of 500, 2500 and 5000 mg/L respectively. The extracts from *Thymus* sp. 01 achieved a 79.3 and 100% inhibitory effect at doses of 2500 and 5000 mg/L, respectively. The extracts from *Verbena officinalis* and *Thymus* sp. 03 significantly diminished MS viability after 72 h of incubation at the 5000 mg/L dose (Fig. 3). The remaining EOs treatments failed to suppress MS viability of *V. dahliae* in naturally infested soil.

Table 3. Mean inhibition (%) of mycelial growth for V004 and V117 pathotypes of *V. dahliae* grown on PDA amended with four doses of essential oils.

Essential oil from	**Isolates**	
	V004	**V117**
Citric acid 01	69.5*	67.9*
Citric acid 02	59.8	55.1
Cymbopogon sp.	40.8	28.8
Eucalyptus sp.	51.9	50.5
Illicium verum	8.9	6.5
Laurus nobilis	11.3	10.7
Melaleuca alternifolia	28.8	25.0
Melaleuca cajeputi	49.3	58.8
Mentha sp.	41.3	19.3
Mirtus communis	13.4	13.0
Origanum vulgare	14.3	14.3
Pinus sp.	45.0	23.8
Rosmarinus officinalis	15.7	14.3
Salvia officinalis	35.5	10.8
Satureja sp.	64.0	47.8
Thymus sp.*01*	61.1	71.5
Thymus sp.*02*	30.6	8.8
Thymus sp.*03*	49.8	48.8
Thymus sp.*04*	41.5	54.9
Verbena officinalis	44.8	34.3
$LSD_{0.05}$	**36.5**	**35.0**

*Mean values are the average of four plant extract doses (5, 50, 500 and 5000 mg/L), five replicated Petri dishes and two experiments. Significant differences between any treatment means are given by the least significant difference (LSD) test critical value at $P = 0.05$.

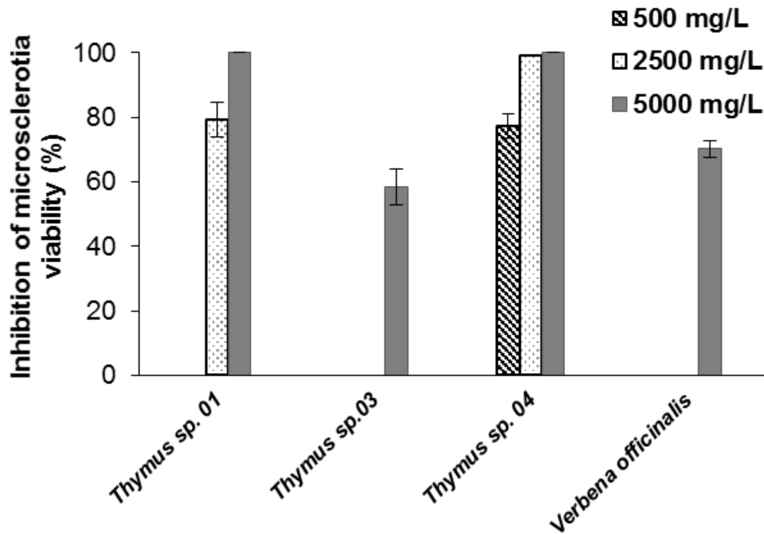

Figure 3. Mean inhibition (%) of *Verticillium dahliae* microsclerotia viability and standard error of the mean in a naturally infested soil treated with plant extracts and essential oils at three doses (500, 2500 and 5000 mg/l). Not shown treatment doses did not inhibit MS viability.

Effect of essential oils on the infection by Verticillium wilt in olive plants

The effect of the treatments on disease severity and the efficacy of the treatments at the end of the experiment are reported in Table 4. The tested products showed phytotoxic symptoms and adverse effects on treated plants at doses higher than 1000 mg/L. Non-inoculated plants in the experiment remained healthy. The earliest symptoms of VW in olive plants growing in soil infested with the D isolate of the pathogen began 28 days after inoculation. Disease symptoms included wilting and drying of shoots, extended necrosis and defoliation of green or necrotic leaves. Both essential oils significantly reduced VW disease, but the extract prepared in the laboratory (*Thymus* sp. 04) showed greater effect than the commercial formulation (*Thymus* sp. 01). For both treatments, RAUDPC, final disease severity and mortality decreased by 65% and 42%, 53% and 46%, and 100% and 50%, respectively (Table 4).

Table 4. Disease-related parameters for olive plants growing in artificially infested soil with the defoliating *Verticillium dahliae* pathotype and treated with essential oils.

EOs treatment	**INCIDENCE (%)[1]**	**MORTALITY (%)[1]**	**DISEASE SEVERITY[2]**	**RAUDPC[2]**
Sterile control	0^{D}	0^{C}	0^{C}	0^{D}
Inoculated control	100^{A}	100^{A}	96.9 ± 2.0^{A}	100 ± 0.0^{A}
Thymus sp. 01	90^{B}	50^{B}	54.4 ± 6.6^{B}	58.3 ± 16.1^{B}
Thymus sp. 04	75^{C}	0^{C}	46.9 ± 6.4^{B}	35.2 ± 8.2^{C}

[1]Percentage of plants ± standard error (SE) showing symptoms or killed by *V. dahliae* 12 weeks after inoculation (n = 20). In each column, mean values followed by the same letter were not significantly different according to the multiple comparisons for proportions test (Zar, 1999) at P = 0.05. [2]Disease severity ± SE 14 weeks after inoculation based on scale of 0 to 16 (0 = no lesions, 16 = 94-100% of affected tissue) and relative area under the disease progress curve (RAUDPC) ± SE developed over the assessment period. In each column, mean values followed by the same letter are not significantly different according to Fisher's protected LSD test at P = 0.05.

DISCUSSION

The application of several plant substances has been successfully used for eradicating or reducing *V. dahliae* microsclerotia in soil or reducing mycelial growth of the pathogen. Additionally, some of these plant products have been able to reduce or delay the incidence of infections and disease onset and development in several herbaceous hosts of the pathogen (Nega, 2014). In woody hosts, such as olive, this kind of experimental research is very scarce (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz et al., 2012) and there is little information about the effect of plant extracts or essential oils on mycelial growth and infective structures (MS) of *V. dahliae*.

The antifungal effect of 44 PEs and 20 EOs pre-selected from a literature survey on VW diseases was assessed in this study. The results showed that the essential oils of *Thymus* sp. were the most effective. In particular, the EOs from *Thymus* sp. 04 prepared in the laboratory showed the most consistent effect, reaching a complete reduction of mycelial growth, germination of MS and reduction of VW development in olive plants of cv. Picual. The inhibitory activity of *Thymus* sp. has been widely investigated, particularly the activity of essential

oils against plant pathogenic fungi (Pina-Vaz et al., 2004; Šegvić Klarić et al., 2007; Numpaque et al., 2011; Elshafie et al., 2015). This antifungal activity is probably related to high doses of monoterpenes and phenolic compounds (Báidez et al., 2007). In fact, the primary constituents of the EOs from *Thymus* sp. are carvacrol and thymol, which are present in the Lamiaceae family (Bakkali et al., 2008). The effect of species from the family Lamiaceae on the mycelial growth of *V. dahliae* was studied *in vitro* by Arslan and Dervis (2010) and Giamperi et al. (2002). However, in the present study, some species of this family such as *Marrubium vulgare*, *Mentha* sp., *Origanum vulgare, Salvia officinalis* and other species such as *Laurus nobilis* and *Rosmarinus officinalis* did not show inhibitory effect against *V. dahliae* in either *in vitro* or *in vivo* conditions.

López-Escudero et al. (2007) demonstrated that the application of dried plant residues of *Thymus mastichina* and *Lavandula stoechas* were highly effective in reducing the viability of *V. dahliae* MS in naturally infested soil. In some cases, the number of MS detected in soil treated with these products was higher than in the control treatment. This could be due to the stimulation of inactive MS by extract compounds or the lethal effect on beneficial soil microorganisms that have been recognized as a pathogen repressing factor (López-Escudero et al., 2007).

The treatments with olive leaf extracts had scarce effect on the mycelial growth of either isolate. Only the 'Frantoio' leaf extract showed an inhibitory effect of about 40% against both isolates of *V. dahliae*. However, in another study, Mulero-Aparicio et al. (2014) were unable to obtain any effect against *V. dahliae* using extracts from 'Frantoio' leaves. These discrepant results could be due to differences in the leaf age and the subsequent differences in the concentration of active compounds at the time of leaf collection.

Extracts of *Allium* plants have not been extensively investigated in VW diseases, although garlic extract or juice showed a strong effect *in vitro* against several fungal pathogens (Curtis et al., 2004; Slusarenko et al., 2008). In this study, the PEs from *Allium sativum* exhibited a low inhibitory effect against *V. dahliae*,

whereas the commercial product from *Allium* sp. achieved an inhibition of mycelial growth higher than 50% for the D isolate and close to 40% for the ND isolate. However, the garlic extracts had not effect on MS viability. This lack of effectiveness could be due to a low release or inactivation of active substances against the pathogen, since in a study with potted garlic plants grown in *V. dahliae* infested soil, we observed 80% inhibition of MS viability (Mulero-Aparicio et al., *unpublished results*).

The biofumigation potential of Brassicaceae green manures against *V. dahliae* is well known in several crops (França et al., 2013; Neubauer et al., 2014) and it has been extensively assessed in olive groves (Bejarano-Alcázar, 2008). In our study, however, the plant extracts and essential oils from species of this plant family did not show effect against *V. dahliae*. This lack of effectiveness could have the same explanation suggested above regarding garlic extracts. These results are according to other studies that had evaluated the effectiveness of cover crops on VW diseases (França et al., 2013; Neubauer et al., 2014).

Although plant extracts are known to be efficient antimicrobial agents, this study showed that most products tested had no effect on *V. dahliae* MS and consequently on disease control; however, an effect on mycelial growth was observed, particularly at the highest dose of 5000 mg/L. Regarding the pathogen life cycle, MS are dispersal and overwintering structures with a melanized, compacted cell layer. It is possible that the primary compound of *Thymus* sp., timol, had detrimental effects on the germination of MS as well as on the newly-formed MS. A reduction in the proportion of viable MS would be expected to result in increased disease control. Because the conidia and mycelium are weaker structures, the documented antifungal effect was higher.

One of the mechanisms of action of PEs and EOs is the induction of resistance, both locally or systemically, in the treated plants (Walters et al., 2005). In this study, we have not considered this option due to its complexity, although this aspect is very important in understanding the full effectiveness of these

products. Therefore, we are currently evaluating the effect of some PEs, EOs and other natural products as inducers of resistance in olive plants grown in soil infested by *V. dahliae* (Varo et al., 2016).

This study demonstrates the efficacy of *Thymus* sp. against *V. dahliae*, and the potential use of essential oils for its control. Although our results demonstrate the antifungal activity of essential oils, the mechanisms of action are not well documented. Thus, to develop EOs as alternatives to synthetic fungicides, further field studies are required to evaluate phytotoxicity and its effects under natural conditions. Research work is in progress on additional opportunities for the integrated control of VW of olive.

CONCLUSIONS

Verticillium wilt of olive trees has been detected in almost all regions where olive is cultivated, causing serious concern to growers, nursery companies and the olive oil industry. No effective control strategy is available for the disease, and the concern about pesticide use is increasing. Natural plant-derived treatments could provide a wide variety of compounds as an alternative strategy for control of this disease. The present study showed that the essential oil of *Thymus* sp. prepared in the laboratory completely inhibited mycelial growth, and reduced microsclerotia viability and VW disease in susceptible inoculated olive plants. We suggest that *Thymus*-based treatment could be integrated into control practices for this disease.

ACKNOWLEDGEMENTS

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7

Main study: Field experiments

7

The effect of potential biocontrol treatments on the development of Verticillium wilt in olive orchards with different inoculum densities.

INTRODUCTION

Verticillium wilt (VW) disease, caused by the widespread soil-borne fungus *Verticillium dahliae,* is one of the most serious worldwide diseases in olive (*Olea europaea* L.), causing severe losses and plant death (López-Escudero and Mercado Blanco, 2011). One concerning observation about this disease in several important Mediterranean olive-growing regions is the rapid spread of *V. dahliae* isolates belonging to a defoliating (D) pathotype, which is more virulent than the dominant non-defoliating (ND) pathotype (López-Escudero et al., 2004).

Management of VW is a challenge for olive growing due to the endophytic growth of the pathogen in the xylem, the ability to infect multiple hosts and the longevity of its propagules in the soil (Alström, 2001). These facts have turned this disease as one of the major threat of the olive crop worldwide.

Currently, there is a lack of effective methods to reduce the inoculum concentration in the soil that motivates the search for new alternative strategies. Other methods, such as the use of tolerant cultivars (Arias-Calderón et al., 2015; Trapero et al., 2015), soil solarisation (Pegg and Brady, 2002) and cultural practices (López-Escudero and Mercado Blanco, 2011), have been tested but they provide only partially effective control of the pathogen.

For this reason, a sustainable, eco-friendly and integrated disease management strategy must be developed. Previous studies conducted in this current work (Chapters 4, 5 and 6) have reported the effective biological control against *V. dahliae* of microorganisms (several fungi and bacteria, and their extracts), selected organic amendments (waste of plants, animals and food industry) and plant

extracts. This massive selection of candidates had been conducted in three stages: i) *in vitro*, by the effect on the mycelial growth and spore germination of the pathogen; ii) in natural infested soil, by the effect on the reduction of microsclerotia of the pathogen and iii) *in planta*, by the effect on the infection of olive plants under controlled conditions. However, an additional study under field conditions (fourth stage) to evaluate the effect of the selected candidates in previous studies on Verticillium wilt of olive (VWO) trees grown in highly infested soil is essential to complete the screening.

Thus, the objective of the present study was to evaluate the effect of 14 candidates selected from among 220 compounds that were previously tested under controlled conditions, by testing their biocontrol effectiveness under three different field conditions.

MATERIALS AND METHODS

Inoculum density

Inoculum density was determined at the initial moment as well as during the experiment to evaluate the inoculum density progression. Soil samples were collected by using a soil probe and three sub-samples were considered per soil sample, at a depth from 25 to 30 cm (Trapero et al., 2013). At the initial moment, soil samples were randomly collected in each experimental field, while during the experiment samples were collected per each treated tree twice a year. Soil samples were separately bulked and thoroughly mixed. Then, the soil from each sample was air-dried. The inoculum density of *V. dahliae* in each soil sample was estimated by wet sieving (Huisman and Ashworth, 1974) using 10 replications of modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977; López-Escudero and Blanco-López, 2007). An amount of 25 g of the sample was suspended in 100 ml of distilled water, shaken at 270 rpm for 1 h at room temperature and filtered through 150 and 35 µm sieves. The residue retained on the 35 µm sieve was recovered in 100 ml of sterile distilled water. Finally, 1 ml of the suspension was plated onto plates (10 replications) of MSPA. After 14 days of

incubation at 22 ± 2ºC in the dark, soil residues were removed with tap water and colonies of *V. dahliae* counted under a stereoscope. The inoculum density in each soil sample was estimated from the number of *V. dahliae* colonies and expressed as the number of microsclerotia (MS) or propagules per gram of air-dried soil (ppg) (López-Escudero et al., 2003).

Plant material

Twelve-month-old rooted olive plants of the susceptible cultivars Picual and Cornicabra, the moderately susceptible cultivar Arbequina and the moderately resistant cultivar Frantoio (López-Escudero et al., 2004; Trapero et al., 2013) were used for the different trials. The cuttings were propagated from *Verticillium dahliae*-free mother olive plants in nursery conditions. At planting time, the plants were 1.0 -1.1 m high with single trunk and three or four secondary branches.

Experimental biocontrol field trials

Based on the previous results (Chapter 4, 5 and 6) where disease suppressiveness was observed, selected biocontrol treatments were tested for their performance against *Verticillium dahliae* under field conditions. The experiments were carried out on three different olive orchards with different inoculum densities, all of them located in Andalusia region (southern Spain). The treatment(s) were applied manually by watering the tree and the dose were calculated per tree. The solid treatments were carefully incorporated to the soil, buried with thin layer of soil and watered with tap water. Two treatment applications were carried out in the beginning of each spring and one in the beginning of each autumn.

Semi-controlled field trial (Cotobajo) (SFT1)

The "Cotobajo" trial was located in a shading structure in the municipality of Guadalcázar (Córdoba province, upper Guadalquivir Valley; UTM coordinates X: 37.774812, Y: -4.960963), from May 2013 to June 2015, protected from rain and excessive sun by a plastic mesh cover. The experiment consisted of 20 l pots filled with a soil naturally infested by *V. dahliae* that was collected from a field

located in the municipality of Utrera (Sevilla, southern Spain). In this area, soils are annually cropped with cotton, tomato and eggplant (all *V. dahliae* hosts), so they are heavily infested with highly virulent strains of *V. dahliae* (cotton-defoliating pathotype) and the incidence and mortality of VWO have been reported to develop quickly (Trapero et al., 2013). A sample of 3.000 kg of soil was collected from this area and used to fill the pots. Subsequently, one olive plant cv. Picual was planted per pot, except an additional treatment with olive plants of susceptible cv. Cornicabra that was also included as a control treatment. Thirteen treatments selected from the 4, 5 and 6 Chapters were tested in this study (Table 1). The experiment was carried out in a randomized complete block design, with seven blocks and two replicates (pots) per block. Non-amended control treatments consisted of sterile soil (was considered as the sterile control) and naturally infested soil with *V. dahliae* (was considered as the inoculated control) (Figure 1).

Table 1. Biolcontrol treatments evaluated for their effectiveness against *Verticillium dahliae* in the semi-controlled field trial 1 (Cotobajo).

CODE	**TREATMENT**	**DOSE**
PICUAL	Water	2 l/plot
CORNICABRA	Water	2 l/plot
Bioten®	*Trichoderma asperellum+T. gamsii*	1:9 (w/w) treatment: soil
CAL03	Olive alperujo waste compost	1:9 (w/w) treatment: soil
FO12	*Fusarium oxysporum* non-pathogenic	1:9 (v:v) treatment:water
LAC02	Dairy waste (Lactic acid)	1:9 (v:v) treatment:water
MAN01	Poultry manure	1:9 (w/w) treatment: soil
MO1	Microorganism mixture EM5®	1:9 (v:v) treatment:water
MO2	Microroganism mixture (Modified EM1®)	1:9 (w/w) treatment: soil
MYCO	Mycorrhiza applied in the planting date	1:9 (w/w) treatment: soil
MYCO21	Mycorrhiza applied 21 days before the planting date	1:9 (w/w) treatment: soil
TEA01	Compost tea	1:9 (v:v) treatment:water
THYM01	Essential oil from *Thymus* sp. 01	1:9 (v:v) treatment:water

Figure 1. Field trial 1 (Cotobajo), Guadalcázar, Córdoba.

Field trial 2 (El Calvario) (FT2)

The "El Calvario" trial was conducted in naturally infested soil under field conditions in the municipality of Villanueva de la Reina (Jaen province, upper Guadalquivir Valley; X: 38.012827, Y: -3.909571) from May 2014 to May 2016. The plot had been cultivated with cotton along the previous 50 years, and it was surrounded by olive orchards severely affected by VWO.

The orchard consisted of 4 rows (4 m distance between rows; 1.5 m distance between plants within each row; 1,667 olive/ha) with a randomized block design with five blocks. Each block, consisted of 12 treatments selected from the chapters 4, 5 and 6 (11 biological treatments and one control) with three replications (olive trees) per treatment of cv. Picual. In addition, four olive trees of three different cultivars, cv. Frantoio, cv. Arbequina and cv. Picual (Table 2) were included in each block separating treatments, in order to evaluate the disease progress in other cultivars (Figure 2). The inoculum density and the pH of the soil of each treatment were recorded twice a year. For soil management, reduced tillage and herbicide applications were used. Biweekly irrigation of 20 L per tree in the dry season was applied.

Table 2. Biocontrol treatments evaluated for their effectiveness against *Verticillium dahliae* in the field trial 2 (El Calvario) or field trial 3 (Guadiana).

CODE	**TREATMENT**	**DOSE**[a]
PICUAL	Olive trees cv. Picual	-
ARBEQUINA	Olive trees cv. Arbequina	-
FRANTOIO	Olive trees cv. Frantoio	-
Bioten®	*Trichoderma harzianum* + *T. viride* Tusal®	1:9 (v:v) treatment:water
CAL03	Olive alperujo waste compost	4 l (amendment)
CALFO12	CALP03 + FO12	4 l (amendment)
MBLAC02	CALP03 + Dairy waste (Lactic acid LAC02)	4 l (amendment)
CGR03	Grape compost Montemayor15	4 l (amendment)
COPP	47% copper oxychloride, 4% nitrogen	0.2:9.8 (v:v) treatment:water
FO12	*Fusarium oxysporum* non-pathogenic 10^6 CFU/ml	1:9 (v:v) treatment:water
FO12 + PF04	*Fusarium oxysporum* non-pathogenic + *Pseudomonas fluorescens*	1:9 (v:v) treatment:water
MO1	Mixture of microorganisms	1:9 (v:v) treatment:water
PF04	*Pseudomonas fluorescens* 10^8 CFU/ml	1:9 (v:v) treatment:water
TEA01	Compost tea	1:9 (v:v) treatment:water

[a]The watering consist of 20 l water per olive tree

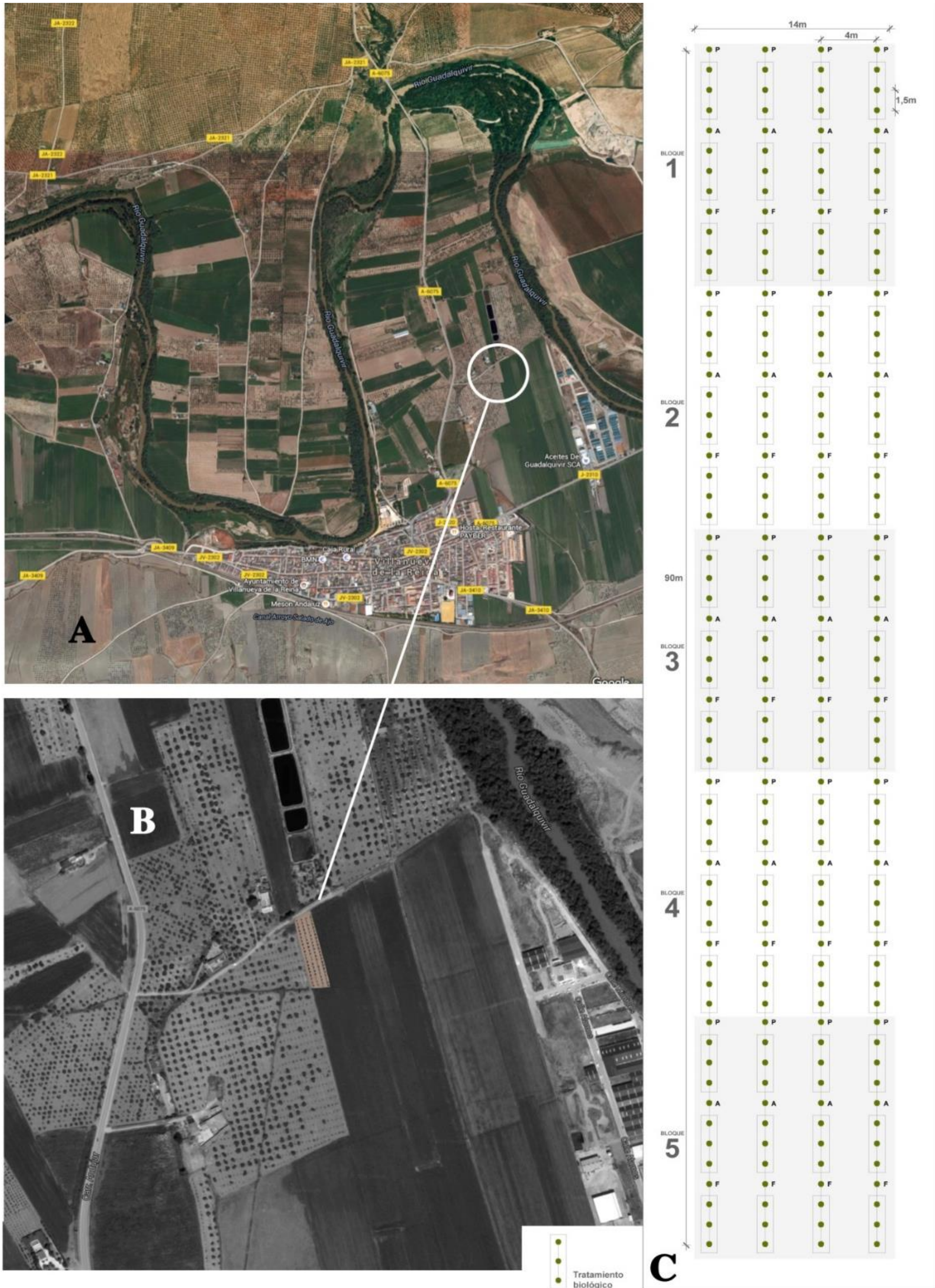

Figure 2. Field trial 2 (El Calvario), Villanueva de la Reina, Jaén. Green spots followed by the letters **P**, **A** and **F** corresponding with olive plants cv. Picual, cv. Arbequina and cv. Frantoio, respectively. The remainder olive plants are cv. Picual.

To determine the relation between the detected incidence of the disease and the inoculum density over time, a correlation of Pearson was performed between the mean values of disease and the corresponding inoculum density, and also between the increase of the disease and the corresponding inoculum density.

Field trial 3 (Guadiana) (FT3)

The “Guadiana” trial was conducted in naturally infested soil in the municipality of Peal de Becerro (Jaen province, upper Guadalquivir Valley; X: 37.909526, Y: -3.232036) from May 2015 up to day. The trial was established in an olive orchard with numerous *V. dahliae* affected olive trees. Olive trees were planted where previously a plant had died, and there were two types of plants: 30-year-old olive cv. Picual and one-year-old olive cv. Picual. Treatments included the non-pathogenic *Fusarium oxysporum* strain FO12, the grape compost CGR03 (Table 2) and an untreated control. The field experiment was performed in a complete block design with 15 blocks. In each block, there were three treatments (two biocontrol treatments and one untreated control) with one replicated tree of each age (Figure 3). The inoculum density and the pH of the soil of each treatment were recorded twice a year. For soil management, reduced tillage and herbicide applications were used according to the traditional management of commercial olive groves in the area. Automatic daily drip irrigation lines of 16 L per tree were used for watering the whole field plot.

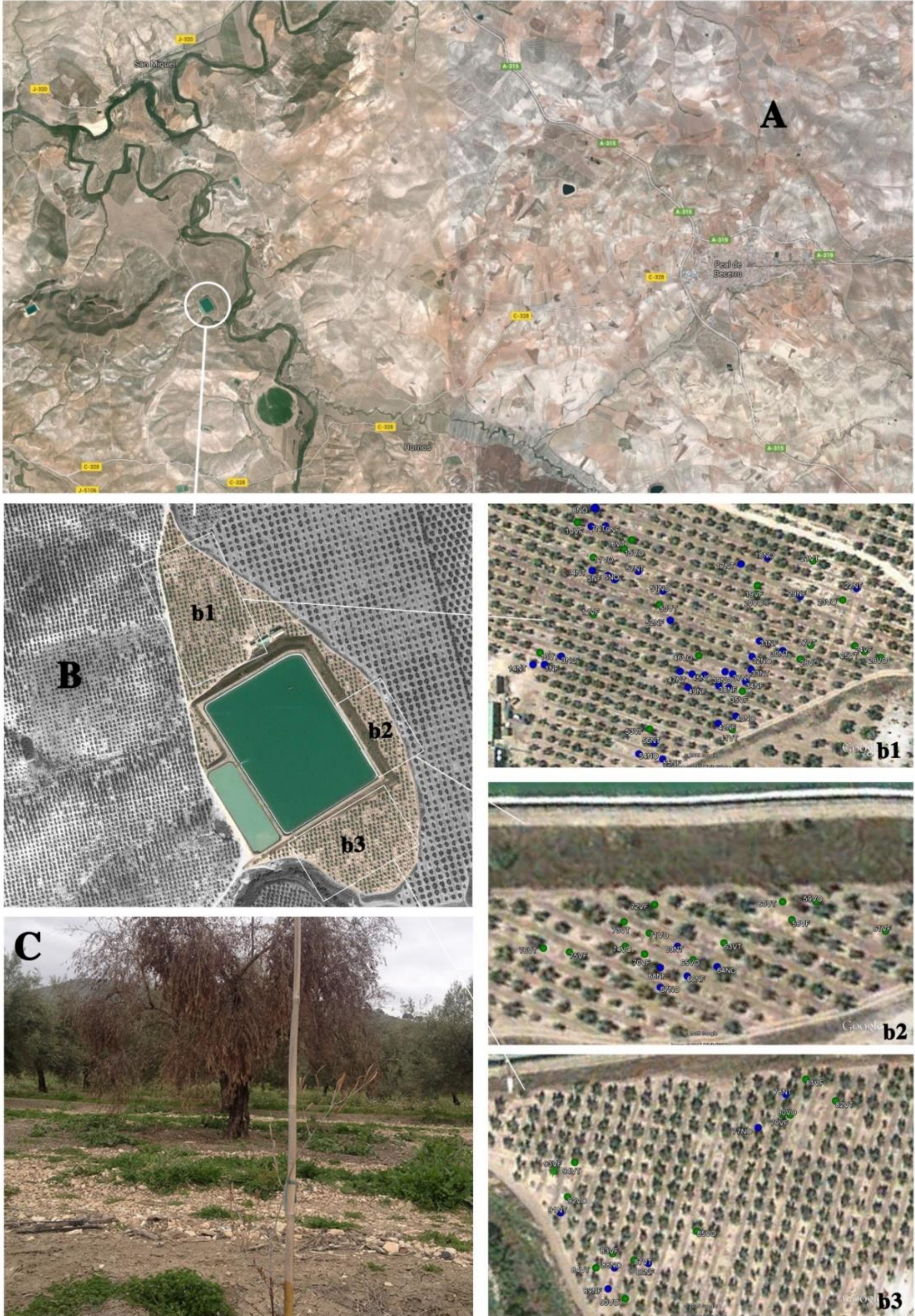

Figure 3. Field trial 3 (Guadiana), Peal de Becerro, Jaén.

Disease assessment

The SFT1, FT2 and FT3 trials were inspected every two weeks for disease symptoms. 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 percentage of affected tissue using four main categories or quarters (≤25, 26-50, 51-75, and 76-100%) with four values per each 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: $Y = 6.25X - 3.125$. 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 incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (Wilhelm and Taylor, 1965; López-Escudero et al., 2004).

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). In brief, affected twigs showing disease symptoms mentioned above were collected from wilted plants (three 20 cm long twigs per tree). When leaf petioles were used, ten defoliated leaves per tree were collected just after affected shoots in the tree were shaken. For pathogen isolation, pieces of collected twigs were washed in running tap water, bark was removed and woody tissues surface disinfesting in 0.5% sodium hypochlorite for 1 min. Chips of wood were placed onto potato dextrose agar (PDA) or acidified PDA. Plates were incubated at 24ºC in the dark for 5-6 days. Leaf petioles cut from defoliated leaves were processed in a similar way.

Data analysis

In the three field trials, analysis of variance (ANOVA) of the inoculum density (ID), final disease severity, the RAUDPC and AUDPC were performed for

each experiment because the values met the assumptions of normality and homogeneity of variances for this analysis.

The inoculum density data were analysed in a completely randomized experimental design. The final disease severity and the RAUDPC were arranged in a randomized block design (seven blocks and two plants per block and per treatment for SFT1 and five blocks and three plants per block and treatments for FT2). The FT3 was arranged in a factorial design with two independent factor age x treatment and 15 blocks and one plant per block, age and per treatment.

When ANOVA showed significant differences among treatments, mean values were compared using Tukey´s HSD test at P = 0.05. Both incidence and mortality were analysed by multiple comparisons for proportions test (P = 0.05) (Zar 1999), which considered the observed and expected frequencies of symptomatic and dead plants, respectively.

RESULTS

Cotobajo trial

No disease symptoms were observed 24 months after the trial establishment. However, significant differences (P < 0.001) on ID were observed between treatments. The initial ID was 5.5 MS g^{-1} and decreased steeply during the 24 months of the experiment in all treatments evaluated. The treatments FO12 and THYM01 were the most effectives on the inhibition of the pathogen in natural infested soil at two as well as at 12 and 24 months after inoculation, showing an ID reduction of 80 and 66.6%, respectively. Moreover, both treatments showed the most consistent results over time of the experiment. In addition, at twelve months the MO1 and CALP03 treatments were also significant differences to the control treatment (Table 3).

Table 3. Relative inhibition (%) of soil inoculum density in potted olive plants treated with biological amendments and maintained in semi-controlled conditions.

Treatment (n=14)	Inhibition (%) of soil inoculum density[a]		
	2 months[b]	12 months	24 months
Bioten®	51.3 b	22.6 cd	3.3 ef
CALP03	0 e	68.1 ab	30.0 cde
FO12	100 a	81.8 a	80.0 a
LAC02	28.2 cd	36.3 bcd	43.3 bc
MAN01	0 e	15.8 d	0 f
MO1	29.5 cd	56.8 abc	40.0 bcd
MO2	10.2 de	26.1 cd	3.3 ef
MYCO21	46.8 bc	36.5 bcd	0 f
TEA01	5.1 e	44.3 bcd	8.3 def
THYM01	100 a	86.3 a	66.6 ab

[a]Relative reduction of inoculum density in soil compared to the control treated with water.
[b]Means in a column followed by the same letter do not differ significantly according to Tukey's honestly significant difference (HSD) test at $P = 0.05$.

El Calvario trial

In this field trial, VWO symptoms with high severity were first observed 14 weeks after planting. Typical symptoms of the disease such as wilting, dieback, and/or defoliation, were observed on affected plants. Wilting primarily started at the lower branches and developed as generalized green leaf defoliation that spreads to the entire tree canopy. Moreover, partial defoliations of green leaves affecting the higher branches were also observed occasionally. Flower mummification occurred during spring and early summer. Occasionally, primarily in early spring, affected plants exhibited wilt, chlorosis and rolling of leaves without the defoliation of green leaves. These plants became completely wilted, and necrotic leaves remained attached to the shoots.

No significant differences on disease suppression was found on treated plants with different biocontrol treatments 24 months after the plantation

establishment (*P* = 0.1015 for RAUDPC). However, data of disease severity at the end of the experiment showed significant differences (*P* = 0.0018) between treatments, but no significant differences with the control treatment by using olive cuttings cv. Picual (PIC). Only the moderately susceptible cv. Arbequina and the resistant cv. Frantoio differed significantly from the control treatment PIC (*P* < 0.001) (Table 4). The incidence of the disease increased quickly showing a high value of RAUDPC, almost 50% of plants were affected, only seven months after the plantation establishment (data not show). This could be due to the mild temperatures in summer 2014 with average temperatures of 30ºC. No significant differences were observed for disease incidence (*P* = 0.2022).

After 24 months, no significant differences (*P* = 0.1851) were observed on the percentage of dead plants between plants treated with biological amendments and control PIC. However, plant mortality on olive cuttings cv. Arbequina (Moderately susceptible) and Frantoio (Moderately resistant) was significantly lower than those observed in all treated plants as well as in control PIC. The mortality in olives cv. Arbequina was slightly higher (10% of dead plants) than those observed in olives cv. Frantoio (5% of dead plants). The time elapsed from the plantation establishment to the point where 50% of trees were diseased (DI_{50}) for biological treatments ranged from 37 to 96 weeks. The TEA01 treatment showed the lowest value of DI_{50} (34) while the COPP01 (96) showed the highest DI_{50} period (Table 4).

Concerning the ID, the biological amendments applied to the olive trees were effective on the reduction of the inoculum (Figure 4). At the beginning of the plantation, the ID showed values of 35 ppg, being reached up to 40 ppg at two months after plantation in control plants. At the same time, MO1 was the most effective treatment showing a reduction of 71.3% of ppg with significant differences (*P* = 0.0327) between the remainder treatments. However, 14 months after plantation, no significant differences were observed between all treatments and control, except the TEA01, which showed up to six times more ppg than those observed in the control treatment. Finally, 19 months after plantation, the

treatments COPP01, MO1, CGR03 and FO12 showed a significant reduction ($P <$ 0.0001) of the ppg: 69.1, 94.4, 94.76 and 96.5%, respectively. The pH of the soil in each treatment ranged between 8.0 and 8.6, and did not show significant differences between treatments.

Table 4. Disease parameters in the olive trial El Calvario established on a soil naturally infested by *Verticillium dahliae* and treated with eleven biological amendments.

TREATMENTS	Incidence (%)[a]	Mortality (%)[a]	DI_{50}[b]	Disease severity[c]	RAUDPC[c]
PIC	77 ab	60 ab	44	70.8 ± 6.7 ab	101.3 ± 12.8 a
ARB	35c	10 c	+96	14.8 ± 6.7 c	25.8 ± 8.9 b
FRA	35 c	5 c	+96	18.3 ± 7.6 c	18.0 ± 7.7 b
Bioten®	87 a	67 ab	39	83.8 ± 7.1 a	121.6 ± 25.9 a
CALP03	93 a	73 a	38	78.3 ± 8.5 ab	154.0 ± 36.4 a
CALP03+FO12	93 a	80 a	37	86.3 ± 6.8 a	188.7 ± 23.9 a
CALPLAC	80 ab	60 ab	50	67.5 ± 10.0 ab	118.9 ± 23.6 a
CGR03	93.3 a	87 a	39	90.2 ± 5.8 a	171.1 ± 26.5 a
COPP01	47 b	27 b	96	36.9 ± 11.6 b	124.9 ± 52.3 a
FO12	87 ab	60 ab	48	61.7 ± 11.6 ab	153.0 ± 29.8 a
FO12+PF04	80 ab	60 ab	48	63.3 ± 11.3 ab	100.3 ± 25.1 a
MO1	60 b	40 b	55	50.0 ± 11.3 ab	90.5 ± 28.9 a
PICF04	87 a	67 ab	53	72.3 ± 9.5 ab	100.7 ± 21.3 a
TEA01	87 a	80 a	34	88.8 ± 6.6 a	170.0 ± 25.8 a

[a]Percentage of plants showing Verticillium wilt symptoms or dead plants 24 months after plantation establishment. Mean values in the same column followed by the same letter are not significantly different according to the multiple comparisons for proportions test at P = 0.05 (Zar, 1999).
[b]DI_{50} = Time in weeks from planting until 50% of the plants were affected.
[c]Final disease severity 24 months after inoculation based on a 0-16 rating scale and relative area under the disease progress curve (RAUDPC) developed over the assessment period. Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at P = 0.05.

There was no correlation between the different inoculum density evaluations and disease incidence values during the study period. This lack of correlation could be due to the high pressure of inoculum density at the beginning of the plantation establishment that caused the early infestation of plants.

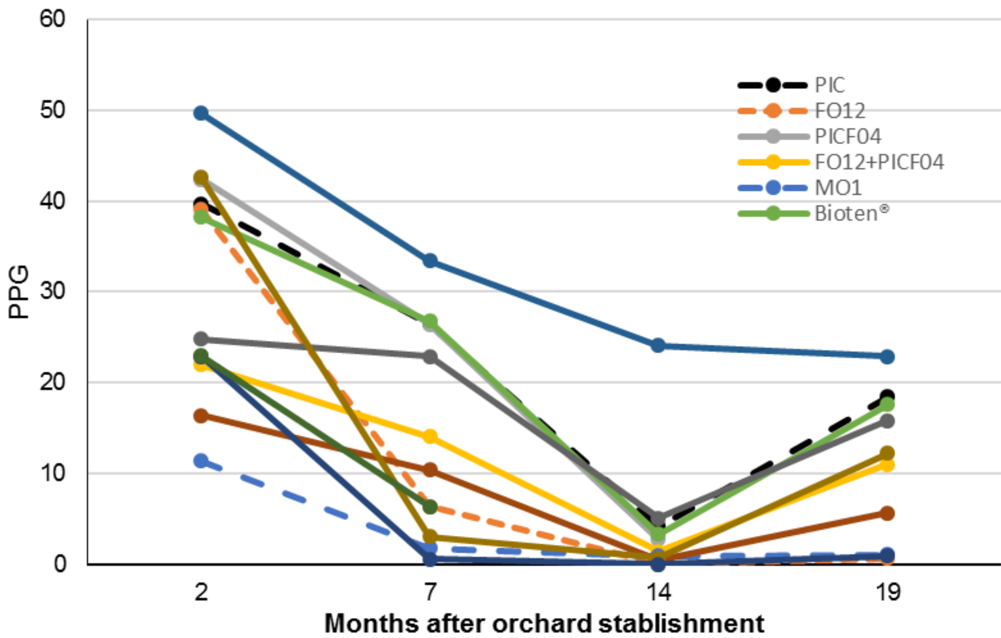

Figure 4. Variation of soil inoculum density over time in the El Calvario trial established on a soil naturally infested by *Verticillium dahliae* and treated with eleven biological amendments or mixes of them.

Guadiana trial

In this field trial, typical symptoms of the disease were observed on affected plants. The first symptoms were observed in early spring, just at 9 months after the plantation establishment. Affected plants exhibited wilt, chlorosis and rolling of leaves or defoliation of green leaves. The onset of the disease was detected in both age olives.

In olive plants, no significant differences for disease suppression were found in the treated plants with both biocontrol treatments regarding the control and tested in this field at 12 months after the plantation establishment (P = 0.5208 for AUDPC and P = 0.5531 for final disease severity). The interaction between age

and treatment did not show significant differences, although two homogeneous group were detected (P = 0.0530).

Treatments with CGR03 on one-year-olive plants only showed 6.7% of symptomatic plants whereas control and FO12 treatments showed 20 and 33.3 % of symptomatic plants, respectively. The differences on mortality observed between treated and control plants depended on the age of trees. One-year-olive plants treated with FO12 showed 13.3% of mortality, whereas no mortality (0 %) was observed for control and CGR03 treatments (Table 5). These are preliminary results. Guadiana trial is a long-term experiment in which the disease symptoms have just merged now. More evaluations will be conducted along the coming years in order to complete this study.

Table 5. Disease parameters in the olive trial Peal established on a soil naturally infested by *Verticillium dahliae* and treated with two biological amendments.

TREATMENTS	**Age plants (year)**	**Incidence (%)[a]**	**Mortality (%)[a]**	**Disease severity[b]**	**AUDPC[b]**
CONTROL	1	20 a	0 a	1.0 a	6.8 a
	30	20 a	6.7 a	9.3 a	128.9 a
FO12	1	33.3 a	13.3 a	19.4 a	234.8 a
	30	26.7 a	0 a	4.3 a	58.3 a
CGR03	1	6.7 a	0 a	1.0 a	2.3 a
	30	20 a	13.3 a	9.8 a	157.6 a

[a]Percentage of plants showing Verticillium wilt symptoms or dead plants 12 months after plantation establishment. Mean values in the same column followed by the same letter are not significantly different according to the multiple comparisons for proportions test at P = 0.05 (Zar, 1999).

[b]Final disease severity 12 months after inoculation based on a 0-16 rating scale and area under the disease progress curve (AUDPC) developed over the assessment period. Means in a column followed by the same letter do not differ significantly according to Tukey's HSD test at P = 0.05.

DISCUSSION

Verticillium dahliae is a great concern to the olive growers, given the lack of control measures to effectively control VW. This led us to invest efforts to focus on an integrated strategy using new biological treatments. The first step to achieve

effective biocontrol is to find a suitable source of potential biological treatments. In this way, in this current study, a total of 14 treatments including biological controls agents, organic amendments and mixtures of them were evaluated in field conditions. All of these products were carefully selected from previous studies conducted under controlled conditions in which a total of 170 treatments were tested. In these previous studies, the 14 treatments selected were effective on the suppression of *V. dahliae* growth *in vitro*, in natural infested soil and also, in planta conditions.

The biological control strategy needs a consistent system that allows for the selection of biological agents *in vitro* or controlled conditions plant bioassays, as well as under field conditions (Daayf et al., 2003). The selection of different types of biocontrol agents against pathogens such as *V. dahliae in vitro* has been actively explored (Berg et al., 2001). However, discrepancy between the antagonistic effects demonstrated *in vitro* and the corresponding effect in microsclerotia and in planta has been repeatedly reported (Weller, 1988; Reddy et al., 1994). For this reason, checking of the suppressive effect observed under controlled conditions of these 14 treatments selected under field conditions is required to complete our study.

The biological treatments used in this study originate from soils with Verticillium susceptible hosts. They are characterized by hold certain biological niches in the olive fields. For example, i) most of these compounds have been previously characterized for their ability to populate rhizosphere (Eparvier & Alabouvette, 1994); ii) *Fusarium oxysporum* FO12 has been described as non-pathogenic (Varo et al., 2016); iii) *Pseudomonas fluorescens* PF04 originate from olive rhizosphere (Mercado-Blanco et al., 2004); iv) the organic amendments such as the alperujo composts originate from the olive cropping ecosystem (Chapter 5, Trillas et al., 2006), whereas CGR03 and LAC amendments not originate from olive cropping system, but their ability to colonize and their suppressive effect have been previously well studied (Markakis et al., 2016).

In SFT1, no disease symptoms were observed on potted plants. We hypothesized that the volume of the plastic pots used in this study (0.7 to 20 l) is not enough to induce disease development in potted plants. In fact, previous studies conducted to evaluate the incidence of VWO under semi-controlled conditions demonstrated that 1.000 l microplots and 10 ppg of inoculum are needed to obtain 100 % of the final incidence of VWO in the susceptible olive cv. Picual (López-Escudero and Blanco-López, 2007; Pérez-Rodríguez et al., 2015). The natural soil used in this trial, is considered conductive because they were collected from olive orchards and cotton fields with a high incidence of the disease. Our results using potted plants contrast with the olive cv. Picual responses to the pathogen under field conditions, where 0.8–4 MS g^{-1} is enough to cause a severe epidemic during the first years after planting (Trapero et al., 2013b; Roca et al., 2016). The great variability in the response of potted olive plants to the pathogen using naturally infested soil may be due to the architecture or development of confined root systems or changes in the chemical stimulants of the root exudates of olive plants. It suggests that the evaluation of VW incidence and severity on potted plants of olive or other woody crops is unfeasible to obtain consistent results. Conversely, when VW severity is evaluated on potted plants by using herbaceous crops such as cauliflower, the results obtained are usually homogeneous (Xiao and Subbarao, 1998). In spite of these difficulties, significant differences were observed in the ID between treatments, being FO12, THYM01, LAC and MO1 the most effective treatments. The possibility to evaluate all of these products tested in SFT1 using potted plants of an herbaceous crop such as cotton could be useful to obtain more homogeneous and consistent results in the future.

Due to the non-homogeneous results obtained in the SFT1 by using potted plants, the FT2 was carried out under field conditions by using olive cuttings planted directly in naturally infected soils. In this trial, the high pressure of inoculum density at the beginning of the plantation establishment that caused the early infestation of plants, and the weather conditions during summer 2014 with moderate temperatures did not allow to obtain successful and consistent results. In

this case, no significant differences were detected in the AUDPC between treatments. However, the ID progress curves showed significant differences between treatments, being CGR03 and FO12 the most effective treatments with 94.76 and 96.5% of ID reduction, respectively. Some of these results are in agreement with those obtained by the chapter 5 of this thesis, where has been demonstrated the suppressive effect of the grape compost to inhibit *V. dahliae* growth *in vitro* and disease development. Non-pathogenic strains of *Fusarium oxysporum* have also been identified as potential BCAs for Verticillium wilt diseases (Angelopoulou et al., 2014; Veloso et al., 2015) One of the potential 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 another species of *Fusarium* (Lemanceau & Alabouvette 1991).

The level of resistance of the three cultivars evaluated under natural conditions cv. Picual, cv. Arbequina and cv. Frantoio is supported by studies showing reduced ability of *V. dahliae* to colonize cv. Arbequina and cv. Frantoio compared to 'Picual' (López-Escudero et al., 2004; Martos-Moreno et al., 2006; López-Escudero et al., 2007; López-Escudero and Mercado-Blanco, 2011; Bubici and Cirulli, 2012; Trapero et al., 2013). However, Frantoio and Arbequina cultivars did not exhibit complete resistance when they were planted in a soil that was heavily infested with *V. dahliae* in the current field study, in accordance with Trapero et al. (2013)

Finally, the FT3 was conducted to evaluate the effectiveness of the best treatments (FO12 and CGR03) resulted of the whole screening work in a natural infested soil with a lower inoculum density. Presently, first disease symptoms have been observed in this trial, but no significant differences for disease suppression have been found yet in the treated plants with both biocontrol treatments with regard to the control. This is a long-term trial in which the evaluations will be completed along the coming years. Thus, the results presented in this study are partial results.

This work demonstrated the difficulties to evaluate the biological treatments against VWO and their application in field conditions. Nevertheless, the results obtained in this study suggest that grape compost amendments or non-pathogenic *F. oxysporum* strains could be useful to reduce the inoculum density of VWO in field conditions. This work provides a practical basis for the potential use of these selected treatments. Their application in the substrate material used during the nursery olive propagation progress as well as in the new stablished plantations could be a potential strategy to reduce the inoculum density of VWO in the soil and consequently the percentage of affected plants. However, further research is needed towards to elucidate the suppressive effect of these compounds in naturally infested soils by VWO and to improve their efficiency in field conditions.

Discusión General

DISCUSIÓN GENERAL

Durante los últimos 35 años, el sector olivarero español ha tenido que enfrentarse a la Verticilosis del olivo sin medidas de control eficaces, por lo que se ha venido recomendando una estrategia de control integrado para paliar en lo posible su rápida expansión con las graves consecuencias para el olivar (Trapero y Blanco, 2008; López-Escudero y Mercado-Blanco, 2011). La ausencia de medidas de control químico para esta enfermedad, junto a las sucesivas restricciones de agroquímicos por parte de la Unión Europea, justificadas en aras de garantizar la sostenibilidad medioambiental y la salud pública, han conducido a profundizar en el estudio del control biológico como solución sostenible y eficaz a las enfermedades de los cultivos. El primer paso para la implementación de una estrategia de naturaleza biológica, dentro de un control integrado de la Verticilosis del olivo, consiste en realizar una selección masiva de potenciales tratamientos biológicos.

Recientes estudios en el control de otras enfermedades de suelo han mostrado que el empleo de agentes de control biológico (ACB), enmiendas compostadas y sustancias naturales surge como una medida de gran interés para el control de la Verticilosis del olivo (Mercado-Blanco et al., 2004; Martos-Moreno et al., 2006; Jiménez Díaz et al., 2009; Markakis et al., 2015). La presente tesis doctoral aborda este reto y representa el primer pilar en el que podrán basarse estudios claves posteriores. Estos se incluirán dentro del marco de medidas de lucha disponibles para solucionar la principal enfermedad que afecta al olivo en la cuenca mediterránea y otras regiones olivareras del mundo.

Esta tesis doctoral presenta un enfoque diferente, en el que se propone identificar los tratamientos más eficaces, entre todos los disponibles, atendiendo a criterios de cercanía en la disponibilidad y viabilidad económica para el agricultor, indistintamente del modo de acción.

Actualmente, junto con la mejora genética, el uso de agentes o tratamientos biológicos para el control de esta enfermedad ha suscitado un gran interés, como indican los trabajos de Mercado-Blanco et al. (2004), Martos-

Moreno et al. (2006), Jiménez Díaz et al. (2009) y Markakis et al. (2015). Una de las limitaciones que surge ante este reto es identificar potenciales tratamientos. Numerosos estudios han mostrado discrepancias entre el efecto antagonista mostrado por determinados tratamientos *in vitro* y los correspondientes en condiciones naturales (Weller and Cook, 1983; Reddy et al., 1994), hecho al que se añade la dificultad de que el cultivo objeto de la evaluación es una especie leñosa (López-Escudero y Mercado-Blanco, 2011). Por ello, surge la necesidad de un rápido y eficaz método de inoculación artificial de plantas de olivo que reproduzca las condiciones naturales de campo y que permita la evaluación de tratamientos biológicos contra *V. dahliae*. En este trabajo se han estudiado y comparado métodos de inoculación que tradicionalmente se han utilizado para olivo u otros cultivos, así como para *V. dahliae* u otros patógenos, de los cuales, el método de infestación del suelo mediante AMA a una dosis del 20% (peso/peso) fue seleccionado en base a la respuesta severa, consistente y homogénea de las plantas y que permitió establecer diferencias entre los tratamientos biológicos. Varios son los métodos de inoculación que se ensayaron sin éxito, por ejemplo, se utilizaron 4 suelos naturales con diferente nivel de inóculo, en macetas de 0.8 l, sin embargo, no se logró la infección de la planta. Hecho que se achaca a la propia arquitectura del sistema radical del olivo y la ausencia de exudados suficientes, ambas causas debidas a la confinación de las raíces de la planta y la ausencia de crecimiento dentro de la maceta.

Además, como paso previo a la comparación de diferentes métodos de inoculación, se llevó a cabo la producción en masa de MS. Las técnicas de producción de ME empleadas por Tjamos y Fravel (1995) y López-Escudero et al. (2006) fueron reproducidas sin éxito para la producción de microesclerocios. Este fue el caso del aislado V117, un aislado defoliante con una patogenicidad ampliamente estudiada y perteneciente a la colección de hongos del Departamento de Agronomía de la Universidad de Córdoba, que tras largo tiempo almacenado y tras sucesivas reutilizaciones, perdió la capacidad para formar microesclerocios. Esta pérdida de capacidad de producir estructuras de

resistencia es común en aislados de *V. dahliae* (Hu et al., 2013; Varo et al., 2016). Por este motivo, se ensayaron 12 medios de producción de ME, de los que se seleccionó el medio MSPM2 con un pH de 11,5, dada la eficiencia de este medio para cepas defoliantes y no defoliantes y la alta producción de ME obtenidos. Además, se detectó, al igual que el estudio llevado a cabo por Hu et al., (2013), que el pH alto fue crucial para para obtener una cantidad de MS suficiente.

Una vez superadas estas limitaciones, se ha comenzado la selección en 4 etapas de los mejores candidatos frente a *V. dahliae*, atendiendo a su efecto sobre el crecimiento micelial, sobre la viabilidad de los MS en un suelo naturalmente infestado, sobre la infección de plantas de olivo en condiciones controladas y finalmente, en campo. No obstante, se ha optado por diferenciar los tres grandes grupos de posibles tratamientos biológicos (ACB, enmiendas compostadas y sustancias naturales) para facilitar la comprensión de este trabajo.

La selección de potenciales tratamientos se realizó tras la evaluación masiva de bacterias, hongos y mezclas de microorganismos, donde la sinergia de los efectos producidos por diferentes microrganismos fue la clave. Así como, enmiendas compostadas mejoradas con ACB y mezclas de sustancias; y tratamientos con aceites esenciales y extractos vegetales. En la primera etapa y evaluación *in vitro* se determinó el efecto antagonista según la reducción del crecimiento micelial de *V. dahliae*. La formación de zonas de inhibición del crecimiento del patógeno en los experimentos de cultivos duales o de biofumigación se debe a la producción de antibióticos, metabolitos tóxicos y/o sideróforos los cuales ejercen como mecanismos de control del patógeno (Swadling y Jeffries, 1996). En esta etapa destacaron varias cepas de *F. oxysporum, Rhizopus* sp., *Trichoderma* sp., los cuales son hongos con una tasa de crecimiento más elevada que *V. dahliae.* También se identificaron por su potencial antagonista el extracto de levaduras procedente de vinagre de vino VIN02, la enmienda animal Gallinaza y el residuo de quesería Plantiforte®, alcanzando un efecto inhibidor del 100%. Resultados muy alentadores se detectaron con los aceites esenciales de *Thymus* sp. y *Citrus* sp., y con extractos

acuosos de *Thymus* sp. Una alta proporción de tratamientos fueron considerados igualmente eficaces, con un efecto inhibidor del crecimiento miceliar del patógeno entre el 90-50%, esta gran cantidad de tratamientos potencialmente eficaces no es extraordinaria, como se ha determinado en investigaciones anteriores (Zheng et al., 2011).

La segunda fase del "screening" masivo de potenciales tratamientos biológicos tuvo mayor importancia que la etapa *in vitro*, debido a que se evaluó el efecto de los tratamientos biológicos sobre las estructuras de resistencia del patógeno, ME, ya que estas estructuras de supervivencia, infección y dispersión del hongo le permiten sobrevivir en el suelo en ausencia de huéspedes durante varios años (Goud et al., 2003). En esta etapa se seleccionaron varias cepas de las especies fúngicas *F. oxysporum* y *G. roseum*, las mezclas de microorganismos MO1 y MO2, y el producto comercial Bioten®. Varias enmiendas orgánicas fueron igualmente efectivas, como son la Gallinaza (aunque el efecto inhibidor decreció con el tiempo), Plantiforte®, el extracto de compost TéCB, el cobre Folicupro® y dos aceites esenciales de *Thymus* sp. Además, hubo un efecto inhibidor en los compost mejorados debido a la sinergia provocada por los beneficios del compost y el aditivo.

Una vez completadas estas dos etapas, se continuó con ensayos en planta. El desarrollo de la metodología de infestación artificial del suelo y las evidencias de efectividad de determinados tratamientos permitió continuar con la selección masiva de una forma eficiente y fiable, permitiendo detectar diferentes niveles de efectividad en planta. En esta etapa se ha permitido, además de evaluar la eficacia de determinados tratamientos, elucidar a grandes rasgos el modo de acción. La cepa del *F. oxysporum* no patógenica FO12, ha surgido como uno de los candidatos más prometedores por su efecto antagonista de *V. dahliae in vitro*, sobre ME y en planta. En un ensayo adicional ha sido evaluada por su efecto de inducción de resistencia sistémica mediante aplicación al suelo y a la parte aérea. Además, tras realizar un extracto crudo, se realizó una separación física del sobrenadante y la cepa para determinar la fracción responsable del efecto contra

V. dahliae. Los resultados obtenidos han determinado que el efecto antagonista de esta cepa se debe a varios mecanismos de acción, ya que, el efecto del extracto crudo fue mayor que las fracciones por separado. Estos resultados coinciden con los trabajos anteriores de Fravel et al. (2003) y Gizi et al. (2011) en los que se determinó el efecto conjunto de mecanismos de antibiosis, micoparisitismo, competición e inducción de resistencia de cepas no patogénicas de *F. oxysporum*.

Otro de los tratamientos más prometedores fue el compost de orujo de vid natural CVID01. Se evaluó natural y tras un proceso de esterilización, libre de microorganismos. Los resultados obtenidos confirmaron que el efecto de control contra *V. dahliae* es debido a los microorganismos que están presentes en el compost, ya que el control de la enfermedad en plantas tratadas con el compost natural fue mucho mayor que las tratadas con el estéril, aunque es necesario elucidar este efecto en futuros ensayos. Es destacable que no existen estudios publicados hasta la fecha, del efecto de compost de orujo sobre *V. dahliae* en olivo.

Con los mejores candidatos seleccionados en los experimentos realizados en condiciones controladas, se han llevado a cabo tres experimentos en campo: uno en condiciones semicontroladas y dos en condiciones naturales. Algunos de los candidatos seleccionados eran originarios de nichos biológicos del olivar y otros tratamientos habían mostrado efectos favorables frente a otras enfermedades vasculares (Eparvier y Alabouvette, 1994; Lemanceau y Alabouvette 1991; Trillas et al., 2006; Varo et al., 2016c; Chapter 5). Concretamente, los tratamientos seleccionados fueron: las mezclas de microorganismos MO1 y MO2, la cepa FO12 del hongo *F. oxysporum*, la cepa PICF4 de la bacteria *P. fluorescens*, un combinado de FO12 + PF04, el producto comercial Bioten®, el residuo de quesería Plantiforte, una enmienda animal a base de gallinaza, el compost de orujo de vid Natural CVID01, los compost mejorados con Plantiforte y con la cepa FO12, el Té de compost TEA01 y, como control, el cobre comercial COPP01.

Los resultados arrojan una eficacia de determinados tratamientos, coincidentes en efectividad con los resultados obtenidos en las anteriores etapas del "screening". Aunque es necesario destacar que la elevada presión del patógeno, con una densidad de inóculo cercano a 35 UFC/g suelo en la segunda finca seleccionada (Villanueva de la Reina, Jaén), provocó un rápido desarrollo de la enfermedad en los primeros meses del experimento, este hecho fue comprobado igualmente por Trapero et al. (2013b). Al final del experimento, la alta densidad de inóculo de este campo no permitió establecer diferencias significativas entre tratamientos respecto a la incidencia y severidad de la enfermedad. Sin embargo, se pudo identificar una notable reducción de la densidad de inóculo del patógeno en el suelo a lo largo del tiempo debida a varios tratamientos. Por ello, se incluyó en la presente tesis doctoral un experimento posterior en Peal de Becerro con dos de estos tratamientos, compost de orujo CVID01 y la cepa FO12, en una finca cuya densidad de inóculo es menor (3.5 UFC/g), los resultados futuros de este trabajo podrán confirmar la eficacia sobre la enfermedad.

Los resultados obtenidos permiten albergar esperanzas con relación al control de la Verticilosis del olivo en campo. No obstante, los mejores candidatos seleccionados en este trabajo deben ser evaluados en varios campos con diferentes densidades de inóculo y condiciones edafoclimáticas, con el fin de determinar su verdadero potencial y sus limitaciones para el control de esta grave enfermedad del olivar. Asimismo, en condiciones controladas, es necesario profundizar en el conocimiento sobre el mecanismo o mecanismos de acción de los mejores candidatos con el objeto de optimizar su modo de aplicación.

Conclusions

Conclusions

1.- In order to implement the biological control of Verticillium wilt of olive (VWO), a mass screening of biological products has been developed. The approach to find potential biocontrol treatments has typically involved processes that require a lot of time and labor. Therefore, an effective method for the selection was developed at the beginning of this study. This massive selection of candidates has convered different microorganisms (various fungi, bacteria and their extracts), organic amendments (OAs) (waste from plants, animals and the food industry) and water extracts and essential oils from several Mediterranean plants for the control of *V. dahliae*.

2.-Mass production of microsclerotia (MS) for artificial infestation of soil is a critical point for the study of epidemiological and control aspects of VWO. To overcome the fail in the production of MS in recalcitrant isolates, a culture medium was optimized and successful inoculation plant experiments were carried out using MS produced *in vitro* (Chapter 2).

3.-The modified sodium polipectate (MSP) medium amended with 0.1% agar was the most suitable medium for the production of MS of *V. dahliae* isolates that have lost the ability to produce MS in the standard culture media (Chapter 2).

4.- Five inoculation methods were compared for screening BCAs. The CMS at 20% was the most effective method and it has been used to evaluate 170 biological treatments for their efficacy against isolates of *V. dahliae* prevalent in southern Spain (Chapter 3).

5.- Overall, the root dip inoculation method is considered a successful method to evaluate the resistance/susceptibility of olive cultivars, but this method has limitations to the screening for BCAs. Also, the inoculation with artificially produced *V. dahliae* MS has important limitations due to the necessary long production period and the lack of productive capacity of the isolates preserved for a long time (Chapter 3).

6.- A total of 47 strains and nine mixtures of microorganisms were evaluated against *V. dahliae*. This screening has resulted in promising fungi and bacteria

strains with antagonistic activity against *Verticillium*, such as two non-pathogenic strains of *F. oxysporum*, one strain of *Phoma* sp., one strain of *P. fluorescens* and two mixtures of microorganisms, which may have multiple modes of action (Chapter 4).

7-. The beginning of the development of a control strategy based on the application of OAs was conducted in this study. Thirty-five OAs and sixteen compost mixtures have been assessed against *V. dahliae.* The overall results showed that VWO was effectively suppressed when plants were grown in a substrate mixed with composted grape marc, or composted alperujo combined with other OAs, such as compost tea and dairy waste. Moreover, the pathogen was significantly reduced in soils that were naturally or artificially infested with the highly virulent pathotype (Chapter 5).

8-. The potential effect of 44 plant extracts and 20 essential oils against *V. dahliae* were evaluated in this study. The results demonstrate the *in vitro* and *in planta* effectiveness of essential oil from *Thymus*, particularly *Thymus* sp. 04 (prepared in the laboratory) and the commercial product *Thymus* sp. 01. The inhibition of mycelial growth and microsclerotia in soil reached 100% in both treatments and achieved a disease reduction in olive plants by 65% and 42% for *Thymus* sp. 04 and sp. 02, respectively. We suggest that Thymus-based treatment could be integrated into the control practices for this disease (Chapter 6).

9-. This is the first report of the use of essential oils to control VW in olive plants. Further studies are warranted to identify the bioactive compounds in the essential oil that control *V. dahliae* and evaluate their potential use as natural fungicides (Chapter 6).

10-. Based on the results of the experiments under controlled conditions, we have selected 14 biological treatments which have been tested in three field experiments, one of these experiments, FT3, is still ongoing. The FO12 and CGR03 treatments are the most promising, but these and other potential treatments must be confirmed in further experiments in field soils with different inoculum densities.

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REGULAR ARTICLE

Kill or cure? The interaction between endophytic *Paenibacillus* and *Serratia* strains and the host plant is shaped by plant growth conditions

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Abstract
Aims Verticillium wilt is difficult to suppress, and causes severe yield losses in a broad range of crops. Five *Serratia* and five *Paenibacillus* endophytic isolates showing antagonistic properties against fungal pathogens were compared for their plant growth-promoting (PGP) potential under different plant growth conditions with the objective of evaluating the PGP of endophytic strains in different *ad planta* systems.
Methods Preselected isolates were applied to the surface-sterilized seeds of oilseed rape and cauliflower using bio-priming. The isolates' PGP effect and root colonization capacities were compared under gnotobiotic conditions. One strain from each genus was selected and tested for its PGP qualities in sterile and non-sterile soil.
Results *Serratia* treatment resulted in different levels of PGP, while *Paenibacillus* strains damaged roots under gnotobiotic conditions. *P. polymyxa* Sb3-1 did not have a significant effect on plant growth in non-sterile soil; however it did promote plant growth in the sterile soil. *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were selected for further testing of their biocontrol effect under field conditions.
Conclusions The choice of growth environments in the investigation of plant-bacterium interaction is crucial. Non-sterile soil is suggested as the ideal medium for use in studying the PGP effect.

Keywords Biocontrol · Bio-priming · Plant growth promotion · PGP · BIOCOMES · *Brassica* · *Serratia* · *Paenibacillus* · Verticillium wilt

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Introduction

Verticillium spp. induce vascular wilting corresponding with high yield losses within a wide range of dicotyledonous plants, including economically important field crops such as oilseed rape (*Brassica napus* L.) and vegetables like cauliflower (*Brassica oleracea* L.) (Debode et al. 2005; Zhou et al. 2006; Dunker et al. 2008). Verticillium wilt caused by *Verticillium dahliae* Kleb. and *Verticillium longisporum* is a severe soil-borne plant disease with no fungicidal control available to date. Due to the genetically heterogeneous and polyphyletic character of *Verticillium* isolates as well as its ecological behavior, the fungus is one of the most challenging phytopathogens to control (Jiménez-Gasco et al. 2014). The Verticillium wilt disease incidents are predicted to increase in future mainly due to decrease in crop rotation time and global warming (Heale and Karapapa 1999; Siebold and Tiedemann 2012). The current trend in plant disease control goes towards

sustainable and environmentally friendly agriculture (directive of the European parliament and of the council 2009/128/EC), so that biological control of Verticillium wilt is especially desirable. Beneficial bacteria have been intensively studied as biocontrol agents against soil-borne diseases including *Verticillium* (Handelsman and Stabb 1996; Weller 1988). They are known not only to promote plant growth and improve soil health, they also induce resistance in plants against pathogens and restrict them from reaching plant roots (Berg 2009). However, inconsistent effects under field conditions are a hurdle in commercialization of many interesting biocontrol systems (Berg et al. 2013). Although rarely reported in literature, biological treatments may also have the opposite effect to that desired, acting against its host, sometimes in combination with pathogens. Recently root endophytes were suggested as promising biocontrol agents against Verticillium wilt in olives (Prieto et al. 2009); they were able to induce resistance in the plant (Cabanás et al. 2014). In addition, fungal endophytes were identified as promising antagonists (Tyvaert et al. 2014). Because endophytes colonize the same microhabitat as *Verticillium*, they probably have substantial, currently under exploited potential to act as biocontrol agents (BCAs) against *Verticillium* spp. in different crops. Owing to their endophytic lifestyle they are better protected against adverse environmental conditions, which should allow more consistent beneficial effects in the field.

Strains from the genera *Serratia* and *Paenibacillus* are widely known for their plant growth promoting (PGP) and biocontrol qualities as well as for their endophytic lifestyle (Petersen and Tisa 2013; Rybakova et al. 2015). For example, *Serratia plymuthica* HRO-C48 has been successfully used for controlling Verticillium wilt and other soil-borne fungi as a soil amendment in strawberry fields (RhizoStar®) (Kurze et al. 2001). The application of *S. plymuthica* HRO-C48 to the seeds of the oilseed rape via bio-priming, pelleting or seed coating was shown to reduce the degree of Verticillium wilt in oilseed rape plants under greenhouse conditions (Müller and Berg 2008). While *Serratia* is a typical inhabitant of *Brassicaceae* (Kalbe et al. 1996), *Paenibacillus* strains have a broader host range and are cosmopolitans. *Paenibacillus* species are world-wide well known as commercially promising BCAs of plant diseases (Berg 2009; Lal and Tabacchioni 2009; Rybakova et al. 2015). One of the main advantages of *Paenibacillus* as a BCA is its ability to build endospores that increase survival of the species in extreme conditions. This provides advantages over the non-spore formers in product formulation and stable maintenance in soil (Emmert and Handelsman 1999). In addition, the broad spectrum of beneficial plant-microbe interaction support the selection of *Paenibacillus* as potential BCA as already shown in detail for *P. polymyxa* E681 (Timmusk and Wagner 1999; Timmusk et al. 2005) Although the biocontrol potential of *Serratia* as well as *Paenibacillus* was identified in scientific studies, *ad planta* systems are necessary to assess these effects.

The objective of the project was to test the effects of the strains at different in vitro and *ad planta* conditions to simplify the selection process for an optimal candidate for protecting oilseed rape and Brassica vegetables against fungal pathogens using seed treatment with beneficial bacteria. In the search for an optimal candidate for the seed treatment of *Brassica* plants we selected five strains of *Paenibacillus* in addition to five strains of *Serratia* (Berg et al. 2002, 2005; Fürnkranz et al. 2012; Köberl et al. 2013; Müller and Berg 2008 and Zachow et al. 2013). Although all strains have different origins, they were mainly isolated from plants and selected according to their antagonistic potential against fungal plant pathogens (Table 1). The selected strains were compared for (1) their ability to inhibit the growth of *Verticillium* spp. in vitro, (2) the efficiency of the bacterial colonization in the oilseed rape and cauliflower and (3) their ability to induce plant growth promotion (PGP) in oilseed rape and cauliflower seedlings. In addition to these studies we compared the colonization patterns of the different BCAs on the roots using confocal laser scanning microscopy (CLSM) combined with fluorescent in situ hybridization and/or using microorganisms labelled with fluorescent markers. Although all *ad planta* systems presented different results, the comparison of the strains' properties and a comparative assessment allowed us to choose one strain of each genus for further testing. As the initial study was performed under soil-less gnotobiotic conditions which may not reflect the natural effects of the BCA on the plant, we additionally evaluated the PGP effects of the selected strains on the plants grown under different artificial *ad planta* conditions. The results observed allowed us to conclude that growth conditions in the investigation of plant-bacterium interaction are crucial and that the same bacteria applied to the seeds may even result in either death of the host plant or in growth promotion depending on plant growth conditions.

Table 1 Selected bacterial isolates and plasmids used in this study

Strains	Closest database match	Environmental source		Reference
Paenibacillus				
Sb3-1	*P. polymyxa*	Agricultural soil		Köberl et al. 2013
Mc2-9	*P. brasilensis*	Chamomile rhizosphere		Köberl et al. 2013
302P5BS	*P. polymyxa*	Lichen		Cernava et al. 2015
Pb71	*P. polymyxa*	Styrian oil pumkin spermosphere		Fürnkranz et al. 2012
GnDWu39	*P. peoriae*	Styrian oil pumkin rhizosphere and spermosphere		Fürnkranz et al. 2012; Liebminger et al. 2011
Serratia				
HRO-C48	*S. plymuthica*	Oilseed rape rhizosphere		Müller and Berg 2008
3Re4-18	*S. plymuthica*	Potato endosphere		Berg et al. 2005; Zachow et al. 2010
3RP8	*S. plymuthica*	Potato rhizosphere		Berg et al. 2002
SP1-3-1	*S. proteamaculans*	Sorgum (primed with primula microbial community) rhizosphere		Zachow et al. 2013
S13	*S. plymuthica*	Summer squash spermosphere		Fürnkranz et al. 2012
Plasmid	Strain	Label	Excitation/Emission optima (nm)	Antibiotic resistance
pSM1890	*S. plymuthica* HRO-C48	EGFP	488 /507	gentamycin
pIN69_mNep	*S. plymuthica* 3Re4-18	mNeptune	532/625	trimethoprim
pIN69_dsRed	*S. plymuthica* 3RP8	DsRed2	563/582	trimethoprim
pIN69_EGFP	*S. proteamaculans* SP1-3-1	EGFP	488/507	trimethoprim
pIN69_EGFP	*S. plymuthica* S13	EGFP	488/507	trimethoprim

Material and methods

Bacterial strains and growth conditions

The bacterial strains used in this study are listed in Table 1. The fungal pathogens used were *V. dahliae* Kleb. V-024 (fungal collection of the Agronomy Dpt. of University of Córdoba) and *V. longisporum* ELV25 Stark (Karapapa et al. 1997) (Messner et al. 1996; strain collection TU Graz, Environmental Biotechnology). *Paenibacillus* and *Serratia* strains were routinely grown on Standard I nutrient agar (NA, SIFIN, Berlin, Germany) at 30 °C. When required, gentamicin or trimethoprim were added at concentrations of 10 and 50 μg ml^{-1}, respectively.

Labelling of the *Serratia* strains with fluorescent proteins for CLSM

In order to study colonization of plant tissue by natural isolates of *Serratia* spp., four strains were transformed with one of the derivates of the rhizosphere-stable plasmid pIN69 (Gasser et al. 2011) carrying genes encoding for the fluorescent proteins DsRed2, EGFP or mNeptune, and a trimethoprim resistance cassette (Table 1). *S. plymuthica* HRO-C48 was labelled with the plasmid pSM1890 conferring a gentamicin resistance and EGFP fluorescence (Haagensen et al. 2002).

In vitro antagonistic assays

Bacterial isolates were screened for their activity towards *V. dahliae* and *V. longisporum* by a dual culture in vitro assay on Waksman agar according to Berg et al. (2002). All strains were tested in three independent replicates.

Seed treatment (bio-priming)

Paenibacillus und *Serratia* strains used for bio-priming were grown on NA. Four to eight plates were used for each priming experiment. Strains were grown for 72 h and harvested following the modified protocol described by D'aes et al. (2011). Cells were scraped from the plates and suspended in sterile 0.85 % (*w/v*) NaCl. Cell concentrations were estimated by measuring their optical density at 600 nm (OD_{600}). The cell concentration was adjusted with sterile 0.85 % (*w/v*) NaCl to an

optical density corresponding to OD_{600} of 10. The final cell concentration was estimated for each strain separately using direct cell counting in a Thoma chamber (depth 0.01 mm). Live cell concentration was measured by successive dilutions of bacterial suspension followed by plating. The winter oilseed rape *Brassica napus* l. partim, "Traviata H 605886" (KWS Saat Einbeck, Germany) or Hybrid cauliflower var. "freedom" (Seminis, Holland) seeds were surface-sterilized for 5 min using 2 % NaOCl. Bio-priming was performed in accordance with the modified protocol of Müller and Berg (2008). Seeds were immersed in the cell suspension for 4 h at 20 °C under agitation. Infiltrated seeds were dried for 1 h at 20 °C until they appeared dry. Seeds incubated with sterile 0.85 % (*w/v*) NaCl solution for 4 h served as a control. Twenty seeds were transferred into 2 ml sterile 0.85 % sodium chloride solution in order to determine cell counts. Each strain was tested in four replicates. Primed seeds were ground for 1 min using an autoclaved mortar and pestle. Suspensions were serially diluted and plated onto NA medium (two replicates per dilution). Plates were incubated for 24–48 h at 30 °C and colony forming units (CFUs) were counted in order to calculate the logarithmic means of CFUs ($\log_{10}$ CFU).

Germination assay

The germination assay was performed following the modified protocol described by Zachow et al. (2013). Germination of oilseed rape seeds primed with *P. polymyxa* Sb3-1 in two concentrations ($\log_{10}$ 5 and $\log_{10}$ 7) was tested on a folded wet filter paper in a (10× 15.5 cm) plastic container at 20 °C. Germination rate was evaluated after 10 days by comparison with the non-inoculated control. The germination rate of the seeds as well as the total weight of the seedlings was then estimated. Each treatment included four replications containing 25 seeds each.

Plant growth promotion and root colonization assays

Plant growth in germination pouches In order to determine the effect of the potential biocontrol strains on the growth of plants in germination pouches, seven bio-primed seeds from each strain and seven non-inoculated seeds (control) were aseptically placed into one germination pouch (Mega International, Minneapolis, USA) which had been filled with 10 ml of sterile water. The pouches were placed upright in sterilized plastic boxes for 14 days. After 14 days, the green parts were gently separated from the roots and the fresh weight of all plant parts was determined gravimetrically. The experiment was carried out in four replicates for each bacterial strain (14 seedlings per replicate). For each replicate, root material from 14 seedlings was sampled into sterile plastic bags and homogenized with mortar after adding 2 ml of NaCl solution (0.85 %). Suspensions were serially diluted and plated on NA as previously described. CFU were determined after 1–3 days of incubation at 30 °C and calculated to a CFU per gram root fresh weight. In experiments with the antibiotic resistant bacteria strains carrying fluorescent tags (Table 1), medium was supplemented with the respective antibiotic.

Plant growth in sterile soil For evaluation of the PGP effect of BCA in the sterile soil, the surface-sterilized oilseed rape seeds were bio-primed independently with $\log_{10}$ 9 for *S. plymuthica* 3RP8 and $\log_{10}$ 7 for *P. polymyxa* Sb3-1. The seeds were sown in autoclaved plastic containers with a volume of 5.6 l containing 1 l propagation compost (Einheitserdewerk, Uetersen, Germany) mixed with vermiculite (4:1, *v/v*). The potting soil mixture was autoclaved in plastic bags twice with a 48-h interval. When the experiments were finished, the plants were weighed for the purpose of analyzing the effects of each BCA on biomass production in comparison to an unprimed control. The experiment was performed in six replicates with 12 seeds planted into each plastic container and repeated at least two times.

Plant growth in non-sterile soil In order to evaluate the PGP effects of BCAs on plants grown in the non-sterile soil oilseed rape seeds were primed independently with $\log_{10}$ 9 for *S. plymuthica* 3RP8 as well as with $\log_{10}$ 5 or $\log_{10}$ 7 for *P. polymyxa* Sb3-1. The seeds were sown in pots (three seeds per pot) with a volume of 250.0 ml containing propagation compost mixed with vermiculite (4:1, *v/v*). They were then grown and evaluated as described above. The seedlings were watered every second to forth day. The experiment was performed in four replicates with nine seedlings each for every bacterial strain and bio-priming concentration, and repeated at least three times.

For all three growth condition experiments the seedlings were kept in a phytochamber (Binder KBWF 720, Tuttlingen, Germany) at 22 °C, day–night regime of 12:12 h (7000 lux) for 14 days.

Isolation of endophytic bacteria from oilseed rape roots grown from seeds bio-primed with *P. polymyxa* Sb3-1

For endophyte enumeration, five 10-day old oilseed rape seedlings grown in germination pouches as described above were surface sterilized for 5 min in a surface sterilization solution (1 % sodium hypochlorite in 0.85 % NaCl solution). During the incubation period the plants were subjected to vortexing for 15 intervals of 10 s duration with 10 s of rest between intervals. This was followed by four washes with sterilized water. The wash solution from the final root rinse (1 ml) was cultured to determine the efficiency of sterilization. Seedlings were then macerated using a mortar, and the numbers of CFU were determined by plating preparations on NA.

Confocal laser scanning microscopy (CLSM) and fluorescent in situ hybridization (FISH)

CLSM was used to study colonization patterns of selected *Paenibacillus* and *Serratia* strains in oilseed rape and cauliflower seedlings grown in germination pouches for 14 days. Plant colonization by *Serratia* strains labelled with fluorescent markers was directly observed with a TCS SPE confocal microscope (Leica Microsystems, Germany) using laser lines/detection wavelengths as described in Table 1 for each fluorescent dye. The plant tissues (autofluorescence) were observed using a 405 nm laser line wavelength and detected at 425–490 nm. Confocal stacks were acquired with Z-step of 0.4–0.5 μm and sequential activation of laser lines/detection windows. Maximum projections of an appropriate number of optical slices were applied to visualize the root sections (confocal stacks).

The FISH technique was utilized in order to study the plant colonization ability of *Paenibacillus* strains. The roots and green parts of the 14 day old seedlings were fixed with 4 % paraformaldehyde/phosphate buffered saline (PBS) (3:1 *vol/vol*). The control group contained roots without bacterial treatment. The fixed samples were then stored in PBS/96 % ethanol (1:1) at −20 °C. The FISH probes for *Firmicutes* genera (LGC354A, LGC354B, LGC354C labelled with FITC; Meier et al. 1999) were used, and the in-tube FISH was performed as described by Cardinale et al. (2008). In this step, 45 % formamide was added to the samples which were then subsequently incubated in a water bath (41 °C) for 90 min. After hybridization, the samples were washed at 42 °C for 15 min. The second hybridization step directing eubacteria included an equimolar ratio of the FISH probes EUB338 (Amman et al. 1990), EUB338 II, and EUB338 III (Daims et al. 1999) labelled with Cy3 followed by another washing step. The unspecific binding of the probes to the plants or bacteria was analyzed by including a negative control sample. Additionally, the seedling roots and green parts were hybridized with NONEUB-FITC and NONEUB-Cy3 probes for the first and second hybridization steps, respectively, following the same protocol as for the positive samples. These samples served as a negative control to detect the unspecific probe hybridization. Microscopy and image capturing were performed as described above.

The evaluation of the colonization preferences of the *Serratia* strains in the oilseed rape and cauliflower seedlings was carried out using Universal Hood transilluminator (Bio-Rad, Austria) using excitation/detection wavelengths as described in Table 1 for each fluorescent dye.

Statistical analysis

The PGP effect of the microorganisms was statistically analyzed using the IBM SPSS program version 20.0 (IBM Corporation, Armonk, NY, USA). The selection of statistical test was done according to Gray and Kinnear (2012). For each of the treatments, at least three replicates were included in the analysis unless otherwise stated. The data was tested for normal distribution by using Q-Q plots and the Shapiro-Wilk test, and the homogeneity of variances was examined using the Levene's test (Bragina et al. 2013). The significance of the differences in plants' weights of the non-inoculated control versus each treatment (Tables 3 and 4) was (pairwise) calculated using a *t*-test with independent samples or by using the non-parametric Mann–Whitney *U* test, depending on the distribution of the variables (normal versus non-normal). The data was expressed as the geometric mean±standard deviation. For the dual culture assay, the data was analyzed for normal distribution and homogeneity of variances as described above. The significance of the differences between zones of inhibition of *Verticillium* growth by different bacterial strains (Table 2) was calculated using one-way ANOVA and Tukey's HSD tests. For both analyses, the P values<0.05 were considered to be significant.

Table 2 Antagonistic activity of preselected *Paenibacillus* and *Serratia* strains towards *V. dahliae* Kleb. and *V. longisporum* Stark ELV25

Strains	*V. longisporum* Stark ELV25 The means of the zones of inhibition (mm)*	*V. dahliae* Kleb. The means of the zones of inhibition (mm)*
P. polymyxa Sb3-1	4.3^{a}	5.7^{ab}
P. peoriae GnDWu39	4.0^{ab}	4.7^{bc}
P. brasilensis Mc2-9	4.0^{ab}	8.7^{a}
P. polymyxa Pb71	3.9^{ab}	2.0^{cde}
P. polymyxa 302P5B5	2.9^{ab}	5.0^{bc}
S. plymuthica 3Re4-18	3.2^{ab}	3.7^{bcd}
S. plymuthica HRO-C48	2.3^{ab}	2.0^{cde}
S. plymuthica 3Rp8	1.2^{ab}	1.7^{cde}
S. plymuthica S13	0.8^{ab}	0.5^{de}
S. proteamaculans SP1-3-1	0^{b}	0^{e}

* The bacteria and *Verticillium* strains were grown on Waksman agar. Zones of inhibition were measured and statistically analysed after 6 days at 20 °C. According to the Tukey's HSD Test at P=0.05 the means from three independent replicates that are followed by a common letter for each isolate do not differ significantly

Results

Characterization and antagonistic effects of preselected *Serratia* and *Paenibacillus* strains towards *Verticillium*

Five selected strains of *Serratia* and *Paenibacillus* (Table 1) were screened for their in vitro activity against two pathogenic *Verticillium* strains: *V. dahliae* Kleb. V-024 and *V. longisporum* Stark ELV25. All strains with the exception of *S. proteamaculans* SP1-3-1 showed inhibition effects on both *Verticillium* strains in vitro (Table 2) while *P. polymyxa* Sb3-1 and *P. brasilensis* Mc2-9 exhibited the highest antagonistic potential for both *Verticillium* strains among tested strains. When *Serratia* strains were compared with each other, *S. plymuthica* 3Re4-18 exhibited the highest antagonistic effect towards both *Verticillium* strains, followed by *S. plymuthica* HRO-C48 and *S. plymuthica* 3Rp8. *S. proteamaculans* SP1-3-1 showed no antagonistic effect to both *Verticillium* strains.

Alteration of the bacterial abundances on the bio-primed seeds and the roots of the seedlings grown in germination pouches

The inoculation of the seeds with the preselected *Paenibacillus* strains with inoculum concentrations spanning from $\log_{10}$ 5.6 to $\log_{10}$ 7.3 CFU ml^{-1} resulted in the attachment of $\log_{10}$ 2.6 to $\log_{10}$ 4.1 CFUs per seed (Table 3). Although we attempted to keep the inoculation concentration constant by using both OD_{600} measurements and a Thoma cell counting chamber to adjust the inoculation concentration, the concentration of the live *Paenibacillus* cells varied. Of interest, the *P. polymyxa* Pb71 that was used for bio-priming at its highest concentration ($\log_{10}$ 7.3 CFU ml^{-1}) had the least abundance of live cells on the seeds after priming ($\log_{10}$ 2.9±0.2 CFU $seed^{-1}$ for oilseed rape and $\log_{10}$ 2.6±0.2 CFU $seed^{-1}$ for cauliflower seeds). We also noted that this strain did not sporulate under conditions used for bio-priming as detected using light microscope (data not shown). On the other hand, *P. polymyxa* GNDwu39 that was inoculated with the lowest concentration among tested *Paenibacillus* strains ($\log_{10}$ 5.6 CFU ml^{-1}) demonstrated the highest abundance of living cells on the seeds after priming ($\log_{10}$ 4.4± 0.1 CFU $seed^{-1}$ for oilseed rape and $\log_{10}$ 4.2± 0 CFU $seed^{-1}$ for cauliflower seeds). The selected *Serratia* strains were applied to the seeds in concentrations ranging from $\log_{10}$ 8.4 to 9.7 CFU ml^{-1}. This resulted in higher abundance of the bacteria on the oilseed rape seeds after priming than observed for *Paenibacillus* strains, ranging from $\log_{10}$ 5.8 CFU $seed^{-1}$ for HRO-C48 to $\log_{10}$ 6.9 CFU $seed^{-1}$ for SP1-3-1 (Table 3). A similar tendency was observed for the bacterial abundancies on the cauliflower seeds (Table 3). The amount of *Serratia* spp. that attached to the seeds during bio-priming was approximately 300 times higher on average when compared to that of the *Paenibacillus* spp.

Table 3 Effect of the 10 selected *Paenibacillus* and *Serratia* strains on the 2 weeks old oilseed rape and cauliflower seedlings grown in germination pouches

		Oilseed rape					Cauliflower				
	Priming concentration (log_{10} CFU ml^{-1})	Abundance on the seed (log_{10} CFU $seed^{-1}$)	Abundance on the root (log_{10} CFU (g) $roots^{-1}$)	Root weight (10 $plants^{-1}$ (mg)	Weight of the green parts (10 $plants^{-1}$ (mg)	Germination (%)	Abundance on the seed (log_{10} CFU $seed^{-1}$)	Abundance on the root (log_{10} CFU (g) $roots^{-1}$)	Root weight (mg 10 $plants^{-1}$)	weight of the green parts (10 $plants^{-1}$ (mg)	Germination (%)
Control	0	0.0±0	0.0±0	243±30	409±9.1	100	0.0±0	6.6±0.9[a]	157±23	351±77	98
Serratia											
HRO-C48	8.8	5.8±0.3	7.7±0.2	276±31	464±38	96	6.3±0.3	7.9±0.1	170±27	408±22	86
S13	9.5	6.4±0.1	8.7±0.2	253±20	429±21	95	6.4±0.1	8.8±0.1	112±26	375±11	91
3RP8	9.5	6.5±0.2	9.0±0.1	325±39*	448±44	93	6.4±0.2	8.9±0.2	201±26	408±58	86
RE4-18	8.4	6.0±0.3	8.4±0.1	337±48*	436±37	98	6.1±0.2	8.8±0.1	149±10	378±12	93
SP1-3-1	9.7	6.9±0.1	8.9±0.2	271±38	451±44	100	6.7±0.2	8.9±0.1	200±32	439±39	89
Paeni bacillus											
Pb71[b]	7.3	2.9±0.2	8.1±0.3	98±58*	154±134*	79	2.6±0.2	0.0±0	0±0	0±0	0*
GnDW-u39	5.6	4.4±0.1	8.4±0.2	44±15*	197±31*	59*	4.2±0	8.5±0.4	34±7	27±25	20*
Sb3-1	7.1	3.9±0.2	8.0±0.1	68±28*	260±30*	75	3.8±0	8.2±0.3	42±15	100±48	27*
Mc2-9	6.1	3.6±0	7.7±0.3	64±5*	271±29*	86	3.6±0.1	7.8±0.6	30±19	57±57	29*
302P5BS	6.5	4.1±0.2	8.2±0.7	46±43*	199±29*	61	4.0±0.1	8.2±0.7	6±11	26±45	11*

[a] Bacteria found on the roots of the seedlings used for negative control were not the strains used for priming

[b] Cauliflower seeds primed with *P. polymyxa* Pb71 did not germinate; For the data evaluating PGP effect (root weight, weight of the green parts and germination columns) the asterisk (*) denotes values that varied significantly from non-primed control group values ($P<0.05$). For the weights of the roots and green parts the fresh weight was determined

After 14 days, the roots of the seedlings were analyzed to determine cell densities. Although the abundances of the bacteria on the roots of the plants fluctuated within each genus, they appeared quite similar when both genera were compared to each other (Table 3). On average, $\log_{10}$ 8.0 *Paenibacillus* and $\log_{10}$ 8.5 *Serratia* CFUs were isolated from 1 g of oilseed rape roots. Cauliflower seedlings contained on average $\log_{10}$ 8.1 and $\log_{10}$ 8.7 CFUs (g) roots $^{-1}$ of *Paenibacillus* and *Serratia* strains, respectively.

Effect of bio-priming with *Serratia* and *Paenibacillus* strains on oilseed rape and cauliflower seedlings grown in germination pouches

The evaluation of the PGP effect of the selected BCAs (Table 1) showed that while *Serratia* treatment resulted in different levels of PGP, the opposite effect was found after *Paenibacillus* evaluation. The treatments with all *Paenibacillus* strains resulted in significant reduction of the growth of the seedling with Sb3-1 being the least damaging for the plant among *Paenibacillus* strains (Table 3). Priming of the oilseed rape and cauliflower seeds with the *S. plymuthica* 3RP8 and 3Re4-18 strains had a significant PGP effect on the root weights of the oilseed rape seedlings, while other *Serratia* strains showed no significant effects on plant growth. In combination with the results of the in vitro dual culture assay, *S. plymuthica* 3RP8 was chosen for further testing.

We found that the root system of the *Paenibacillus* primed oilseed rape and cauliflower seedlings under the described conditions was stunted and appeared damaged (Fig. 1b). The macroscopic appearance of the roots was similar for seedlings primed with bacteria of the same genera (data not shown). The influence of each of the tested strains showed similar tendencies when comparing oilseed rape and cauliflower with each other. Cauliflower seedlings, however, appeared to be more negatively affected by priming with *Paenibacillus* strains than oilseed rape seedlings.

The germination rate of cauliflower seedlings in germination pouches was negatively affected by the bio-priming with *Paenibacillus* strains resulting in a 60–80 % seed germination rate, while no reduction in germination rate was observed when seeds were primed with *Serratia* strains. With respect to the results, the *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were chosen for further experiments.

Effect of bio-priming with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 on oilseed rape seedlings grown in sterile and non-sterile soil

Because priming with *Serratia and Paenibacillus* spp. had a controversial effect on the seedlings grown in artificial gnotobiotic conditions, we decided to evaluate their effect on plants grown in soil. Therefore the oilseed rape seeds primed with *S. plymuthica* 3RP8 and *P. polymyxa* Sb3-1 were sown in either sterile or non-sterile soil. We found that the average fresh weight of the plants primed with *S. plymuthica* 3RP8 did not differ significantly from that of the unprimed seedlings grown in non-sterile and sterile soil (Fig. 1a). Plants primed with *P. polymyxa* Sb3-1 and grown in non-sterile soil were also not significantly different from the unprimed control. Interestingly, the evaluation of the fresh weights of oilseed rape seedlings grown in sterile soil showed an opposite correlation to the germination pouch experiment: seedlings primed with *P. polymyxa* Sb3-1 had significantly higher average weight when compared to the non-primed control (Fig. 1a).

Effect of the priming concentration of the P. polymyxa Sb3-1 on the seed germination and weight of the oilseed rape seedlings

In order to investigate whether the deleterious effect of *Paenibacillus* spp. observed when seedlings were grown in germination pouches correlates with the concentration of the inoculate, we applied $\log_{10}$ 5 and $\log_{10}$ 7 CFU ml^{-1} of *P. polymyxa* Sb3-1 to the surface-sterilized oilseed rape seeds in two independent experiment sets. The seedlings were grown under semi-sterile conditions on the folded filter paper for 10 days in one experiment set (germination assay) and in the non-sterile soil for 14 days in another experiment set. The weights of the seedlings grown on the filter paper was significantly reduced in comparison to the unprimed control in which seeds were primed with *P. polymyxa* Sb3-1 (Table 4). However, no significant differences in the weights of the plants grown in either non-sterile soil or on filter paper was observed when seedlings primed with different bacterial concentrations were compared to one another.

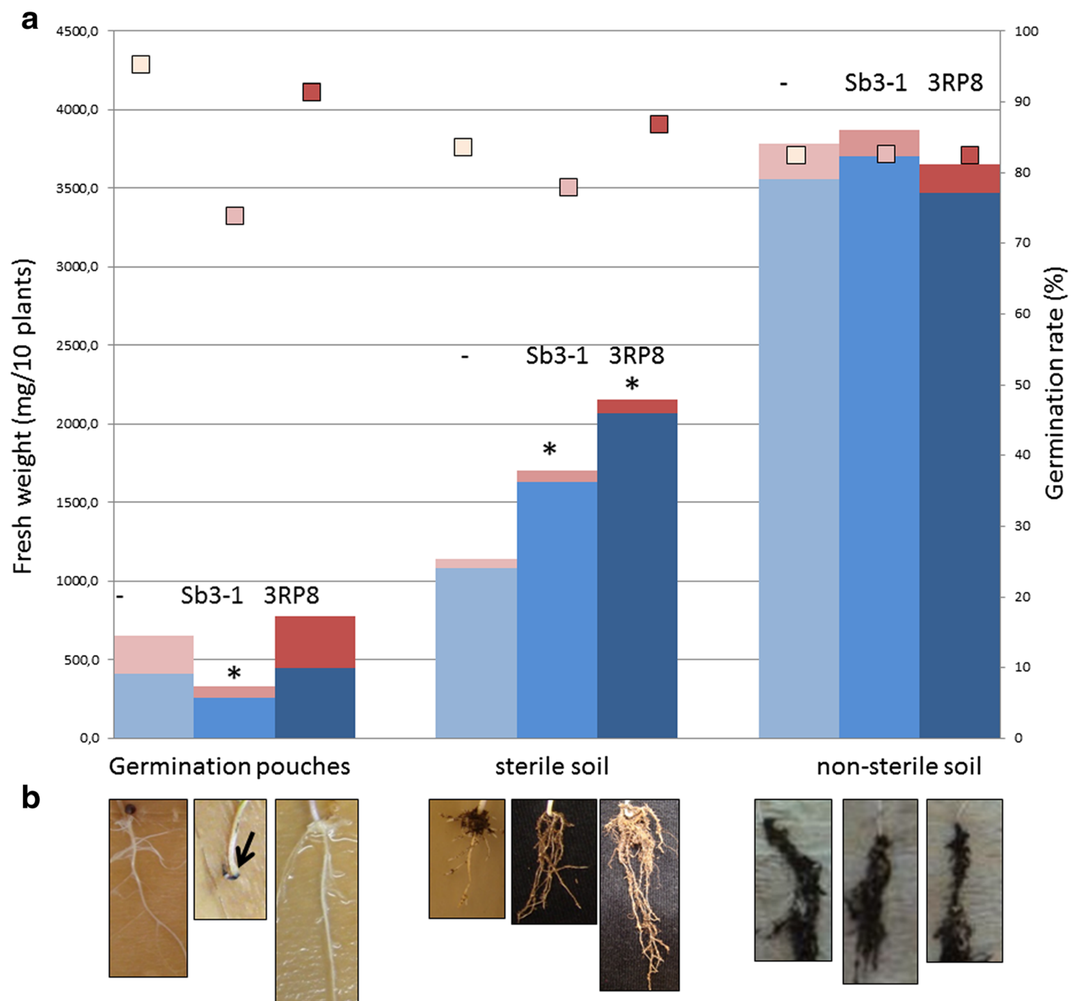

Fig. 1 **a** Comparison of the green part weights of oilseed rape seedlings after 2 weeks of growth in different conditions (sterile germination pouches, sterile soil and non-sterile soil). The seeds were primed with either NaCl solution (negative control, labelled "-") or *P. polymyxa* Sb3-1 (labelled "Sb3-1") or *S. plymuthica* 3RP8 (labelled "3RP8"). The *blue columns* denote mean weights of *green* parts (mg per 10 plants), *red columns* signify mean weights of roots (mg per 10 plants). The *squares* symbolize means of germination rate for each strain and experimental design in % (second axis). For details please refer to Tables 3 and 4. **b** Representative images of root bases of the oilseed rape seedlings corresponding to each bar from (**a**). *Arrow* denotes a stunned root system that was typical for seedlings primed with *Paenibacillus* spp. grown in germination pouches. The *asterisk* (*) denotes values that were significantly different from the non-primed control group values ($P<0.05$)

Table 4 Germination rate and total weight of the oilseed rape seedlings primed with different concentrations of *P. polymyxa* Sb3-1 grown in the non-sterilized soil (14 days) and under semi-sterile conditions on filter paper (10 days)

Inoculum concentration	Non-sterile soil		Semi-sterile filter paper	
	total weight / 10 plants^{-1} (mg)	Germination (%)	total weight / 10 plants^{-1} (mg)	Germination (%)
Non-innoculated control (0)	3536.9±511	83	375.4±32	97
$log_{10}5$	3514.7±492	90	285.6±6*	90
$log_{10}7$	3552.0±429	85	288.2±9*	96

The asterisk (*) denotes values significantly different from non-primed control group ($P<0.05$)

Colonization patterns of BCAs in the plant tissue of the seedlings grown in germination pouches

The ability of the biocontrol strains to colonize plants and their interactions were additionally assessed using the CLSM. *Serratia* isolates were tagged with either DsRed2, GFP or mNeptune enabling direct visualization of the bacterial colonies in the plant tissue (Table 1). *Paenibacillus* isolates were visualized using FISH with genera specific probes. The evaluation of the colonization preferences of the *Serratia* strains in the oilseed rape seedlings showed that fluorescent cells were mostly observed on the upper parts of the roots (Fig. 2a). This was confirmed by the screening of different parts of the root using CLSM. Within the root system, *Serratia* cells were found in either the upper parts of the root, or in the middle part of the root in fewer quantities, but not in the root tips (data not shown). The *Serratia* cells were either found as clouds around the roots, or they formed large micro-colonies in the root tissue (Fig. 2b). The cells were mostly observed in either the rhizosphere or in the intercellular space inside of the root tissue. Similarly shaped colonies, in a reduced abundance, were also found in the leaf tissue of the oilseed rape (Fig. 2c). The *Paenibacillus* colonies were often detected in the areas surrounding damaged root and leaf tissues (Fig. 2d) or in cavities (Fig. 2e and f) where they formed large micro-colonies. Similarly to the *Serratia cells*, the majority of the *Paenibacillus* cells were observed in the upper parts of the root. Bacterial colonization patterns within the same genus appeared to be similar. Furthermore, no notable differences between oilseed rape and cauliflower colonization patterns were observed (data not shown).

We tested whether *P. polymyxa* Sb3-1 was indeed capable of colonizing oilseed rape endophytically in

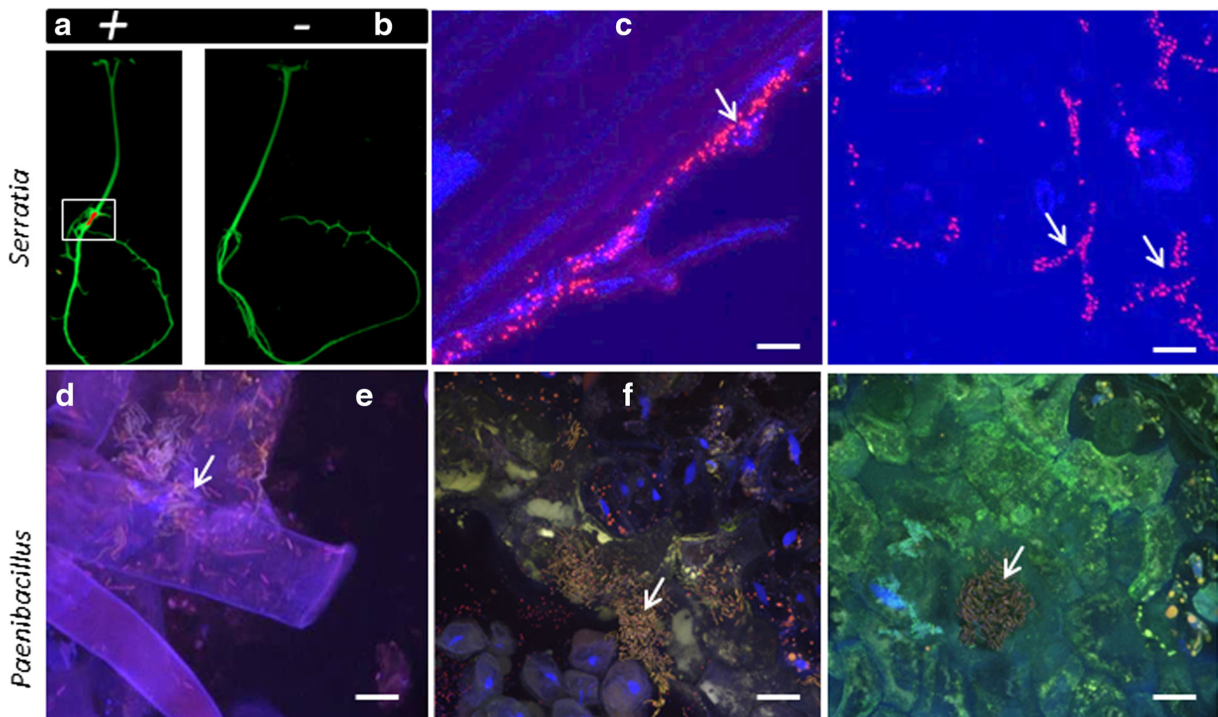

Fig. 2 Visualization of 14 day old oilseed rape seedlings grown in sterile germination pouches primed with either DsRed transformed *S. plymuthica* 3RP8 (labelled as "+") or with NaCl solution (labelled as "-") using universal hood transilluminator (**a**). The *red coloration* at the root base (highlighted with a *white square*) indicates a high saturation level of colonizing bacteria labelled with DsRed. Observations in (**b**) and (**c**) were made with CLSM and show DsRed transformed *S. plymuthica* 3RP8 colonizing the root base (**b**) and leaves of the oilseed rape seedling (**c**). *Paenibacillus* strains were visualized using FISH-CLSM using an equimolar ratio of the *Firmicutes*-specific FISH probes LGC354A, LGC354B and LGC354C labeled with the fluorescent dye FITC (**d**-**f**). *P. polymyxa* Mc2-9 colonies are denoted with *arrows*. The image (**d**) shows *P. polymyxa* Mc2-9 macrocolonies detected in the cavities of the damaged oilseed rape root. Images (**e**-**f**) show *P. polymyxa* Mc2-9 colonies detected in the cavities of the oilseed rape leafs. Images are a projection of 27–77 adjacent confocal optical sections. *Arrows* denote bacterial colonies. Bar represents 25 μm

order to confirm observations made by CLSM where the selected strains were found not only on the surfaces of plants but also in the plant tissues. The surface of the oilseed rape seedlings was sterilized followed by an extensive wash. Only 2.6 CFU per seedling were found in the final washing step in average, indicating an almost perfect sterilization of the plant surface. The homogenized tissues of the seedlings, on the other hand, contained 403 CFU per seedling which suggested that *P. polymyxa* Sb3-1 is capable of an endophytic lifestyle.

Discussion

In our study we were able to evaluate the PGP potential of endophytic *Serratia* and *Paenibacillus* strains that were in vitro shown to be *Verticillium* antagonists. This potential was strain-specific and depended on the plants' growth conditions. However, a comparative assessment as well as additional experiments allowed a selection of optimal candidates for biocontrol agents against Verticillium wilt in oilseed rape and cauliflower. The results of this study could contribute to the development of an environmentally friendly seed treatment against the high risk pathogen *Verticillium* (Zeise and Steinbach 2004).

All tested strains except for *S. proteamaculans* SP1-3-1 showed different degrees of antagonism against *V. longisporum* and *V. dahliae*. This was consistent with the observations made by Zachow et al. (2013) where *S. proteamaculans* SP1-3-1 was reported not to be antagonistic towards *V. longisporum*. Among tested isolates all *Paenibacillus* strains scored better than the *Serratia* strains in the in vitro test. This was probably due to their strong antagonistic properties such as their ability to produce large amounts of soluble and volatile antifungal metabolites (reviewed by Raza et al. 2008; Rybakova et al. 2015).

The efficiency of the bacterial colonization of the environment is proposed to be a crucial factor with regard to the efficacy of microorganisms as suppressors of soil-borne diseases (Weller 1988). Therefore, the next step for our investigation was to compare the colonization properties of the preselected strains with each other. We noticed that *P. polymyxa* Pb71 did not sporulate under the conditions used for bio-priming, while the other four *Paenibacillus* strains showed high degrees of sporulation (data not shown). Further experiments with endospore-forming bacteria exhibiting different degrees of sporulation are necessary to prove whether the ability of a bacterial strain to produce spores may improve its capacity to attach to the seed.

We found that the average number of the *Paenibacillus* cells that attached to the seeds of both cauliflower and oilseed rape was almost 300 times less when compared to abundance of *Serratia* on seeds. This may in part be explained by a lower priming concentration used for *Paenibacillus* spp. Interestingly, the abundancy of bacterial cells isolated from the roots of the bio-primed seedlings was quite similar for both genera. On average, $\log_{10}$ 8.1 CFU g^{-1} roots fresh mass for *Paenibacillus* and $\log_{10}$ 8.6 g^{-1} roots fresh mass for *Serratia* were found on the oilseed rape and cauliflower roots. This suggests the existence of a saturation level for bacterial colonization of the roots of the plant grown under given conditions. This suggestion is consistent with the observations described by Müller and Berg (2008). The authors applied *S. plymuthica* HRO-C48 to the oilseed rape seeds with initial bacterial cell numbers ranging from $\log_{10}$ 3.0 to 7.0 CFU $seed^{-1}$ and observed no significant differences in the plate counts of bacteria re-isolated from the seedling roots. The difference between the final abundance of the bacteria on the roots described in this work ($\log_{10}$ 7.7–9.0 CFU g^{-1} roots fresh mass) and bacterial abundancy on the roots reported by Müller and Berg ($\log_{10}$ 4.7 CFU g^{-1} root fresh mass) is most probably due to the different plant growth conditions. In this study the bacterial abundancy on the roots was tested for plants that were grown in germination pouches, while in the experiments described by Müller and Berg (2008), plants were grown in non-sterile soil under greenhouse conditions.

While, similar to other studies (Kurze et al. 2001; Müller and Berg 2008), *Serratia* strains either promoted the growth of the seedlings or had no effect on the seedlings growth under all tested conditions, this effect was only statistically significant for seedlings grown in sterile soil. On the other hand, we found significant differences in the effects of the tested *Paenibacillus* strains on the plant growth depending on the growth conditions. We found that all *Paenibacillus* strains tested damaged roots when plants were grown in the germination pouches. *P. polymyxa* Sb3-1 did not have a significant effect on plant growth in non-sterile soil, however, they significantly promoted plant growth in the sterile soil. The alterations in the initial priming concentration of *P. polymyxa* Sb3-1 ranging from $\log_{10}$ 4 to $\log_{10}$ 7 did not have any significant effects on plant

growth when plants were grown in non-sterile soil and on the filter paper. These results indicated that the choice of the optimal plant growth condition is more crucial for evaluation of the PGP effect of microorganisms than the choice of a bacterial concentration to be used for bio-priming. We have to notice that in all *ad planta* systems addressed in this study the PGP effects were evaluated at early seedling stage, and we do not know the effects of the BCAs on the later stage of the plants development yet.

The question as to why *Paenibacillus* may have a deleterious effect on the plants has been thoroughly studied by Timmusk et al. (2005 and 2015) and was recently discussed in detail by Rybakova et al. (2015). *Paenibacillus* applied to the roots of *A. thaliana* ecotype C24 seedlings was previously reported to damage plants that were grown in gnotobiotic conditions by degrading plant root cells as shown by CLSM (Timmusk et al. 2005). Indeed, the roots of seedlings grown from seeds bio-primed with each of the five *Paenibacillus* strains evaluated in this study appeared stunted and were significantly reduced in weight and length (Fig. 1). Moreover, the CLSM images showed that the roots of the plants were highly damaged and large macro-colonies of *Paenibacillus* cells were found in the cavities remaining after cell degradation both in roots and the leaves (Fig. 2). A similar effect of *Paenibacillus* on the *A. thaliana* seedlings was reported by Timmusk et al. (2005); however, in that case no *Paenibacillus* cells were detected in the leaves. Furthermore, we detected considerably more *Serratia* and *Paenibacillus* cells at the base of the seedlings' roots than in the root tips. The colonization patterns of *Serratia* strains observed by CLSM were similar to those observed for *Serratia* strains in the sugar beet rhizosphere (Zachow et al. 2010). Timmusk and co-workers, on the other hand, observed bacterial accumulation of *P. polymyxa* cells mainly around the root tip of *A. thaliana.* This difference may be due to the different plant cultivars used in these studies. The other possible explanation for the different character of spreading of bacteria to the plant tissues observed in this study and by Timmusk et al. (2005) is the use of different methods of applying bacteria to the plant. In our study seeds were submersed in a suspension of bacterial cells (bio-priming), while Timmusk and coworkers dipped the roots of the *A. thaliana* seedlings into the bacterial suspension. We speculate that it is easier for bacterial cells to spread to the green parts of the plant from the inoculated seed than in the case where bacterial cultures were applied to the roots of the seedlings. The accumulation of the bacterial cells at the root base may also be linked to its spatial proximity to the area of bio-priming.

It has been suggested that a paradoxical *Paenibacillus*-plant relationship may occur when the balance between *Paenibacillus* spp. and the soil microbiome in gnotobiotic conditions is upset. As a result, instead of protecting plants from pathogens, *Paenibacillus* spp. degrades root cells probably using the released metabolites as a nutrition source (Rybakova et al. 2015). *P. polymyxa* Sb3-1 was shown to be an endophytic bacterium for oilseed rape by both CLSM and seedlings' surface-sterilization following by cell count analysis. The ability of endophytic bacteria to destroy plant cell walls in order to enter into plant was linked to their ability of degrading pectin (Anand et al. 2006). It has been speculated that this is the way how endophytic bacteria may avoid cell defense mechanisms as the breakdown products of cell wall components, like pectin, induce systemic disease responses in plants (Anand et al. 2006). It is plausible that the plants grown artificially under gnotobiotic conditions in germination pouches without soil are weakened, and their defense system is impaired. This may result in a shift of the balance between the plant and endophytic bacteria in favor of bacteria resulting in damage of plant cells. This theory is also supported by the different effects of the same endophytic strain observed in oilseed rape and cauliflower seedlings in which cauliflower seedlings were much more strongly affected by the *Paenibacillus* spp. than oilseed rape seedlings. It has also been reported that morphological changes of the root have been associated with auxin production and excretion by PGP bacteria like *Paenibacilus* spp. Auxin has been shown to promote the sensitization of the host towards the bacterial pathogen and results in the development of disease symptoms (reviewed by Ludwig-Müller 2014 and Rybakova et al. 2015). Additionally, the non-ribosomal peptide/ polyketide synthases originated compounds produced by *P. polymyxa* has recently been shown to be partly or even fully responsible for its deleterious influence (Timmusk 2015). The local oversaturation of *Paenibacillus*-derived secondary metabolites in the rhizosphere of the seedlings grown in germination pouches may result in the observed deleterious effect.

When plants are grown under less artificial conditions, for example in non-sterile soil, *Paenibacillus* spp. can produce soluble and volatile metabolites that inhibit the growth of pathogens and also induce plants' defense

mechanisms resulting in changes in plant gene expression (Timmusk and Wagner 1999). They also build a biofilm around the roots that functions as a protective layer to prevent access by pathogens (Timmusk et al. 2005). These interactions may result in a PGP and a biocontrol effect of the *Paenibacillus* spp. as it was described for several *Paenibacillus* strains (Rybakova et al. 2015, and references therein). The question arises as to why the significant PGP effect of *P. polymyxa* Sb3-1 compared to the untreated control occurred only in sterile soil and not in the non-sterile soil. The PGP effect of some *P. polymyxa* spp. and diverse microbial communities on the host plants like Tobacco (*Nicotiana tabacum*) or *A. thaliana* grown in sterile soil has been shown by several groups (e.g., Phi et al. 2010 and Carvalhais et al. 2013). The situation where a PGP effect occurred in sterile soil while it was not observed in the non-sterile soil has to the best of our knowledge not been documented before. On the contrary, Kloepper and Schroth (1980) compared the PGP effects of several rhizobacteria on radish seedlings grown under gnotobiotic conditions in germination pouches, in sterile soil, and in non-sterile soil. The authors found that the same rhizobacteria increased plant growth in non-sterile conditions, while no significant effect on plant grown in sterile soil was observed. Of interest, some of the rhizobacteria studied by the authors also damaged roots of the seedlings grown in germination pouches. Another study (Li et al. 2012) describes an endophytic actinobacterial strain that stunted the root development of *Artemisia annua* seedlings grown under sterile conditions when inoculated at higher concentrations. The same strain did not show effects on the growth of *A. annua* under greenhouse conditions. One possible answer is that *Paenibacillus* as a facultative soil bacterium can easily survive in soil by using nutrients present in the soil for its own metabolism. Plant cells do not have to be degraded in order for the released metabolites to be used as a nutrition source for *Paenibacillus*. In this case, the positive effects of the *Paenibacillus* on the plant override possible negative effects and so the plant can profit from this relationship. The PGP is probably achieved by the production of plant growth hormones by the *Paenibacillus* rather than its biocontrol properties because possible pathogens are missing in the sterile soil conditions. The differences observed in PGP effect of *P. polymyxa* Sb3-1 on plants grown in sterile and non-sterile soil is most probably linked to the shift in the soil microbiome that occurs when other microorganisms are present, as in the case of non-sterile soil (Erlacher et al. 2014). For example, it is possible that the existing community in the non-sterile soil is sufficient for the optimal growth of the plant so that no effect of the treatment with the BCA can be observed.

In our study we were able to confirm some observations from previous studies, like the in vitro antagonistic effect towards *Verticillium* of the nine out of ten selected strains and the deleterious effect of *P. polymyxa* on the roots of the plants grown in gnotobiotic soil-free conditions (Timmusk et al. 2005). Moreover, we found that in contrast to other published bacteria-plant relationships (for example, Kloepper and Schroth 1980), *P. polymyxa* Sb3-1 enhanced the growth of oilseed rape seedlings in sterile soil conditions, while no effect was observed in the non-sterile soil.

Our results have shown that in the search for an ideal biocontrol strain it is not sufficient to perform only the in vitro tests or to exclusively study the interaction of the plant with the bacterium in artificial gnotobiotic conditions. This study suggests that the natural non-sterile soil is the best medium for studying plant-bacterium interaction as it reflects the field conditions on the best way. It is also apparent that further testing of the selected strains for their biocontrol effects against *Verticillium* wilt in *Brassica* spp. as well as further greenhouse experiments and field trials are necessary in order to fully evaluate the biocontrol effect of the selected strains. In conclusion, our study has not only contributed to the development of a sustainable and environmentally friendly solution to the as yet untreatable disease on *Brassica* plants, it has also allowed provided greater insight into a specific and controversial plant-endophyte interaction.

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