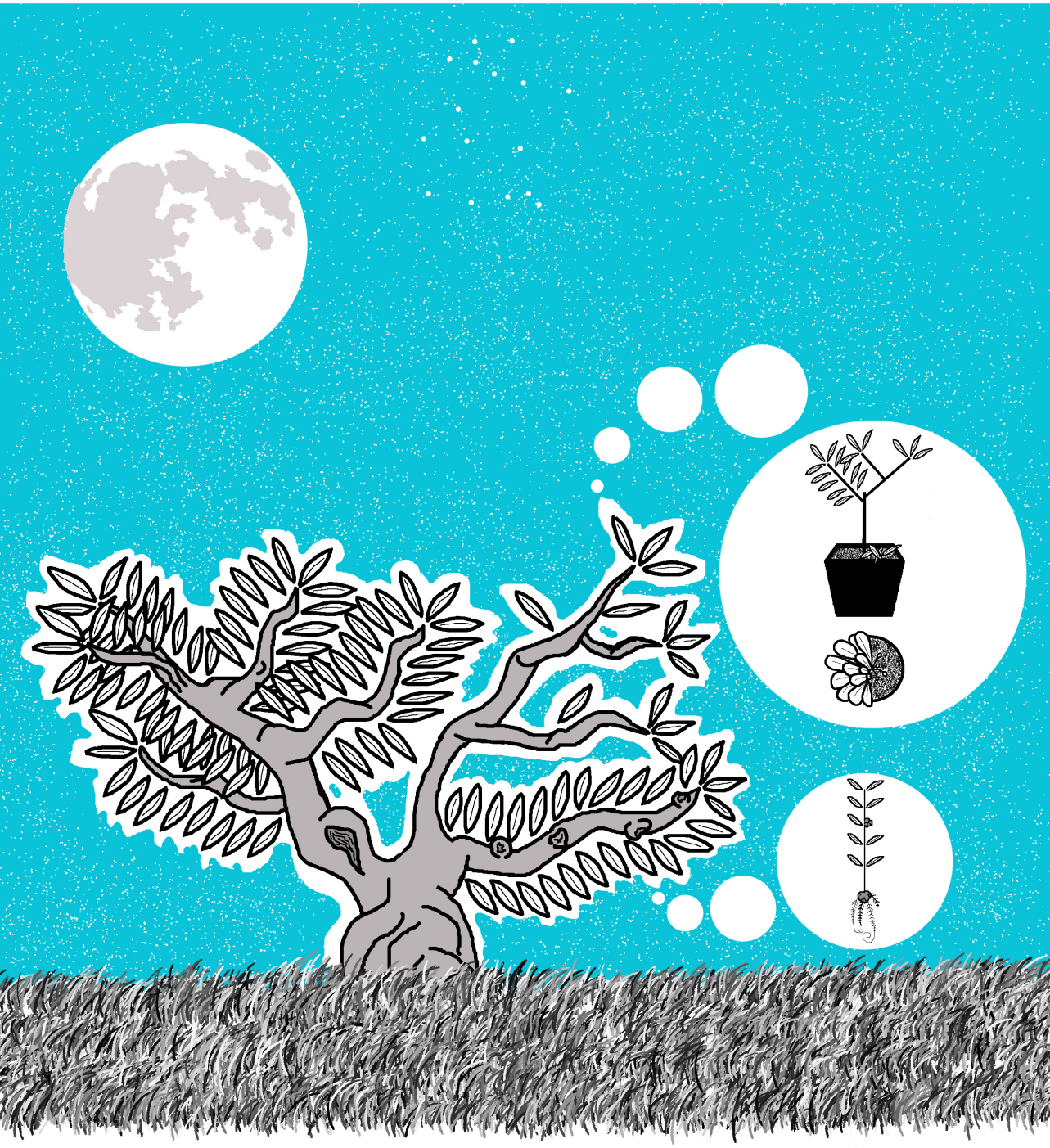
Biological control and endophytism of the olive root bacterium *Pseudomonas fluorescens* PICF7



AUTHOR: Mª MERCEDES MALDONADO GONZÁLEZ
SUPERVISOR: JESÚS MERCADO BLANCO

TITULO: *Biological control and endophytism of the olive root bacterium Pseudomonas fluorescens PICF7*

AUTOR: *Mª Mercedes Maldonado González*

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

www.uco.es/publicaciones
publicaciones@uco.es

UNIVERSIDAD
D
CÓRDOBA

Tesis doctoral

Biological control and endophytism of the olive root bacterium *Pseudomonas fluorescens* PICF7

Doctoranda

Mª Mercedes Maldonado González

Director

Jesús Mercado Blanco

Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (IAS), Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC)

Tesis presentada por compendio de artículos para la obtención del Título de Doctor con Mención Internacional por la Universidad de Córdoba

Córdoba, 2015

TÍTULO DE LA TESIS:
Biological control and endophytism of the olive root bacterium *Pseudomonas fluorescens* PICF7

DOCTORANDO/A:
Mª Mercedes Maldonado-González

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

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

El trabajo titulado "Biological control and endophytism of the olive root bacterium *Pseudomonas fluorescens* PICF7", realizado por Mª Mercedes Maldonado González, se considera correctamente finalizado y reúne los requisitos necesarios para su exposición y defensa como Tesis Doctoral.

El trabajo se ha llevado a cabo en el Departamento de Protección de Cultivos del Instituto de Agricultura Sostenible (IAS) de Córdoba, perteneciente a la Agencia Estatal Consejo Superior de Investigaciones Científicas (CSIC). El grado de consecución de los objetivos inicialmente planteados ha sido muy satisfactorio. Se ha avanzado en el conocimiento de la interacción tripartita olivo-*Verticillium dahliae*-*Pseudomonas fluorescens* PICF7. Se ha determinado el nivel de implicación de determinados fenotipos bacterianos, tanto en la capacidad endófita de la cepa PICF7 como en el control biológico que ejerce frente a una de las enfermedades más importantes que afecta al olivar. Además del conocimiento fundamental adquirido, se han generado herramientas biotecnológicas que permitirán avances tanto en el contexto general de la interacción planta-microorganismo como en el desarrollo de nuevas medidas de control dentro de una estrategia de manejo integrado de la Verticilosis del olivo.

Los trabajos efectuados han quedado reflejados en numerosas contribuciones científicas. Son de destacar tres artículos en revistas de prestigio pertenecientes al área de Microbiología e incluidas en los dos primeros cuartiles (dos en Q1 y una en Q2) de la relación publicada por el '*Journal Citation Report*' (JCR). Además, la doctoranda es coautora de otro artículo de investigación (publicado en revista Q2) no recogido en esta Tesis y ha presentado cuatro comunicaciones orales en dos congresos nacionales y dos internacionales. También es co/autora de otras nueve comunicaciones presentadas en varias reuniones científicas. Por último, ha efectuado durante el periodo de ejecución del trabajo tres estancias en un centro de reconocido prestigio, la Universidad de Utrecht (Países Bajos), que le han permitido complementar su formación como investigadora.

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

Córdoba, 29 de junio de 2015

Firma del/de los director/es

Fdo.: Jesús Mercado Blanco

Mención Internacional

La presente Tesis cumple con las directrices establecidas según RD 99/2011 para la obtención del Título de Doctor con Mención Internacional por la Universidad de Córdoba:

1. Realización de una estancia de 8 meses divida en tres períodos (6 Octubre-29 Diciembre, 2010; 28 Julio-11 Noviembre, 2011; y 2 Junio-29 Julio, 2013) fuera de España en el grupo de Plant-Microbe Interaction en la Universidad de Utrecht bajo la supervisión del Dr. Peter A. H. M. Bakker.

2. Redacción y presentación de la Tesis en inglés.

3. Informe oficial previo de la Tesis realizado por dos doctores/as expertos con experiencia investigadora acreditada pertenecientes a alguna institución de educación superior o instituto de investigación distinto de España:

   - ✓ Dra. Leah Tsror, Agricultural Research Organization (ARO), Department of Plant Pathology and Weed Research Gilat Center, MP Negev 85280, Israel.

   - ✓ Dr. Sotiris Tjamos, Plant Pathology, Agricultural University of Athens 75 Iera Odos str, Athens 11855, Greece.

4. Un doctor/a perteneciente a alguna institución de educación superior o centro de investigación no española, y distinto del responsable de la estancia de investigación, formará parte del tribunal evaluador de la tesis.

   - ✓ Prof. Gabriel Berg, Graz University of Technology (TU Graz), Institute of Environmental Biotechnology, Petersgasse 12, A-8010 Graz, Austria.

La doctoranda:

Fdo.: Mª Mercedes Maldonado González

Tesis como compendio de publicaciones

La presente Tesis está constituida por 3 artículos publicados en revistas incluidas en los tres primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por el Journal Citation Reports (JCR):

1. Maldonado-González, M. M., Schilirò, E., Prieto, P., and Mercado-Blanco, J. (2015). Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. *Environmental Microbiology*, (on-line first). DOI: 10. 1111/1462-2920.12725.

   Datos de 2014 (JRC): Índice de impacto 6.201, posición 12/119 y primer cuartil (Q1) en el área temática de Microbiología.

2. Maldonado-González, M. M., Bakker, P. A., Prieto, P., and Mercado-Blanco, J. (2015). *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. *Frontiers in Microbiology*, *6,* 266. DOI: 10.3389/fmicb.2015. 00266.

   Datos de 2014 (JRC): Índice de impacto 3.989, posición 27/119 y primer cuartil (Q1) en el área temática de Microbiología.

3. Maldonado-González, M. M., Prieto, P., Ramos, C., and Mercado-Blanco, J. (2013). From the root to the stem: interaction between the biocontrol root endophyte *Pseudomonas fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB 3335 in olive knots. *Microbial Biotechnology*, *6*(3), 275-287. DOI: 10.1111/1751-7915.12036.

   Datos de 2014 (JRC): Índice de impacto 3.081, posición 39/119 y segundo cuartil (Q2) en el área temática de Microbiología.

La doctoranda:

Fdo.: Mª Mercedes Maldonado González

Funding

This thesis was funded by grants AGL2009-07275 ('Verticilosis del olivo: bases moleculares de tolerancia natural y de resistencia inducida por bacterias') and AGL2011-30343-C02-01 ('Análisis funcional de factores de virulencia y determinantes del espectro de huésped en *Pseudomonas savastanoi'*) from Spanish MICINN/ MINECO and grants P07-CVI-02624 ('Identificación de los mecanismos implicados en el control de la Verticilosis del olivo por cepas de *Pseudomonas* spp. colonizadoras naturales de raíces de olivo'), P08-CVI-03475 ('Genómica funcional y proteómica de transportadores MDR bacterianos implicados en infecciones de plantas') and P12-AGR-667 ('*Pseudomonas* spp. efectivas en el biocontrol de la Verticilosis del olivo: bases de su endofitismo, efectos sobre la planta y microbiota asociada, rango de colonización y aplicaciones prácticas') from J. Andalucía (Spain), all of them co-financed by ERDF of the EU.

Agradecimientos

Siete años después, tras mucho trabajo y aprendizaje, puedo afirmar que el doctorado me ha cambiado en muchos sentidos. Me ha permitido viajar a otros países, conocer multitud de personas, desarrollar mi vida como investigadora y, sobre todo, crecer. Por eso ¡gracias!. Gracias a todos aquellos que han participado de alguna manera en este largo camino. Gracias a mi madre, Mercedes, por poder costearme la Licenciatura en Biología y sobre todo por haberme dado la mejor vida hasta que fui capaz de volar. Gracias a mis hermanos, Pedro y Elvira, y a todo lo vivido con ellos que no ha sido poco. Gracias a mi padre, soy como soy en gran parte por ti. Y, gracias a toda mi familia, en especial a mi abuela Eulogia a la que tengo muy presente. Siempre me apoyasteis y animasteis. Gracias a mi director, Jesús, me has dado la oportunidad de seguir evolucionando como persona tanto a nivel profesional como personal. Gracias también a todos aquellos investigadores que han participado en mi formación a lo largo de este periplo, Cayo, Encarnita, Leire, Pilar y Peter. Peter, hasta luego cocodrilo!, hasta luego Lucarrrrrrr! 'but always see you later'. Thanks to Corné, David, Guido, Saskia and again Peter for the great ambient that I enjoyed in all my stays in your awesome lab in the Netherlands. Mención especial a Eli y David por ser mentores en lo profesional y amigos en lo personal. Gracias a todos los amigos a lo largo de este período. A los del principio, Adri, David, Rebeca y Zori, con vosotros el comienzo de mi aventura fue lo mejor que me pudo pasar. Y, gracias a todos los que habéis ido llegando y saliendo de mi vida pero que siempre formareis parte de mi historia, tantos que no sé si cabréis Álvaro, Ana, Ángel, Antonio, Antonio (vecino), Carlos, Carlos (Mejicano), Carmen, Carmen (Paraguaya), Carmen (Sevilla), David, Estefanía, Eva, Javi, Julia, Liz, Lola, Lola (Gaditana), Manolo, María, Macmolder, Melania, Paco, Rocío, etc. ¡Os quiero chicos!. Agradecida sin medida a Thaïs, me cambiaste amor. También a Ana, Marta y Sheila. Todas, sin duda, mujeres con mayúsculas. Likewise, thanks to all my friends in the Netherlands Ainoa, Amparo, Carmen, Chiel, Christos, David, Dieu, Dimitri, Fernando, Hamid, Iraria, Iván, Javi, Leti, Mikko, Nora, Pim and, especially, to Roeland (love you men). You all guys make me feel at home in the Netherlands. I´ve never thought how much that experience could change me. También, agradecer a mis amigos del 'Algafequi' Alicia, Andrea, Pablo, Quique, Torqui y Victoria por esas horas de circo y de no circo. También a mis parejas pasadas y presente porque todos habéis sido importantes en diferentes momentos de mi vida. Gracias por aprender a conocerme a través de vosotros. De nuevo, ¡GRACIAS A TODOS!.

Content

List of figures

List of tables

List of abbreviations

ACB	Agente de control biológico
AFLP	Amplified fragment length polymorphism
Amp	Ampicillin
ANOVA	Analysis of variance
Aux	Auxotroph
BCA	Biological control agent
Ca	Calcium
CAS	Chrome azurol S
Cfu	Colony forming units
Chl	Chloramphenicol
Chx	Cycloheximide
CLSM	Confocal laser scanner microscopy
CSIC	Centro Superior de Investigaciones Científicas
Cys	Cysteine
D	Defoliating
DAB	Days after bacterization
DAI	Days after inoculation
DI	Disease incidence
DII	Disease intensity index
DKW	Driver-Kuniyuki walnut
EGFP	Enhanced green fluorescent protein
ein2	ET insensitive2, *Arabidopsis thaliana* mutant affected in the protein EIN2
ET	Ethylene
FAO	Food and Agriculture Organization of the United Nations
fliI	Mutant impaired in swimming motility
GFP	Green fluorescence protein
Gm	Gentamicin
Gm^R	Gentamicin resistant
gltA	Mutant with diminished *in vitro* growth in potato dextrose agar medium
IAA	Indole-3-acetic acid
IDM	Integrated disease management
IFAPA	Instituto de Formación Agraria y Pesquera de Andalucía
IGS	Intergenic spacer
ISR	Induced Systemic Resistance

ITS	Internal transcribed spacer
JA	Jasmonic acid
jar1	*Arabidopsis thaliana* mutant affected in jasmonyl isoleucine conjugate synthase 1
K	Potassium
KBA	King's medium B agar
LAMP	Loop-mediated isothermal amplification
LB	Luria-Bertani
LBA	Luria-Bertani agar
LSD	Least significant difference
MATE	Multidrug and toxic compound extrusion transporter
ME419	*gltA*
ME424	*fliI*
ME589	*pvdI*
ME1508	Cys Aux
Mg	Magnesium
MS	Microsclerotia
myb72	*Arabidopsis thaliana* mutant affected in R2R3-MYB-like transcription factor protein
N	Nitrogen
Nal	Nalidixic acid
Nat	Natamycin
ND	Non-defoliating
NPR1	NONEXPRESSOR OF *PR* GENES1
OKD	Olive knot disease
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PGPR	Plant growth promoting rhizobacteria
PR	Pathogen-related
Psv	*Pseudomonas savastanoi* pv. savastanoi
Pvd	Pyoverdine; pioverdina
pvdI	Mutant impaired in siderophore pyoverdine production
QNRT-PCR	Quantitative nested real time polymerase chain reaction
RAPD	Random amplified polymorphic DNA
Rf	Rifampicin
RFLP	Restriction fragment length polymorphism
RFP	Red fluorescent protein
RT-PCR	Real time polymerase chain reaction
RT-qPCR	Real time quantitative polymerase chain reaction

SA	Salicylic acid
SAR	Systemic Acquired Resistance
SAUDPC	Standardized area under the disease progress curve of *DII* plotted over time
sid1	*Arabidopsis thaliana* mutant defective in a member of the MATE family
sid2	*Arabidopsis thaliana* mutant affected in isochorismate synthase
SM	Swimming medium
SNP	Single nucleotide polymorphism
SSM	Standard succinate medium
Tc	Tetracycline
Tc^R	Tetracycline resistant
TO	Tuberculosis del olivo
VBNC	Viable but non-culturable
VCG	Vegetative compatibility group
VO	Verticilosis del olivo
VOCs	Volatile organic compounds
VW	Verticillium wilts
VWO	Verticillium wilt of olive

Summary

Olive (*Olea europaea* L.) has always been a fundamental crop in the Mediterranean Basin. Driven by the fact, among others, that an increasing number of scientific reports highlight the benefits that olive oil consumption has for human health, olive tree cultivation has spread worldwide to other regions with Mediterranean-type climate. Two relevant pathogens affecting olive trees are the hemibiotrophic soil-borne fungus *Verticillium dahliae* and the bacterium *Pseudomonas savastanoi* pv. savastanoi (Psv), causal agents of Verticillium wilt of olive (VWO) and olive knot disease (OKD), respectively. Effective control of these pathogens must rely on integrated disease management strategies, with emphasis in preventive, cost-effective and environmentally-friendly measures. Among the available control tools, the use of microbial antagonists with biocontrol potential emerges as a promising strategy to implement either alone or in combination with other disease management measures. Moreover, the use of biological control agents (BCA) native from the host plant, and therefore adapted to the target ecological niche where their benefits can be deployed, is a reasonable approach. In previous studies, *Pseudomonas fluorescens* PICF7, an indigenous inhabitant bacterium of the olive rhizosphere, was confirmed as an effective BCA against the defoliating, highly-virulent pathotype of *V. dahliae*. This bacterial strain displays endophytic lifestyle in olive roots, produces the siderophore pyoverdine (Pvd), and is able to induce a broad range of defense responses in both roots and above-ground organs when colonizing olive roots. Even though the information so far gathered has increased in the last few years, the study of the PICF7-*V. dahliae*-olive tripartite interaction is complex and an in-depth knowledge on the relationships established among the three partners is needed. On the one hand, working with a woody, long-living plant such as olive poses difficulties inherent to the own characteristics of the plant. On the other hand, the genetic and molecular mechanisms underlying VWO biocontrol exerted by strain PICF7 are still unknown. To shed light for the first time on PICF7 traits involved in the control of *V. dahliae* as well as in the ability of this BCA to colonize the interior of olive roots, four PICF7 phenotypes were studied by mutant analysis. Thus, two mutants affected in traits usually related to colonization ability and biological control of plant pathogens (i.e. swimming motility and siderophore pyoverdine production) and two altered in nutritional requirements (i.e. growth delay in PDA medium and cysteine auxotrophy) were generated, characterized and evaluated in both *in vitro* and in *in planta* assays. In addition, this thesis also investigated whether the model plant *Arabidopsis thaliana* can be used to facilitate the identification of *P. fluorescens* PICF7 traits involved in both *V. dahliae* biocontrol and endophytic lifestyle. The aim was to compare the behavior of the same PICF7 mutant phenotypes in olive and Arabidopsis. Furthermore, since strain

PICF7 is able to induce systemic defense responses in olive aerial tissues, this thesis also aimed to assess whether mechanisms such as Induced Systemic Resistance (ISR) and/or Systemic Acquired Resistance (SAR) are triggered in olive upon PICF7 root colonization. To prove this, spatial separation of the BCA and the pathogen is required. This prerequisite is difficult to fulfill in this tripartite interaction since both microorganisms share the same ecological niche – the olive roots. To achieve that, two different approaches were followed in this thesis. On the one hand, biocontrol effectiveness of strain PICF7 was evaluated against OKD, inoculating the BCA in the roots and Psv in the stems. As a complement to these bioassays both microorganisms were also co-inoculated in stems in order to study the potential effects that the presence of strain PICF7 might cause in tumor development. On the other hand, by using diverse mutants of the model plant *A. thaliana* affected in defense signaling pathways, the ability of PICF7 to trigger ISR/SAR responses when applied to the roots was evaluated against the foliar fungal pathogen *Botrytis cinerea*. In these studies confocal laser scanner microscopy (CLSM) and fluorescently-labeled bacterial derivatives were used to monitor: (i) the colonization ability of *P. fluorescens* PICF7 in *A. thaliana* to check whether this BCA can establish endophytically in this host; and (ii) the localization and possible interaction of Psv and PICF7 in olive stem tissues and during olive tumor development. Results demonstrated a similar behavior of *P. fluorescens* PICF7 and its mutants in both olive and *Arabidopsis* plants. Thus, although all mutants colonized the roots of both hosts the cysteine auxotroph mutant always displayed the lowest population size. Regarding to biocontrol ability, cysteine auxotrophy but not swimming motility nor pyoverdine production seemed to be related to VW suppression in both hosts. Results from CLSM showed that all PICF7 mutants evaluated in this thesis colonized endophytically olive root tissues to the same extent than the wild type. In contrast, no evidence of endophytic colonization of *A. thaliana* was found for PICF7 and its mutants, but only colonization of the rhizoplane. Evidence of an ISR response triggered by strain PICF7 was found against *B. cinerea* in *A. thaliana*. In contrast, colonization of olive roots by PICF7 did not hinder knot development produced by Psv in olive. However, when strain PICF7 was co-inoculated with Psv in stems a transient decrease of the pathogen population size, a reduction of tumor necrosis, and changes in the knot inner anatomy were observed. These findings indicated that presence of PICF7 altered the normal development of the tumor. Besides, CLSM imagery revealed that Psv cells within the hyperplasic tissue showed a different location depending on whether PICF7 was absent (Psv cells at the knot surface) or present (Psv cells mostly located in the inner regions of the tumors). By using this methodological approach it was also shown for the first time that pathogen cells can colonize the vascular tissue outside the hyperplasic tissue, indicating that Psv can potentially move systemically.

Resumen

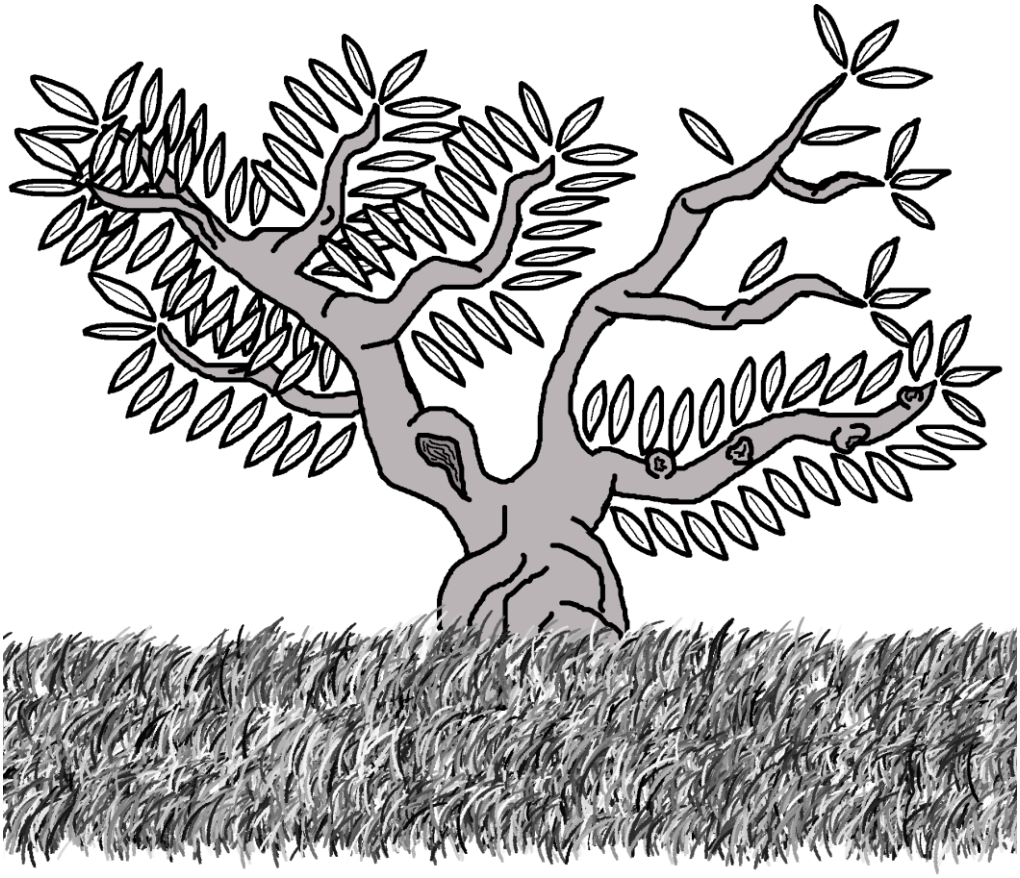
El olivo (*Olea europaea* L.) es un cultivo fundamental de la Cuenca Mediterránea. En estas últimas décadas se ha extendido a otras regiones que disfrutan de clima Mediterráneo debido, entre otros factores, al creciente número de investigaciones científicas que subrayan los efectos beneficiosos derivados del consumo del aceite de oliva en nuestra salud. Sin embargo, este cultivo se encuentra amenazado por diversos factores a/bióticos. De entre los diversos estreses bióticos cabe destacar al hongo hemibiotrofo de suelo *Verticillium dahliae*, causante de la enfermedad más importante que afecta a este cultivo, la Verticilosis del olivo (VO). Otra seria amenaza para la olivicultura es la Tuberculosis del olivo (TO) causada en este caso por la bacteria *Pseudomonas savastanoi* pv. savastanoi (Psv). Para un control efectivo de cualquier fitopatología, entre ellas la VO y la TO, es aconsejable llevar a la práctica una estrategia basada en el manejo integrado de la enfermedad, teniendo en especial consideración aquellas medidas preventivas que son tanto rentables como respetuosas con el medio ambiente. Entre las herramientas de control disponibles, el uso de microorganismos antagonistas (agentes de control biológico [ACBs]) emerge como una medida prometedora. Los ACBs poseen la ventaja de que pueden aplicarse tanto de forma individual como en combinación con otras medidas de manejo de la enfermedad, además de actuar de manera beneficiosa en su propio nicho ecológico al cual se encuentran plenamente adaptados. En estudios previos, *Pseudomonas fluorescens* PICF7, bacteria indígena de la rizosfera de raíz, confirmó ser un ACB efectivo frente al patotipo defoliante, altamente virulento, de *V. dahliae*, además de producir el sideróforo pioverdina (Pvd), inducir un amplio rango de respuestas defensivas tanto en raíces como en órganos aéreos y comportarse como endófita en la raíz del olivo. Sin embargo, el conocimiento en lo referente a la interacción tripartita PICF7-*V. dahliae*-olivo es todavía muy escaso, dificultado en parte por las características propias de un huésped leñoso y longevo como el olivo. Asimismo, los mecanismos genéticos y moleculares que subyacen en el control biológico de la VO por parte de la cepa PICF7 son todavía desconocidos. Para dilucidar qué caracteres de PICF7 pueden estar implicados en el control de *V. dahliae*, así como la capacidad de este ACB para colonizar el interior de las raíces de olivo, se seleccionaron cuatro fenotipos de PICF7 mediante el empleo de mutantes. Para ello, se generaron, caracterizaron y evaluaron tanto *in vitro* como en ensayos en planta dos mutantes afectados en rasgos habitualmente relacionados con la capacidad de colonización y el control biológico de fitopatógenos (motilidad tipo 'swimming' y producción del sideróforo pioverdina) y dos alterados en requerimientos nutricionales (retraso en el crecimiento en medio PDA y auxotrofía en cisteína). Además, la presente tesis también investigó la posibilidad de que la planta modelo *Arabidopsis thaliana*

pudiera ser un posible sustituto y/o complemento que facilitase la identificación de los caracteres de *P. fluorescens* PICF7 involucrados tanto en el biocontrol de *V. dahliae* como en el endofitismo. Para ello, se comparó el comportamiento de los cuatro fenotipos mutantes de PICF7 mencionados con anterioridad en ambas plantas huésped, olivo y *Arabidopsis*. Además, puesto que la cepa PICF7 es capaz de inducir respuestas de defensa sistémica en tejidos aéreos del olivo, esta tesis también tuvo como objetivo evaluar si los mecanismos tales como la resistencia sistémica inducida ('Induced Systemic Resistance'; ISR) y/o la resistencia sistémica adquirida ('Systemic Acquired Resistance'; SAR) se activan en olivo tras la colonización de la raíz por parte de PICF7. Para la consecución de lo anterior es requisito indispensable la separación espacial del ACB y del patógeno. Esta premisa es difícil de cumplir en esta interacción tripartita ya que PICF7 y *V. dahliae* comparten el mismo nicho ecológico, las raíces del olivo. Por tanto, se llevaron a cabo dos enfoques diferentes. Por un lado, se evaluó la eficacia de la cepa PICF7 en el biocontrol de la TO (enfermedad que afecta a la parte aérea) al inocular el ACB en las raíces y a la bacteria fitopatógena (Psv) en tallo. Con el fin de complementar estos bioensayos ambos microorganismos se co-inocularon en tallo para así estudiar los efectos que la presencia de la cepa PICF7 podría causar en el desarrollo del tumor. Por otro lado, mediante el uso de diversos mutantes afectados en rutas de señalización relacionadas con la defensa de la planta huésped en la planta modelo *A. thaliana*, se evaluó la capacidad de PICF7 para desencadenar respuestas ISR y/o SAR tras su aplicación en raíz frente al hongo patógeno foliar *Botrytis cinerea*. En estos estudios se usó la microscopía confocal láser de barrido ('Confocal Laser Scanner Microscopy'; CLSM) junto con derivados bacterianos marcados con fluorescencia para observar: (i) la capacidad de colonización de *P. fluorescens* PICF7 en *A. thaliana* y comprobar si puede establecerse endofíticamente en este huésped; y (ii) la localización y posible interacción de Psv y PICF7 en los tejidos del tallo y el efecto que la presencia del ACB pudiera tener en el desarrollo del tumor. Los resultados obtenidos demostraron un comportamiento similar de *P. fluorescens* PICF7 y sus mutantes tanto en olivo como en *Arabidopsis*. Todos los mutantes fueron capaces de establecerse en las raíces de ambos hospedadores. Sin embargo, el mutante auxótrofo en cisteína siempre mostró un tamaño poblacional menor. En cuanto a la capacidad de biocontrol, la auxotrofía en cisteína pero no la motilidad tipo 'swimming' ni la producción de pioverdina parecen estar relacionadas con la supresión de la VO en ambos hospedadores. Los resultados obtenidos gracias a la CLSM mostraron que tanto PICF7 como sus mutantes colonizan endofíticamente el tejido radical del olivo. Por el contrario, no se obtuvo evidencia de colonización endofítica por parte de PICF7 y sus mutantes en *A. thaliana*, sólo colonización del rizoplano. Igualmente, se demostró que la cepa PICF7 provoca una respuesta ISR frente a *B. cinerea* en *A. thaliana*. Por otro lado, la

colonización de las raíces de olivo por parte de PICF7 no impidió el desarrollo de tumores tras la inoculación de Psv en tallo. Sin embargo, la co-inoculación en tallo de ambas bacteria produjo una disminución transitoria del tamaño poblacional del patógeno, una reducción de la necrosis tumoral y cambios en la anatomía interna del tumor. Estos hallazgos indican que la presencia de PICF7 altera el desarrollo normal del tumor. Además, las imágenes de CLSM revelaron que las células de Psv en el tejido hiperplásico muestran una ubicación diferencial definida por la ausencia de la cepa PICF7 (células de Psv en la superficie del tumor) o presencia de la misma (células de Psv mayoritariamente localizadas en las regiones internas del tumor). Además, el uso de este enfoque metodológico demostró claramente por primera vez que las células del patógeno pueden colonizar el tejido vascular fuera del tumor, lo que indica que Psv puede desplazarse potencialmente de manera sistémica.

CHAPTER 1

General introduction



1.1. Olive history and importance

Olive (*Olea europaea* L.) cultivation was established and spread throughout the Mediterranean Basin since at least 5000 years ago (Connor, 2005) by Phoenicians, Greeks and Romans. The original area of domestication of this tree, from wild olive (*Olea europaea* var. *sylvestris*) (Moazzo et al., 1994; Teofrasto, 1988), is uncertain and controversial. While some authors affirm that cultivated olive originated from the Eastern Mediterranean Basin (Zohary and Spiegel, 1975; Angiolillo et al., 1999), this assumption being supported by archeological evidence, others suggest that domestication took place in the Western Mediterranean area (Magdelaine and Ottaviani, 1984; Terral and Arnold-Simard, 1996; Terral, 1997). More recent surveys using molecular techniques propose that cultivar selection was multilocal, taking place in different areas of the Mediterranean region (Besnard et al., 2001). After the spread of olive cultivation in the Mediterranean Basin, this crop was introduced into other countries situated between latitudes 30°- 45° at both Northern and Southern Hemispheres in Mediterranean-type climatic regions (**Figure 1.1**). Thus, following the discovery of the Americas, olive cultivation was expanded into both south and north of the continent (Peru, Argentina, Chile, USA, and Mexico). Thereafter, in the XIX century, olive crop reached Australia. Since olive oil benefits for human health are increasingly established based on a number of scientific reports (Owen et al., 2000; Amiot, 2014), the list of regions where this tree is being cultivated has steadily grown over the last two centuries: South Africa, China, Japan, Pakistan and Australia (Connor, 2005).

Figure 1.1. Olive cultivation in the world. Grey rectangle and solid circles, regions where olive tree is being cultivated. Grey rectangle frames the Mediterranean Basin. Solid circles mark the remaining areas. Based on http://www.zonu.com/fullsize/2009-11-05-10853/Mapa-Mudo-del-Mundo.html.

Regarding to olive world production and cultivation acreage, Spain is the leading country in both aspects, accounting for 7.9 x 10^6 tons (39% of the world production) and 2.5 x 10^6 ha, (24% of the world acreage) (**Table 1.1**) (FAO, 2013). Production is diversified in table olives, oil mill olives and olives with double aptitude (7.6 x 10^4, 2.5 x 10^6 and 7.1 x 10^4 ha, respectively) (MAGRAMA, 2014). In particular, the region of Andalusia (Spain) is the main producer with a registered cultivated area of 1.5 x 10^6 ha (5.5 x 10^4, 1.4 x 10^6 and 6.7 x 10^4 ha devoted to table olives, oil mill olives and olives with double aptitude, respectively) (MAGRAMA, 2014).

Table 1.1. Production and area devoted to olive cultivation worldwide.

Country	Production[a]		Cultivation area[a]		VWO[1] first report
	Tons (x1000)	%	Ha (x1000)	%	
Spain	7875.80	38.71	2500.00	24.40	Caballero et al., (1980)
Italy	3022.89	14.86	1125.00	10.98	Ruggieri, (1946)
Greece	2000.00	9.83	930.00	9.08	Zachos, (1963)
Turkey	1676.00	8.24	825.83	8.06	Saydam and Copcu, (1972)
Morocco	1181.68	5.81	922.24	9.00	Serrhini and Zeroual, (1995)
Syrian Arab Republic	1000.00	4.91	690.00	6.74	Al-Ahmad and Mosli, (1993)
Tunisia	963.00	4.73	1800.00	17.57	Triki et al., (2006)
Egypt	510.00	2.51	52.10	0.51	
Algeria	395.00	1.94	330.00	3.22	Bellahcene et al., (2000)
Portugal	350.90	1.72	347.30	3.39	Gouveia and Coelho, (2007)
Argentina	172.00	0.85	63.00	0.61	Docampo et al., (1981)
USA	145.00	0.71	17.00	0.17	Snyder et al., (1950)
Libya	138.00	0.68	210.00	2.05	
Jordan	128.19	0.63	62.39	0.61	Mamluk et al., (1984)
Albania	125.00	0.61	48.00	0.47	
Lebanon	97.00	0.48	58.00	0.57	Makhlouf and Geagea, (2005)
Australia	93.52	0.46	42.00	0.41	Morschel, (1961)
Occupied Palestinian Territory	76.00	0.37	51.00	0.50	
Chile	74.30	0.37	18.31	0.18	
Israel	67.00	0.33	34.00	0.33	Levin et al., (2003a)
Peru	57.77	0.28	16.44	0.16	

Iran (Islamic Republic of)	36.00	0.18	22.00	0.21	Sanei et al., (2004)
Croatia	34.27	0.17	18.59	0.18	
France	26.85	0.13	17.17	0.17	Vigouroux, (1975)
Mexico	26.30	0.13	6.92	0.07	
Iraq	19.00	0.09	4.65	0.05	Al-Taae and Al-Taae, (2010)
The former Yugoslav Republic of Macedonia	13.00	0.06	6.00	0.06	
El Salvador	10.25	0.05	5.00	0.05	
Cyprus	8.88	0.04	10.40	0.10	
Afghanistan	6.60	0.03	2.20	0.02	
Uruguay	6.30	0.03	3.00	0.03	
Montenegro	2.90	0.01	2.40	0.02	Latinovic and Vucinic, (2010)
China	2.00	0.01	0.27	0.00	
China, Taiwan Province of	2.00	0.01	0.27	0.00	
Slovenia	1.50	0.01	0.90	0.01	
Azerbaijan	0.84	0.00	1.74	0.02	
Brazil	0.30	0.00	0.10	0.00	
Bosnia and Herzegovina	0.15	0.00	0.11	0.00	
Uzbekistan	0.10	0.00	0.10	0.00	
Kuwait	0.06	0.00	0.04	0.00	
Malta	0.01	0.00	0.01	0.00	Porta-Puglia and Mifsud, (2005)
WORLD	20346.34	100.00	10244.46	100.00	

[a] 2013 data obtained from the Food and Agriculture Organization of the United Nations (FAO) (http:// faostat3.fao.org/browse/Q/*/E)
[1] VWO, Verticillium wilt of olive

1.2. Principal diseases affecting olive

Olive cultivation is threatened by a multitude of diverse (a)biotic stresses. Known biotic disorders are summarized along with their importance in the Mediterranean Basin in **Table 1.2**.

Table 1.2. Principal biotic disorders affecting olive cultivation.

Disease	Causal agent	Importance[1]
Aerial mycoses		
Peacock eye	*Fusicladium oleagineum*(= *Cyclonconium oleaginum, = Spilocaea oleagina*)	H
Anthracnose	*Colletotrichum acutatum, C. gloesporioides* (=*gloeosporium olivarum*)	H
Leaf spot	*Pseudocercospora cladosporioides* (= *Cercospora cladosporioides*)	M
Prill	*Capnodium elaeophilum*	L
Canker	*Botryosphaeria dothidea* (=*Camarosporium dalmaticum*)	L
Leprosy	*Phlyctema vagabunda* (=*Glieosporium olivae*)	L
Other fruit rots	*Alternaria, Aspergillus, Cladosporium, Diplodia, Geotrichum, Fusarium, Phomopsis*, etc.	L
Other leaf mycosis	*Leveillula, Phyllactinia, Stictis panizzei*, etc.	L
Cankers	*Neofusicoccum mediterraneum, Eutypa lata, Phoma incompta*	L
Wood decay	*Fomes, Formitiporia, Phellinus, Polyprous, Stereum*, etc.	L
Vascular mycosis		
Verticillium wilt	*Verticillium dahliae*	H
Root diseases		
Rot of thin roots	Oomycetes: *Phytophthora, Pythium,*etc. Fungi: *Cylindrocarpon, Fusarium*, etc.	M-L
Rot of thick roots	*Armillaria mellea, Rosellinia necatrix, Omphalotus olearius*	L
Bacterial diseases		
Olive tuberculosis (= olive knot disease)	*Pseudomonas savastanoi* pv. savastanoi	H-M
Olive quick decline syndrome	*Xylella fastidiosa*	H
Virus and phytoplasma diseases		
Malformations, Yellowness	Virus and phytoplasmas not identified	W
Latent infections, Yellowness	*Nepovirus, Cucumovirus, Oleavirus*, etc.	W
Nematodes		
Nodules/Root damage	*Meloidogyne, Pratylenchus*, etc.	W
Phanerogams		
Mistletoe, Red-berry Mistletoe, Dodder	*Viscum*, *Cuscuta*	W

Based on Trapero et al., (2010)

[1]H=high; M=moderate; L=low; W=without general practical importance, although severe attacks have been observed occasionally

Two biotic constraints affecting olive cultivation in almost all olive growing areas worldwide, in particular within the Mediterranean Basin, are *Verticillium dahliae* (López-Escudero and Mercado-Blanco, 2011; Tsror, 2011; Jiménez-Díaz et al., 2012) and *Pseudomonas savastanoi* pv. savastanoi (Psv) (Quesada et al., 2012; Ramos et al., 2012). The gradual shift from traditional olive cultivation to high-tree density orchards (up to 2,000 trees/ha) with drip-irrigation systems (Tous et al., 2010) may introduce new concerns, yet insufficiently evaluated, about the incidence and severity of traditional and/or emerging olive pests and diseases. Thus, dense foliage and narrow alleys in olive hedgerow orchards may provoke high humidity, mild temperature (favorable to disease development) and lower penetration of fungicide treatments (Connor et al., 2014) thereby increasing the incidence of attacks by air-borne fungi such as *Spilocaea oleagina* (olive peacock spot), *Pseudocercospora cladosporioides* (olive cercosporiose), and *Colletotrichum* spp. (olive anthracnose) (Trapero et al., 2009). Furthermore, it has been suggested that damage caused by harvesting machinery commonly used in this type of super-intensive cropping systems can enhance the probability of olive knot disease attacks (see below) (Trapero et al., 2009).

This thesis is focused on Verticillium wilt of olive (VWO) and olive knot disease (OKD), and a brief summary of aspects such as importance, aetiology, symptomatology, epidemiology, life cycle and diversity of the causal agents, mainly for VWO, will be presented in the next sections. This basic knowledge is obviously of relevance for the development and success of control measures within integrated disease management strategies. Among the control measures, biological control emerges as a promising one. Therefore, emphasis will be on the use of biological control agents (BCA_s) against VWO, presenting available antecedents, particularly on the potential that bacterial endophytes have to confront VWO, such as strain *P. fluorescens* PICF7. Succinct information on OKD will be also presented since biocontrol of Psv by strain PICF7 has been explored. Besides, the use of *Arabidopsis thaliana* as a study system to find out whether systemic defense responses are elicited by strain PICF7 will be briefly introduced as well.

1.3. Importance of Verticillium wilt of olive

Verticillium wilt of olive (VWO), caused by *Verticillium dahliae* Kleb. (Klebahn, 1913), is one of the most important diseases affecting olive cultivation (**Table 1.1**). This syndrome was described for the first time in Italy by Ruggieri, (1946). Since then,

VWO has been reported in almost all areas where olive crop is present (**Table 1.1**), causing severe losses in some of them and a great concern in the olive cultivation sector and derivative industries. In Spain, the first observation was reported in 1979 in experimental fields near to Córdoba (Andalusia, Southern Spain) (Caballero et al., 1980). Further surveys performed in the three major olive-growing provinces (Córdoba, Jaén and Seville) in Andalusia confirmed the presence of the disease (Blanco-López et al., 1984). The extension, distribution and severity of VWO attacks have been thoroughly reviewed (López-Escudero and Mercado-Blanco, 2011; Tsror, 2011; Jiménez-Díaz et al., 2012). Recent surveys and epidemiological studies have added novel information about the disease, confirming the spread of the pathogen. For instance, a survey in the Guadalquivir Valley (Andalusia, Southern Spain) by López-Escudero et al., (2011) (**Figure 1.2**) showed that VWO is alarmingly spreading in the region with a mean disease incidence (*DI*) of 20.4% (9000 examined trees) (25.7, 23.7 and 12%, for Jaén, Córdoba and Seville provinces, respectively). These authors concluded that the *DI* was dependent on several agronomical factors. For example, significant higher *DI* values were found in irrigated, non-tilled, low density (<200 trees per ha) or young (<25 year-old) orchards as well as in groves located nearby areas where *V. dahliae* susceptible hosts were cultivated or close to the Guadalquivir river (<10 km). Besides, 'Picual' was the most frequently affected cultivar (cv.) by the disease, and highly-virulent, defoliating (D) isolates being the most commonly-found pathotype. A broader survey study carried out by Jiménez-Díaz et al., (2011) in Andalusia demonstrated the prevalence of the vegetative compatibility group (VCG, see below) 1A (highly virulent) (78.1%) of *V. dahliae* isolates, followed by VCG2A, VCG4B and VCG2B (19.8%, 1.4% and 0.6%, respectively). Analysis by PCR (polymerase chain reaction)-based procedures confirmed VCG1A as D pathotype while the other VCG's group isolates are characterized as non-defoliating (ND, moderately severe symptoms) pathotype (see below). Furthermore, it was common the prevalence of a single VCG among surveyed orchards, although two and three VCGs were also identified, being VCG1A the prevalent VCG in the three most important olive-growing provinces (Córdoba, Jaén, and Seville). In addition, agricultural factors including irrigation management, source of irrigation water and planting stock plus soil cropping history were significantly associated with the occurrence of VCG1A compared to VCG2A (Jiménez-Díaz et al., 2011). A study carried out only for Granada province highlighted that prevalence (percentage of olive trees from which the pathogen was isolated) of VWO was higher in valleys with irrigated cropland history, especially those ones where herbaceous crops were previously cultivated in addition to saline, alkaline, and sloping soils (Rodríguez et al., 2011). These authors stressed the relevance of using pathogen-free certified planting

material to avoid the spreading of the disease, a key measure for a successful integrated management of VWO (López-Escudero and Mercado-Blanco, 2011). Out of Spain, both the severity and the number of affected plants have increased markedly in recent years in some regions. For instance, a traditional olive producing area in Northwest Argentina (Department of Arauco, Province of La Rioja) was surveyed. Results showed that VWO incidence varied from 0 to 9% and that the disease also caused death of trees in some cases (Ladux et al., 2012).

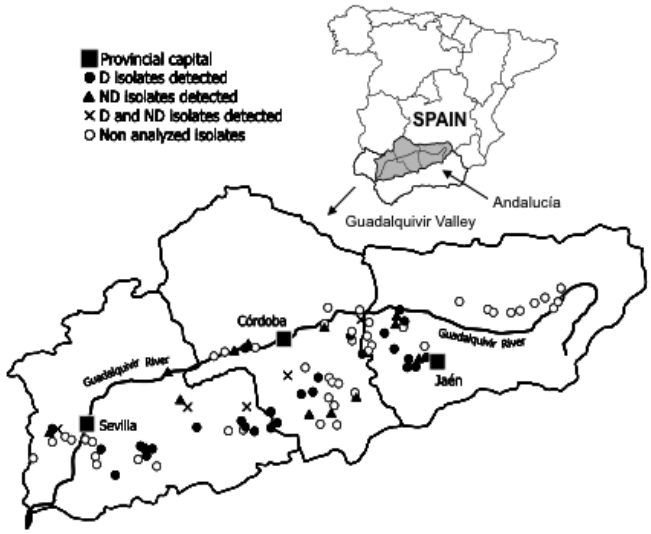


Figure 1.2. Distribution of *Verticillium dahliae* isolates in olive orchards surveyed throughout the Guadalquivir Valley (Southern Spain). D, Defoliating isolates; ND, non-defoliating isolates. Figure reproduced from López-Escudero et al., (2011), *Phytopathologia Mediterranea, 49*(3), 370-380, DOI 10.14601/Phytopathol_Mediterr-3154

1.4. Symptoms and causal agent of Verticillium wilt of olive

1.4.1. Symptomatology

Two different syndromes are traditionally recognized as induced by *V. dahliae* infections in olive, apoplexy (acute form) and slow decline (chronic form) (**Figure 1.3**). In the apoplexy syndrome, which takes place mainly in late winter to early spring, leaves undergo a process of chlorosis, turn color to light-brown and roll back, remaining bound to the branches, followed by a dieback of branches and twigs. Moreover, this syndrome may lead to the death of young trees (Blanco-López et al., 1984; Rodríguez-Jurado et al., 1993; Jiménez-Díaz et al., 1998; López- Escudero and

Blanco-López, 2001; López-Escudero and Mercado-Blanco, 2011). In contrast, in the slow decline syndrome, occurring in spring and gradually progressing to early summer, the main symptoms are necrosis of inflorescences, mummification of fruits and defoliation of green or dull-green leaves excluding in some cases those at the distal end of the branches (López-Escudero and Mercado-Blanco, 2011). Interestingly enough, and regardless of the syndrome, any of these symptoms may concur in the same tree. Infected olive trees overcoming the disease may show the so-called phenomenon of natural recovery, and new healthy suckers can emerge from the base of the trunk or infected branches (López-Escudero and Blanco-López, 2005a; Markakis et al., 2009; Bubici et al., 2014) (see below).

Figure 1.3. Symptoms observed in apoplexy (**A**) and slow decline syndromes (**B-D**) in olive trees. **A**. Die-back of twigs and branches affecting partially the tree. **B**. Defoliation of green or dull-green leaves. **C**. Necrosis of inflorescences. **D**. Mummification of fruits. Images reproduced from López-Escudero and Mercado-Blanco, (2011), *Plant and Soil, 344*(1-2), 1-50, DOI 10. 1007/ s11104-010-0629-2

1.4.2. Ethiology of Verticillium wilt of olive

The causal agent of VWO, *Verticillium dahliae* Kleb. (Klebahn, 1913) (Division, Ascomycota; Class, Sordariomycetes; Order, Phyllachorales), is a soil-borne, hemibiotrophic, haploid and asexually reproducing fungus (Pegg and Brady, 2002; Barbara and Clewes, 2003; Fradin and Thomma, 2006; Klosterman et al., 2009). Recently, evidence of ancestral or cryptic sexual stage has been reported (see below) (Milgroom et al., 2014; Short et al., 2015). Under microscopy, *V. dahliae* conidiophores possess a hyaline structure (**Figure 1.4A**). Conidia are produced at the tip of flask-shaped phialides disposed in whorls on erect and unbranched conidiopho-

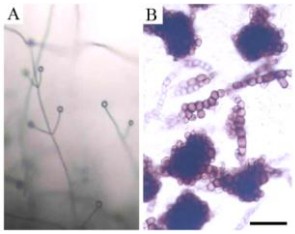


Figure 1.4. *Verticillium dahliae* conidiophores (verticils) exhibiting conidia at the tips of phialides (**A**) and melanized microsclerotia (**B**). The fungus was grown on potato dextrose agar (PDA) plates. Scale bar represents 50 µm. Images reproduced from López-Escudero and Mercado-Blanco, (2011), *Plant and Soil, 344*(1-2), 1-50, DOI 10.1007/ s11104-010-0629-2

res so-called verticils. This species differentiates morphologically from other phytopathogenic representatives within the genus *Verticillium* (i.e. *V. albo-atrum*) by the generation of microsclerotia (MS) (**Figure 1.4B**). These are small, multicellular and heavily melanized resting structures (Soesanto and Termorshuizen, 2001) produced by *V. dahliae* in senescent or dead tissues of infected plants at the end of the parasitic phase of its life cycle or under adverse environmental conditions (see below). These structures of resistance may remain dormant in the soil for prolonged periods of time (>15 years) (Wilhelm, 1955). From a phytopathological point of view, this species is a remarkable member of the *Verticillium* genus because of its broad host range. It can produce severe vascular wilt diseases in many plant species (>400) worldwide, including economically-important herbaceous and woody crop species, landscape trees and weeds (Pegg and Brady, 2002; Fradin and Thomma, 2006; Inderbitzin et al., 2011).

1.5. Diversity of *Verticillium dahliae*

Verticillium dahliae displays a broad morphological, physiological, genetic, molecular and pathological variability (Pegg and Brady, 2002; Papaioannou et al., 2013; Papaioannou and Typas, 2014). For instance, fungal colonies show numerous shapes and colors depending on the growth media used, as well as a wide diversity of conidiophores and MS (Pegg and Brady, 2002). Regarding to virulence, for instance, olive and cotton (*Gossypium hirsutum* L.) infecting *V. dahliae* isolates are traditionally divided into two different pathotypes (Rodríguez-Jurado et al., 1993): a) highly virulent isolates, usually producing defoliation of (green) leaves and drastic weight and height reduction in the host, are considered as belonging to the D pathotype; and b) isolates which provoke moderate symptoms (milder defoliation, necrosis and chlorosis) which are assigned to the ND pathotype (López-Escudero and Blanco-López., 2001). Nevertheless, it is necessary to emphasize that a clear-cut division between these two virulence groups is not always easy to establish attending to the symptoms/syndrome they caused. Occasionally, D and ND isolates may show similar virulence degree in artificial inoculation experiments, a so-called continuum of virulence (Dervis et al., 2010). Overall, however, infections by the D pathotype are more severe, and can eventually lead to the death of the tree, while plants infected by ND isolates may have more probabilities to overcome the infection and recover from symptoms. It is worth mentioning that a correlation between cotton ND and D isolates with tomato races 1 and 2, respectively, has been recently reported (Subbarao et al., 2015).

Before the availability of specific molecular markers, *V. dahliae* isolates were also genetically classified according to their assignment to the so-called VCGs. Two *V. dahliae* isolates are said to belong to the same VCG (compatible isolates) if they show the ability to undergo hyphal anastomosis and form stable heterokaryons (Leslie, 1993; Katan, 2000). The classification of VCGs (VCG1A, VCG1B, VCG2A, VCG2AB, VCG3, VCG4A, VCG4B and VCG6) has been established from *V. dahliae* isolate collections infecting diverse hosts (see, for instance, Strausbaugh et al., 1992; Bhat et al., 2003; Tsror and Levin, 2003; Jiménez-Díaz et al., 2006; Omer et al., 2008). Nevertheless, only representatives of the VCG1A, VCG2A, VCG2B, and VCG4B have been so far identified among *V. dahliae* isolates infecting olive worldwide (Cherrab et al., 2002; Tsror and Levin, 2003; Collado-Romero et al., 2006; Dervis et al., 2007). Interestingly, VCG1A isolates correspond to the olive and cotton D pathotype (Pérez-Artés et al., 2000; Collado-Romero et al., 2006; Dervis et al., 2010). It is worth mentioning that the virulence displayed by any given *V. dahliae* isolate (or pathotype or VCG representative) may vary depending on the host plant. For instance, cotton/olive VCG1A isolates elicit severe defoliation in cotton but not in artichoke (Jiménez-Díaz et al., 2006). Moreover, the use of molecular markers (see below) has shown *V. dahliae* populations as highly clonal and hence most of known VCGs correlate with clonal lineages (Dobinson et al., 1998; Collado-Romero et al., 2006, 2008; Dung et al., 2013). Nonetheless, some exceptions have been found (Jiménez-Díaz et al, 2006; Jiménez-Gasco et al., 2014). Indeed, recent reports suggest that this relationship is more complex and it is possible that sexual recombination might have occurred in the past (Milgroom et al., 2014; Short et al., 2015; **Figure 1.5**).

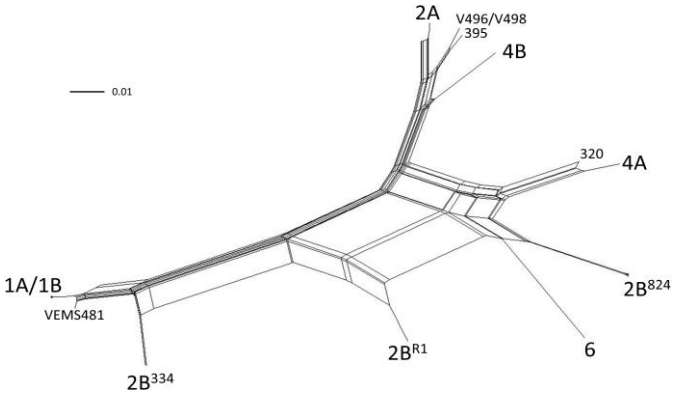


Figure 1.5. A neighbor-net network of all lineages and recombinant haplotypes of *Verticillium dahliae*, based on 26,748 SNPs. Images reproduced from Milgroom et al., (2014), *PloS ONE*, *9*(9), e106740. DOI 10.1371/ journal.pone.0106740

A possible correlation among VCG, host specificity, virulence and geographical distribution may help in adopting disease control measures; for instance, in the prediction of the disease and/or in breeding for VWO resistance

programs. Some studies support that an association between VCG and host specificity exists. Hence, prevalence of VCG4 isolates in potato (*Solanum tuberosum* L.) (Joaquim and Rowe, 1991; Strausbaugh et al., 1992; Korolev et al., 2000), VCG1A (D-pathotype) in cotton (Daayf et al., 1995; Zhengjun et al., 1998; Korolev et al., 2001, 2008; Dervis et al., 2008), or VCG2A and 2B in artichoke (*Cynara scolymus* L.) (Jiménez-Díaz et al., 2006) were observed. However, other studies do not support this VCG-host correlation (Elena, 1999; Korolev et al., 2000, 2001). Furthermore, the existence of slight complementation between different VCGs deriving in heterokaryons, or the occurrence of hybrids from interspecific origin (Daayf et al., 1995; Hiemstra and Rataj-Guranowska, 2003; Joaquim and Rowe, 1990; Strausbaugh et al., 1992), seem to suggest a continuum of genetic variation enhancing variability and source of evolution for this genus (Collado-Romero et al., 2010). Thus, although VCG classification offers valuable information about *V. dahliae* populations, it poses deficiencies to establish correlations with virulence degree or host specificity (Elena, 1999; Korolev et al., 2000, 2001).

To better elucidate the intricate structure of *V. dahliae* populations more powerful methods such as a repertory of molecular approaches have been used. Thus, analysis and comparison of restriction fragment length polymorphism (RFLP) (Carder and Barbara, 1991; Typas et al., 1992; Carder et al., 1994; Okoli et al., 1994; Dobinson et al., 2000), random amplified polymorphic DNA (RAPD) (Messner et al., 1996; Koike et al., 1996; Bhat and Subbarao, 1999; Zeise and Von Tiedemann, 2002), amplified fragment length polymorphism (AFLP) (Collins et al., 2003; Fahleson et al., 2003; Radišek et al., 2003; Collado-Romero et al., 2006), DNA sequences such as the intergenic spacer (IGS), internal transcribed spacer (ITS) regions of genes encoding ribosomal RNA and repeated DNA sequences (Morton et al., 1995a,b; Subbarao et al., 1995; Dobinson et al., 1998; Pramateftaki et al., 2000; Jiménez-Gasco et al.,2014) have been used. Besides, molecularly-based methods also provide relevant information about phylogenetic relationships among *V. dahliae* VCGs (Collado-Romero et al., 2008, 2010; Jiménez-Gasco et al., 2014). Finally, molecular markers are of great interest for both *in planta* and in soil detection/diagnosis procedures (see below). The variety of available markers, derived from these previous, fundamental studies to detect and discriminate *V. dahliae* isolates is now broad enough. Indeed, a number of VCG- or pathotype-specific primers to detect, for instance, *V. dahliae* isolates infecting olive or other hosts (see below) have been designed and implemented in specific, rapid and effective detection protocols (Pérez-Artés et al., 2000; Mercado-Blanco et al., 2003b; Collado-Romero et al., 2006, 2009).

1.6. Verticillium wilt of olive disease cycle

The life cycle of *V. dahliae* comprised three different phases: dormant, parasitic and saprophytic (**Figure 1.6**). In the dormant stage, MS present in infested soil are inhibited from germinating as a result of mycostasis or microbiostasis (Huisman, 1982), enduring in a state of dormancy for several years (> to 15 years) (Wilhelm, 1955). MS germination was demonstrated as non-host specific (Pegg and Brady, 2002). It takes place under favorable environmental conditions and triggered by root exudates, a rich source of carbon and nitrogen (Huisman, 1982; Mol and Scholte, 1995). Prieto et al., (2009) have described in detail the colonization and infection process (parasitic phase) of olive plants by a D representative of *V. dahliae* using confocal laser scanner microscopy (CLSM) and a fluorescently-tagged *V. dahliae* derivative (**Figure 1.6**). Thus, after MS germination (**Figure 1.6A**) conidia colonize profusely the root surface (**Figure 1.6B**) followed by a massive inter- and intracellular proliferation of the pathogen hyphae into the cortex and xylem (**Figure 1.6C**). Later, *V. dahliae* hyphae systemically colonize the vascular system (**Figure 1.6D**). Rapid upwards conidia transportation from the root to aerial tissues by the transpiration stream has been reported in other hosts, including woody species (Pegg and Brady, 2002). Eventually, conidia are trapped in bordered pits or at vessel end walls germinating into new hyphae invading adjacent vessel elements leading to an increase in the infection (Schnathorst, 1981). The general colonization of the aerial tissue elicits development of disease symptoms (e.g. wilting and drying of shoots, defoliation of leaves, inflorescence mummification; **Figure 1.6E,F**), the pathogen entering in the saprophytic phase. The cycle is completed by the formation of new MS in the dying plant tissues that are released into the soil upon decomposition of plant debris, thereby increasing the number of infective propagules (Navas-Cortés et al., 2008; **Figure 1.6G**).

1.7. Epidemiology of Verticillium wilt of olive: a brief overview

A number of studies have evaluated diverse factors which, alone or in combination, help to explain the persistence, increase and/or dispersion of *V. dahliae* infecting propagules in the soil. On the one hand, continuous cultivation of *V. dahliae*-susceptible crops can increase the number of pathogen propagules (Wilhelm and Taylor, 1965; Blanco-López et al., 1984; Tjamos and Botseas, 1987; Tjamos and Tsougriani, 1990; Bejarano-Alcázar et al., 1996; **Figure 1.7B**). Related to this, it is important to emphasize the abovementioned ability of the pathogen to colonize a wide range of plants, including wild flora. On the other hand, inherent characteristics

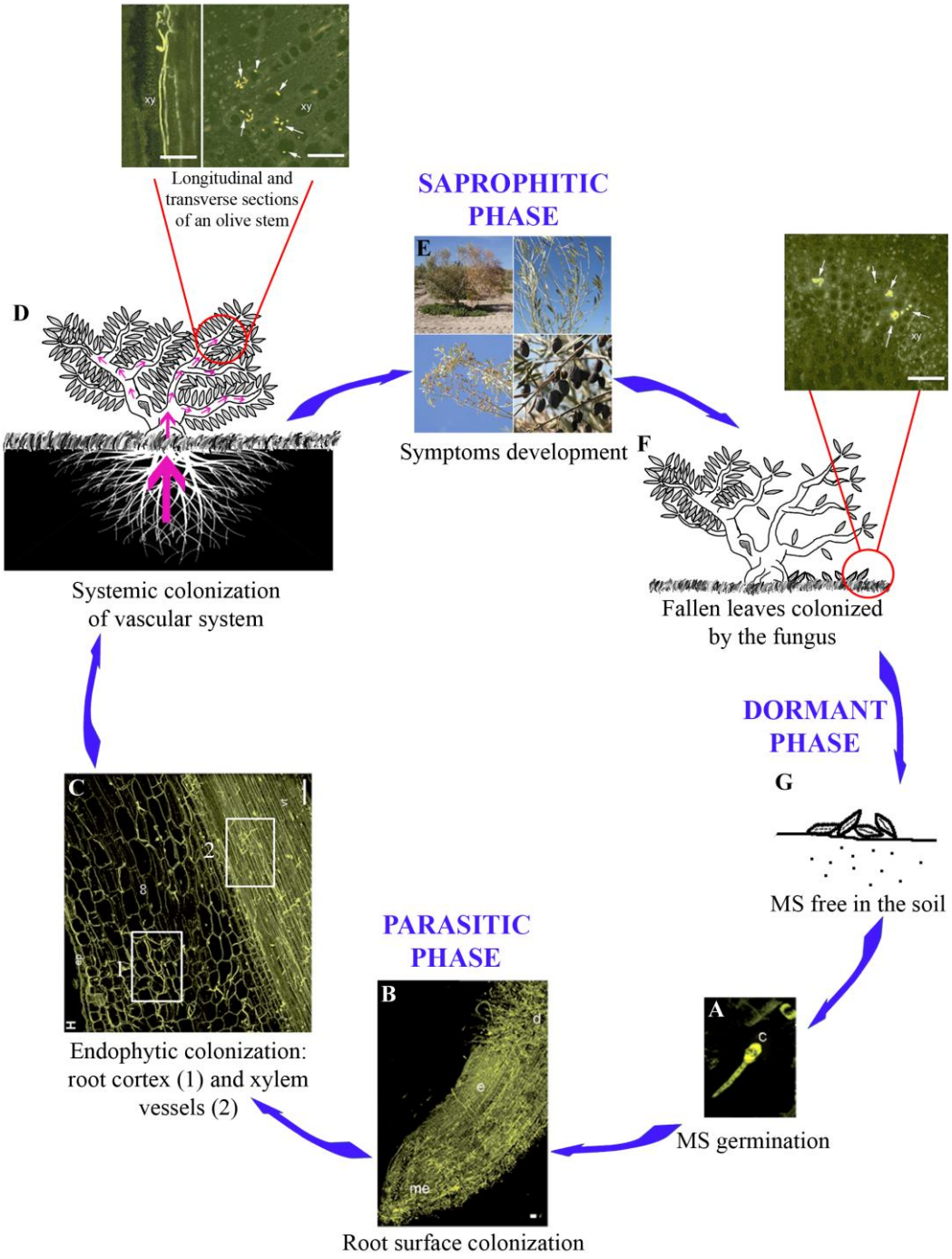


Figure 1.6. *Verticillium dahliae* life cycle and interaction with olive tissues. CLSM images are reproduced from Prieto et al., (2009), *Microbial Biotechnology, 2*(4), 499-511, DOI 10.1111/j.1751-7915.2009.00105.x

of *V. dahliae* such as the ability to endure for many years in the soil in the form of MS (see above). Infective propagules present in soil and/or in the plant debris may be dispersed by, among other means, wind (Easton et al., 1969), runoff, irrigation (Thanassoulopoulos et al., 1981; López-Escudero and Blanco-López, 2005b; López-Escudero et al., 2009), contaminated vehicles (Al-Ahmad and Mosli, 1993; Serrhini and Zeroual, 1995; **Figure 1.7A**) and tillage tools (López-Escudero et al., 2008), and/or human transport of infected plant residues or crops (Wilhelm and Taylor, 1965; Schnathorst and Sibbett 1971a,b; Tjamos and Botseas, 1987; Tjamos and Tsougriani, 1990; Navas-Cortés et al., 2008).

A general correlation between losses caused by the disease in several crops and water irrigation regimes has been observed (Ruggieri, 1948; Cirulli, 1981; Schnathorst, 1981; Blanco-López and DeVay, 1987; Pegg and Brady, 2002). In olive, lower yields can be explained by a shift from dry-farming to irrigation and/or high watering frequencies in orchards where the presence of pathogen propagules was relevant or in environments/periods favorable for *V. dahliae* attacks (Blanco-López et al., 1984; López-Escudero and Blanco-López, 2005b; López-Escudero et al., 2009). Thus, due to the abovementioned factors higher *DI* values have been reported in several countries in the Mediterranean Basin such as Morocco (Serrhini and Zeroual, 1995), Syria (Al-Ahmad and Mosli, 1993) and Spain (Blanco-López et al., 1984; López-Escudero et al., 2009; Pérez-Rodríguez et al., 2013). This situation can be exacerbated when olive trees are planted at high densities (Rodríguez et al., 2008). High irrigation regimes can boost *V. dahliae* inoculum density in soil. This can be due either to the presence of new pathogen propagules in the irrigation water or to the fact that higher humidity can contribute to increase the pre-existing inoculum. Besides, inadequate watering management may trigger VWO onset and/or its development as well as enhance root growth, thereby increasing the surface of contact with the pathogen (Huisman, 1982; Fernández et al., 1991; Xiao et al., 1998; Xiao and Subbarao, 2000; López-Escudero and Blanco-López, 2005b).

On the other hand, with the aim to obtain early production and high yields, excess of nitrogen (N) fertilization has been commonly used in olive cultivation practices. However, it is known that an excess of N may increase the susceptibility to diseases (Agrios, 1997), as observed for Verticillium wilt in cotton (El-Zik, 1985). In contrast, several studies demonstrated that the influence of N fertilization in disease susceptibility is dose dependent or linked to the chemical form of the N used. Thus, ammonia and nitrous acid inputs were shown to produce a decrease in the number

of *V. dahliae* propagules in soil (Tenuta and Lazarovits, 2002) and, in addition, an increase of the biological activity of pathogen antagonists (Pegg and Brady, 2002).

Tilling has also been described as a relevant mechanism for *V. dahliae* dispersion within and among cultivated plots in both herbaceous and woody hosts (Hiemstra and Harris, 1998; Pegg and Brady, 2002). In addition, plowing can inflict root damage favoring the penetration of the pathogen, as it has been reported in cotton (Schnathorst and Mathre, 1966) and olive (Tjamos, 1993). Then, preserving vegetation covers appears as a positive measure since, furthermore, it is associated with water conservation, protection against erosion, improvement of soil structure and organic matter contents in olive crop (Gómez et al., 2009; Rodríguez-Lizana et al., 2008). However, no till farming may be a problematic issue from a phytopathological point of view since some weeds may act as reservoirs of the pathogen and hence the use of herbicides is recommended.

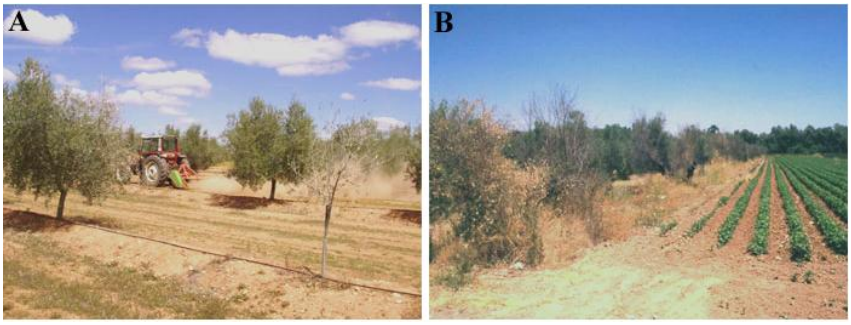


Figure 1.7. Factors increasing *V. dahliae* distribution (**A**) and inoculum density (**B**). **A**. Use of contaminated vehicles. **B**. Cultivation of *V. dahliae*-susceptible crops such as cotton. Images reproduced from López-Escudero and Mercado-Blanco, (2011), *Plant and Soil, 344*(1-2), 1-50, DOI 10.1007/ s11104-010-0629-2

The use of soil amendments can also influence pathogen's inoculum density in soil, either directly by eliminating pathogen propagules or indirectly by favoring conditions propitious to microbial antagonists development (see below). Nevertheless, some organic amendments can act as a pathogen inoculum source. This is the case of amendments based on dung from sheep fed in fields where the presence of *V. dahliae*-infected plant material had been confirmed (Al-Ahmad et al., 1992; López-Escudero and Blanco-López, 1999; Markakis et al., 2014).

The most favorable temperature for VWO development ranges from 22 to 25°C (Garber and Presley, 1971). Severity caused by *V. dahliae* attacks is stimulated

by air temperatures of 20-25°C during spring season, followed by summers with a wide thermal range but never exceeding 30-35°C (Wilhelm and Taylor, 1965; López-Escudero and Blanco-López, 2001). Temperatures above 25°C for long periods usually affect negatively both pathogen colonization and symptoms development. Moreover, soil temperature has been suggested to influence disease progress. Thus, it was highlighted that infections by D and ND *V. dahliae* isolates are favored under certain soil temperatures in cv. Picual and Arbequina (Calderón et al., 2014).

Edaphic characteristics are also implicated in VWO epidemiology. However, little knowledge is available in this regard. This disease is usually favored in neutral to alkaline soils (pH 6-9) as acidic pH's (<5.5) have demonstrated to inhibit pathogen growth as well as diminish MS production and endurance. Likewise, accumulation of Mn and Al in host plant tissues at low pH produces a toxic impact in *V. dahliae* causing suppression in mycelium growth. In contrast, soils with low Ca or high K or Mg levels diminish disease severity (Pegg and Brady, 2002). Nevertheless, the actual effect of these factors on the pathogen and the disease can be further influenced by soil texture, rate of organic matter mineralization, soil microbiota activity, etc. (Gamliel et al., 2000; Lazarovits et al., 2000; Goicoechea, 2009). Finally, natural saline soils or saline stress induced by the recycling of salt-rich irrigation water have been shown to enhance the incidence and severity of Verticillium wilt in several herbaceous and woody hosts (Pegg and Brady, 2002). For instance, olive cultivars Barnea, Picual and Souri planted in a sandy-loam saline soil with high salt content and irrigated with saline water showed as highly susceptible to VWO (Levin et al., 2003a,b, 2007).

1.8. Olive responses to *Verticillium dahliae* attacks

Vascular pathogens may survive and proliferate in the vascular system of their host plants causing vascular wilt diseases (e.g. *V. dahliae*). Preference of these pathogens to proliferate in the nutrient-poor xylem vessels could possibly be due to the fact that death xylem cells (tracheary elements) are more easily colonized (low osmotic pressure) than living cells (high osmotic pressure) of the phloem (Yadeta and Thomma, 2013). Currently, the most successful strategy to control vascular infections is the use of resistant host plants (see below). Since the pathogen can be detected in the above-ground organs of resistant cultivars some authors prefer to use the term tolerance instead of resistance (Robb, 2007) when referring to VWO. Nevertheless, histopathological observations and *V. dahliae* DNA quantification suggest that pathogen penetration and spreading can be actively prevented, hindered or

restricted in olive (Rodríguez-Jurado, 1993; López-Escudero et al., 2004; López-Escudero and Blanco-López, 2005a; Antoniou et al., 2008; Markakis et al., 2009). Resistance/tolerance to VWO has been reported for several commercial cultivars and rootstocks (López-Escudero and Mercado-Blanco, 2011; Bubici and Cirulli, 2012; Trapero et al., 2013; García-Ruiz et al., 2014; Arias-Calderón et al., 2015).

Under field conditions, woody hosts infected by *V. dahliae* may overcome the disease and display the so-called phenomenon of natural recovery (Shigo, 1984; Hiemstra, 1998; Tippett and Shigo, 1981). In olive, VWO recovery is characterized by a decrease in *DI* and development of new suckers at the base of the trunk or at infected brunches. Natural recovery has been observed in naturally-infected olive trees under field conditions as well as in artificially-inoculated, nursery-produced young olives under controlled conditions (Wilhelm and Taylor, 1965; Vigouroux, 1975; Tjamos et al., 1991; Rodríguez-Jurado et al., 1993; Mercado-Blanco et al., 2001a; Levin et al., 2003b; López-Escudero and Blanco-López, 2001, 2005a; Markakis et al., 2009; Bubici and Cirulli, 2014). The reduction of VWO symptoms may be related to responses deployed by olive trees after pathogen infection, including enclosure of the necrotic xylem vessels, fungus inactivation in the xylem, prevention of new infections, and/or increase of diverse phenolic compounds (Wilhelm and Taylor, 1965; Tjamos et al., 1991; Rodríguez-Jurado, 1993; López-Escudero and Blanco-López, 2001, 2005a; Baídez et al., 2007; Markakis et al., 2010). Moreover, VWO recovery is strongly influenced by several decisive factors including the level of resistance of the olive genotype, environmental conditions, virulence and spreading of the pathogen, etc (Martos-Moreno et al., 2001; López-Escudero et al., 2004; López-Escudero and Blanco-López, 2001, 2005a). Yet, our knowledge on olive resistance to VWO as well as on the mechanisms underlying the natural recovery phenomenon is scant.

1.9. Control of Verticillium wilt of olive: the importance of an integrated disease management strategy

The approaches used to control plant pathogens can be divided in pre-planting (preventive) and post-planting (palliative or curative) measures (**Figure 1.8**). These actions must be based on accurate knowledge of the pathogen, the host plant, and the epidemiological factors contributing to the disease. Preventive and curative measures must be combined within an integrated disease management (IDM) strategy aiming to the exclusion and/or eradication and/or reduction of the

pathogen, as well as to reduce the efficacy of the initial inoculum, to increase the resistance of the host and to delay the disease onset and potential secondary cycles (Agrios, 1997). This combination of control measures is particularly needed for diseases such as VWO since none of the available tools have so far shown effective when implemented individually (López-Escudero and Mercado-Blanco, 2011). Moreover, any integrated control program should guarantee the sustainability of the crop at both economic and environmental levels in addition to its durable effectiveness (Caballero and Murillo, 2003). The use of preventive measures is obviously the most plausible strategy from economical and environmental perspectives. It should be stressed that many of the pre-planting measures can also be used as palliative tools, reinforcing the efficacy of any IDM programme.

1.9.1. Pre-planting control measures

The use of pathogen-free propagation material is crucial in a control strategy of VWO. Consequently, fast and consistent *in planta* pathogen detection methods are of high importance in the management of *V. dahliae* (López-Escudero and Mercado-Blanco, 2011). Traditional detection methods based on pathogen isolation have thus been replaced/complemented by PCR-based techniques which are less time-consuming, more consistent (Morera et al., 2005) and, in some cases, able to differentiate among *V. dahliae* pathotypes (Mercado-Blanco et al., 2003b). Conventional PCR using primers based in molecular markers such as, among others, RAPDs (Triki et al., 2011) or AFLPs (Gharbi et al., 2015) have been developed for the specific detection of *V. dahliae* in olive. For *in planta* detection, nested-PCR protocols provide more accurate and sensitive results than approaches based on one single PCR (Mercado-Blanco et al., 2001a, 2002). The refinement of this technique allows to identify two or more *V. dahliae* isolates in infected tissues in a single reaction, either by duplex nested-PCR (Mercado-Blanco et al., 2003b) or multiplex nested-PCR (Collado-Romero et al., 2009). These methods are qualitative but RT(real time)-q(quantitative)PCR approaches, in contrast, offer the advantage of quantitative, rapid and accurate detection of target DNA(s). Different available variants of this technique have been successfully used to quantitatively detect soil-borne fungal pathogens originating from diverse environmental samples (Schena et al., 2004b). This has been the case of *V. dahliae* in different host plants (Atallah et al., 2007; Gayoso et al., 2007; Duressa et al., 2012; Wei et al., 2015) including olive (Schena et al., 2004a; Ceccherini et al., 2013; Gramaje et al., 2013), and even at the pathotype level (Mercado-Blanco et al., 2003a; Markakis et al., 2009). Furthermore, the sensitivity of RT-qPCR can be enhanced by applying a nested approach, QN(quantitative

nested)RT-PCR. This procedure has been used to show Verticillium wilt progress over time in a woody plant such as the smoke tree (*Cotinus coggygria*) (Wang et al., 2013), and can be potentially applied in olive.

The evaluation of *V. dahliae* inoculum in soil is also important in a VWO integrated control framework. Similarly to detection in plant tissues, PCR-based methods have been developed and applied to detect and quantify *V. dahliae* in soil samples advantageously over traditional detection procedures (López-Escudero and Mercado-Blanco et al., 2011). Recently, a loop-mediated isothermal amplification (LAMP) assay using LAMP primers has been developed to detect *V. dahliae* pathotypes in soil samples without prior DNA purification (Moradi et al., 2014). Finally, a density flotation-based extraction of MS method followed by RT-PCR showed reproducible and sensitive results in the detection of diverse *Verticillum* species, including *V. dahliae*, in artificially and naturally infested soils (Debode et al., 2011).

Soil disinfestation prior to the establishment of a new olive orchard using physical, cultural, biological or chemical methods is highly recommended to eliminate or reduce the presence of soil-borne pathogens. Soil solarization has shown to be a major measure in the control of soil-borne pathogens (Katan, 2015) as well as in diverse Verticillium wilts of herbaceous and woody hosts (Pegg and Brady, 2002). This method, however, has not been widely used in olive as a pre-planting measure possibly due to the fact that olive groves generally cover vast areas that would need a high economical investment, among others limitations (López-Escudero and Blanco-López, 2001). Regarding to the application of chemicals, although its effectiveness against *V. dahliae* has been confirmed in different crops (Goicoechea, 2009), its use is discouraged because of public concerns on human and animal health as well as their unwished effect to the environment and soil beneficial microbiota. Therefore, a dose reduction and/or combination with other less harmful soil disinfestation methods such as soil amendments derived from animal or plant organic residues (organic amendments, see below) are recommended (Hamblin, 1995; Goicoechea, 2009). Cultural and biological practices that promote and activate antagonistic microbiota have demonstrated some success in VWO suppression (Devay and Pullman, 1984; López-Escudero and Mercado-Blanco, 2011). Such practices include the retardation in olive grove planting along with crop rotation, cropping of non-susceptible species, and the use of organic amendments (biofumigation, see below) combined or not with BCA (Hao et al., 2011; Vitullo et al., 2013).

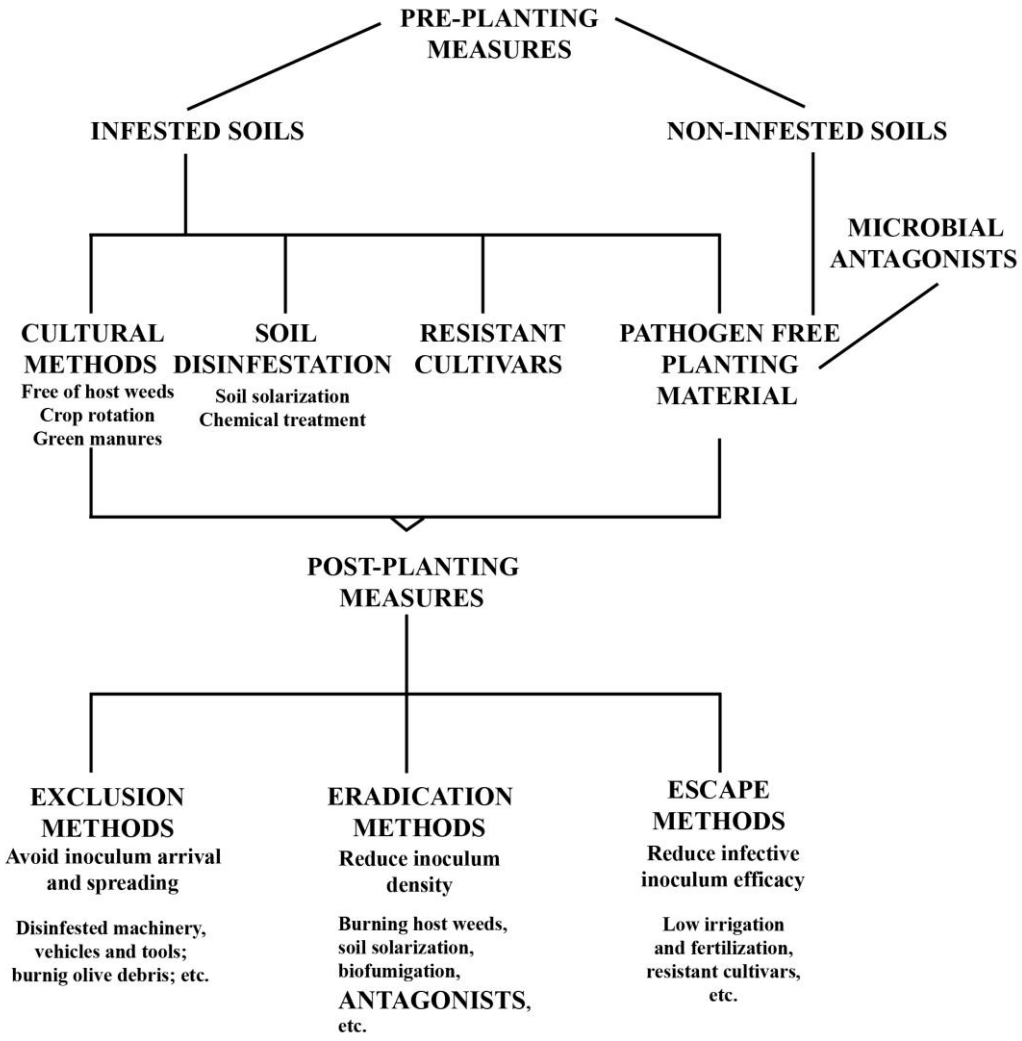


Figure 1.8. Proposed scheme for an integrated control of Verticillium wilt of olive. Based on Blanco-López and Jiménez-Díaz, (1995), Trapero and Blanco-López, (2008) and, López-Escudero and Mercado-Blanco, (2011).

Obtaining and using VWO resistant, tolerant or low susceptible cultivars by means of breeding programs is probably the most economically effective and environmentally-friendly control measure to be implemented wherever olive is an important commodity. So far, however, the majority of olive cultivars inspected, the most economically and historically relevant ones, are susceptible to *V. dahliae* (Martos-Moreno, 2003; López-Escudero et al., 2004; Martos-Moreno et al., 2006). Besides, breeding for resistance is hampered when working with a woody crop such as olive that needs several years to produce fruits. Nevertheless, recent studies have

reported interesting results with the use of selected cultivars in breeding programs aiming to generate new olive varieties resistant to *V. dahliae* (Arias-Calderón et al., 2015; Trapero et al., 2015). Another promising approach is the use of resistant olive rootstocks, which hamper pathogen progress to grafted scions of agronomical interested as observed in artificial inoculation experiments (Porras-Soriano et al., 2003; Bubici and Cirulli, 2012) and under field conditions (Hartmann et al., 1971). However, mores studies are still needed to obtain WVO resistant varieties.

1.9.2. Post-planting control measures

Palliative measures are used when the pathogen is already established in the olive grove, an issue that has been extensively reviewed (López-Escudero and Mercado-Blanco, 2011; Tsror, 2011; Jiménez-Díaz et al., 2012). Post-planting control can be divided in exclusion, eradication and evasive measures (**Figure 1.8**). Briefly, these measures are intended to prevent the onset and/or progress of the disease, and/or to diminish seasonal *DI* values, the severity of the symptoms and percentage of affected/dead plants. Accordingly, exclusion methods aim to avoid propagation and influx of additional propagules into established orchards by using, for instance, disinfested machinery, vehicles and tools. The new orchard should be established far from susceptible crops as well, and must avoid herbaceous *V. dahliae* hosts in the vicinity of olive groves when conducting intercropping. Eradication measures intend to hinder the pathogen growth and spread within/between neighboring plots using physical, cultural, biological and chemical methods. Cultural practices such as the elimination (i.e. burning) of potential *V. dahliae*-host weeds and infested plant parts (twigs, shoots, branches and, if economically affordable, fallen green leaves) are recommended as they are pathogen inoculum sources (Tjamos and Botseas, 1987; Tjamos and Tsougriani, 1990; Rodríguez-Jurado et al., 2002; Navas-Cortés et al., 2008). Moreover, when moderate or low level of pathogen inoculum is present the use of soil solarization is recommended. This physical control method can obviously be applied as a pre-planting measure for VWO control (see above), but also as a post-planting method for eradicating/diminishing *V. dahliae* MS in soils (i.e. when replacing dead trees at specific spots). Besides, it can be combined with other palliative measures such as application of BCAs and biofumigation (Tjamos and Paplomatas, 1987). Soil amendments based on a wide range of organic matter such as composted materials, green or animal manures, plant extracts and sewage sludge (residues or final products from wastewater treatment processes), can also be used as a curative measure modifying the soil physical structure, releasing chemical

compounds with fungicide/fungistatic properties or favoring the development of antagonistic microorganisms (Lazarovits et al., 2000; Bhat and Subbarao, 2001; Huang et al., 2006; Termorshuizen et al., 2006; Tsror et al., 2007; Avilés et al., 2011; Korthals et al., 2014; Neubauer et al., 2014). Chemical control of *V. dahliae*, both in soil and in *planta*, is problematic and has proven so far ineffective due to localization of the pathogen within the xylem and the endurance of MS in soil. Consequently, although some studies about the application of systemic fungicides to control *V. dahliae* control have been conducted, limited success was obtained (Tjamos, 1993; Trapero and Blanco-López, 2008). The use of microbial antagonists as a post-planting measure in controlling VWO has been proposed, mainly during first years after planting (López-Escudero and Mercado-Blanco, 2011). However, effective biological control under field conditions is highly difficult to accomplish, particularly when dealing with woody plants such as olive (e.g. long-living, large root system, etc.), although some promising results have been recently obtained (Markakis et al., 2015). In relation to escape (evasive) methods, little information is available. These means are aimed to decrease the effectiveness of the pathogen infection by influencing pathogen behavior (e.g. proper irrigation dosages and nitrogen fertilization) or reducing the probability of contact between the pathogen and plant roots. However, mechanisms underlying these strategies are still poorly understood (El-Zik, 1985; Blanco-López and Jiménez-Díaz, 1995; Arbogast et al., 1999).

1.10. Biological control and its potential to manage Verticillium wilt of olive

Biological control of plant diseases is an environmentally-friendly measure based on the utilization of beneficial microorganisms, ideally originating from the host plant and/or the target niche where they will be eventually released, that can be used as both pre-planting and post-planting method. Biological control relies on a fine and intricate equilibrium of interactions of four players: the pathogen, the BCA, the host plant and the environment (Fravel, 1988; Weller, 1988; Thomashow and Weller, 1996). Besides, the beneficial effect displayed by a BCA against any phytopathogen involves a prior successful colonization of the niche (e.g. the rhizosphere, the inner plant root, etc.), reaching a specific (threshold) population size that is determined by various factors which are dynamically interacting, conforming the so-called 'rhizosphere competence' phenomenon (Mercado-Blanco, 2015a). The success of any BCA in the rhizosphere is thus influenced by the soil (pH, type, temperature, water content and accessibility, etc.), the host plant (root exudates composition, genotype, etc.), the own BCA characteristics (motility, biosurfactants, antibiotics and/or exo-enzymes production, etc.), and the microbiome associated to the plant

(rhizoplane, endorhizosphere) (Berg et al., 2006; Costa et al., 2006; Mercado-Blanco, 2015a). Therefore, it is essential to study each factor alone and in relation to the others as well as an in-depth knowledge of the competence occurring in the rhizosphere (O'Sullivan and O'Gara, 1992; Lugtenberg et al., 2001; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007; Raaijmakers et al., 2009).

The available knowledge on biological control of soil-borne pathogens affecting woody plants is lower compared to that on herbaceous species. One explanation is that research with woody hosts poses a number of difficulties which are consequence of factors such as the tree longevity, more complex anatomy, big root systems, etc. (Kawaguchi et al., 2012; Romeralo et al., 2015; Santiago et al., 2015; Nakaew et al., 2015). Regarding to biological control of Verticillium wilts, most of the studies have focused on herbaceous hosts as well, and only few BCAs have being described, either fungi (i.e. *Talaromyces* and *Trichoderma*, and *Glomus* spp., respectively) or bacteria (i.e. *Achromobacter*, *Bacillus*, *Paenibacillus*, *Pseudomonas*, *Serratia*, *Streptomyces*) (see, for instance, Garmendia et al., 2004, 2005; Tjamos et al., 2004, 2005; Kobra et al., 2009; Erdogan and Benlioglu, 2010).

Nevertheless, promising results on biocontrol of VWO have been reported. For instance, *Serratia plymuthica* strain HRO-C48 (Kalbe et al., 1996), a BCA exhibiting control of fungal diseases including *V. dahliae* in different host plants (Kurze et al., 2001; Müller and Berg, 2008; Erdogan and Benlioglu, 2010), showed prolonged colonization of the olive rhizosphere and effective control of a D isolate in artificially-inoculated (soil inoculation) olive plants (cv. Arbequina), although results varied depending on the infestation method (pathogen soil inoculation or root dipping) used (Müller et al., 2007). Various mechanisms have been proposed to be involved in Verticillium wilt biocontrol by *S. plymuthica*, such as swimming motility, production of antibiotics (prodigyosine and pyrrolnitrin), lytic enzymes (chitinases and glucanases), siderophores, small volatile organic compounds (VOCs) (sodorifen) and/or phytohormones (indole-3-acetic acid, IAA) (Kalbe et al., 1996; Berg, 2000; Frankowski et al., 2001; Kamensky et al., 2003; Weise et al., 2014). However, none of these traits have so far been investigated as involved in biocontrol of VWO.

Trichoderma spp. is a fungus widely distributed in many ecological niches. A number of non-phytopathogenic strains of this genus have been used as BCA against numerous soil-borne plant phytopathogenic fungi (Prasad and Naik, 2008). Biocontrol exerted by *Trichoderma* spp. can be due to mechanisms such as antibiosis, nutrient

and space competition, and mycoparasitism (reviewed by Benítez et al., 2004). Bioformulations consisting of two species of *Trichoderma* (*T. asperellum* and *T. gamsii*) were tested against a D isolate of *V. dahliae* under controlled and field conditions. Results showed VWO suppression depending on the infestation method used (Jiménez-Díaz et al., 2009a). Other studies have shown the potential of combining *Trichoderma* with other control measures to manage *V. dahliae*. Thus, combination of organic amendments (olive oil by-products) or soil solarization with *Trichoderma* provoked a significant decrease of *V. dahliae* MS in soil (Lima et al., 2007; Otero et al., 2012).

A few reports have used root endophytic fungi as BCA against *V. dahliae* (Matta and Garibaldi, 1977; Narisawa et al., 2002, 2004). They can play interesting roles for the plant besides their suppressive effect against Verticillium wilt such as improving host resistance to diverse (a)biotic stresses (Arnold, 2007; Mejía et al., 2008; Scheffer et al., 2008; Rodríguez et al., 2009). Related to this, previous inner 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).

Mycorrhizal fungi have also been used to control soil-borne pathogens, including *V. dahliae* (Porras-Soriano et al., 2006), in addition to facilitate olive plant establishment (Estaún et al., 2003; Calvente et al., 2004; Binet et al., 2007) and protection against (a)biotic stresses (Castillo et al., 2006, 2010; Dag et al., 2009; Medda-Hamza et al., 2010; Bompadre et al., 2014). For instance, endomycorrhizal-inoculated nursery olive plants showed better growth parameters on both the roots and above-ground organs (Chliyeh et al., 2014). Similarly, inoculation with *Glomus intraradices* diminished disease severity produced by *Phytophthora* spp. in olive seedlings when compared to control plants (non-inoculated) (Guerrero, 1999). On the contrary, the same *G. intraradices* suspension was not able to suppress VWO (Jiménez-Díaz et al., 2009b; Porras-Soriano et al., 2006). *Glomus intraradices* was also evaluated alone or together with *G. mosseae* regarding to their ability to improve olive growth and development (4-year-old plants) following the transplant into a saline water-irrigated, *V. dahliae*-inoculated soil under arid conditions (Kapulnik et al., 2010). Results showed that despite plant growth increased and adaptation to arid conditions improved after mycorrhization, olive tolerance to *V. dahliae* was not enhanced.

Finally, *Pseudomonas* spp. have been widely studied and used as effective BCAs of diverse plant diseases as well as plant growth promoters (O'Sullivan and O'Gara, 1992; Mercado-Blanco and Bakker, 2007; Weller, 2007; Höfte and Altier, 2010; Mercado-Blanco, 2015b). Different *Pseudomonas* spp. strains have been identified and evaluated as BCA against VWO (Sanei and Razavi, 2011; Triki et al., 2012). Even though information on the use of beneficial pseudomonads as BCA of VWO is scarce, some valuable information has been gathered during the last decade regarding to the potential of native olive rhizosphere *Pseudomonas* spp. strains in controlling infections by the D pathotype of *V. dahliae* in nursery-produced plants (see below).

Considering results from available studies, the use of BCAs to control VWO seems a promising strategy, particularly at the nursery propagation stage or soon after planting. Besides, combination of a BCA with other control measures, or the combination of diverse BCA's is an interesting scenario yet to be explored. For instance, diverse BCA's would colonize different regions of the root system (rhizoplane, interior) and deploy distinct and effective biocontrol mechanisms to suppress the target pathogen (e.g. induction of systemic resistance and antibiosis). However, the design of effective bioformulations still needs of in-depth studies since adverse interactions among the BCAs, and between the BCA and the native beneficial microbiota and/or the host plant may take place in the target niche (Whipps, 2001).

1.11. Olive knot disease

Pseudomonas savastanoi pv. savastanoi (Psv), the causal agent of OKD, is a relevant pathogen in most of the important olive-growing areas (Young, 2004; Quesada et al., 2010a). It has been also detected in commercial olive orchards in Egypt (Ahmad et al., 2009), Nepal (Balestra et al., 2009), Southern Australia (Hall et al., 2004), and Turkey (Basim and Ersoy, 2001). Although this disease causes serious problems, there is no precise estimation of production losses in the available bibliography (Young, 2004; Ramos et al., 2012; Quesada et al., 2012) except for two surveys conducted in California (USA) and Spain. In California, Schroth et al., (1973) reported no differences in plant vigor in 40-year-old trees when they were artificially inoculated with Psv in a commercial orchard. However, a correlation between number of knots in branches and crop losses, as well as significant differences in fruit weight between lightly- and moderately-infected olive trees were found. Conversely, the vigor of 7-year-old trees in a high-density olive grove (cv. Arbequina) in Spain was higher in

non-inoculated plants (control), although the presence of Psv did not significantly influence fruit yield (Quesada et al., 2010a). The influence of Psv infections in olive oil quality has also been investigated (Schroth et al., 1968, 1973; Tjamos et al., 1993) but further research is needed to better understand the effects of Psv on commercial olive production.

1.12. Aetiology, symptomatology, epidemiology and control of olive knot disease: a brief introduction

Olive knot disease is possibly the first plant disease clearly described in ancient texts. Theophrastus (370-286 BC) (Iacobellis, 2001) described this disease in '*De historia et de causis plantarum'* as 'olive suffers from the nail, that some other call it fungus or little bowl'. The causal agent of OKD was first isolated, described and named by Luigi Savastano as *Bacillus oleae tubercolosis* (1889). This bacterium is a member of the *P. syringae* complex comprising up to ten *Pseudomonas* species and more than 60 pathovars (Gardan et al., 1999; Bull et al., 2010; Young, 2010). Nomenclature and classification of this pathogen is under revision and *P. savastanoi* pv. savastanoi and *P. syringae* pv. savastanoi are currently considered as synonymous. Several herbaceous and woody plant species have been reported as hosts (Apocinaceae, Fabaceae, Myrtaceae, Oleaceae and Rhamnaceae) of this pathogenic bacterium (Gardan et al., 1992)

Infections by Psv produce the development of hyperplasic growth (called knots, galls or tumors) on stems (**Figure 1.9**) and branches, preferably on woody rather than herbaceous tissue and, to a lesser extent, on leaves and fruits in olive trees (Smith, 1920; Varvaro and Surico, 1978; Sisto and Iacobellis, 1999). Severe attacks in olive trees may produce death of branches and consequently an onward declining of the plant, resulting in loss of tree vigor (stunting) and yield (Tjamos et al., 1993). In addition, Psv may generate a minor size and lower quality of fruits (Schroth et al., 1973; Young, 2004; Quesada et al., 2010a).

The disease cycle, extensively described by Temsah et al., (2008), starts when the pathogen invades natural openings or wounds inflicted in the plant by leaf scars, freezing and pruning. Thereafter, Psv penetrates into the plant invading the intercellular spaces of the cortical parenchyma. Once therein, the bacterial pathogen releases primary cell walls degrading enzymes (cellobiase, cellulase, peptinase and xylanase) (Magie, 1963; Quesada et al., 2012), producing cavities where Psv increases its population (**Figure 1.9C**). Moreover, Psv synthesizes phytohormones such as IAA

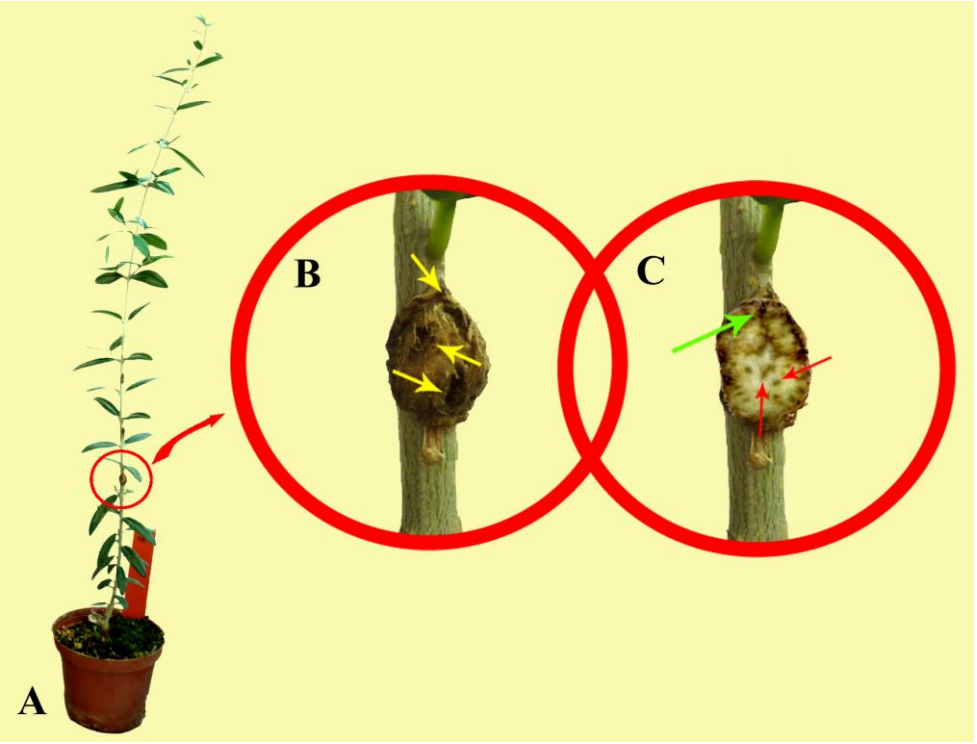


Figure 1.9. An olive plant artificially inoculated with Psv (**A**) and external (**B**) and internal (**C**) appearance of an olive knot.
A. One-year-old 'Arbequina' plant showing tumors 92 days after inoculation with Psv (intentional wounding).
B. Magnification of one tumor showing the corked surface and the superficial cavities (yellow arrows).
C. Longitudinal cut of the knot showing inner cavities (red arrows) and one open cavity to the exterior (green arrow).

and cytokinin (Smidt and Kosuge, 1978; Comai and Kosuge, 1980; Surico et al., 1985; Iacobellis et al., 1994) which interfere with plant endogenous signals inducing cambium activity of diverse host tissues increasing their size and cell division (hypertrophy and hyperplasia, respectively) (Surico et al., 1985; Sisto et al., 2004). Simultaneously, several cell tissues dedifferentiate to produce xylem elements and periderm (cork; **Figure 1.9C**). At later stages of knot development, hyperplasic activity breaks knot surface presenting to the exterior cavities full of bacteria (**Figure 1.9B**) thus favoring Psv dissemination.

The pathogen is able to endure endophytically inside the tumors across seasons occasionally migrating systemically (Marchi et al., 2009) and producing secondary knots in new wounds (Penyalver et al., 2006). Furthermore, Psv may survive as an epiphyte in olive. Thus, appropriate humidity conditions stimulate

exudate production which contains an important amount of bacteria (Wilson, 1935; Ercolani, 1978; Quesada et al., 2007). Furthermore, survival of this pathogen upon and/or inside insects and birds has been suggested although no conclusive results have been yet provided (Wilson, 1935; Quesada et al., 2010a). Rapid spread of Psv from affected to non-affected trees has been reported by Quesada et al., (2010a). Thus, long distance bacterial cells dispersal may occur by introducing infected planting material in newly-established orchards. Short distance dispersal can be explained by factors such as raindrop splashing, wind-blown aerosols, insects and cultural practices (e.g. high tree density, severe pruning and harvesting) (Horne et al., 1912; Wilson, 1935; Tous et al., 2007).

Regarding to agronomical factors affecting OKD it has been shown that this disease can be promoted by excess of N fertilization (Paoletti, 1933; Young, 1987; Baratta and Di Marco, 1981) increasing, for instance, Psv population on the olive phylloplane (Balestra and Varvaro, 1997). Moreover, specific range of temperature (22 to 25°C; Wilson, 1935) may also initiate tumor development. However, it should be noted that Psv may establish in olive trees with temperatures ranging from 5 to 37 °C. Therefore, this bacterial pathogen may first colonize olive at any season to further elicit tumor formation when proper temperature occurs (Wilson, 1935).

Effective control of OKD must rely in the implementation of pre- and post-planting measures. Preventive measures, particularly the use of pathogen-free planting material, are more convenient since Psv cannot be eradicated once established in olive (Young, 2004; Quesada et al., 2010a,b; Ramos et al., 2012). Certification of pathogen-free olive propagation material is compulsory and has been traditionally performed by conventional detection methods (i.e. culturing and isolation, pathogenicity tests, biochemical or serological techniques) (Surico and Lavermicocca, 1989; Young and Triggs, 1994). However, PCR-based protocols are more sensitive and less time-consuming (Penyalver et al., 2000; Bertolini, 2003a,b; Bella et al., 2009; Tegli et al., 2010; Gori et al., 2012). Breeding for OKD resistance is the most interesting control strategy. However, information about olive cultivar resistance to Psv is scant and mostly derives from field observations (Wilson, 1935; Barranco, 1998; Trapero and Blanco, 1998) and from comparative inoculation experiments restricted to few cultivars (Varvaro and Surico, 1978; Panagopoulos, 1993; Benjama, 1994; Marcelo et al., 1999; Hassani et al., 2003; Young et al., 2004; Catara et al., 2005). However, it is worth mentioning the detailed research conducted by Penyalver et al., (2006) where the influence on symptoms development of diverse

variables (cultivar, plant age, development of secondary knots, etc.) was assessed using a range of olive cultivars and Psv strains.

The importance of chemical control and its inconsistency in suppression of OKD under field conditions have been highlighted (Quesada et al., 2012). Thus, copper-based chemical treatments (combined or not with organic compounds) showed an important reduction of epiphytic Psv populations as well as a prevention of Psv entry through the injuries. In addition, cases of resistance to these chemicals were not observed in surviving Psv bacteria. Quesada et al., (2010b) reported that copper-based bactericides significantly diminished pathogen population soon after inoculation. Finally, antibiotics and oil-water emulsion containing hydrocarbon-based treatments have been proposed (Scrivani and Bugiani, 1955; Ark and Thompson, 1960; Schroth and Hildebrand, 1968; Trapero and Blanco, 1998). Nevertheless, so far, the use of antibiotics is banned by the EU legislation in the treatment against plant pathogenic bacteria.

Regarding to cultural practices it is advisable to harvest and prune healthy trees first (Wilson, 1935), and to avoid procedures such as knocking down with wooden poles which can produce injuries on olive branches thereby facilitating the entrance of Psv (Krueger et al., 1997). It is therefore more appropriate the manual harvesting (milking method) or the use of mechanical vibration (Civantos et al., 2008). To reduce Psv inoculum two approaches can be followed: the complete elimination of the tree (highly-affected tree) (Wilson, 1935; Penyalver et al., 1998) or pruning Psv-infected branches (mildly-affected tree). The removal of individual knots should be avoided since this action produces more wounds and hence increases the risk of new infections (Wilson, 1935; Quesada et al., 2010a). Similarly to VWO, Psv-infected plant material should be eliminated, for instance by burning (Trapero and Blanco, 1998).

Concerning to biological control methods to manage OKD, only limited success has been reported. For instance, *P. syringae* pv. ciccaronei has been shown to inhibit the growth and reduce the endurance of epiphytic Psv on leaves and twigs of artificially-infected olive plants by the production of bacteriocins (Lavermicocca et al., 2002, 2003). These proteinaceous toxins are valuable candidates as natural products and showed to be highly specific. Similarly, a proteinaceous compound synthesized by *Bacillus subtilis* F1 isolated from olive leaves significantly reduced the weight of knots (Krid et al., 2010). While these bacterial strains diminish symptoms of

OKD, the mechanisms explaining their effectiveness against Psv are still mostly unknown (Lavermicocca et al., 2002; Krid et al., 2012).

1.13. Bacterial endophytes and biocontrol

The term endophyte derives from two Greek words (*endon* and *phyton*), and strictly means 'in the plant'. Endophytes are defined as 'bacteria and fungi that can be detected at any moment within the tissues of apparently healthy plant hosts without producing disease symptoms' (Schulz and Boyle, 2006; Mercado-Blanco and Lugtenberg, 2014). It has been postulated that, most likely, all plants host microbial endophytes (Rosenblueth and Martínez-Romero, 2006; Mercado-Blanco and Lugtenberg, 2014). This association may be considered as a super-organism in which the plant and its associated inner microbiome work coordinately to maintain this outstanding ecosystem (Podolich et al., 2015). From this association, the plant can benefit in aspects such as growth promotion and phytopathogens control while endophytes live in a 'comfortable' niche that provides nutrients and shelter against (a)biotic stresses (see below) (Bacon and Hinton, 2006; Mercado-Blanco and Lugtenberg, 2014).

A microorganism is recognized as an endophyte when recovered by traditional culture-dependent methods from the host tissue after surface sterilization. However, inner tissue re-colonization of the host as well as visualization by microscopy must be proven to claim true endophytism (Reinhold-Hurek and Hurek, 1998; Rosenblueth and Martínez-Romero, 2006; Mercado-Blanco and Lugtenberg, 2014). The use of culture-independent approaches has revealed that most bacterial endophytes are non-culturable or viable but non-culturable (VBNC), and that diversity of bacterial endophytes is much higher than that revealed by traditional culture-dependent methods (Handelsman, 2004; Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Podolich et al., 2015; Berg et al., 2014).

Bacterial endophytes can colonize diverse plant organs and tissues such as roots, stems, leaves, flowers, fruits, seeds, ovules, tubers and the xylem sap (Berg and Hallmann, 2006, Rosenblueth and Martínez-Romero, 2006; Mercado-Blanco and Bakker, 2007; Compant et al., 2010; Malfanova et al., 2013). However, they are predominantly found in the roots in comparison to other plant parts (Mercado-Blanco and Lugtenberg, 2014). The rhizosphere is considered the main entrance site for endophytes. However, endophytic populations are generally lower compared to microbial rhizosphere populations. Indeed, it is assumed that endophytes derive

from an active selection of plant growth promoting rhizobacteria (PGPR) by the plant (Rosenblueth and Martínez-Romero, 2004). The host plant attracts specific rhizobacteria by, for instance, root exudates production (chemotaxis), thereby shaping its rhizosphere-associated microbiome (Berendsen et al., 2012). Occasionally, some of these PGPR attach to the root surface (Lugtenberg and Kamilova, 2009) and gain entrance to the plant interior establishing themselves as endophytes (Zhang et al., 2014). The main entry points for endophytic bacteria in roots are wounds and breaks produced by arthropods, microbes and nematodes, or at the emergence sites of lateral roots. Other penetration areas can be the root development regions (apical, differentiation and elongation root zones), roots hairs and intercellular spaces of the root epidermis (Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011; Mercado-Blanco and Lugtenberg, 2014). Besides, this passive mode of entry, active penetration may be facilitated by the production of bacterial cell wall-degrading exo-enzymes, such as cellulases and pectinases (Reinhold-Hurek and Hurek, 2011).

Endophytic bacteria can provide benefits to the host plant such as enhanced growth and protection against different (a)biotic stresses. It is plausible to assume that mechanisms involved in the promotion of plant growth and protection against phytopathogens are similar to those displayed by free-living beneficial rhizobacteria (Mercado-Blanco and Lugtenberg, 2014). Whilst the host plant provides to their associated endophytic bacteria a stable nutrient source, reduction of microbial competition and alleviation of (a)biotic stresses usually found in the rhizosphere (Bacon and Hinton, 2006), the host plant benefits from growth promotion traits deployed by its microbial residents (**Figure 1.10**). Thus, bacterial endophytes can directly enhance plant growth *via* biofertilization and phytoestimulation, providing essential nutrients and increasing phytohormones production respectively; or indirectly, for example by rhizoremediation (inactivation of pollutants) (Afzal et al., 2014) and/or protection against abiotic stresses (e.g. drought, saline soils, etc.) (Hardoim et al., 2008; Yang et al., 2009; Azcón et al., 2013). Indirect plant growth can also be promoted by bacterial endophytes through phytopathogen suppression that is, exerting biological control. Biocontrol mechanisms deployed by endophytes are not sufficiently elucidated but it can be assumed they are similar to those observed for rhizosphere beneficial microbes: nutrient and niche competition, antibiosis, and/or elicitation of plant defense responses such as induced systemic resistance (ISR, see below) (Mercado-Blanco and Lugtenberg, 2014).

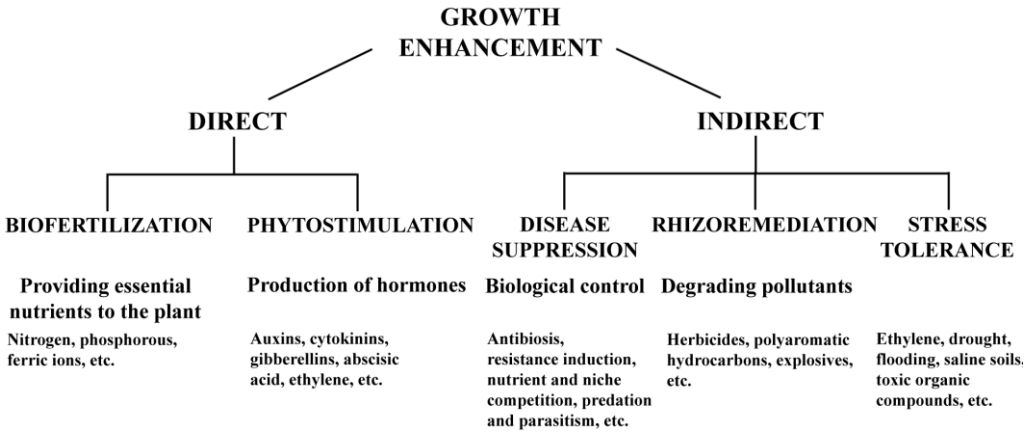


Figure 1.10. Benefits deployed by bacterial endophytes to their host plants. Based on Mercado-Blanco and Lugtenberg, (2014).

1.14. Indigenous olive roots *Pseudomonas* spp. as effective biocontrol agents against VWO

Pseudomonas spp. are a group of Gram-negative, aerobic, chemoheterotrophic, motile, bacillary bacteria profusely found in nature and able to colonize a wide range of niches due to their wide metabolic diversity and simple nutritional requirements (Spiers et al., 2000; Silby et al.,2011). A number of native *P. putida* and *P. fluorescens* strains were previously isolated from olive root tissues. Moreover, some of them were confirmed as effective BCAs against *V. dahliae* when young nursery-produced olive plants cv. Picual (highly susceptible) were artificially inoculated with a representative isolate of the D pathotype (Mercado-Blanco et al., 2004). Several traits traditionally involved in biocontrol (i.e. *in vitro* antagonism, production of the siderophore pyoverdine [= pseudobactin] [Pvd], salicylic acid [SA], or hydrogen cyanide) (Mercado-Blanco and Bakker, 2007) were demonstrated for some of these strains. These phenotypes can therefore be potentially involved in suppression of *V. dahliae* in olive, although definitive proofs are still needed to elucidate the mechanisms implicated in VWO biocontrol. Moreover, effective colonization of olive roots and endurance in this organ have been shown, indicating that these BCAs are well adapted to the target niche (Mercado-Blanco et al., 2004).

One of the most promising isolates is *P. fluorescens* PICF7. This strain displayed effective control of VWO in different nursery-produced olive cultivars, either micro-propagated or self-rooted and under controlled (growth-chamber) or semi-controlled (greenhouse) growth conditions (Mercado-Blanco et al., 2004; Prieto

et al., 2009). Further work using CLSM, vibratome plant tissue sectioning and a fluorescently-tagged PICF7 derivative enabled to demonstrate that this bacterium is able to endophytically colonize root hairs as well as the intercellular spaces of the olive root cortex (Prieto and Mercado-Blanco, 2008; **Figure 1.11**). This lifestyle confers additional advantages to this BCA since strain PICF7 is adapted to the ecological niche where it can display its biocontrol effect against *V. dahliae*: the olive roots. Besides, superficial and endophytic colonization of undamaged roots by PICF7 appeared to be critical for the effective biocontrol of VWO. That is, previous colonization of this niche by the BCA before penetration (i.e. by root injures) of *V. dahliae* into the roots seems to be essential to counteract the deleterious effects of the pathogen (Prieto et al., 2009). The use of the microscopy and biotechnological tools mentioned above evidenced that root hairs play a key role in the inner colonization of the roots by this BCA (Prieto et al., 2011; **Figure 1.11A**). More recently, it has been demonstrated that strain PICF7 induces a wide range of respon-

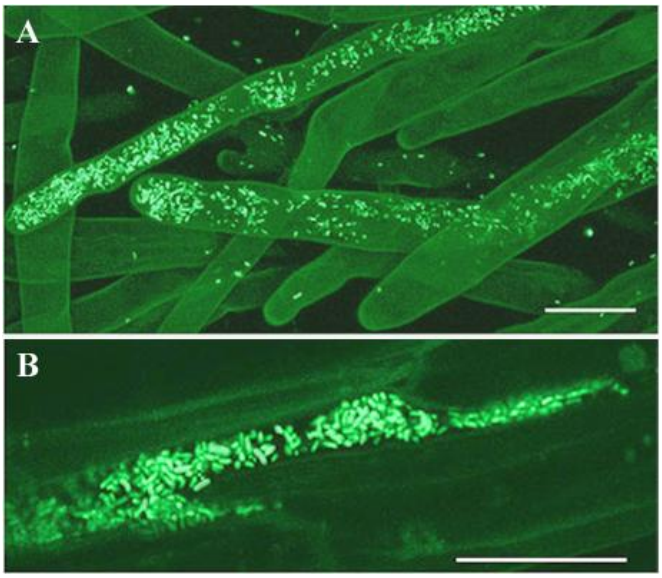


Figure 1.11. CLSM images showing endophytic colonization of a fluorescently-tagged PICF7 derivative in olive roots.
A. Inner colonization of root hairs.
B. Intercellular colonization of the spaces of the root cortex. Images reproduced from Prieto et al., (2011), *Microbial Ecology*, *62*(2), 435-445, DOI 10.1007/s00248-011-9827-6

ses, many of them related to defense to different stresses, both at local (roots) (Schilirò et al., 2012) and systemic (above-ground organs) (Gómez-Lama Cabanás et al., 2014) level. These responses may explain, at least to some extent, the biocontrol

activity exerted by strain PICF7 as well as its endophytic colonization. However, nothing is known yet about PICF7 traits involved in both biocontrol and endophytism, although the recent release of its complete genome will be a valuable tool in future studies (Martínez-García et al., 2015).

1.15. Induced resistance mechanisms in plants against pathogens: an introductory summary

Plant defense is multilayered, conformed as walls that the pathogen attempts to overthrow. Once the pathogen defeats non-specific defense mechanisms such as plant structural (cuticle, cell wall, etc.) and chemical barriers (antimicrobial chemicals) it has to cope and overcome so-called induced immune mechanisms deployed by the plant after pathogen recognition through highly-conserved, pathogen-associated signals (Pieterse et al., 2009; Boller and Felix, 2009). One of these defense responses is SAR (Systemic Acquired Resistance) that should not be confounded with ISR. While both responses enhance the basal defensive capacity of the plant against a wide range of microorganisms, SAR is generally triggered by pathogens (or insects) (Vlot et al., 2009; Spoel and Dong, 2012; Pieterse et al., 2014) and ISR is elicited by non-pathogenic microorganisms (bacteria and fungi), including rhizobacteria and endophytes (Bakker et al., 2007; Mercado-Blanco and Bakker, 2007; Walters et al., 2013). It should be emphasized that SAR and ISR responses may show hormone(s)-mediated cross talk (Pieterse et al., 2009). Moreover, the redox-regulated protein NONEXPRESSOR OF *PR* GENES1 (NPR1) is implicated in both responses (Dong, 2004). However, while this protein is a transcriptional co-activator of PR genes in the nucleus in SAR response, NPR1 has been detected in the cytosol but with an unknown function in ISR (Pieterse et al., 2014). Furthermore, SAR is SA-dependent and activates pathogen-related (PR) genes that produce PR proteins related to antimicrobial activity (Gaffney et al., 1993; Sticher et al., 1997). In contrast, ISR is generally jasmonic acid (JA)/ethylene (ET)-dependent and does not activate PR-genes (Pieterse et al., 2014). Several ISR-elicitors (determinants) have been proposed including flagella, lipopolysaccharides, exopolysaccharides, siderophores, iron-regulated metabolites, antibiotics, biosurfactants and VOCs (De Vleesschauwer and Höfte, 2009, Pieterse et al., 2014). Apparently most ISR-inducing bacteria exhibit multiple determinants triggering ISR (Bakker et al., 2003). On the other hand, improvement of ISR may be carried out by combining different inducing agents usually showing higher ISR response in comparison to single application (Jetiyanon et al., 2003; Alizadeh et al., 2013).

Arabidopsis thaliana (L.) Heyhn (2n=10) is an annual small flowering plant member of the mustard family (Brassicaceae). Considered in the past as a weed, with no major agronomic significance, *Arabidopsis* was acquiring importance progressively in plant science becoming essential when it was the first plant with its total genome sequenced by the *Arabidopsis* Genome Initiative, (2000). Besides, this plant exhibits important advantages such as one of the smallest plant genomes (approx. 157 Mbp) (Bennett et al., 2003), rapid generation time (6 to 12 weeks), high seed production (several thousand per plant), and the ease for genetic manipulation. In this regard, the use of *Agrobacterium tumefaciens* as a DNA vector (Feldmann and Marks, 1987; Bechtold et al., 1993; Clough and Bent, 1998), among other methods, has led to the generation of insertion mutants for most of the genes, which converted *A. thaliana* as a valuable genetic model organism to unravel the mechanisms underlying many plant processes. An in-depth understanding of the interactions established between plants and microbes is very difficult for many crops, particularly in woody plants as olive with long generation time, large genome (aprox. 2200 Mb) and insufficient genetic knowledge (Muñoz-Mérida et al., 2013). The use of *A. thaliana* would therefore facilitate the study of the genetic and molecular bases of plant-*V. dahliae*-BCA interaction (Meschke and Schrempf, 2010; Tjamos et al., 2005), as well as to elucidate whether ISR/SAR responses are implicated in the biological control exerted by the beneficial endophyte *P. fluorescens* PICF7 against *V. dahliae*.

Objectives of this Thesis

Considering the above, the general objectives of this thesis are to perform, for the first time, an in-depth study on defined bacterial traits potentially involved in the endophytic behavior of strain *Pseudomonas fluorescens* PICF7 in olive roots as well as on its effective biocontrol activity against the olive soil-borne pathogen *Verticillium dahliae*. The study of the involvement of selected bacterial traits will be done both in olive (natural host) and *Arabidopsis thaliana* (model plant). Moreover, the biocontrol range of strain PICF7 was also explored by assessing its effectiveness as BCA against another relevant olive pathogen: *Pseudomonas savastanoi* pv. savastanoi, the causal agent of OKD.

Efforts to reach these objectives led to the publication of three articles which are compiled and presented in this thesis as chapters 2, 3 and 4:

Chapter 2 main objective → To evaluate the involvement of specific traits of *P. fluorescens* strain PICF7 on olive roots colonization ability and effective control of VWO.

Sub-objectives:

- ✓ Generation of a random transposon insertion mutant bank of strain PICF7
- ✓ Identification and characterization of strain PICF7 mutants impaired in traits related with rhizosphere/root colonization and biocontrol activity: motility, siderophore production and nutritional requirements
- ✓ Root colonization ability and biocontrol behavior of selected PICF7 mutant phenotypes *in planta*

Chapter 3 main objective 2 → To assess whether *A. thaliana* is a suitable host to assist in the identification of traits involved in *V. dahliae* biocontrol deployed *P. fluorescens* PICF7, and to investigate the possible involvement of induced systemic resistance responses in its biocontrol activity.

Sub-objectives:

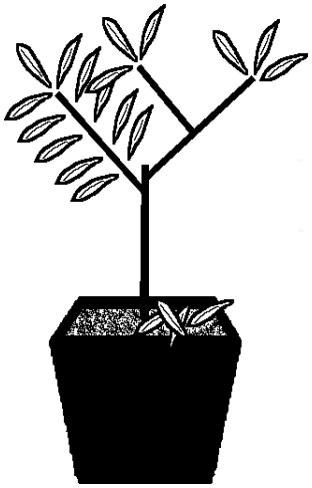
- ✓ Pathogenicity and virulence exerted by *V. dahliae* olive pathotypes (defoliating and non-defoliating) in diverse *A. thaliana* accessions
- ✓ Colonization and biocontrol behavior of strain PICF7 and mutant phenotypes previously tested in olive in several *A. thaliana* genotypes
- ✓ Ability to elicit induced systemic resistance responses in *A. thaliana* by strain PICF7 against the leaf pathogen *B. cinerea*

Chapter 4 main objective → To explore the potential biocontrol activity of *P. fluorescens* PICF7 against the olive pathogen *P. savastanoi* pv. savastanoi.

Sub-objectives:

- ✓ Assessment of an effective long-distance biocontrol of *P. savastanoi* upon inoculation of strain PICF7 in roots (natural niche)
- ✓ Effects of the presence of strain PICF7 in olive knot development: a micro and macroscopic study of the interaction between *P. savastanoi* and PICF7

CHAPTER 2



Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility

This chapter has been published as:

Maldonado-González, M. M., Schilirò, E., Prieto, P., and Mercado-Blanco, J. (2015). Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. *Environmental Microbiology*, (on-line first). DOI: 10. 1111/1462-2920.12725.

2.1. Summary

Pseudomonas fluorescens PICF7 is an indigenous inhabitant of olive (*Olea europaea* L.) rhizosphere, able to display endophytic lifestyle in roots, to induce a wide range of defence responses upon colonization of this organ and to exert effective biological control against Verticillium wilt of olive (VWO) (*Verticillium dahliae*). We aimed to evaluate the involvement of specific PICF7 phenotypes in olive root colonization and VWO biocontrol effectiveness by generating mutants impaired in swimming motility (*fliI*) or siderophore pyoverdine production (*pvdI*). Besides, the performance of mutants with diminished *in vitro* growth in potato dextrose agar medium (*gltA*) and cysteine (Cys) auxotrophy was also assessed. Results showed that olive root colonization and VWO biocontrol ability of the *fliI*, *pvdI* and *gltA* mutants did not significantly differ from that displayed by the parental strain PICF7. Consequently, altered *in vitro* growth, swimming motility and pyoverdine production contribute neither to PICF7 VWO suppressive effect nor to its colonization ability. In contrast, the Cys auxotroph mutant showed reduced olive root colonization capacity and lost full biocontrol efficacy. Moreover, confocal laser scanning microscopy revealed that all mutants tested were able to endophytically colonize root tissue to the same extent as wild-type PICF7, discarding these traits as relevant for its endophytic lifestyle.

2.2. Introduction

Verticillium dahliae Kleb. is a soil-borne phytopathogenic fungus causing vascular diseases collectively known as Verticillium wilts in a wide range of plant species (Pegg and Brady, 2002). A susceptible host for *V. dahliae* is olive (*Olea europaea* L.), which is one of the most important woody crops in the Mediterranean Basin. Olive cropping is threatened by diverse (a)biotic menaces of variable importance which highly depend on a number of factors (soil characteristics, climatic conditions, soil resident microbiota, agricultural practices, etc.). One of the most important biotic constraints for olive cultivation is *V. dahliae*. This disease has been steadily spreading in many areas where olive is a relevant commodity, causing great concern to farmers and the olive oil industry (López-Escudero and Mercado-Blanco, 2011).

The effective control of Verticillium wilt of olive (VWO) is very difficult due to several factors (López-Escudero and Mercado-Blanco, 2011). Because of this complex scenario, an integrated disease management strategy has been proposed as the only plausible way to control VWO, combining physical, chemical, biological and agronomical measures. Within this framework, preventive measures (pre-planting) are mostly encouraged, although palliative actions (post-planting) are also needed to limit the expansion of the pathogen or to alleviate losses caused by the disease in established orchards (Tjamos, 1993; López-Escudero and Mercado-Blanco, 2011).

The use of effective biological control agents (BCAs) is a promising tool that can be used both as a preventive and palliative measure. For instance, application of microbial antagonists in pathogen-free certified olive plants during the propagation process at nurseries has been proposed (Tjamos, 1993). There are only few reports on the identification and characterization of potential BCAs against VWO, although mostly preliminary or showing limited success (Müller et al., 2007; Aranda et al., 2011; Sanei and Razavi, 2011; Mercado-Blanco and López-Escudero, 2012). Moreover, very little is known about the mechanisms involved in biocontrol activity exerted by these microbes.

A well-known effective BCA against VWO is *Pseudomonas fluorescens* PICF7 (Mercado-Blanco et al., 2004; Prieto et al., 2009). This strain, originally isolated from roots of nursery-propagated olive plants, displays an endophytic lifestyle (Prieto and Mercado-Blanco, 2008; Prieto et al., 2009, 2011), produces the siderophore pyoverdine (= pseudobactin) (Pvd) *in vitro* (Mercado-Blanco et al., 2004), and induces

a broad range of defence responses in both roots (Schilirò et al., 2012) and above-ground organs (Gómez-Lama Cabanás et al., 2014) of olive. Nevertheless, nothing is known about PICF7 traits involved in both endophytic colonization and biocontrol effectiveness against *V. dahliae*.

Efficient colonization and persistence on/within the targeted plant tissue where biocontrol pseudomonads may deploy its biocontrol activity is essential for successful crop protection (Lugtenberg et al., 2001; Mercado-Blanco and Bakker, 2007). While bacterial traits involved in colonization of the rhizosphere and plant root surface have been studied in some detail, little is known about determinants implicated in the active colonization of the root interior by biocontrol endophytic bacteria (Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011). For instance, it is suggested that diverse cell wall-degrading enzymes and motility are involved in the endophytic colonization process (Hallmann et al., 1997; Compant et al., 2005; Reinhold-Hurek and Hurek, 2011). Bacterial endophytes are adapted to live within a protected niche, less exposed to (a)biotic stresses and relying on a constant source of nutrients provided by the host plant (Bacon and Hinton, 2006). In contrast, they must cope with the defence barriers deployed by the plant to confront this 'non-hostile' colonization (Wang et al., 2005; Conn et al., 2008; Schilirò et al., 2012). Therefore, beneficial bacterial endophytes effective in promoting plant growth and suppressing deleterious microorganisms are an excellent source of biotechnological weapons to be exploited in agro-ecosystems (Mercado-Blanco and Lugtenberg, 2014). For instance, endophytic *Pseudomonas* spp. have proven to provide benefits for the host plant in several cases (Chen et al., 1995; Nejad and Johnson, 2000; Kuklinsky-Sobral et al., 2004; Prieto et al., 2009). As mentioned for the colonization process, biocontrol mechanism(s) deployed by endophytes also remain largely unknown. However, since bacteria able to develop an endophytic lifestyle usually originate from the rhizosphere, it is plausible to assume that their beneficial effects may operate similarly to those described for rhizosphere-associated bacteria (Kloepper and Ryu, 2006; Mercado-Blanco and Lugtenberg, 2014).

The present study is, therefore, aiming to shed light on determinants of PICF7 potentially involved in superficial and endophytic colonization of olive roots and biocontrol activity against *V. dahliae*. We have particularly focused on the implication of motility and siderophore production. Motility is one of the most important traits for efficient rhizosphere colonization by specific *Pseudomonas* spp. strains (Navazo et al., 2009). Yet the actual contribution of bacterial motility in rhizosphere (and

endophytic) colonization may vary among plant-bacteria interactions. Thus, non-motile derivatives or mutants with reduced motility can be impaired in competitive colonization of the rhizosphere by beneficial bacteria in several plants (Lugtenberg et al., 2001; Ormeño-Orrillo et al., 2008). In contrast, *P. fluorescens* Q8r1-96 exhibiting decreased motility was not impaired in rhizosphere colonization (Mavrodi et al., 2006). Plant beneficial *Pseudomonas* spp. may synthesize a wide diversity of siderophores, iron (Fe3+)-chelating compounds frequently related with the biocontrol activity exerted by many strains of this genus (Mercado-Blanco and Bakker, 2007). Nevertheless, the true implication of *Pseudomonas*-produced siderophores in biological control of soil-borne pathogens is a controversial issue (Cornelis and Matthijs, 2002; Weller, 2007; Lemanceau et al., 2009; Bakker et al., 2014).

In order to elucidate potential mechanisms implicated in olive root colonization (even endophytically) and suppression of VWO by *P. fluorescens* PICF7, a mutant bank of strain PICF7 was generated by random transposon insertion. A set of generated mutants was mainly screened for siderophore production and swimming motility. Selected mutants were further characterized and assessed for their ability to colonize olive root tissues and for their VWO biocontrol performance. Colonization and disease suppression bioassays were performed under non-gnotobiotic conditions, a scenario closer to the environmental conditions where the interaction between olive roots and *P. fluorescens* PICF7 takes place.

2.3. Material and methods

2.3.1. Bacteria, culturing media and production of bacterial inocula

Pseudomonas fluorescens PICF7 (Mercado-Blanco et al., 2004) and several mutants and fluorescently tagged derivatives of this strain were used in this study (**Table 2.1**). Tn5-tetracycline resistant (Tc^R) transposon insertion mutants ME419, ME424, ME589 and ME1508 were constructed by biparental matings (see below). Tc^R and/or gentamicin resistant (Gm^R), green fluorescence protein (GFP)-labelled derivatives of strain PICF7, used to monitor olive root colonization by confocal laser scanner microscopy (CLSM; see below), were named as PICF7-G, ME419-G, ME424-G, ME589-G and ME1508-G (**Table 2.1**). *Pseudomonas* strains were always grown at 25°C in King's medium B (King et al., 1954) agar (KBA) plates. When needed, antibiotics were added at the following concentrations (mg l^{-1}): nalidixic acid (Nal), 10; tetracycline

(Tc), 20; gentamicin (Gm), 10; ampicillin (Amp), 50; chloramphenicol (Chl), 13; and cycloheximide (Chx), 100. *Escherichia coli* strains (**Table 2.1**), S17-1 harbouring the suicide plasmid pJQ18 which contains transposon Tn5 (Hynes et al., 1989) and DH5α harbouring plasmid pLMR1, were grown at 37°C in Luria-Bertani (Miller, 1972) agar (LBA) amended with Tc (20 mg l^{-1}) and Gm (50 mg l^{-1}) respectively. *Pseudomonas* strains inocula were prepared as described in Maldonado-González and colleagues (2013). Bacterial cell densities required for each experiment were determined spectrophotometrically (A600 nm) by building up standard curves and culturing viable cells from serial dilution series onto KBA plates (to count PICF7 wild-type colonies), or KBA plates supplemented with the antibiotics Tc (for Tn5-Tc^R derivatives), Gm (for GFP-labelled PICF7 derivative) or Tc plus Gm (for GFP-labelled Tn5-Tc^R mutants).

2.3.2. GFP labelling of *Pseudomonas*

Strain PICF7 and selected Tn5-Tc^R insertion mutants were transformed with plasmid pLRM1 (Gm^R, GFP) (Rodríguez-Moreno et al., 2009). Electrocompetent cells of each bacterial strain were transformed with plasmid pLRM1 by electroporation as described by Prieto and Mercado-Blanco (2008). Transformation frequency (transformants/µg plasmid DNA) was calculated for each strain. The presence of plasmid pLMR1 in selected transformed derivatives was further confirmed by plasmid purification (FavorPrep Plasmid DNA Extraction Mini Kit, Ping-Tung, Taiwan) and restriction analysis with EcoRI (New England BioLabs, Beverly, MA). Plasmid pLRM1 stability in each transformed derivative was assessed by continuous growth of exponential-phase cultures kept under vigorous shaking (250 rpm) without antibiotic (Gm) selection pressure for 6 days at 25°C (approximately 120 generations). Serial dilution series of these cultures were plated onto LBA plates every 24 h and incubated at 25°C during 48 h. Subsequently, 100 randomly selected colonies were individually transferred to LB agar plates amended with Gm. The number of Gm-resistant colonies for each strain was counted and the percentage of plasmid loss (Gm-sensitive colonies) scored. The rate of plasmid loss/generation was calculated according to Durland and Helinski (1987) equation, which is percent plasmid loss/generation = [1 - (*R*N/*Ri*)1/N] x 100, where *R* is the frequency of the plasmid-mediated resistance in the population, *i* is initial, and N is the number of generations elapsed.

Table 2.1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Characteristics	Reference or source
Bacterial strains		
P. fluorescens		
PICF7	Wild-type PGPR	Mercado-Blanco et al., 2004
ME419	PICF7 Tn5 (Tc^R) *in vitro* growth mutant derivative, $GltA^-$	This work
ME424	PICF7 Tn5 (Tc^R) motility mutant derivative, $FliI^-$	This work
ME589	PICF7 Tn5 (Tc^R) siderophore mutant derivative, $PvdI^-$	This work
ME1508	PICF7 Tn5 (Tc^R) auxotroph Cys mutant derivative	This work
PICF7-G	PICF7 (Gm^R) GFP-labeled mutant derivative	This work
ME419-G	ME419 (Tc^R and Gm^R) GFP-labeled mutant derivative	This work
ME424-G	ME424 (Tc^R and Gm^R) GFP-labeled mutant derivative	This work
ME589-G	ME589 (Tc^R and Gm^R) GFP-labeled mutant derivative	This work
ME1508-G	ME1508 (Tc^R and Gm^R) GFP-labeled mutant derivative	This work
Escherichia coli		
S17-1	*thi pro recA hsdR hsdM* RP4-2-Tc,Mu-Km, Tp^RSm^R	Simon et al., 1983
DH5α	*recA1 endA1* Φ80d *lacZ dam-15*	(Clontech)
Plasmids		
pJQ18	pSUP5011 derivative; carries Tn5-Mob-Tc	Alfred Pühler
pLRM1	pBBR1-MCS5 carrying a fusion of the $P_{A1/04/03}$ promoter to the *gfpmut3** gene	Rodríguez-Moreno et al., 2009

GltA, Type II citrate synthase; FliI, flagellum-specific ATP synthase; PvdI, putative pyoverdine non-ribosomal peptide synthetase; Tc, tetracycline; Cys, cysteine; Gm, gentamicin; GFP, Green Fluorescence Protein

2.3.3. *Verticillium dahliae* growth conditions, pathogen inoculum production and plant material

Verticillium dahliae V937I, a highly virulent isolate representative of the defoliating (D) pathotype (Collado-Romero et al., 2006), was used in VWO biocontrol assays.

Pathogen inoculum was prepared as described in Mercado-Blanco and colleagues (2004). The number of conidia per millilitre was scored in a Neubauer chamber and adjusted to the required working concentration. Biological control assays were carried out using nursery-produced, 3-month-old olive plants cv. Picual, qualified as highly susceptible to the D pathotype (López-Escudero et al., 2004). Plants were originated from two different commercial nurseries located in Córdoba province (Southern Spain). Previous to starting the bioassays, plants were kept at least 1 month within a controlled-growth chamber at 25 ± 1°C with a 14-h photoperiod and a light intensity of 65 µmol m^{-2} s^{-1} for acclimatization. Plants used for CLSM were 2-month-old and were acclimated for 2 weeks under the same environmental conditions.

2.3.4. Generation of a *Pseudomonas fluorescens* PICF7 mutant bank

A random transposon insertion mutant bank of *P. fluorescens* PICF7 was generated by using a Tn5 derivative carried in the suicide plasmid pJQ18 (Simon et al., 1983) (**Table 2.1**) and biparental mating as described in Mercado-Blanco and colleagues (2001b). More than 9000 transconjugants were obtained, mixed and cryopreserved in 30% glycerol at -80°C. The percentage of auxotroph mutants in the mutant bank was checked by replicating a fair number (>500) of Tc^R colonies in both standard succinate medium (SSM) and LBA that were incubated at 25°C during 72 h. Mutants unable to grow in SSM but capable to do so in LBA were considered as auxotrophs, and the percentage of transposon insertions leading to auxotrophy was calculated.

2.3.5. Phenotypic characterization of *Pseudomonas fluorescens* PICF7 mutants

The screening of mutants altered in swimming motility was as follows. In a first round, 2000 Tc^R colonies previously grown overnight on KBA plates (amended with Tc) at 25°C were inoculated (35 mutants/plate) in square 'swimming medium' (SM) (1% tryptone, 0.5% NaCl, 0.3% agar) (Déziel et al., 2001) agar plates (12 x 12 cm) along with the parental strain PICF7 (used as a control). Swimming motility was revealed after overnight incubation at 25°C. Transconjugants showing altered behavior (lack, increase or decrease of swimming motility compared with the wild type) were further tested individually (one mutant per plate together with a colony of PICF7) in SM agar. This check step was repeated twice for each mutant. Other types of bacterial motilities, such as swarming (Overhage et al., 2007) and twitching (Alit-Susanta and Takikawa, 2006), were also tested in appropriate media. Data on

colony diameter of putative mutants were subjected to analysis of variance (ANOVA), and means were compared with that of the parent strain (PICF7) using two-sided Dunnett's multiple comparisons with a control at $P < 0.05$. Mutants displaying a significantly altered swimming motility phenotype were selected for further characterization and stored in 30% glycerol at -80°C.

Two different culturing media were used to screen PICF7 mutants altered in siderophore production. In a first round, a set of 2000 transconjugants were tested in parallel using KBA and the universal siderophore detection medium chrome azurol S (CAS) agar (Schwyn and Neilands, 1987). Production of the major siderophore Pvd in KBA was observed as a green fluorescence when grown colonies were submitted to UV light. Production of iron-chelating compounds in CAS agar plates is revealed by the production of orange haloes around siderophore-producing colonies. A colony of strain PICF7 was included in all plates for comparison. Mutants (Tc^R colonies) previously grown in KBA plates (amended with Tc) (25°C, 24 h) were individually transferred to KBA and CAS plates (49 mutants/plate) without Tc (to allow growth of a colony of PICF7 used as a reference) and incubated at 25°C for 24 h. Absence, decrease or increase of fluorescence under UV irradiation in KBA and orange haloes produced on CAS plates were scored for each single mutant. A preselected set of colonies showing altered siderophore production phenotypes was checked in the same media by plating cell suspensions (5 µl) of each mutant individually, per triplicate, onto CAS and KBA, along with a suspension of *P. fluorescens* PICF7 cells (control). This step was repeated at least twice. Thus, fluorescence in KBA was checked again and the relative halo size [(halo diameter - colony diameter)/ halo diameter] produced in CAS media was calculated for each mutant. Relative haloes size data were subjected to ANOVA and means were compared with strain PICF7 mean using two-sided Dunnett's multiple comparisons with a control at $P < 0.05$.

To screen for *P. fluorescens* PICF7 mutants potentially altered in *in vitro* antagonism against *V. dahliae*, the following experimental approach was carried out. In a first screening round, mutants (2000) and *V. dahliae* isolate V937I were confronted as follows: potato dextrose agar (PDA) medium was prepared, cooled down to 45°C and mixed with a conidial suspension of V937I to yield a final concentration of approximately 1×10^4 conidia ml^{-1}. Once agar plates solidified, individual colonies (35 mutants/plate) were inoculated with a sterile toothpick and incubated for 4 days at 28°C. The presence of haloes around bacterial colonies (inhibition or retardation of the fungal growth) was checked periodically. Each plate

contained a colony of PICF7 as a reference. Mutants showing a different behaviour compared with that of the parental strain were pre-selected, and the procedure was repeated but each 'candidate' mutant was individually plated as a 5 µl drop of bacterial suspension together with a suspension of PICF7. This check test was repeated twice.

Finally, selected mutants impaired in siderophore production, swimming motility and/or altered behavior in *in vitro* antagonism assay against *V. dahliae* were further tested for their ability to grow in liquid and solid SSM (pH 7.0) (Meyer and Abdallah, 1978) for 20 h or 48 h respectively. Mutants with the absence or decrease in green fluorescence (Pvd production) or unable to grow in this minimal medium (auxotroph) were selected. This assay was done at least twice for each mutant.

2.3.6. Identification of transposon insertion sites in selected PICF7 mutants

A collection of selected mutants (43) altered in one of the phenotypes mentioned above were analyzed to determine the gene disrupted by Tn5 transposon insertion. A combination of arbitrary and nested-PCR was implemented according to Caetano-Anollés (1993). Total DNA of selected mutants was extracted and purified according to i-genomic CTB Extraction Mini Kit (Intron Biotechnology, European Biotech Network, Belgium). A first round of amplification was accomplished by using total DNA from each mutant as a template and two primers, one arbitrary (ARB1; 5'-GGCCACGCGTCGACTAGTACNNNNNNNNNNGATAT-3') and another specific to the internal right end of the transposon Tn5 (Tn5Ext; 5'-GAACGTTACCATGTTAGGAGGTC-3'). The first PCR round consisted of 5 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 55°C and 2 min at 72°C, followed by an extension period of 5 min at 72°C. The final volume was 30 µl (1 x *Taq* DNA polymerase buffer, 3 mM MgCl2, 2 mM dNTP, 0.8 µM of each primer, 1 U μl^{-1} of *Taq* DNA polymerase and 0.5 ng of extracted DNA). One microlitre of the previous reaction was submitted to a nested-PCR reaction with specific primers, ARB2 (5'-GGCACGCGTCGACTAGTAC-3') and Tn5Int (5'-CGGGAAA GGTTCCGTTCAGGACGC-3'), the sequences of which corresponded to the conserved region of ARB1 and to the right end of Tn5Ext respectively. Nested-PCR conditions were as follows: 2 min at 95°C; 30 cycles of 30 s at 95°C, 30 s at 57°C, 2 min at 72°C, followed by 3 min at 72°C. Final volume was 30 µl (1 x Taq DNA polymerase buffer, 3 mM MgCl2, 2 mM dNTP, 0.8 µM of each primer and 1 U μl^{-1} of *Taq* DNA polymerase). Amplicons were electrophoresed in 0.8% agarose gels, and the observed band was extracted from gel and purified (FavorPrepTM GEL/ PCR Purification Mini Kit).

Adjacent DNA region to the Tn5 insertion site of each selected mutants was sequenced (Sistemas Genómicos S.L., Paterna, Valencia, Spain) using primer Tn5Int. DNA sequences were compared against available databases (GenBank and *Pseudomonas* Genome Database) using the BLASTX and BLASTN programmes (Altschul et al., 1997) available at the NCBI network service, and against the complete PICF7 genome sequence (deposited in GenBank, CP005975) (Martínez-García et al., 2015).

2.3.7. L-Cysteine cross-feeding assay

Growth of mutant ME1508 was impaired in SSM and sequence analysis revealed possible auxotrophy in Cysteine (Cys) biosynthesis. To check this mutant phenotype, a cross-feeding experiment was carried out. Bacterial suspensions of strain PICF7 (positive control) and mutant ME1508 originating from fresh colonies grown in LBA were prepared in SSM and inoculated (350 µl) in 5 ml of SSM (control) and SSM amended with increasing (1, 2, 5, 10 and 20 mg l^{-1}) concentrations of L-Cys (Sigma-Aldrich, St Louis, MO) (final OD600 = 0.1). Cultures were grown in an orbital shaker at 25°C for 48 h (150 rpm). Cell growth and restoration of Pvd production by ME1508 in liquid SSM upon L-Cys addition were determined as previously described (Mercado-Blanco et al., 2004). This experiment was repeated twice.

2.3.8. Verticillium wilt of olive biocontrol experiments

Three independent bioassays were conducted to assess the effectiveness of four PICF7 mutants altered in different phenotypes to control VWO. Bioassays were carried according to the procedure described by Mercado-Blanco and colleagues (2004). The carefully washed (tap water) root systems of nursery-produced olive plants (cv. Picual) were dipped in suspensions of each bacterial strain (cell densities ranging from 5.8 x 10^8 to 1.7 x 10^9 cfu [colony-forming units]ml^{-1}, 15 plants) or 10mM $MgSO_4 \cdot 7H_2O$ (control, 9-12 plants) for 15 min. Then plants were transplanted into clay pots filled with soil mixture (sand/ loam, 2:1,vol/vol) thoroughly mixed with a conidia suspension (ranging from 3.2 x 10^5 to 2.7 x 10^6 conidia g^{-1} soil) of *V. dahliae* D isolate V937I (Collado-Romero et al., 2006) or distilled sterile water (control treatment). Plants were kept in a growth chamber (conditions describe above) during at least 110 days after inoculation (DAI). Disease symptoms, such as defoliation, chlorosis and wilting, were scored along the bioassay using a 0-4 rating scale according to the percentage of affected leaves and twigs (0, no symptom; 1, 1-33%;

2, 34-66%; 3, 67-100%; and 4, dead plant) at weekly intervals after inoculation with the pathogen for the first 2 months, and thereafter every 10 days.

Disease severity data were used to determine the following: (i) a disease intensity index (*DII*) defined as $DII = (\sum Si \times Ni)/(4 \times Nt)$, where *Si* is severity of symptoms, *Ni* is the number of plants with *Si* symptoms severity, and *Nt* the total number of plants; (ii) final disease incidence (*DI*) established as the percentage of affected plants at the end of the bioassays; and (iii) standardized area under the disease progress curve of *DII* plotted over time (days) (*SAUDPC*) calculated according to Campbell and Madden (1990). *SAUDPC* data were subjected to ANOVA, which was calculated using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$.

2.3.9. Olive root colonization ability of PICF7 mutants

To assess whether PICF7 mutant derivatives were affected in their ability to colonize olive roots tissues, even endophytically, two approaches were followed. On the one hand, population size of introduced bacteria was checked on/in root samples from each biocontrol experiment approximately at 110 days after bacterization (DAB). To do so, root systems of three plants per treatment were kindly uprooted and immersed in tap water to remove the excess of soil particles at the end of each experiment. Subsequently, plants were deposited over filter paper and the roots were air-dried and weighted. Then, root tissue samples (1 g) of each plant were thoroughly ground using a mortar in 5 ml of 10 mM $MgSO_4 \cdot 7H_2O$, and serial dilutions of root macerates were plated in KBA plates amended with Amp, Chl and Chx (for PICF7 cells counting), plus Tc (for Tc^R mutant cells counting). Plates were incubated at 25°C for 24 h and cfu g^{-1} fresh root tissue was estimated for each strain. Data were subjected to ANOVA and means were compared with strain PICF7 using two-sided Dunnett's multiple comparisons with a control at $\alpha = 0.05$.

On the other hand, to monitor both surface and inner olive root colonization by PICF7 and its Tc^R mutants, GFP (Gm^R) derivatives (see above), vibratome (Vibratome Series 1000plus, TAAB Laboratories Equipment, Aldermaston, UK) root sectioning and CLSM (Axioskop 2 MOT microscope, Carl Zeiss, Jena GmbH, Germany) were used. The Gm^R GFP-labelled mutants were applied to olive root systems as previously indicated for biocontrol assays. One to two weeks after bacteria

inoculation, fresh and healthy roots from two-three plants per treatment were manipulated, longitudinally sectioned and visualized by CLSM according to Prieto and Mercado-Blanco (2008). Finally, to count viable cells of each Gm^R GFP-labelled mutant, 1 g from root tissue of each plant analyzed by CLSM was ground as mentioned above, and serial dilutions of the macerate were plated onto KBA amended with Amp, Chl, Chx and Gm, and grown at 25°C during 48 h.

In order to assess the possible translocation of *P. fluorescens* PICF7 cells from the roots to aerial tissues of inoculated plants, the following experimental set-up was followed. Two-month-old 'Picual' plants were carefully bacterized, avoiding cross-contamination of above-ground organs, by watering pots with freshly prepared suspensions of the GFP-labelled PICF7 Gm^R derivative (log_{10} 8.8- 9.7 cfu ml^{-1}, 120 ml pot^{-1}) three consecutive times (day 0, 5 and 10). Subsequent watering of bacterized plants and that of control, non-bacterized plants, was carried out with tap water. Root and stem samples of two bacterized plants were checked by CLSM (see above) at 14, 21, 60 and 90 DAB. Sampled plants were carefully uprooted, the roots dipped in tap water to remove soil excess and allow to air-dry over filter paper. Each plant was divided into above-ground part and root system. On the one hand, and for assessing PICF7 viable cells, a sample of root tissue (1 g), two segments of the stem (1 cm each) and 4 half-leaves with their petioles originating from the basal zone of each sampled plant were weighted and macerated in 10 ml of 10 mM $MgSO_4.7H_2O$. Serial dilutions of root and basal aerial sections macerates were plated per duplicate in KBA amended with Amp, Chl and Chx (to count indigenous bacteria and PICF7 Gm^R) and with these antibiotics plus Gm (to count PICF7 Gm^R) and incubated at 25°C for 48 h. On the other hand, vibratome sections of roots (longitudinal), stems and petioles (transversal) were analyzed by CLSM as mentioned above. Mutants population size data were compared with strain PICF7 using Student's t-test ($\alpha = 0.05$) (**Table 2.S1**).

2.4. Results

2.4.1. Construction of a mutant library of *Pseudomonas fluorescens* PICF7

A total of 9300 *P. fluorescens* PICF7 Tc^R colonies were obtained after biparental mating between *Escherichia coli* DH5α (pJQ18) and PICF7 (**Table 2.1**). Transposon Tn5-Tc^R insertion frequency was calculated as 2×10^{-5}. The number of mutants thus obtained was theoretically enough to have more than one random insertion every 1

kb throughout the genome of *P. fluorescens* PICF7 (≈ 6.1 Mbp) (Martínez-García et al., 2015). The percentage of insertions leading to auxotrophy was 1.36%.

2.4.2. Screening and selection of PICF7 mutants affected in swimming motility, siderophore production or antagonism against *V. dahliae*

Results showed that *P. fluorescens* PICF7 exhibited swimming motility when tested in SM (**Figure 2.1**). Clear evidence of swarming or twitching motilities was not obtained for strain PICF7 under tested conditions, and thus they were not further investigated. A set of 2000 Tn5-Tc^R insertion mutants were then checked for the loss/alteration in (i) swimming motility, (ii) siderophore production and (iii) *in vitro* antagonism against *V. dahliae* D pathotype. From a preliminary screening, 55 Tc^R mutants affected in one or more of these phenotypes were finally selected for further characterization (**Table 2.S2**). Screening of this subset of mutants revealed that 14 of them displayed altered swimming motility phenotypes compared with that of the parental strain. Five mutants showed no motility or less than 10% than wild-type PICF7, while nine mutants displayed a reduction ranging from 10% to 70% of the wild-type phenotype (**Table 2.S2**).

Siderophore production by Tn5-Tc^R insertion mutants was assessed in different media (CAS, KBA and SSM). A total of 46 mutants were found to exhibit a modified siderophore-producing phenotype depending on the media used. For instance, 10 mutants were completely impaired in Pvd production, since neither green fluorescence nor production of an orange halo around the colonies was found in any of the culturing media tested in comparison to the wild-type phenotype of strain PICF7 (**Table 2.S2**). Mutants partially altered in siderophore production (i.e. reduced orange halo) were not considered for further characterization in the present study.

The screening for *in vitro* antagonism exerted by Tn5-Tc^R insertion mutants against the *V. dahliae* D isolate V937I (highly virulent) was carried out in PDA medium. After evaluating a set of 2000 insertion mutants, none of them were inhibiting *V. dahliae* growth inhibition halo, similarly to the phenotype displayed by the parental strain PICF7. During this screening process, one Tn5-Tc^R colony showed reduced growth on PDA (**Figure 2.1B**). This altered phenotype was not observed in KBA, LBA and nutrient agar media (**Figure 2.1**). This mutant was also selected for further characterization (see below).

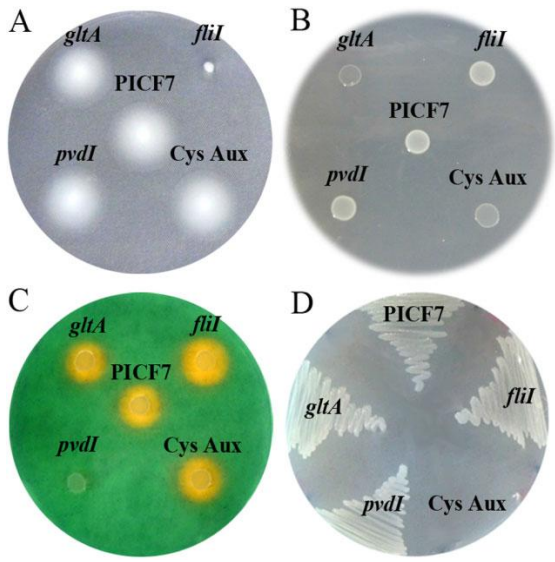


Figure 2.1. Phenotypes of *Pseudomonas fluorescens* PICF7 and selected Tn5-Tc^R insertion mutants in SM (**A**), PDA (**B**), CAS (**C**) and SSM (**D**) agar media. *gltA*, mutant ME419 showing *in vitro* growth delay in PDA; *fliI*, mutant ME424 impaired in swimming motility; *pvdI*, mutant ME589 impaired in pyoverdine (Pvd) production; and Cys Aux, mutant ME1508 (cysteine auxotroph).

A. Individual colonies from overnight cultures of strain PICF7 and mutant derivatives grown on KBA plates were inoculated using a toothpick onto SM and incubated overnight at 25°C. Strains PICF7, *gltA*, *pvdI* and Cys Aux showed swimming motility around the inoculation point but not the *fliI* mutant.

B. Five-microlitre drops of overnight cultures of each strain were deposited over PDA medium and incubated at 25°C for 4 days. Mutant *gltA* showed reduced growth compared with strain PICF7 and other mutants.

C. Five-microlitre drops of fresh cell suspensions of strain PICF7 and its mutants were deposited over CAS medium agar plates and incubated overnight at 25°C. All mutant derivatives but mutant *pvdI* produced similar orange haloes as that observed for wild-type strain PICF7.

D. Growth of PICF7 and its mutant derivatives on SSM after 48 h at 25°C.

2.4.3. Identification of genes disrupted in selected PICF7 mutants

Among the 55 pre-selected mutants, 44 were finally confirmed as affected in just one of the phenotypes under study. Localization of the Tn5-Tc^R insertion in these 44 mutants was performed by nested-PCR analysis. Nine mutants amplified a single band that was eluted, purified and sequenced. Based on the altered phenotype, the presence of a unique amplicon after nested-PCR, and the disrupted gene identified, four mutants (**Figure 2.1**) were finally selected for evaluation in subsequent olive root colonization and VWO biocontrol bioassays. Mutant ME424 has completely lost swimming motility (**Figure 2.1**), and transposon insertion was located within a putative *fliI* gene homologue, coding for a flagellum-specific ATP synthase (**Table**

2.2). Mutant ME589 was totally impaired in Pvd production (no halo on CAS medium and no green fluorescence in KBA/SSM media) (**Figure 2.1**) and harbored the Tn5 insertion in a putative *pvdI* gene homologue that codes for a Pvd non-ribosomal pep-

Table 2.2. Identification of genes disrupted in selected PICF7 mutants.

Mutant	Amplicon size	Mutant type	Accession number	Closest species/strain	Query cover	E-value	Identity %	Function
ME419	354 bp	Altered *in vitro* growth	AHF49667.1	*Pseudomonas* sp. RM12EL_44B	87%	3e-46	74	Type II citrate synthase
ME424	426 bp	Swimming motility	WP_010208746.1	*Pseudomonas* sp. R81	84%	2e-145	100	Flagellum-specific ATP synthase
ME589	185 bp	Pyoverdine production	1476433	*Pseudomonas fluorescens* A506	49%	2e-08	64	Putative pyoverdine non-ribosomal peptide synthetase
ME1508	420 bp	Cysteine auxotroph	WP_012723853.1	*Pseudomonas fluorescens*	99%	2e-95	100	Putative sulfite reductase

Disrupted genes identification was carried out by obtaining amplicons of the flanking Tn5-regions in PICF7 Tc^R-mutant derivatives by a combination of arbitrary and nested-PCR followed by sequence comparison in available databases using the BLASTX Program (see text for details).

tide synthetase (**Table 2.2**). Mutant ME419, which displayed growth delay in PDA medium (**Figure 2.1**), carries the Tn5-Tc^R insertion in a putative homologue of the *gltA* gene, potentially coding for a type II citrate synthase (**Table 2.2**). Finally, mutant ME1508 was randomly selected from auxotroph mutants obtained during the mutagenesis process (**Figure 2.1**). Sequence analysis of adjacent regions to the Tn5-Tc^R insertion in this mutant revealed that the transposon was located in a gene coding for a putative sulfite reductase involved in Cys biosynthesis (**Table 2.2**). Indeed, *in vitro* cross-feeding assays showed that addition of L-Cys to SSM medium (up to 20 mg l^{-1}) fully restored ME1508 growth and Pvd production ability in SSM to wild-type PICF7 levels (**Table 2.3**). Thus, in addition to *pvdI* (Pvd defective, ME589) and *fliI* (swimming motility defective, ME424) mutants, the *gltA* (reduced *in vitro* growth, ME419) and Cys auxotroph (ME1508) mutants were selected to be included in the *in planta* bioassays as examples of metabolism-altered phenotypes. Thus, *in vitro* growth delay, Cys auxotrophy, siderophore-mediated Fe3+ competition and

motility were evaluated as per their potential role in rhizosphere competence, olive root colonization and/or biocontrol ability of strain PICF7.

Table 2.3. Bacterial growth (OD_{600}) and pyoverdine production ($OD_{400/600}$) by strain PICF7 and its mutant derivative ME1508 in SSM supplemented with L-Cys.

L-Cys (ng/ml)	Bacterial Growth (OD_{600})[1,3]		Pyoverdine ($OD_{400/600}$)[2,3]	
	PICF7	ME1508	PICF7	ME1508
0	0.40 ± 0.03	0.06 ± 0.01*	3.0 ± 0.15	1.5 ± 0.32*
1	0.44 ± 0.11	0.09 ± 0.01*	2.9 ± 0.11	2.2 ± 0.27*
2	0.43 ± 0.14	0.15 ± 0.02*	2.7 ± 0.11	2.3 ± 0.19*
5	0.46 ± 0.05	0.34 ± 0.03*	2.8 ± 0.17	2.2 ± 0.09*
10	0.38 ± 0.04	0.42 ± 0.15	3.1 ± 0.22	2.5 ± 0.16*
20	0.41 ± 0.03	0.43 ± 0.04	2.9 ± 0.14	2.9 ± 0.20

[1] Bacterial growth (OD_{600}) 48 h after strain PICF7 and mutant ME1508 inoculation (5.7×10^8- 1.4×10^9 cfu/ml) in SSM (control) and SSM with increasing (1, 2, 5, 10 and 20) L-Cys concentration (final $OD_{600} \approx 0.1$).

[2] Production of pyoverdine calculated according to Djavaheri et al*.,* (2012).

[3] Data are means of three repetitions performed in three independent experiments. Means in a column followed by asterisk are significantly different according to Fisher´s protected LSD test ($P < 0.05$).

2.4.4. Olive root colonization ability of *P. fluorescens* PICF7 mutants

To determine whether Tc^R mutant derivatives ME424, ME589, ME419 and ME1508 colonize olive roots to the same extent as strain PICF7, three roots per treatment and per biocontrol assay (see below) were examined at the end of the experiments (approximately 110 DAB). In experiment I, population sizes of introduced bacteria were not significantly different ($P = 0.34$), although mutants always displayed lower population size values compared with strain PICF7 (**Table 2.4**). In bioassay II, however, a significantly ($P < 0.05$) lower population size was found for the Cys auxotroph mutant ME1508 compared with that exhibited by strain PICF7 but not with the other mutants (**Table 2.4**). Finally, in bioassay III, population sizes of *fliI* and Cys auxotroph mutants were significantly ($P < 0.05$) lower than that of strain PICF7 (**Table 2.4**).

Table 2.4. Root colonization ability and Verticillium wilt of olive biocontrol performance of *Pseudomonas fluorescens* PICF7 and its Tc^R mutant derivatives.

Experiment[1]	Treatment[2]	Disease assessment[3]			Bacterial population (log_{10} cfu g^{-1} of fresh root)[4]
		SAUDPC	DII	DI (%)	
I	Control	0.36[a]	0.59	93.33	
	P. fluorescens				
	PICF7	0.09[c]	0.24	78.57	4.3 ± 0.5[a]
	ME419 (*gltA*)	0.24[abc]	0.44	93.33	2.9 ± 0.8[a]
	ME424 (*flil*)	0.16[bc]	0.43	84.62	3.3 ± 0.9[a]
	ME589 (*pvdI*)	0.17[bc]	0.33	78.57	3.4 ± 0.7[a]
	ME1508 (Cys Aux)	0.34[ab]	0.51	86.67	3.1 ± 0.3[a]
II	Control	0.28[ab]	0.46	100	
	P. fluorescens				
	PICF7	0.24[b]	0.43	80	4.3 ± 0.2[a]
	ME419 (*gltA*)	0.20[b]	0.33	71.43	3.9 ± 0.6[ab]
	ME424 (*flil*)	0.29[ab]	0.38	84.62	3.5 ± 0.8[ab]
	ME589 (*pvdI*)	0.23[b]	0.35	76.92	3.9 ± 0.5[ab]
	ME1508 (Cys Aux)	0.47[a]	0.53	100	2.9 ± 0.4[b]
III	Control	0.32[a]	0.40	60	
	P. fluorescens				
	PICF7	0.05[b]	0.11	43.75	4.1 ± 0.6[a]
	ME419 (*gltA*)	0.15[ab]	0.31	66.67	3.5 ± 0.3[ab]
	ME424 (*flil*)	0.05[b]	0.13	53.33	3.3 ± 0.2[b]
	ME589 (*pvdI*) [5]	-	-	-	3.5 ± 0.1[ab]
	ME1508 (Cys Aux)	0.10[b]	0.23	71.43	2.6 ± 0.2[c]

[1]Three independent experiments were carried out spanning 110 days after inoculation (DAI) (experiment I), 118 DAI (experiment II) and 113 DAI (experiment III).

[2] The root system of three-month-old olive plants were dipped in bacterial suspensions (5.8×10^8 - 1.7×10^9 cfu/ml) for 15 min and then transplanted into autoclaved soil for colonization assay, or autoclaved soil artificially infested with 3.2×10^5 - 2.7×10^6 conidia $g^{-1}$of the defoliating *Verticillium dahliae* isolate V937I for biocontrol assays. Plants were grown in a growth chamber under controlled conditions (see text for details).

[3] *SAUDPC*, standardized area under the disease progress curve of *DII* (disease intensity index) plotted over time. *DI*, final disease incidence (percentage of affected plants at the end of the experiment). Means in a column followed by different letters are significantly different according to Fisher´s protected LSD test ($P < 0.05$).

[4] Cell counts of *Pseudomonas* strains were determined on modified King´s medium B agar (PICF7) or on modified KBA amended with Tc (Tc^R PICF7 mutant derivatives). Data are means of three root samples (1 g each). Means followed by different letters are significantly different according to Two-sided Dunnett's Multiple Comparisons with a Control (PICF7) at $\alpha = 0.05$.

[5] Plants bacterized with ME589 and transplanted to soil infested with *V. dahliae* V937I unexpectedly died. However, plants treated with ME589 alone (9) showed healthy and therefore were kept and used for viable bacteria counts.

2.4.5. Root endophytic colonization ability is not affected in *P. fluorescens* PICF7 mutants

To visualize olive roots surface/inner colonization GFP-labelled derivatives of PICF7 and the selected Tc^R mutants were generated. Transformation frequency of plasmid pLRM1 ranged from 4.7 x 10^2 to 7.6 x 10^3 transformants/µg plasmid DNA. Introduction of plasmid pLRM1 did not affect the behavior of the mutants, and all selected GFP-labelled clones showed similar phenotypes as their parents (**Figure 2.1; table 2.S2**). While plasmid pLRM1 revealed as not completely stable neither in PICF7 nor in their mutants (6.14% plasmid loss/generation), the presence of a GFP-labelled bacterial cells within olive root tissues for each strain was clear (15 DAB). Moreover, population sizes of introduced bacteria at 15 DAB reached similar values for all strains but for mutant ME589-G (*pvdI*), which colonized olive roots/rhizosphere at a significantly ($P < 0.05$) lesser extent (**Table 2.S1**). CLSM imagery obtained from root tissue samples during 15 DAB showed that PICF7, as well as all mutant strains evaluated, were able to endophytically colonize the root interior predominantly among the intercellular spaces of the root cortex. Remarkably, however, GFP-labelled cells could also be localized within the root vascular system for all strains but for the *fliI* mutant (**Figure 2.2**). Taking into account this finding, an experiment aimed to demonstrate possible active/passive movement of PICF7 cells form root xylem vessels to aerial tissues was carried out. When GFP-tagged PICF7 Gm^R cells were applied to the root system of 'Picual' plants (irrigated three times at days 0, 5 and 10 with a bacterial cells suspension), no tagged bacteria could be retrieved from aerial tissues, indicating that there was no transport to upper parts of the plants through the xylem vessels. In contrast, PICF7 successfully colonized the roots of the examined plants (average population sizes of $\log_{10}$ 5.2 cfu g^{-1} of fresh root tissue).

2.4.6. Biological control activity of strain PICF7 is determined neither by pyoverdine production nor swimming motility

Three independent experiments were carried out to assess biocontrol performance of PICF7 mutants. Results showed, overall, that swimming motility (impaired in mutant ME424) and Pvd production (abolished in mutant ME589) are not needed for the effective biocontrol exerted by strain PICF7 against *V. dahliae* (**Table 2.4**). However, differences were found among experiments. Thus, in bioassay I (spanning 110 DAI), PICF7-bacterized plants displayed a significant ($P < 0.05$) decrease in the *SAUDPC* in comparison to non-bacterized plants (control). Besides, a decrease in *DII* and the final *DI* values were observed (**Table 2.4**). Mutants ME424 and ME589

showed a similar behaviour than the parental strain, and *SAUDPC* was also significantly ($P < 0.05$) lower than in non-treated plants, although *DII* and *DI* values for ME424-treated and ME589-treated plants were higher than that for PICF7-bacterized plants (**Table 2.4**). In contrast, neither *P. fluorescens* PICF7 nor its *pvdI* and *fliI* mutant derivatives displayed biocontrol activity in bioassay II (118 DAI), likely due to a high disease pressure scored in this experiment (i.e. 100% final DI in control plants) (**Table 2.4**). Finally, in bioassay III (113 DAI), strain PICF7 and mutant ME424 controlled Verticillium wilt epidemics effectively. Indeed, *SAUDPC* decreased significantly ($P < 0.05$) in both treatments compared with non-bacterized plants (**Table 2.4**). The *DII* was also reduced in PICF7-treated and ME424-treated plants in comparison to that observed for control plants. Regarding the final *DI* values, no differences were found between PICF7 and ME424, and both treatments reduced the number of diseased plants at the end of the experiment in comparison to non-bacterized plants (**Table 2.4**). It must be mentioned that the effect of ME589 could not be evaluated in this bioassay because most of the *V. dahliae*-inoculated plants in this treatment unexpectedly died after manipulation. In summary, mutations in the putative *pvdI* and *fliI* genes did not seem to affect biocontrol activity in mutants ME589 and ME419 respectively.

2.4.7. Cys auxotrophy in strain PICF7 diminished its VWO biocontrol effectiveness

Besides mutants impaired in swimming motility and Pvd production, two additional mutants, one affected in growth on PDA plates (ME419) and another showing Cys auxotrophy (ME1508), were included in VWO biocontrol experiments. Results also varied among bioassays. Overall, the behavior of mutant ME419 did not significantly ($P > 0.05$) differ from that of PICF7 for any of the disease parameters analyzed in the three experiments (**Table 2.4**). Similarly, *SAUDPC*, *DII* and final *DI* from ME419-treated plants were never found to be different from that of non-bacterized plants (**Table 2.4**). Therefore, mutation in the putative *gltA* gene did not seem to affect biocontrol activity in mutant ME419, despite of the fact that growth of this mutant *in vitro* was evidently altered (**Figure 2.1**). On the other hand, ME1508 mutant (Cys auxotroph) showed a significantly ($P < 0.05$) higher *SAUDPC* than that scored for PICF7-treated plants in bioassays I and II. Likewise, *DII* (0.51 and 0.53) and final *DI* (86.7% and 100%) were higher in ME1508-treated plants than in PICF7-treated plants (**Table 2.4**). This indicated that Cys auxotrophy negatively influenced biocontrol performance in mutant ME1508. However, this mutant behaved similarly to PICF7, ME419 and ME424 in bioassay III, and no significant ($P = 0.29$) differences were

scored among treatments (**Table 2.4**). It is worth mentioning here that final *DI* in bioassay III was considerably lower in the control (non-bacterized; 60%) treatment than in bioassays I (93.3%) and II (100%).

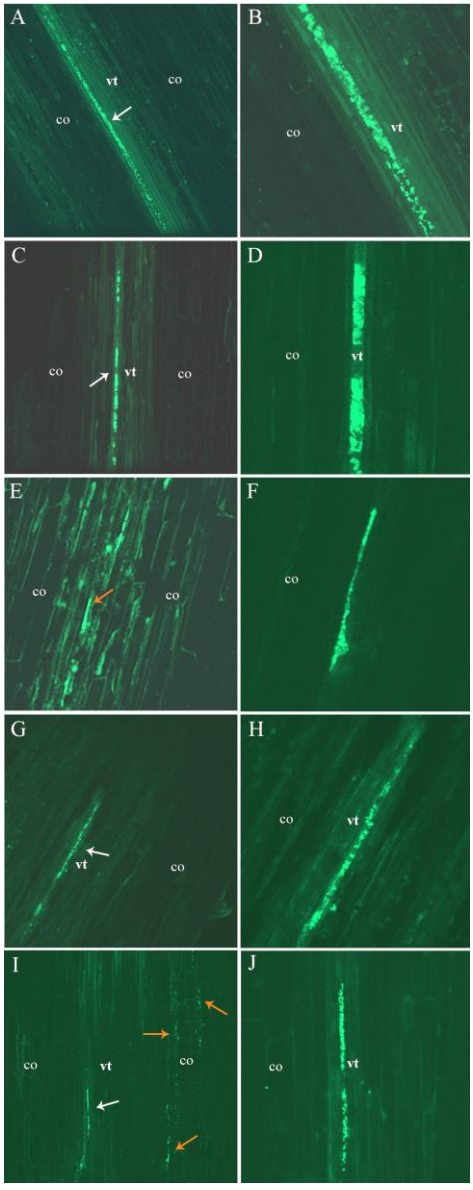


Figure 2.2. Confocal laser scanning microscopy images of longitudinal vibratome root sections (40 µm thick) showing localization of *Pseudomonas fluorescens* PICF7 and its Tn5-Tc^R insertion mutant derivatives GFP-labelled. Images were taken from one to two weeks after root-dip bacterization with *Pseudomonas*-GFP. Strain PICF7 (**A**,**B**), *gltA* (**C**,**D**), *fliI* (**E**,**F**), *pvdI* (**G**,**H**) and Cys auxotroph (**I**,**J**). Pictures show that all strains endophytically colonize olive roots: vascular tissue (white arrows), and intercellular spaces of the cortex (red arrows). Scale bar represents 100 µm in **A**, **C**, **E**, **G** and **I** (left panels), and 30 µm in **B**, **D**, **F**, **H** and **J** (right panels); co, cortical cells; vt, vascular tissue.

Discussion

Effective control of VWO is highly difficult by a number of reasons comprehensively reviewed elsewhere, and must thus rely on an integrated disease management

strategy with emphasis in pre-planting measures (López-Escudero and Mercado-Blanco, 2011). A promising preventive action is the use of native microbial antagonists able to efficiently colonize the target niche (i.e. soil, rhizosphere, roots, etc.). However, our knowledge on the mechanisms involved in suppression of *V. dahliae* by microbial antagonists is still very limited, not only for the particular case of VWO but also for many crops that can be infected by this pathogen (Alström, 2001; Tjamos et al., 2005; Berg et al., 2006; Antonopoulos et al., 2008; El Hadrami et al., 2011; Meschke et al., 2012).

One of the best BCA of VWO so far studied is the olive root endophyte *P. fluorescens* PICF7 (Mercado-Blanco et al., 2004; Prieto et al., 2009, 2011). Strain PICF7 is able to trigger a broad range of defence responses in both root (Schilirò et al., 2012) and aerial (Gómez-Lama Cabanás et al., 2014) tissues, pointing to a scenario in which VWO biocontrol by PICF7 could be mediated by induced resistance mechanism(s) (Pieterse et al., 2014). We have recently shown, however, that the presence of PICF7 cells in root tissues does not suppress olive knot disease in stems caused by *Pseudomonas savastanoi* pv. savastanoi (Maldonado-González et al., 2013). Nothing is currently known on which PICF7 traits could be involved in triggering such responses or whether additional biocontrol mechanisms (i.e. antibiosis, competition, etc.) might be effective against *V. dahliae*. Likewise, knowledge on PICF7 phenotypes involved in olive rhizosphere colonization and endophytic lifestyle is absent. Therefore, the objective of the present study was to examine whether selected traits play a role in root colonization and VWO biocontrol abilities exerted by strain PICF7. The approach followed was to generate mutants in specific phenotypes, and to assess their colonization and biocontrol performance *in planta* under non-gnotobiotic conditions. This means that introduced bacteria faced a situation closer to a natural environment (i.e. nursery-produced plants carrying a highly-diverse microbiome) than that found in axenic systems normally used in this type of studies. Besides, this work has been carried out using a woody plant relevant in Mediterranean agro-ecosystems instead of a model plant.

Successful biocontrol of soil-borne phytopathogens by any given BCA must be preceded by the efficient colonization of the target niche (i.e. rhizosphere soil, root surface, root interior) (Mercado-Blanco and Bakker, 2007). A number of *Pseudomonas* spp. traits involved in rhizosphere and/or root colonization have been studied (Lugtenberg et al., 2001), and some have shown as key elements for the subsequent biocontrol efficacy exerted by specific strains (Chin-A-Woeng et al.,

2000). Motility and chemotaxis are thus considered essential for root colonization by *P. fluorescens* strains (Lugtenberg and Bloemberg, 2004), although flagella-driven motility has been demonstrated to be necessary for root colonization in some cases (Capdevila et al., 2004; Martínez-Granero et al., 2006) but not in others (Howie et al., 1987). The involvement of swimming motility in colonization and biocontrol has been proven for some pseudomonads. Thus, a triple mutant strain (KSW) of *P. fluorescens* F113 affected in *kinB*, *sadB* and *wspR* showed increased swimming motility and rhizosphere colonization ability than that of the wild-type strain, as well as improved biocontrol activity against *Fusarium oxysporum* f.sp. *radicis lycopersici* in tomato (*Solanum lycopersicum* Mill.) and *Phytophthora cactorum* in wild strawberry (*Fragaria vesca* L.). (Barahona et al., 2011). Recently, Sang and Kim (2014) have also suggested that biocontrol activity of *Pseudomonas corrugata* CCR04 and CCR80 in pepper (*Capsicum* sp.) plants against the soil-borne oomycete *Phytophthora capsici* can be mediated by successful root colonization through biofilm formation and swimming and swarming motilities. Our results demonstrated that *P. fluorescens* PICF7 displays swimming motility. This trait seems to contribute to the full colonization ability of olive rhizosphere/roots by strain PICF7. Indeed, mutant ME424 always showed lower population sizes than that of the parental strain in all experiments, although it was significantly different only in bioassay III (**Table 2.4**). On the contrary, lack of swimming motility did not hinder mutant ME424 to colonize the root interior nor diminished its biocontrol efficiency against *V. dahliae* (see below).

Population sizes associated with roots scored for all mutants tested were always lower, either significantly (i.e. mutant ME1508 in bioassays II and III) or just showing a trend, compared with that observed for strain PICF7 (**Table 2.4**). However, this decline in population size over time observed for mutants ME424 (*fliI*) and ME589 (*pvdI*) did not affect their ability to control *V. dahliae*. Therefore, we conclude that neither Pvd production nor swimming motility is implicated in VWO suppression. Regarding ME419 (*gltA*), growth problems observed in PDA medium for this mutant did not affect root colonization ability nor biocontrol performance compared with PICF7. On the contrary, Cys auxotrophy significantly affected the root colonization ability of mutant ME1508, which overall displayed the lowest populations sizes at the end of the bioassays (>100 DAI). Moreover, mutant ME1508 had lost wild-type VWO biocontrol phenotype. Importance of amino acid synthesis has been earlier shown for the colonization of tomato roots by *P. fluorescens* strain WCS365 (Simons et al., 1997). Plant roots produce exudates composed of a broad range of low-molecular (i.e. amino acids, organic acids, sugars, phenolics, etc.) and high-molecular [i.e.

mucilage (polysaccharides), proteins, etc.] weight compounds (Bais et al., 2006). Soil microorganisms are chemically attracted by root exudates, which serve as an important source of nutrients, including amino acids. Among amino acids synthesized by roots, Cys and cystine (oxidation of two Cys molecules covalently linked via disulfide bond) have been detected in root exudates of several plant species (Gitte et al., 1978; Gaworzewska and Carlile, 1982; Dennis et al., 2010). A gene coding for a putative sulfite reductase, an enzyme related with Cys metabolism, has been identified in mutant ME1508. Moreover, this mutant was unable to grow in SSM in contrast to nutrient-rich media (i.e. LBA and KBA) where ME1508 grew normally. Amendment of L-Cys to SSM restored the ability of ME1508 to grow and produce Pvd. It is plausible to think that low availability of Cys in the olive rhizosphere makes it ME1508 less efficient in root/rhizosphere colonization, and consequently in VWO suppression effectiveness. Nevertheless, its ability to colonize inner root tissues remained unaffected, in spite of the fact that rhizosphere populations of ME1508 were overall significantly lower than that of the parental strain.

Mechanisms involved in the endophytic colonization of roots by bacteria, including beneficial *Pseudomonas* spp., are mostly unknown (Hardoim et al., 2008; Reinhold-Hurek and Hurek, 2011). Recent comparative genomics and bioinformatics approaches may shed light on the identification of specific traits linked to endophytism by beneficial bacteria (Mitter et al., 2013; Ali et al., 2014). So far, however, only a few bacterial characteristics have been shown, mostly by mutational studies, as truly implicated either on gaining entrance into the root interior, spreading to distant organs, or endurance within plant tissues (Reinhold-Hurek and Hurek, 2011). For instance, a *pilT* mutant of *Azoarcus* sp. BH72 unable of twitching motility was impaired in the endophytic colonization of rice (Böhm et al., 2007). Twitching and swarming motilities have not been demonstrated in strain PICF7 under tested conditions. While swimming motility has been shown for PICF7, our results indicate that this phenotype is not relevant for inner colonization of olive roots by strain PICF7. The same accounts for Pvd production, *in vitro* growth delay and Cys auxotrophy. Indeed, all PICF7 mutants tested in this study could be clearly observed colonizing the intercellular spaces of the root cortex. Interestingly enough, root tissue sectioning and CLSM imagery allowed to demonstrate that PICF7, ME419, ME589 and ME1508 were also able to colonize root xylem vessels, a location not detected in previous studies (Prieto and Mercado-Blanco, 2008; Prieto et al., 2009, 2011). However, no evidence of PICF7 movement from roots to above-ground organs using the lumen of the xylem vessels was obtained under experimental conditions

used here, corroborating previous results using *in vitro*-propagated olive plants and a root-dip inoculation protocol (Maldonado-González et al., 2013).

Pseudomonas spp. produce a large variety of siderophores to cope with iron-limiting conditions (Bultreys, 2007), Pvds being the prevalent class (Meyer, 2000). In addition, many plant beneficial *Pseudomonas* spp. strains produce additional, secondary siderophores (Buysens et al., 1996; Mercado-Blanco et al., 2001b; Loper et al., 2012). The involvement of *Pseudomonas*-produced siderophores in biological control is controversial. They may act through competition of Fe3+, thereby limiting its availability for pathogens. Besides, disease suppression mediated by siderophores has been shown to play an important role in some cases (Buysens et al., 1996; Audenaert et al., 2002), but minor (or no involvement at all) in others (Hamdan et al., 1991; Ongena et al., 1999; Djavaheri et al., 2012). Some *Pseudomonas*-produced siderophores have also been suggested to suppress plant diseases via induction of systemic resistance, although this issue is also controversial (Djavaheri et al., 2012; Bakker et al., 2014). *Pseudomonas fluorescens* PICF7 produces Pvd (Mercado-Blanco et al., 2004; this study), although production of additional, secondary siderophore(s) cannot be completely ruled out according to PICF7 genome data (Martínez-García et al., 2015). In fact, a large number of PICF7 mutants with altered phenotypes in iron-chelating ability have been generated in this study. However, we focused our attention on a mutant fully impaired in Pvd production (ME589), a phenotype corroborated by cultivation of this mutant in different growing media and by the identification of the gene disrupted in its genome (a putative *pvdI* homologue). Mutant ME589 showed a similar behaviour than PICF7 regarding root colonization ability, endophytism and biocontrol performance. It can, therefore, be concluded that Pvd production does affect neither VWO biocontrol effectiveness nor endophytic colonization by strain PICF7.

Finally, attention should be called here to the frequently observed biocontrol inconsistency/variability, a phenomenon amply referred in the literature (Lindow, 1988; Kraus and Loper, 1992). Biocontrol/colonization assessment carried out in this study showed variable results among bioassays (**Table 2.4**), a situation previously found when characterizing biocontrol strains from olive roots, including PICF7 (Mercado-Blanco et al., 2004). It is therefore compulsory, in our opinion, to present results from different independent biocontrol assays before to state sound conclusions regarding biocontrol performance of any claimed BCA. This is particularly true when the niche where the host plant, the pathogen, the introduced BCA and the

resident microbiota interact is the rhizosphere. This is a complex, highly dynamic scenario where a multiplicity of trophic interactions takes place, thereby influencing the fitness and performance of the BCA (Berg et al., 2006; Raaijmakers et al., 2009). In our case, this scenario poses the added experimental difficulties of dealing with a nursery-propagated woody host plant used under non-gnotobiotic conditions.

In summary, results presented here shed light, for the first time, on the actual involvement of specific *P. fluorescens* PICF7 phenotypes in olive root colonization and biocontrol against *V. dahliae*. Mutant analysis showed that swimming motility and Pvd production are not implicated in VWO suppression and endophytic behaviour displayed by PICF7. Cys auxotrophy compromised wild-type phenotypes such as VWO control and root colonization ability but did not hinder inner colonization of olive root tissues. The availability of a PICF7 mutant library will allow the screening of more phenotypes in the future, aiming to unravel the underlying mechanisms of PICF7 biocontrol and endophytism in a woody long-living plant such as olive.

Acknowledgements

This research was funded by Grant AGL2009-07275 from Spanish MICINN/MINECO and Grant P07-CVI-02624 from J. Andalucía (Spain), both co-financed by ERDF of the EU. Thanks are due to Prof. Antonio Martín for the use of the CLSM facilities and Mr. Antonio Valverde for his technical assistance.

Supplementary data

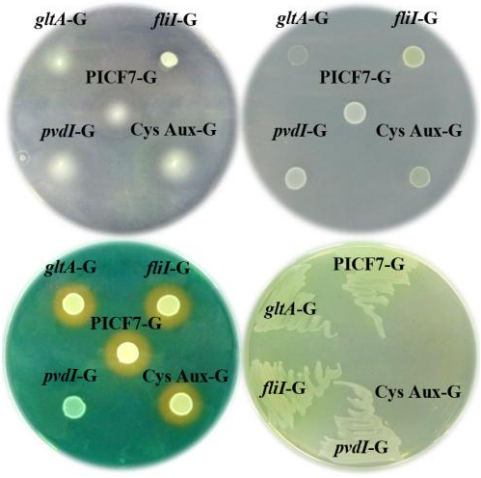


Figure 2. S1. Phenotypes displayed by *Pseudomonas fluorescens* PICF7 mutant derivatives carrying plasmid pLRM1 in SM (up left), PDA (up right), CAS (down left) and SSM (down right) agar media. As observed for strain PICF7 and its Tc^R mutant derivatives (results shown in Fig. 1), the GFP-labelled ME419 mutant (*gltA*-G) displayed growth delay in PDA. The GFP-labelled ME424 mutant (*fliI*-G) showed lack of swimming motility in SM. The GFP-labelled ME589 mutant (*pvdI*-G) was impaired in pyoverdine (Pvd) synthesis in CAS. Finally, the GFP-labelled ME1508 mutant (Cys Aux-G) displayed cysteine auxotrophy in SSM.

Table 2. S1. Root colonization ability of GFP-labeled (Gm^R) *Pseudomonas*.

Treatment[1]	Bacterial population (log_{10} cfu g^{-1} of fresh root)[2]
P. fluorescens	
PICF7-G	6.2 ± 0.1
ME419-G	6.0 ± 0.4
ME424-G	5.6 ± 0.4
ME589-G	4.5 ± 0.2*
ME1508-G	6.2 ± 0.5

[1] The root system of three-month-old olive plants were dipped in Gm^R mutant suspensions (1.4 - 2.5 x 10^9 cfu/ml) for 15 min and then transplanted into autoclaved soil. Plants were grown in a growth chamber under controlled conditions (see text for details).

[2] Cell counts of *Pseudomonas* mutants were determined on modified King´s medium B agar amended with Gm. Data are means of two root samples (1 g each). Means followed by asterisk are significantly different according to Student's *t*-test (α= 0.05)

Table 2. S2. Phenotypes of pre-selected *Pseudomonas fluorescens* PICF7 mutant derivatives.

Strain	Swimming motility[1]	Siderophore production[2]		
Code	SM (%)	CAS (%)	KB	SSM
PICF7	+	+	++	++
ME75	69*	+	++	++
ME78	+	+	++	+
ME81	+	+	+	++
ME93	-*	-*	-	Aux
ME95	-*	+	-	+
ME98	+	+	++	+
ME150	+	+	+	++
ME173	+	119*	++	++
ME177	+	+	+	+
ME180	+	+	+	++
ME195	+	+	-	++
ME210	+	+	+	+
ME302	-*	23*	-	Aux
ME326	+	+	+	++
ME419[3]	**+**	**+**	**++**	**++**
ME424	**-***	**+**	**++**	**++**
ME432	70*	+	+	+
ME433	+	83*	-	+
ME436	+	+	+	++
ME452	+	115*	+	++
ME458	+	116*	++	++
ME502	35*	+	++	++
ME503	38*	+	++	++
ME524	+	-*	-	-

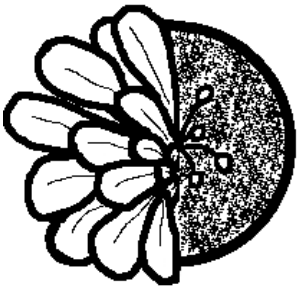
ME537	+	+	-	-
ME589	**+**	**-***	**-**	**-**
ME670	64*	+	++	+
ME720	+	+	+	+
ME859	+	+	+	++
ME886	+	-*	-	++
ME1045	+	+	+	++
ME1068	+	+	+	++
ME1144	+	78*	+	+
ME1157	+	+	+	++
ME1178	+	+	+	+
ME1186	+	-*	-	-
ME1255	70*	+	+	++
ME1321	+	83*	+	Aux
ME1355	61*	69*	+	+
ME1431	51*	+	++	++
ME1434	+	117*	++	++
ME1508	**+**	**+**	**++**	**Aux**
ME1578	+	-*	-	-
ME1586	+	-*	-	-
ME1622	64*	+	+	+
ME1688	+	-*	-	-
ME1690	+	+	++	+
ME1694	+	-*	-	-
ME1726	+	62*	+	+
ME1854	+	-*	-	-
ME1906	-*	+	++	Aux
ME1919	+	57*	+	+
ME1946	+	-*	-	-
ME1966	+	-*	-	-

[1] Swimming motility was assessed after overnight incubation (25°C) of each mutant along with the parent strain PICF7 in SM (swimming motility medium) per triplicate. Percentage of swimming motility was calculated comparing diameter of both mutant and strain PICF7 assigning the value of 100% to the parental strain. Means followed by asterisk are significantly different according to Two-sided Dunnett's Multiple Comparisons with a Control (PICF7) at $\alpha = 0.05$. +, motility similar to strain PICF7; -, no motility.

[2] Siderophore production of pre-selected mutants (55) was observed in different media such as CAS (Chrome azurol S), KBA (King´s B agar) and SSM (standard succinate medium) per triplicate (see text for details). In CAS medium iron-chelating compounds synthesis was established as production of orange haloes around siderophore-producing colonies ([halo diameter - colony diameter]/halo diameter) expressed in percentage when compare to wild type PICF7 (100%). In KBA and SSM media siderophore synthesis was revealed by fluorescence under UV irradiation. Relative haloes size data were subjected to analysis of variance (ANOVA) and means were compared to strain PICF7 mean using Two-sided Dunnett's Multiple Comparisons with a Control at $P < 0.05$. ++, wild type; +, reduced fluorescence; -, no fluorescence; Aux, auxotrophy.

[3] This mutant was selected according to its reduced growth on PDA (see main text for details).

CHAPTER 3



Arabidopsis thaliana as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7

This chapter has been published as:

Maldonado-González, M. M., Bakker, P. A., Prieto, P., and Mercado-Blanco, J. (2015). *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae* biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. *Frontiers in Microbiology*, *6,* 266. DOI: 10.3389/fmicb.2015. 00266.

3.1. Summary

The effective management of Verticillium wilts (VWs), diseases affecting many crops and caused by some species of the soil-borne fungus *Verticillium*, is problematic. The use of microbial antagonists to control these pathologies fits modern sustainable agriculture criteria. *Pseudomonas fluorescens* PICF7 is an endophytic bacterium isolated from olive roots with demonstrated ability to control VW of olive caused by the highly-virulent, defoliating (D) pathotype of *Verticillium dahliae* Kleb. However, the study of the PICF7-*V.dahliae*-olive tripartite interaction poses difficulties because of the inherent characteristics of woody, long-living plants. To overcome these problems we explored the use of the model plant *Arabidopsis thaliana*. Results obtained in this study showed that: (i) olive D and non-defoliating (ND) *V. dahliae* pathotypes produce differential disease severity in *A. thaliana* plants; (ii) strain PICF7 is able to colonize and persist in the *A. thaliana* rhizosphere but is not endophytic in *Arabidopsis*; and (iii) strain PICF7 controls (VW) in *Arabidopsis*. Additionally, as previously observed in olive, neither swimming motility nor siderophore production by PICF7 are required for VW control in *A. thaliana*, whilst cysteine auxotrophy decreased the effectiveness of PICF7. Moreover, when applied to the roots PICF7 controlled *Botrytis cinerea* infection in the leaves of *Arabidopsis*, suggesting that this strain is able to induce systemic resistance. *Arabidopsis thaliana* is therefore a suitable alternative to olive bioassays to unravel biocontrol traits involved in biological control of *V. dahliae* by *P. fluorescens* PICF7.

3.2. Introduction

Verticillium spp. are soil-borne, cosmopolitan ascomycete fungi producing vascular wilts and severe losses in many economically-relevant crops worldwide (Pegg and Brady, 2002; Fradin and Thomma, 2006; Inderbitzin et al., 2011). *Verticillium dahliae* Kleb. causes most of the known Verticillium wilts (VWs; Agrios, 1997; Jiang et al., 2005). It seriously compromises olive (*Olea europaea* L.) cultivation in many countries of the Mediterranean Basin, producing Verticillium wilt of olive (VWO). Effective control of this disease is difficult because of a number of contributing factors (Tsror, 2011). In fact, none of the currently-available measures are completely successful when applied individually. Therefore, the implementation of an integrated disease management strategy is proposed as the most effective way to control VWO, with emphasis on preventive (pre-planting) actions (López-Escudero and Mercado-Blanco, 2011). One of these measures is the use of antagonistic rhizobacteria as biological control agents (BCA), particularly in pathogen-free certified olive plants at the nursery-production stage (Tjamos, 1993). Beneficial rhizosphere *Pseudomonas* spp. strains have been extensively studied and used as BCA, exploiting a range of mechanisms including production of antibiotics, competition for nutrients and/or colonization sites, and induced systemic resistance (Weller et al., 2002; Haas and Défago, 2005; Mercado-Blanco and Bakker, 2007). Selected strains of *Pseudomonas* spp. have thus shown successful in suppressing VW in different susceptible hosts, including olive (Berg et al., 2006; Debode et al., 2007; Uppal et al., 2008; Erdogan and Benlioglu, 2010; Sanei and Razavi, 2011; Triki et al., 2012).

The olive root endophyte *P. fluorescens* PICF7 is an effective BCA of VWO caused by the defoliating (D, highly virulent) pathotype of *V. dahliae* in nursery-propagated olive plants (Mercado-Blanco et al., 2004; Prieto et al., 2009; Maldonado-González et al., 2015). Upon olive root colonization, strain PICF7 elicits a broad range of defense responses both locally (roots) (Schilirò et al., 2012) and systemically (aerial organs) (Gómez-Lama Cabanás et al., 2014). Results from these studies indicated that systemic defense responses, either SAR (Systemic Acquired Resistance) (Durrant and Dong, 2004) and/or ISR (Induced Systemic Resistance) (Bakker et al., 2007), can be triggered in olive tissues after inoculation with PICF7. SAR and ISR are phenotypically similar, leading to an enhanced resistance state of the plant. While salicylic acid (SA) plays a major role in SAR (Gaffney et al., 1993; Sticher et al., 1997), ISR works through jasmonic acid (JA) and ethylene (ET) signaling pathways in most cases (Pieterse et al., 2014), although there are exceptions (Audenaert et al., 2002). Remarkably, SA, JA

and ET pathways have undefined boundaries at some points and can show cross-talk between them (Kunkel and Brooks, 2002; Koornneef and Pieterse, 2008; Zamioudis and Pieterse, 2012). This situation has been observed in plant defense responses triggered by beneficial endophytic bacteria (Conn et al., 2008), including the interaction olive-*P. fluorescens* PICF7 (Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2014). From a practical perspective, simultaneous triggering of the SA and the ET/JA signaling pathways can lead to enhanced disease suppression thereby improving implementation of biological control (Van Wees et al., 2000).

To demonstrate ISR-mediated disease suppression the BCA and the pathogen need to be spatially separated throughout the experiment to rule out direct interaction between the microorganisms (Van Loon et al., 1998). Whether ISR is involved in biocontrol of *V. dahliae* by strain PICF7 in olive is difficult to assess since both microorganisms share the same ecological niche -the root system. One possibility would be the use of a split-root system, but this approach is complicated in olive. Another possibility is to evaluate PICF7 biocontrol performance against olive pathogens affecting above-ground organs (Maldonado-González et al., 2013). Here we use the model plant *Arabidopsis thaliana,* with a range of available mutants in defense signaling pathways, to unravel the involvement of induced resistance (Segarra et al., 2009). *A. thaliana* has previously been used to evaluate efficacy of BCAs (Meschke and Schrempf, 2010), including ISR-mediated biocontrol of *V. dahliae* (Tjamos et al., 2005).

Without excluding additional mechanisms (i.e. antibiosis, competition, etc.) induction of plant defense response seems to explain the biocontrol activity exerted by strain PICF7 (Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2014). However, little is known about PICF7 traits implicated in biocontrol efficacy. Mutant analysis have recently revealed that production of the siderophore pyoverdine (Pvd) and swimming motility are not required for control of VWO nor for endophytic colonization by PICF7 (Maldonado-González et al., 2015). Strain PICF7 exhibits good and prolonged surface and endophytic colonization abilities in different olive cultivars and under diverse experimental conditions (Mercado-Blanco et al., 2004; Prieto and Mercado-Blanco, 2008; Prieto et al., 2011). Moreover, colonization ability of strain PCF7 is not limited to olive root tissues since our previous studies have demonstrated that it can colonize and persist in olive stems (Maldonado-González et al., 2013) and even in the root system of sunflower (*Helianthus annuus* L.) (Maldonado-González et al., 2012). Inner and rhizoplane colonization of olive roots

by PICF7 seems to be crucial for VWO biocontrol efficacy of strain PICF7 (Prieto et al., 2009).

Verticillium dahliae isolates infecting olive have been classified into D and non-defoliating (ND, moderately virulent) pathotypes (López-Escudero and Mercado-Blanco, 2011), which correlates with their genetic and molecular differences (Mercado-Blanco et al., 2003b; Collado-Romero et al., 2006). Differential virulence displayed by isolates that infect olive was shown also in cotton (*Gossypium hirsutum* L.) (Schnathorst and Sibbett, 1971; Dervis et al., 2010). However, D-pathotype isolates do not behave as the most virulent group in artichoke (*Cynara scolymus* L.) (Jiménez-Díaz et al., 2006). Pathogenicity of *A. thaliana* by *V. dahliae* has been demonstrated earlier (Soesanto and Termorshuizen, 2001; Veronese et al., 2003; Tjamos et al., 2005; Zhao et al., 2014). However, there is no information on whether *V. dahliae* olive D and ND pathotypes induce the same differential virulence in *A. thaliana* plants than that observed in olive and cotton.

The main objective of this study was to assess whether the model plant *A. thaliana* can be used to identify *P. fluorescens* PICF7 traits involved in the control of *V. dahliae*. To achieve this, several sub-objectives were pursued: (i) to determine whether *V. dahliae* olive pathotypes (D and ND) cause differential virulence in *A. thaliana*; (ii) to assess whether *P. fluorescens* PICF7 colonizes and persists in the root system of different *A. thaliana* genotypes; (iii) to check whether strain PICF7 is able to endophytically colonize *A. thaliana* roots; (iv) to investigate whether strain PICF7 is able to control VW in different *A. thaliana* genotypes; (v) to determine whether specific PICF7 phenotypes behave in *A. thaliana* as previously observed in olive plants; and (vi) to find out if PICF7 is able to elicit an ISR response in *A. thaliana* using the leaf pathogen *Botrytis cinerea*.

3.3. Materials and methods

3.3.1. Bacterial strains, fungal isolates, growth conditions and inoculum production

Pseudomonas fluorescens PICF7 (Mercado-Blanco et al., 2004; Martínez-García et al., 2015), four Tn5-Tc^R (tetracycline-resistant) transposon insertion mutants (Maldonado-González et al., 2015), a PICF7 fluorescently-tagged derivative (Prieto and Mercado-Blanco, 2008) and a *P. fluorescens* WCS417 rifampicin-resistant spontaneous mutant (WCS417r) (Lamers et al., 1988) were used in this study (**Table**

3.1). Strain PICF7 mutant ME424 is impaired in swimming motility, mutant ME589 lacks siderophore Pvd production, mutant ME419 shows growth delay in potato dextrose agar (PDA) medium, and mutant ME1508 is a cysteine (Cys) auxotroph (Maldonado-González et al., 2015; **Table 3.1**). To determine strain PICF7's ability to colonize roots of *Arabidopsis*, a Tc^R enhanced green fluorescent protein (EGFP)-labeled derivative (harboring plasmid pMP4655) (Bloemberg et al., 2000; Prieto and Mercado-Blanco, 2008) was used in confocal laser scanning microscopy (CLSM) experiments (see below). To evaluate possible systemic defense responses strain WCS417r was used. All bacterial strains were grown at 28°C on King's medium B (King et al., 1954) agar (KBA) plates, when needed supplemented with antibiotics at the following concentrations (mg l^{-1}): tetracycline (Tc, 20); ampicillin (Amp, 50); chloramphenicol (Chl, 13); natamycin (Nat, 100) and rifampicin (Rf, 50).

Table 3.1. Bacterial strains and plasmids used.

Strains or plasmids	Characteristics	Reference or source
Bacterial strains		
P. fluorescens		
PICF7	Wild-type olive root endophyte	Mercado-Blanco et al., (2004)
ME419	PICF7 Tn5 (Tc^R) *in vitro* growth mutant derivative, $GltA^-$	Maldonado-González et al., (2015)
ME424	PICF7 Tn5 (Tc^R) motility mutant derivative, $FliI^-$	Maldonado-González et al., (2015)
ME589	PICF7 Tn5 (Tc^R) siderophore mutant derivative, $PvdI^-$	Maldonado-González et al., (2015)
ME1508	PICF7 Tn5 (Tc^R) auxotroph Cys mutant derivative	Maldonado-González et al., (2015)
PICF7 (pMP4655)	PICF7 (Tc^R) EGFP-labeled derivative	Prieto and Mercado-Blanco, (2008)
WCS417r	Spontaneous rifampicin mutant of strain WCS417	Lamers et al., (1988)
Plasmids		
pMP4655	oriBBR1, oriVS1, oriT (p15A), *lac::eGPF*, Tc^R	(Bloemberg et al., 2000)

GltA, type II citrate synthase; FliI, flagellum-specific ATP synthase; PvdI, putative pyoverdine non-ribosomal peptide synthetase; Tc, tetracycline; Cys, cysteine; EGFP, Enhanced Green Fluorescence Protein.

Pseudomonas inoculum was prepared as described by Maldonado-González et al., (2013). Bacterial cell densities required for each experiment were adjusted spectrophotometrically (A600 nm) by building up standard curves and culturing viable cells from serial dilution series onto KBA plates (to count PICF7 wild type colonies), or KBA plates supplemented with Tc or Rf (for Tn5 mutant and EGFP-labeled PICF7 derivatives and WCS417r, respectively).

Four isolates of *V. dahliae*, three representative of the D pathotype (V150I and V937I isolated from olive and V138I originated from cotton, all belonging to the vegetative compatibility group [VCG] 1A) and one of the ND pathotype (V789I, belonging to VCG4B and isolated from olive) (Collado-Romero et al., 2006), were used in this study. These isolates are deposited in the culture collection of the Department of Crop Protection, Institute for Sustainable Agriculture (CSIC), Córdoba, Spain. Inocula of *V. dahliae* isolates were prepared as described by Mercado-Blanco and co-workers (2004).

The necrotrophic fungus *B. cinerea* was used to carry out ISR assays. A conidial suspension (100 µl) of the pathogen (stored at -80°C) was inoculated on half-strength PDA plate and grown at 22°C for one month at 9.5-h photoperiod (100 µmol m^{-2} s^{-1}). Then, 5-10 ml of half-strength PDB was added to the plates, and conidia were released from the mycelium by scraping with a sterile glass rod. The conidial suspension was filtered through sterile glass wool and the density was adjusted with sterile half-strength PDB.

3.3.2. Plant material and plant growth conditions

Several genotypes of *A. thaliana* were used: wild-type Col-0 and its derivatives *ein2* (ET insensitive2, affected in the protein EIN2, central component in the ET signal transduction pathway and first positive regulator in the route) (Guzmán and Ecker, 1990)*, jar1* (affected in jasmonyl isoleucine conjugate synthase 1, enzyme essential in the production of JA) (Pieterse et al., 1998), *myb72* (affected in R2R3-MYB-like transcription factor protein, unable to elicit ISR response) (Van der Ent et al., 2008), *sid1* (defective in a member of the MATE [multidrug and toxic compound extrusion transporter] family, required for SA accumulation, no SAR response) (Serrano et al., 2013), and *sid2* (isochorismate synthase mutant, unable to elicit SAR response) (Nawrath and Métraux, 1999). Seeds were carefully distributed over wet river sand supplemented with half-strength Hoagland nutrient solution contained in a small tray. This setup, conveniently moist, was placed within a covered tray. After 2 or 3 weeks in a growth chamber at 21 ± 1°C, 100% relative humidity and 8-h photoperiod (200 µmol m^{-2} s^{-1}), seedlings were used for colonization/biocontrol assays or pathogenicity tests. After transplanting, plant growth conditions for all experiments were 21°C ± 1°C, 70 % relative humidity and 8-h photoperiod of fluorescent light as indicated above.

3.3.3. Verticillium wilt development in *Arabidopsis thaliana*: pathogenicity tests

To determine whether olive- and cotton-infecting *V. dahliae* isolates produce disease symptoms in *A. thaliana* plants, isolates V138I, V150I, V937I and V789I (see above), were tested in accession Col-0 and its mutant derivatives *ein2*, *jar1* and *sid1* (see above). Three-week-old *Arabidopsis* plants (20) of each genotype were inoculated by dipping their root system in a conidial suspension (7.5×10^5 - 2.9×10^6 conidia ml^{-1}) of each *V. dahliae* isolate or in distilled sterile water (control treatment). *Arabidopsis* seedlings were then gently transplanted to soil (potting soil:river sand, 12:5) previously autoclaved twice for 20 min with a 24-h interval. Plants were grown in a growth chamber under controlled conditions described above. Disease incidence was scored as the percentage of diseased leaves of the total number of leaves infection according to the following scale: 0, no symptom; 1, 1-33%; 2, 34-66%; 3, 67-100%; and 4, dead plant. Disease score was performed twice a week after pathogen inoculation during the first month and, onwards, every 7 days (14, 18, 21, 25, 32 and 39 days after inoculation [DAI]).

Data were submitted to analysis of variance (ANOVA). Disease severity data were used to calculate: (i) a disease intensity index (*DII*) defined as $DII = (\Sigma Si \times Ni)/(4 \times Nt)$, where *Si* is severity of symptoms, *Ni* is the number of plants with *Si* symptoms severity, and *Nt* the total number of plants; (ii) the final disease incidence (final *DI*) determined as the percentage of affected plants; and (iii) the standardized area under the disease progress curve of *DII* plotted over time (days) (*SAUDPC*) (Campbell and Madden, 1990). ANOVA was calculated by means of Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared using Fisher's protected least significant difference (LSD) test at $\alpha = 0.05$.

3.3.4. Colonization of *Arabidopsis thaliana* rhizosphere by *Pseudomonas fluorescens* PICF7 and its mutant derivatives

To demonstrate whether strain PICF7 is capable to colonize and persist in the rhizosphere/roots of *A. thaliana* plants, three *Arabidopsis* genotypes where used (Col-0 and its mutant derivatives *myb*72 and *sid*2). Colonization ability of PICF7 mutants ME419, ME424, ME589 and ME1508 (**Table 3.1**) was also evaluated in Col-0 plants. Prior to transplantation of 2-week-old *Arabidopsis* seedlings, the soil (potting soil:river sand, 12:5) was bacterized with a cells suspension of PICF7 or each mutant derivative (8.0×10^7- 2.8×10^8 cfu [colony-forming units] g^{-1} soil) as described by

Djavaheri et al., (2012). Plants were kept in a growth chamber (conditions describe above) for 3-4 additional weeks. Then, three root systems per bacterial treatment were harvested and shaken for 1 minute in 5 ml of 10 mM $MgSO_4 \cdot 7H_2O$ containing 0.5 g of glass beads (Pieterse et al., 1996). For bacteria counts, 10 µl-drops from serial dilutions of root macerates were deposited onto the surface of KBA plates (two per dilution) supplemented with Amp, Chl and Nat for PICF7, plus Tc in the case of Tc^R mutant derivatives. Bacterial colonies were scored after incubation at 28°C during 24 h. This experiment was performed three times. Data were subjected to ANOVA and means were compared to colonization of strain PICF7 in Col-0 plants using Two-sided Dunnett's Multiple Comparisons with a Control at $\alpha = 0.05$.

3.3.5. Verticillium wilt of *Arabidopsis thaliana* biocontrol experiments

Bioassays were carried out to evaluate the ability of *P. fluorescens* PICF7 to control *V. dahliae* in *A. thaliana*. Likewise, the biocontrol performance of selected PICF7 mutants (ME419, ME424, ME589 and ME1508) was also tested. To assess whether *P. fluorescens* PICF7 and its mutants exerted biocontrol against *V. dahliae*, *A. thaliana* Col-0 and its mutants *myb72* (no ISR response) and *sid2* (no SAR response) were used. Two-week-old seedlings were transplanted to *Pseudomonas*-bacterized soil (potting soil:river sand, 12:5) (8.0×10^7- 2.8×10^8cfu g^{-1} soil) or 10 mM $MgSO_4 \cdot 7H_2O$ (control). Seedlings were grown for 1 week under controlled conditions as previously mentioned. After that, plants (20-25) were uprooted, rinsed with tap water and their root systems dipped in a conidial suspension (3.9×10^5- 4×10^6 conidia ml^{-1}) of the olive D isolate *V. dahliae* V937I. Control plants (10) were immersed in sterile distilled water.

Disease symptoms (chlorosis, wilting) were scored along the experiments twice a week according to the scale ranged from 0 to 4 previously described, and *SAUDPC*, *DII* and final *DI* were calculated (see above). Biocontrol bioassays were repeated three (for PICF7 evaluation) or two (for mutant derivatives assessment) times. *SAUDPC* data were subjected to ANOVA and means were compared using Fisher's protected LSD test at $\alpha = 0.05$.

3.3.6. *Botrytis cinerea* ISR bioassay

To assess whether *P. fluorescens* PICF7 was able to elicit ISR in *A. thaliana* the foliar pathogen *B. cinerea* was used. Two independent bioassays were carried out with *A. thaliana* Col-0 plants. Bioassays were accomplished using 7-week-old *Arabidopsis*

plants (20) previously grown either in control soil or in *P. fluorescens* PICF7- or WCS417r- (positive control) treated soil (9 x 10^7- 2 x 10^8 cfu g^{-1} soil) for 5 weeks. Then, six to eight well-developed leaves were inoculated by applying 5 µl droplets of a conidial suspension of *B. cinerea* (1.7 - 7.5 x 10^5 conidia ml^{-1} half strength PDB) (Djavaheri et al., 2007). Plants were then kept at 100% relative humidity for 2 to 4 days and disease symptoms scored according to the following scale: 0, no symptoms; 1, small non-spreading lesion; 2, small non-spreading lesion with chlorosis; 3, spreading lesion with chlorosis; 4, spreading lesion and leaf completely chlorotic or dead. Severity data were used to calculate percentage of disease leaves per plant and then subjected to ANOVA. Data means were compared using Fisher's protected LSD test at $\alpha = 0.05$.

3.3.7. Confocal laser scanning microscopy

In order to assess the colonization ability of *P. fluorescens* PICF7 in *A. thaliana* roots, experiments using *in vitro*- and pot-grown *Arabidopsis* plants (Col-0, *myb72* and *sid2*) were conducted. The experiment with *in vitro*-propagated plants was performed using seeds of each *A. thaliana* genotype dipped in 500 µl of an EGFP-tagged PICF7 derivative (**Table 3.1**) bacterial suspension (1.3 x 10^9 cfu ml^{-1}), contained in microfuge tubes, and incubated at 25°C, 400 rpm for 4 h. Then, bacterized seeds (20) of each genotype were placed separately in two different lines (10 per line) on the surface of a square water-agar plate (12 cm x 12 cm). All plates were kept in a growth chamber at 23 ± 2°C in the dark. In experiment with plants grown in pots, sterile mixed soil (potting soil:river sand, 12:5) supplemented with half-strength Hoagland solution (70 ml Kg^{-1}) was inoculated with the EGFP-tagged PICF7 derivative (8.4 x 10^8 cfu ml^{-1}) and placed into pots. Then, six 2-week-old plants per genotype were placed individually per pot and incubated in a growth chamber at 23 ± 2°C with a 8-h photoperiod of fluorescent light (65 µmol m^{-2} s^{-1}), 100% relative humidity.

To visualize EGFP-tagged PICF7 cells, two plants per genotype were removed from the corresponding substrates (water agar or mixed soil) and the aerial part excised. In the case of seedlings explanted from pots, roots were carefully rinsed with water to eliminate soil particles. Then, fresh and intact roots were visualized under Axioskop 2 MOT microscope (Carl Zeiss, Jena GmbH, Germany) set with a krypton and an argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss) at time points 12, 15, 18, 29 days after bacterization (DAB) for the *in vitro* assay and 25 DAB (final time) for the *in planta* experiment. CLSM captures

were transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss). Processing of images was carried out by AdobePhotoshop CS version 8.0.1 software (Adobe Systems, San Jose, CA, USA).

3.4. Results

3.4.1. *Verticillium dahliae* olive D and ND pathotypes are differentially virulent on *Arabidopsis thaliana*

Pathogenicity test carried out in *A. thaliana* Col-0 and its mutant derivatives *ein2* and *jar1*, insensitive to ET and JA, respectively, and *sid1* impaired in SA biosynthesis showed that all *V. dahliae* isolates tested produced VW symptoms in all *A. thaliana* genotypes. Interestingly enough, differences in symptoms appearance and severity (chlorosis, wilting and growth delay; **Figure 3.1**) were found depending on the infecting pathotype. Overall, disease symptoms developed earlier in plants inoculated with D isolates (V138I, V150I and V937I) compared to ND-inoculated plants (isolate V789I) (first symptoms observed at 7 and 14 DAI, respectively). Thus, severe to moderate disease symptoms were observed in all *A. thaliana* genotypes when inoculated with D isolates (*SAUPDC* values ranged from 0.43 to 0.86; *DII* 0.55 to 0.96; Final *DII* 85-100%; **Table 3.2**). In contrast, plants inoculated with isolate V789I

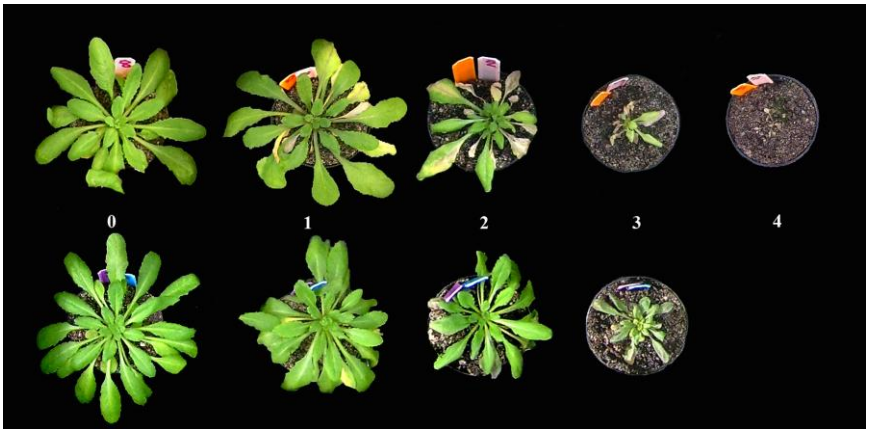


Figure 3.1. Scale of symptoms (chlorosis, wilting) produced by the defoliating isolate V937I (**A**) and the non-defoliating isolate V789I (**B**) of *Verticillium dahliae* in *Arabidopsis thaliana* Col-0 plants. Numbers represent the percentage of diseased leaves of the total number of leaves: 0, no symptom; 1, 1-33%; 2, 34-66%; 3, 67-100%; and 4, dead plant. Severity of symptoms produced by isolate V937I (0-4; **A**) was always higher than that observed for V789I-inoculated plants (0-3; **B**). These symptoms were observed in all *A. thaliana* genotypes analyzed in this study (see text for details).

Table 3.2. Pathogenicity test of selected isolates of olive defoliating (D) and non-defoliating (ND) *Verticillium dahliae* pathotypes carried out in different *Arabidopsis thaliana* accessions.

A. thaliana **genotype**	***V. dahliae*** **isolate**	**Disease assessment**		
		SAUDPC	***DII***	**Final *DI* (%)**
Col-0	V138I (D)	0.80[a]	0.95	100
	V150I (D)	0.86[a]	0.95	100
	V937I (D)	0.53[cd]	0.70	100
	V789I (ND)	0.48[de]	0.39	95
ein2	V138I (D)	0.80[a]	0.94	100
	V150I (D)	0.83[a]	0.95	100
	V937I (D)	0.75[ab]	0.87	100
	V789I (ND)	0.36[ef]	0.24	80
jar1	V138I (D)	0.74[ab]	0.89	95
	V150I (D)	0.65[bc]	0.74	95
	V937I (D)	0.43[d]	0.55	85
	V789I (ND)	0.23[f]	0.19	50
sid1	V138I (D)	0.85[a]	0.96	100
	V150I (D)	0.74[ab]	0.93	100
	V937I (D)	0.49[d]	0.65	85
	V789I (ND)	0.44[d]	0.41	85

Standardized area under the disease progress curve (*SAUDPC*) of a *DII* (disease intensity index) plotted over time and final disease incidence (*DI*, percentage of affected plants) were calculated at the end of the experiment (38 days after pathogen inoculation). Means in a column followed by different letters are significantly different in accordance to Fisher's protected LSD test ($P < 0.05$). See Section "Materials and Methods" for details.

(ND) always showed lower disease parameters (i.e. *SAUPDC* values varied from 0.23 to 0.48; *DII* 0.19 to 0.41; Final *DII* 50-95%) than D isolates (**Table 3.2**). Disease

symptoms produced by *V. dahliae* V937I (D) were intermediate and, for instance, *SAUDPC* data were not significantly different from that of V789I (ND) in both Col-0 and *sid1* plants (**Table 3.2**). Disease severity caused by *V. dahliae* isolates was also different depending on the *A. thaliana* genotype tested. Thus, disease symptoms (*SAUDPC*) produced by isolate V150I (D) were significantly ($P < 0.05$) less severe in *jar1* than in Col-0 and *ein2* plants (**Table 3.2**); isolate V937I (D) induced significantly ($P < 0.05$) higher disease severity in *ein2* plants (*SAUDPC* 0.75) than in the other tested genotypes; or isolate V789I (ND) was significantly ($P < 0.05$) less virulent in *jar1* in comparison to Col-0 and *sid2* plants (**Table 3.2**). Overall, V138I and V150I behaved as the most virulent isolates in all *A. thaliana* genotypes tested (**Table 3.2**).

3.4.2. *Pseudomonas fluorescens* PICF7 colonizes and persists on *Arabidopsis thaliana* roots but is not endophytic

Strain PICF7 was able to colonize and persist on roots of Col-0, *myb72* and *sid2* plants as bacterial counts shown after 32-40 DAB in three experiments carried out. PICF7 population sizes observed (**Table 3.3**) were not significantly different ($P = 0.10$; $P = 0.64$ and $P = 0.95$, respectively) among *A. thaliana* genotypes analyzed. Population sizes of native rhizobacteria found in control treatment plants were always significantly ($P < 0.05$) lower than PICF7 population sizes found in PICF7-bacterized plants but for *sid2* plants in experiment I. Native bacteria seemed to be displaced by introduced PICF7 cells since population sizes of the former in PICF7-treated plants were negligible and/or impossible to determine (**Table 3.3**).

In order to assess the ability of strain PICF7 to endophytically colonize *A. thaliana* plants, roots of plants from different genotypes (Col-0, *myb72* and *sid2*), bacterized with an EGFP-labeled PICF7 derivative, and grown either on water agar or in soil (pots) conditions were analyze by CLSM. Root samples were visualized by CLSM at 12, 15, 18 and 29 DAB (water agar) or at 25 DAB (soil). Under these experimental conditions, evidence of endophytic colonization of root tissues was not found for any of the examined *A. thaliana* genotypes, nor at any observation time. However, the rhizoplane of bacterized plants was profusely colonized by PICF7 cells (**Figure 3.2A,B**). In contrast, PICF7 is able to colonize the intercellular spaces of the olive root cortex (Prieto and Mercado-Blanco, 2008) as shown for comparative purpose in **Figure 3.2C**.

Table 3.3. *Pseudomonas fluorescens* PICF7 root colonization ability and biocontrol performance of *V. dahliae* in different *A. thaliana* accessions.

Experiment[1]	*A. thaliana* genotype[2]	Treatment	Disease assessment[3]			Bacterial population (log_{10} cfu g^{-1} of fresh root)[4]	
			SAUDPC	*DII*	Final *DI* (%)	PICF7	Native
1	Col-0	Control	0.56^{a}	0.75	94.44	na	5.0 ± 0.1^{d}
		P. fluorescens PICF7	0.25^{b}	0.51	76.47	6.6 ± 0.1^{ab}	-
	*myb*72	Control	0.49^{a}	0.74	77.78	na	5.7 ± 0.3^{c}
		P. fluorescens PICF7	0.18^{bc}	0.33	44.44	6.3 ± 0.3^{b}	-
	*sid*2	Control	0.24^{b}	0.35	38.89	na	6.5 ± 0.4^{ab}
		P. fluorescens PICF7	0.02^{c}	0.03	5.88	6.9 ± 0.3^{a}	-
2	Col-0	Control	0.39^{abc}	0.55	80.00	na	3.7 ± 0.0^{b}
		P. fluorescens PICF7	0.33^{bc}	0.38	79.17	5.6 ± 0.2^{a}	-
	*myb*72	Control	0.54^{a}	0.74	92.00	na	2.8 ± 0.8^{c}
		P. fluorescens PICF7	0.43^{ab}	0.56	72.00	5.3 ± 0.4^{a}	-
	*sid*2	Control	0.27^{c}	0.37	68.00	na	2.9 ± 0.2^{bc}
		P. fluorescens PICF7	0.33^{bc}	0.47	72.00	5.5 ± 0.2^{a}	-
3	Col-0	Control	0.17^{b}	0.27	58.82	na	4.3 ± 0.1^{c}
		P. fluorescens PICF7	0.14^{b}	0.27	72.22	7.2 ± 0.4^{a}	-
	*myb*72	Control	0.14^{b}	0.29	55.00	na	5.9 ± 0.5^{b}
		P. fluorescens PICF7	0.12^{b}	0.27	60.00	7.0 ± 0.4^{a}	-
	*sid*2	Control	0.37^{a}	0.64	77.78	na	5.6 ± 0.4^{b}
		P. fluorescens PICF7	0.22^{bc}	0.43	63.16	7.0 ± 0.1^{a}	-
4	Col-0	Control	0.12^{b}	0.22	47.37	na	nd
		P. fluorescens PICF7	0.12^{b}	0.19	23.81	nd	nd
	*myb*72	Control	0.27^{ab}	0.35	47.37	na	nd
		P. fluorescens PICF7	0.14^{b}	0.23	40.00	nd	nd
	*sid*2	Control	0.39^{a}	0.55	70.00	na	nd
		P. fluorescens PICF7	0.15^{b}	0.27	42.11	nd	nd

[1]Four independent experiments were conducted spanning 32 DAI (Experiment 1), 40 DAI (Experiments 2 and 3) or 37 DAI (Experiment 4).

[2] The root system of 2-week-old *Arabidopsis* seedlings were transplanted into PICF7-bacterized soil (8.0×10^{7} - 2.8×10^{8} cfu g^{-1} soil) previously autoclaved for colonization assays. In biocontrol experiments, 1 week after PICF7-treatment plants were uprooted and their root systems dipped in a *V. dahliae* isolate V937I (D pathotype) conidia suspension (3.9×10^{5}- 4×10^{6} conidia ml^{-1}) or distilled sterile water (control treatment) for 15 min. Plants were then grown in a growth chamber under controlled conditions. See Material and Methods for details.

[3]Standardized area under the disease progress curve of *DII* plotted over time. Final *DI*, final disease incidence (percentage of affected plants at the end of the assay). Means in a column followed by different letters are significantly different in accordance to Fisher's protected LSD test ($P < 0.05$).

[4] Strain PICF7 and native bacteria cells counts were carried out on modified King's medium B agar. Data are means of three root samples (1 g each). Means followed by different letters are significantly

different in accordance to Two-sided Dunnett's Multiple Comparisons with a Control (Col-0 plants) at α = 0.05. na, not applicable; nd, not determined in this experiment; -, native population not detected or negligible in PICF7-bacterized plants.

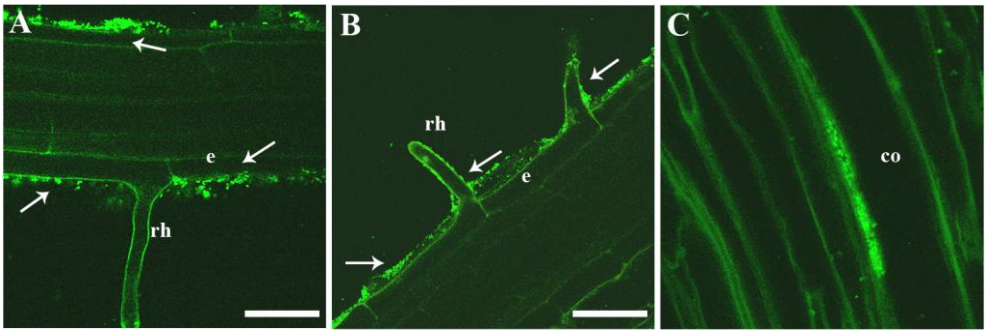


Figure 3.2. Confocal laser scanning microscopy (CLSM) images of intact roots from two *A. thaliana* genotypes [Col-0 (**A**) and *myb72* (**B**)] seedlings showing *Pseudomonas fluorescens* PICF7 (EGFP-labeled) cells location. Microphotographs show that PICF7 successfully colonizes the root surface of Arabidopsis (white arrows) but not the root interior. For comparison purposes, endophytic colonization of the root cortex of olive by PICF7 is also shown in (**C**), (see Prieto and Mercado-Blanco [2008] for technical details). CLSM images were taken approximately two weeks after seed (**A,B**) or root-dip (**C**) bacterization with strain PICF7. Scale bar represents 50 µm in (**A,B**); and 15 µm in (**C**); co, cortical cells; e, epidermis; rh, root hair; vt, vascular tissue.

3.4.3. *Pseudomonas fluorescens* PICF7 decreases Verticillium wilt symptoms in *Arabidopsis thaliana*

To determine whether strain PICF7 is able to control *V. dahliae* in *Arabidopsis* bioassays were conducted in which both the pathogen (isolate V937I, D pathotype) and the BCA were root inoculated. Results from four independent experiments indicated that strain PICF7 showed a trend to suppress the disease, although results varied among bioassays (**Table 3.3**). Thus, PICF7 was able to significantly ($P < 0.05$) suppress the disease in all *A. thaliana* genotypes (Col-0, *myb72*, and *sid2*) assessed in Experiment 1 (**Table 3.3**). VW control was more consistently observed in *sid2* plants (Experiments 1, 3 and 4) as revealed by *SAUDPC* values significantly ($P < 0.05$) lower in PICF7-bacterized plants compared to non-treated plants. Similarly, *DII* and final *DI* values were lower in these bioassays, but not in Experiment 3 (**Table 3.3**). Overall, disease parameters observed in experiments were lower in PICF7-treated plants compared to that in non-bacterized (control) plants, although differences were not statistically significant except for the cases mentioned above (**Table 3.3**). For instance, presence of PICF7 in *myb72* roots/rhizosphere produced a substantial decrease in all disease parameters analyzed in Experiments 2 and 4 (**Table 3.3**).

3.4.4. Behavior of *Pseudomonas fluorescens* PICF7 mutants in *Arabidopsis thaliana*

To assess whether *A. thaliana* can be used in the evaluation of PICF7 traits potentially involved in the biocontrol of *V. dahliae*, selected PICF7 mutants impaired in swimming motility or Pvd production, altered growth on PDA medium, or displaying Cys auxotrophy were used in three independent bioassays. Overall, results showed that neither swimming motility nor Pvd production are implicated in the effective biocontrol of VW in *Arabidopsis* by strain PICF7, but mutations affecting its growth in PDA (mutant ME419) or Cys auxotrophy (mutant ME1508; **Table 3.4**). Differences in biocontrol performance were found among experiments, though. For instance, ME1508-bacterized (Experiment 1) and ME419-treated (Experiment 2) plants showed a significant ($P < 0.05$) increase in *SAUDPC* compared to plants pre-treated with the parental strain PICF7 (**Table 3.4**). Furthermore, all PICF7 mutants displayed higher *DII* values than that scored for strain PICF7 in bioassays I and II (**Table 3.4**). Results from bioassay III did not show any significant difference among treatments. However, higher final *DI* percentages were observed for all mutant treatments compared to PICF7-treated plants (**Table 3.4**). Low disease pressure in this bioassay compared to that scored in the other two experiments could explain lack of significant differences. Finally, all bacterial strains colonized and persisted on roots of Col-0 plants. Nevertheless, some differences were found among experiments. Thus, mutant population sizes of all strains were not significantly different at the end of Experiments 1 and 2. Nonetheless, PICF7 showed a significantly ($P < 0.05$) larger population size in Experiment 3. Interestingly, mutant ME1508 (Cys auxotrophy) showed the lowest population size in all experiments (**Table 3.4**).

3.4.5. *Pseudomonas fluorescens* PICF7 elicits systemic defense responses against *Botrytis cinerea* in *Arabidopsis thaliana*

To determine whether *P. fluorescens* PICF7 can elicit systemic defense responses in aerial tissues upon colonization of the root system, disease development by *B. cinerea* inoculated on the leaf was determined in Col-0 plants in two independent experiments. Results showed that presence of strain PICF7 in roots reduced disease incidence caused by *B. cinerea* in Col-0 plants in both experiments and to the same extent as strain WCS417r, although this decrease was significant ($P < 0.05$) only in Experiment 2 (**Table 3.5**).

Table 3.4. Root colonization ability and *V. dahliae* biocontrol performance of *P. fluorescens* PICF7 and their mutant derivatives in Col-0 *A. thaliana* plants.

Experiment[1]	Treatment[2]	Disease assessment[3]			Bacterial population (log_{10} cfu g^{-1} of fresh root)[4]
		SAUDPC	*DII*	Final *DI* (%)	
1	Control	0.37[ab]	0.61	73.08	nd
	P. fluorescens				
	PICF7	0.25[b]	0.41	60.00	5.7 ± 0.2
	ME419 (*gltA*)	0.33[ab]	0.60	79.17	5.5 ± 0.3
	ME424 (*fliI*)	0.35[ab]	0.63	79.17	5.6 ± 0.1
	ME589 (*pvdI*)	0.35[ab]	0.50	65.38	5.7 ± 0.3
	ME1508 (Cys Aux)	0.45[a]	0.65	84.00	5.3 ± 0.2
2	Control	0.18[ab]	0.27	58.82	nd
	P. fluorescens				
	PICF7	0.15[b]	0.27	72.22	7.2 ± 0.4
	ME419 (*gltA*)	0.36[a]	0.55	84.00	6.6 ± 0.3
	ME424 (*fliI*)	0.25[ab]	0.44	66.67	7.2 ± 0.2
	ME589 (*pvdI*)	0.28[ab]	0.44	68.00	7.0 ± 0.4
	ME1508 (Cys Aux)	0.33[ab]	0.50	65.22	6.4 ± 0.2
3	Control	0.13[a]	0.22	47.37	nd
	P. fluorescens				
	PICF7	0.12[a]	0.19	23.81	7.5 ± 0.4
	ME419 (*gltA*)	0.11[a]	0.20	36.36	6.5 ± 0.2[*]
	ME424 (*fliI*)	0.22[a]	0.31	40.00	6.4 ± 0.1[*]
	ME589 (*pvdI*)	0.22[a]	0.43	56.00	6.8 ± 0.4[*]
	ME1508 (Cys Aux)	0.14[a]	0.24	36.00	6.0 ± 0.4[*]

[1]Three independent experiments were performed. Experiment 1 and 3 spanned 36 days and Experiment 2 39 days.

[2] The root system of 2-week-old *Arabidopsis* seedlings were transplanted into PICF7 or Tc^R-mutant derivatives bacterized soil (1.0 - 2.8 x 10^8 cfu g^{-1} soil) previously autoclaved for colonization experiment. For biocontrol assays, one week after bacterization plants were uprooted and their roots dipped in a *V. dahliae* isolate V937I (D pathotype) conidia suspension (3.9 x 10^5- 1.7 x 10^6 conidia ml^{-1}) or distilled sterile water (control treatment) for 15 min. Plants were grown in a growth chamber under controlled conditions. See Section "Materials and Methods" for details.

[3]Standardized area under the disease progress curve of *DII* plotted over time. Final *DI*, final disease incidence (percentage of affected plants at the end of the experiment). Means in a column followed by different letters are significantly different in accordance to Fisher's protected LSD test ($P < 0.05$).

[4] *Pseudomonas* cell counts were conducted on modified King's medium B agar. Data are means of three root samples (1 g each). Means followed by an asterisk are significantly different in accordance to Two-sided Dunnett's Multiple Comparisons with a Control (PICF7) at $\alpha = 0.05$. nd, not determined.

Table 3.5. *Botrytis cinerea* biocontrol by *P. fluorescens* PICF7 and WCS417r in *A. thaliana* plants.

Experiment[1]	Treatment[2]	Diseased leaves (%)[3]
1	Control	60.18[a]
	WCS417r	50.00[b]
	PICF7	58.55[ab]
2	Control	76.82[a]
	WCS417r	69.57[ab]
	PICF7	66.90[b]

[1]Two independent experiments were carried spanning 7 weeks.

[2]Two-week-old Arabidopsis Col-0 seedlings were transplanted into control soil or PICF7- or WCS417r-bacterized soil (9×10^7 - 2×10^8 cfu g^{-1} soil) previously autoclaved. Five weeks later, six to eight well-developed leaves were inoculated by applying 5 µl droplets of a conidial suspension of *B. cinerea* (1.7 - 7.5×10^5 conidia ml^{-1} half strength PDB). Plants were grown in growth chamber under controlled conditions.

[3]Percentage of diseased leaves. Means followed by different letters are significantly different in accordance to Fisher's protected LSD test ($P < 0.05$).

3.5. Discussion

Control of VWO is difficult, encouraging the implementation of an integrated disease management strategy (López-Escudero and Mercado-Blanco, 2011; Tsror, 2011). The use of microbial antagonists is gaining attention as an environmentally friendly approach for VWO control, particularly as a preventive measure (Mercado-Blanco and López-Escudero, 2012). Previous studies have shed light on potential mechanisms of *P. fluorescens* PICF7 involved in *V. dahliae* control and the endophytic lifestyle this bacterium shows in olive roots (Prieto et al., 2009; Schilirò et al., 2012; Gómez-Lama Cabanás et al., 2014; Maldonado-González et al., 2015). However, traits responsible for the successful biocontrol of VWO exerted by PICF7 remain mostly unknown. Furthermore, they are very complex to elucidate because of, among other factors, the idiosyncrasy of the host plant (i.e. longevity, large size, long duration of bioassays, lack of mutants, etc.). Therefore, the present research aimed to explore whether the short-living, genetically well known, and easy-to-manipulate model plant *A. thaliana* was amenable to facilitate and expedite the search for strain PICF7 traits implicated in VW suppression, and whether results obtained with this model system are similar to that observed in the natural tripartite interaction olive-*P. fluorescens* PICF7-*V. dahliae*.

Verticillium dahliae isolates infecting olive (and cotton) are classified into D and ND pathotypes, the former being generally more virulent than the latter (Schnathorst and Mathre, 1966; Mercado-Blanco et al., 2003b; López-Escudero et al., 2004). Nevertheless, a complete correspondence between molecular/genetic/ pathogenic groups (Collado-Romero et al., 2006) is not always found, and a continuum of virulence has been reported (Dervis et al., 2010). Furthermore, they can differ in pathogenicity and virulence depending on the host (Jiménez-Díaz et al., 2006). Since we aimed to assess whether *A. thaliana* can be used for the evaluation of the VWO biocontrol performance of *P. fluorescens* PICF7, it was necessary to determine the pathogenicity and virulence of selected D and ND in this model plant. Results showed that all D isolates originating from cotton (V138I) or olive (V150I and V937I) used in this study caused more severe disease symptoms than the olive ND pathotype (V789I). Therefore, virulence displayed by *V. dahliae* isolates in *Arabidopsis* plants correlated to that observed in olive. Interestingly, isolate V937I had an intermediate virulence and no difference was found between this D representative and isolate V789I in Col-0 and *sid1* plants, suggesting that the continuum of virulence previously observed in olive (Dervis et al., 2010) is also found in *Arabidopsis*. The fact that ND and D olive isolates were pathogenic in *Arabidopsis* and that both pathotypes showed the same differential virulence in this host and in olive meant that the first objective of our study was accomplished. In order to avoid excessive disease pressure that could potentially mask disease suppression effectiveness by the BCA, isolate V937I was selected for subsequent biocontrol experiments.

Efficient colonization of the target plant tissue by a BCA is a prerequisite for effective biocontrol (Lugtenberg et al*.,* 2001; Mercado-Blanco and Bakker, 2007). Furthermore, endophytic lifestyle displayed by some rhizobacteria leading to benefits to the plant is an interesting biotechnological potential to be explored (Mercado-Blanco and Lugtenberg, 2014). The biocontrol strain PICF7 colonizes and persists on/in olive root tissues (Mercado-Blanco et al., 2004; Prieto and Mercado-Blanco, 2008). It can also persist in olive stems after artificial inoculation (Maldonado-González et al., 2013), and it efficiently colonizes the root system of an unrelated species such as sunflower (Maldonado-González et al., 2012). Results from this present study demonstrated that strain PICF7 is also able to colonize and persist on roots of *A. thaliana* genotypes, indicating that this BCA has a wide host colonization range. This apparent broad colonization ability makes it strain PICF7 as an excellent candidate to be studied as a model bacterium in plant-microbe interactions.

However, no evidence of endophytic colonization was found under experimental conditions used. Indeed, while PICF7 is able to internally colonize the root hairs (Prieto et al., 2011), the intercellular spaces of the root cortex (Prieto and Mercado-Blanco, 2008; **Figure 3.2C**), and the root vascular tissue (Maldonado-González et al., 2015) of olive, endophytic lifestyle of PICF7 seems to be hindered in *Arabidopsis* (**Figure 3.2A,B**).

Pseudomonas fluorescens PICF7 is able to induce a multiplicity of defense responses in olive root tissues upon root inoculation (Schilirò et al., 2012). Recently, defense responses were shown to be also induced systemically, and it has been hypothesized that both SA- and JA/ET-mediated signaling responses can be involved in biocontrol exerted by PICF7 (Gómez-Lama Cabanás et al., 2014). However, actual implication of ISR and/or SAR responses in suppression of VWO has not yet been demonstrated. This is hampered because both strain PICF7 and *V. dahliae* share the same niche (roots). Spatial separation of the pathogen and the BCA is needed to prove ISR. Our previous works have aimed to assess the effectiveness of systemic defense responses mediated by PICF7 against another olive pathogen affecting above-ground organs (*Pseudomonas savastanoi* pv. savastanoi; Psv) and producing olive knot disease (Ramos et al., 2012). However, root colonization by PICF7 did not impair development of tumors in Psv-inoculated olive stems (Maldonado-González et al., 2013). Thus, even though PICF7 triggers a wide range of systemic defense responses (Gómez-Lama Cabanás et al., 2014), they do not seem to be effective against Psv. *Arabidopsis* has been earlier used to prove the involvement of ISR against *V. dahliae* mediated by the BCA *Paenibacillus alvei* K165 (Tjamos et al., 2005). Moreover, *A. thaliana* has also served to prove that an endophytic strain (*P. fluorescens* FPT9601-T5) originating from tomato (*Solanum lycopersicum* Mill) is able to trigger systemic defense responses effective against *Pseudomonas syringae* pv. tomato (Wang et al., 2005). Consequently, two different approaches were followed. On the one hand, to evaluate whether presence of PICF7 in *Arabidopsis* roots can control disease caused by the foliar necrotrophic fungus *B. cinerea*. On the other hand, to assess whether PICF7 biocontrol performance against VW was affected in *A. thaliana* mutants unable to trigger ISR (*myb72*) or SAR (*sid2*). In the first approach, spatial separation of the BCA and the pathogen is guaranteed particularly because no evidence of endophytic colonization of *Arabidopsis* tissues by PICF7 was obtained. Therefore, mechanisms such as competition and/or antagonism can be excluded in this case. Despite the fact that results varied between bioassays, PICF7 was able to significantly decrease symptoms caused by *B. cinerea* to the same extent as *P.*

fluorescens WCS417r (**Table 3.5**), as previously demonstrated for this strain (Van der Ent et al., 2008). This suggests that an effective systemic defense response is induced by PICF7 when present on *Arabidopsis* roots, corroborating previous findings found in olive aerial tissues (Gómez-Lama Cabanás et al., 2014). In addition, the use of *Arabidopsis* mutants revealed that strain PICF7 has the capability to control *V. dahliae* in different *A. thaliana* genotypes, although VW suppression was more consistently observed in *sid2* plants (**Table 3.3**). This may suggest that low levels of SA may help to increase the biocontrol performance of PICF7. Nevertheless, results from these bioassays were not consistent enough and VW biocontrol in *Arabidopsis* by PICF7 may rely on mechanisms and/or abilities (i.e. root endophytic colonization) that are not operative in this host in contrast to olive (Prieto et al., 2009). Moreover, mechanisms other than induced resistance could also be involved in suppression of *V. dahliae* since in *Arabidopsis* mutants impaired in either ISR or SAR disease control was still observed.

An additional objective of this study was to evaluate whether the use of the study system here developed can facilitate the identification of bacterial traits involved in VW biocontrol by strain PICF7. The colonization and VWO biocontrol abilities of PICF7 mutants affected in swimming motility (ME424), Pvd production (ME589), *in vitro* growth delay in PDA (ME419), or Cys auxotrophy (ME1508) were previously analyzed in olive (Maldonado-González et al., 2015). Here we examined the behavior of these mutants in *A. thaliana* Col-0. Population sizes of mutants did not significantly differ from that of strain PICF7 but in one experiment, stressing the variability also scored in bioassays carried out with olive plants. Interestingly, the Cys auxotroph mutant ME1508 always displayed the lowest population sizes, a similar behavior found in olive. Regarding to biocontrol performance, swimming motility and Pvd production of strain PICF7 seemed not to be crucial for VW suppression in *A. thaliana*, as also found in olive (Maldonado-González et al., 2015). However, both ME419 and ME1508 mutants did not control VW in some of the experiments (**Table 3.4**), suggesting that the nutritional requirements affected in these mutants can play a role in both colonization and biocontrol. An important outcome is that, overall, the behavior of the PICF7 mutants was similar to that previously reported in olive.

We conclude that the model plant *A. thaliana* provides a suitable and complementary approach to study *P. fluorescens* PICF7 traits involved in biocontrol of *V. dahliae*. In *Arabidopsis* the D and ND pathotypes of the pathogen showed a behavior similar to that in olive. PICF7 colonizes and persists in the *Arabidopsis*

rhizosphere, and it decreases VW symptoms in this model plant. Moreover, the behavior of four selected PICF7 mutants affected in different traits was similar to that previously demonstrated in olive. These findings encourage the use of *A. thaliana* both for pathogenicity and virulence assessment of *V. dahliae* isolates and for the evaluation of large numbers of PICF7 mutant phenotypes related with biological control, saving time and space. In contrast, since we have not been able to demonstrate endophytism of strain PICF7 in *A. thaliana*, bacterial traits involved in this lifestyle cannot be evaluated in this plant. However, the different behavior that PICF7 displays in olive and *Arabidopsis* offers good opportunities to unravel mechanisms underlying endophytism by this bacterium.

Acknowledgments

This research was funded by grants AGL2009-07275 from Spanish MICINN/MINECO and P07-CVI-02624 and P12-AGR-667 from J. Andalucía (Spain), both co-financed by ERDF of the EU.

CHAPTER 4

From the root to the stem: interaction between the biocontrol root endophyte *Pseudomonas fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB 3335 in olive knots

This chapter has been published as:

Maldonado-González, M. M., Prieto, P., Ramos, C., and Mercado-Blanco, J. (2013). From the root to the stem: interaction between the biocontrol root endophyte *Pseudomonas fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB 3335 in olive knots. *Microbial Biotechnology*, *6*(3), 275-287. DOI: 10.1111/1751-7915.12036.

4.1. Summary

Olive knot disease, caused by *Pseudomonas savastanoi* pv. savastanoi, is one of the most important biotic constraints for olive cultivation. *Pseudomonas fluorescens* PICF7, a natural colonizer of olive roots and effective biological control agent (BCA) against Verticillium wilt of olive, was examined as potential BCA against olive knot disease. Bioassays using *in vitro*-propagated olive plants were carried out to assess whether strain PICF7 controlled knot development either when co-inoculated with the pathogen in stems or when the BCA (in roots) and the pathogen (in stems) were spatially separated. Results showed that PICF7 was able to establish and persist in stem tissues upon artificial inoculation. While PICF7 was not able to suppress disease development, its presence transiently decreased pathogen population size, produced less necrotic tumors, and sharply altered the localization of the pathogen in the hyperplasic tissue, which may pose epidemiological consequences. Confocal laser scanning microscopy combined with fluorescent tagging of bacteria revealed that when PICF7 was absent the pathogen tended to be localized at the knot surface. However, presence of the BCA seemed to confine *P. savastanoi* at inner regions of the tumors. This approach has also enabled to prove that the pathogen can moved systemically beyond the hypertrophied tissue.

4.2. Introduction

Pseudomonas savastanoi pv. savastanoi (Psv) (Gardan et al., 1992; Sisto et al., 1999) is the causal agent of olive (*Olea europaea* L.) knot disease (Kennelly et al., 2007; Ramos et al., 2012) and an unorthodox member of the *Pseudomonas syringae* complex, encompassing at least 60 pathovars and several other *Pseudomonas* species (Gardan et al., 1999; Bull et al., 2010; Young, 2010). Infection of olive by Psv results in overgrowth formation (tumors, galls or knots) on the stems and branches of the host plant and, occasionally, on leaves and fruits. Olive knot is worldwide distributed and it is considered one of the most important diseases of this woody crop. Although losses caused by olive knot are difficult to assess and greatly depend on geographical location and olive cultivar, tree vigour, growth and yield have been reported to be moderately or severely reduced in infected trees, as well as the size and quality of fruits (Schroth et al., 1973; Young, 2004; Quesada et al., 2010a). The pathogen does not survive for long in soil, and is normally found as an epiphyte and also endophytically (Ercolani, 1978; Penyalver et al., 2006; Quesada et al., 2007), being able to move over short distances within olive orchards through dissemination of epiphytic bacteria (Quesada et al., 2010a). Natural isolates of Psv are phenotypically and genotypically heterogeneous, exhibiting broad virulence diversity (Penyalver et al., 2006) as well as variation in size and morphology of induced tumors (Pérez-Martínez et al., 2007). Psv NCPPB 3335, a highly virulent strain both in adult trees (Pérez-Martínez et al., 2007) and in micropropagated olive plants (Rodríguez-Moreno et al., 2008, 2009), is being used as a model organism for the study of the molecular basis of the disease onset and development (tumor formation) in woody hosts. The draft genome sequence of NCPPB 3335 (Rodríguez-Palenzuela et al., 2010) and the closed sequence of its three native plasmids (Bardaji et al., 2011) have been recently obtained.

Olive knot cannot be eradicated once established in plants, and its control must therefore be based on preventive measures (Young, 2004; Quesada et al., 2010a,b; Ramos et al., 2012). However, from an integrated disease management strategy perspective only a few control measures have proved to be effective. For instance, olive cultivars completely resistant to the pathogen are not yet available (Penyalver et al., 2006). Thus, chemical control involving regular application of copper compounds has been traditionally used to manage the disease (Teviotdale and Krueger, 2004; Young, 2004; Quesada et al., 2010b), posing environmental risks and enhancing the likelihood of pathogen resistance. Regarding to biological control of olive knot disease, antagonistic bacteria against Psv have been isolated,

comprising a bacteriocin-producing *P. syringae* pv. ciccaronei strain (Lavermicocca et al., 2002), a collection of fluorescent *Pseudomonas* strains isolated from the rhizosphere of different plants (Rokni-Zadeh et al., 2008), including a *Pseudomonas putida* isolate producing a salicylate-containing antibiotic (Vlassak et al., 1992; Li et al., 2011), and several *P. fluorescens* and *Bacillus subtilis* strains isolated from olive knots and from leaves of Psv-infected trees (Krid et al., 2010). Although some of these strains have shown to reduce olive knot symptoms (Lavermicocca et al., 2002; Krid et al., 2012), little is known about the *in planta* community interplay between these antagonistic bacteria and the pathogen in the development of the disease.

Pseudomonas fluorescens PICF7 is a natural inhabitant of the olive rhizosphere isolated from roots of nursery-propagated olive plants (cv. Picual) (Mercado-Blanco et al., 2004). This strain has been shown to be an effective biological control agent (BCA) against Verticillium wilt of olive (Mercado-Blanco et al., 2004; Prieto et al., 2009), a disease caused by the soil-borne fungal pathogen *Verticillium dahliae* Kleb., and currently considered one of the most important biotic constraints for this woody crop (López-Escudero and Mercado-Blanco, 2011). Strain PICF7 has also been shown to develop an endophytic lifestyle within olive root tissues under diverse experimental conditions (Prieto and Mercado-Blanco, 2008; Prieto et al., 2009, 2011). Recent functional genomics analysis has revealed that root colonization by PICF7 induces a broad range of defence responses in olive root tissues as well as the activation of diverse transcription factors known to be involved in systemic defence responses (Schilirò et al., 2012). This depicts a scenario where PICF7 might be an effective BCA against other pathogens infecting olive, although additional biocontrol mechanisms (i.e. antibiosis) deployed by PICF7 and operating *in planta* cannot be completely ruled out.

In this study we evaluate the potential of *P. fluorescens* PICF7 to be used as a BCA against olive knot disease both in *in vitro*-propagated explants and in lignified, pot-acclimated plants. We tested the hypothesis whether PICF7, a natural inhabitant of olive roots, can be effective against a pathogen which affects above-ground organs of the same host under two different situations: (i) the BCA applied to the roots (its natural niche) and the pathogen inoculated into the stems, and (ii) both microorganisms co-inoculated in artificially produced wounds on the favourable, natural environment of the pathogen (stems). We assessed strain PICF7's ability to: (i) colonize and persist in olive stem tissues; (ii) influence the establishment of the pathogen on/in its target niche; and (iii) affect olive knot development. The

interaction between *P. fluorescens* PICF7 and Psv NCPPB 3335 was investigated *in planta* at both macro- and microscopic levels.

4.3. Material and methods

4.3.1. Bacterial strains, growth conditions and inocula production

Pseudomonas fluorescens strain PICF7 (Mercado-Blanco et al., 2004), Psv strain NCPPB 3335 (Pérez-Martínez et al., 2007) and their fluorescently tagged derivatives-PICF7 carrying plasmid pMP4662 (Bloemberg et al., 2000), which harbours the red fluorescent protein (RFP) marker (Prieto and Mercado-Blanco, 2008), and NCPPB 3335 transformed with the plasmid pLRM1 harbouring the green fluorescent protein (GFP) marker (Rodríguez-Moreno et al., 2009), were used in this study. Growth conditions for *P. fluorescens* and Psv strains, and assessment of the stability of plasmids pMP4662 and pLRM1 in their respective hosts, have been earlier described by Prieto and Mercado-Blanco (2008) and Rodríguez-Moreno and colleagues (2009) respectively. Bacterial inocula were prepared from cultures previously grown on King's B Agar (KBA; King et al., 1954) or Luria-Bertani Agar (LBA; Miller, 1972) plates at 25-28°C for 24 h. Bacterial cells were resuspended in 10 mM $MgSO_4 \cdot 7H_2O$ by scraping bacterial lawns off with a sterile rod, washed twice (4500 rpm, 10 min) and resuspended in sterile 10 mM $MgSO_4 \cdot 7H_2O$. Bacterial cell densities required for each experiment were established spectrophotometrically (A600 nm) by building up standard curves and culturing viable cells from serial dilution series onto KBA or LBA media (wild type) or KBA supplemented with the antibiotics tetracycline (20 mg l^{-1}) (PICF7-RFP) or gentamicin (10 mg l^{-1}) (NCPPB 3335-GFP).

4.3.2. Plant material and plant growth conditions: *in vitro* propagated and lignified, pot-acclimated plants

Olive plants were micropropagated and rooted in Driver-Kuniyuki walnut (DKW) medium (Driver and Kuniyuki, 1984) from an *in vitro* germinated seed originated from a cv. 'Arbequina' plant (Rodríguez-Moreno et al., 2008) at the Instituto de Formación Agraria y Pesquera de Andalucía (IFAPA, Junta de Andalucía, Churriana, Málaga, Spain). Explants were transferred to sterile glass tubes with DKW without hormones and grew for at least 2 weeks in a growth chamber at 25 ± 1°C with a 16-h photoperiod. The length of plants used at the time of the bioassays was 80-100 mm long, with stems of 1-2 mm in diameter and always displaying 3-5 internodal segments.

For assays carried out with lignified plants (1 and 2 years old), plants were granted by the Instituto de Formación Agraria y Pesquera de Andalucía (IFAPA, Junta de Andalucía, Churriana, Málaga, Spain).

4.3.3. *In vitro* antagonism of *P. savastanoi* NCPPB 3335 by *P. fluorescens* PICF7

To demonstrate whether *P. fluorescens* PICF7 exhibits *in vitro* antagonism against Psv, four drops (5 µl, 10^8 cfu [colony-forming units]ml^{-1}) of strain PICF7 were placed on the surface of KBA, LBA and Potato Dextrosa Agar (PDA) media plates previously inoculated with 100 µl of bacterial suspensions of strain NCPPB 3335 (ranging from 10^5 to 10^8 cfu ml^{-1}). Two series of plates (two per assayed media) were incubated at 25 and 28°C, respectively, and after 72 h growth inhibition halos around PICF7 colonies were scored. Relative size of inhibition haloes was calculated according to the formula (halo diameter - colony diameter)/halo diameter. The experiments were carried out twice.

4.3.4. Assessment of the colonization ability of *Pseudomonas fluorescens* PICF7 on/in tissues of *in vitro*-propagated Arbequina plants

To determine whether *P. fluorescens* PICF7 colonizes and persists on/in roots of *in vitro*-propagated 'Arbequina' explants, 36 plants in total were uprooted from the DKW medium and their root systems dipped in a *P. fluorescens* PICF7 cells suspension (3.8 x 10^8 cfu ml^{-1}) (24 plants) or 10 mM $MgSO_4 \cdot 7H_2O$ (control treatment) (12 plants) for 15 min (Mercado-Blanco et al., 2004). After that, bacterized and non-treated roots were placed on top of several sheets of sterile filter paper 3 min to remove the excess of bacteria suspension or 10 mM $MgSO_4 \cdot 7H_2O$.

To assess colonization and persistence on/in stems, drops (2 µl) of a PICF7 cells suspension (8.3 x 10^7 cfu ml^{-1}) were applied to intentional wounds made after removing a petiole of one intermediate leaf per plant (24 plants per treatment) with a sterile scalpel (Pérez-Martínez et al., 2010). After treatment, plants were placed again into sterile glass tubes containing DKW medium.

PICF7 populations on/in plant tissues were determined throughout both experiments by sampling three plants at 0, 3, 5, 7, 15, 30, 40 and 60 days after inoculation (DAI). Thus, three root samples (100 mg) and three stem fragments

(spanning 1 cm above and below from the inoculation point) from six independent plants were crushed in 1 ml of 10 mM $MgSO_4 \cdot 7H_2O$ under sterile conditions.

In addition, to verify whether PICF7 cells translocate from artificially bacterized roots to the stems, 12 plants were processed as described above. Root systems were dipped in a PICF7 cells suspension (4.0×10^8 cfu ml^{-1}) for 15 min. Then, plants were placed into sterile wide-mouthed bottles containing water agar medium to avoid PICF7-contamination of stems. Roots and stems of each plant were separately analyzed at 15, 30, 40 and 50 DAI. Thus, the root system of each plant was removed and the stem was divided into three segments (i.e. basal, intermediate and apical sections). Subsequently, roots and stem segments were crushed in 1 ml of 10 mM $MgSO_4 \cdot 7H_2O$. Serial dilutions of tissue macerates were plated onto KBA and incubated at 25°C for 48 h. After that, PICF7 colonies were counted and bacterial populations were determined along time. This assay was performed twice.

Manipulation of plants during bacteria inoculation, sampling and cell counting procedures were always conducted under sterile conditions within a laminar air flow cabinet.

4.3.5. Olive-*Pseudomonas fluorescens*-*Pseudomonas savastanoi in vitro* bioassays

Two different types of bioassays (I and II) were conducted to investigate whether *P. fluorescens* PICF7 control olive knot disease of *in vitro*-propagated 'Arbequina' plants. On the one hand, *P. fluorescens* PICF7 and Psv NCPPB 3335 were applied separately in different tissues (roots and stems respectively) to explore the possibility that the BCA could elicit a defence systemic response in 'Arbequina' plants against the pathogen (type I bioassays). On the other hand, the BCA and the pathogen were simultaneously inoculated (cells suspension mix) in intentionally produced wounds in the stems (type II bioassays).

For type I bioassays, 48 explants (24 per treatment) were carefully uprooted from the growth media and dipped for 15 min in a bacterial suspension of strain PICF7 (3.8×10^8 cfu ml^{-1}) (BCA treatment) or 10 mM $MgSO_4 \cdot 7H_2O$ (control treatment), according to Mercado-Blanco and colleagues (2004). All plant manipulations were performed as indicated above except that, to avoid accidental contamination of stems with the BCA after root dip inoculation, bacterized plants were gently introduced in wide-mouthed bottles containing sterile water-agar (7 g agar l^{-1} distilled water) where they remained for the rest of the bioassay. One week after

PICF7 treatment, stems were wounded once by excision of an intermediate leaf with a sterile scalpel and, immediately, a drop (2 µl) of a Psv suspension (7.7 x 10^7 cfu ml^{-1}) was applied to the wound under sterile conditions (Pérez-Martínez et al., 2010). Population size of strain NCPPB 3335 was assessed along the experiment, sampling stem fragments spanning 1 cm above and below the pathogen inoculation point at 0, 3, 5, 7, 15, 30, 40 and 60 DAI in control and PICF7-treated plants. Tissue manipulation was performed as indicated above. In addition, population of *P. fluorescens* PICF7 was also monitored in bacterized roots at the same time-points. For that purpose, root tissue samples (100 mg) were sampled and manipulated as previously indicated. Three independent plants were examined at each sampling time-point. Bacteria counts were performed as indicated for colonization assays (see above). The assay was performed twice.

In type II bioassays a drop (2 µl) of a bacterial suspension containing a mixture of *P. fluorescens* PICF7 (6.8 - 10^7 cfu ml^{-1}) and Psv NCPPB 3335 (2.86 x 10^7 cfu ml^{-1}) were applied to intentional wounds made on the stems of 24 *in vitro*-propagated 'Arbequina' plants as previously indicated (see above). A group of 24 additional plants were inoculated only with a suspension of Psv cells (9.4 x 10^7 cfu ml^{-1}) (control treatment). As in type I bioassays, population size of NCPPB 3335 was evaluated at 0, 3, 5, 7, 15, 30, 40 and 64 DAI in both control plants (only inoculated with NCPPB 3335) and plants co-inoculated with the BCA (PICF7) and the pathogen (NCPBB 3335). Population size of strain PICF7 was also score at the same time-points. Stem tissue segments (1 cm above and below the inoculation point) were crushed in 1 ml of 10 mM $MgSO_4 \cdot 7H_2O$ under sterile conditions. Serial dilutions of macerates were plated onto KBA and incubated at 25°C for 48 h as describe in colonization assay. This bioassay was performed four times.

Mean values of population size of Psv scored throughout experiments I and II in absence and presence of PICF7 were compare using Student's *t*-test ($\alpha = 0.05$) along the experiments.

Development of knot disease symptoms on *in vitro* 'Arbequina' plants were captured with a digital camera (Panasonic FS 42, Lumix) and processed using PHOTOSHOP 4.0 software (Adobe Systems, San Jose, CA, USA).

For all bioassays involving *in vitro*-propagated 'Arbequina' plants, glass tubes or wide-mouthed recipients containing explants were always kept within controlled-

growth chambers at 25 ± 1°C with a 16-h photoperiod and a light intensity of 65 µmol m^2 s^{-1}.

4.3.6. Epifluorescence and confocal laser scanning microscopy

Epifluorescence and confocal laser scanning microscopy (CLSM), combined with fluorescent tagging of bacteria, were used to examine presence of the BCA, the pathogen and their potential interactions in *in vitro*-propagated 'Arbequina' plant tissues. Thus, in order to confirm changes in Psv NCPBB 3335 population levels in the presence of the BCA (see *Results* section), a specific bioassay was designed. Drops (2 µl) of suspensions of either GFP-tagged NCPPB 3335 alone (final cell density 4.8 x 10^7 cfu ml^{-1}) or a mix of GFP-tagged NCPPB 3335 (final cell density 1.3 x 10^8 cfu ml^{-1}) and strain PICF7 (final cell density 3.5 x 10^7 cfu ml^{-1}) were applied to intentionally made wound performed on the stems of *in vitro*-propagated plants as indicated above. Twelve plants per treatment were used. Development of knots was observed along 4 weeks after inoculation. Two plants were examined at 2, 3, 6, 8, 13 and 27 DAI using a stereoscopic fluorescence microscope (Leica MZ FLIII, Leica Microsystems, Wetzlar, Germany) equipped with a 100 W mercury lamp and a GFP2 filter (excitation, 480/ 40 nm). In addition, progress of knot symptoms was photographed with visible light using the same equipment. All images (epifluorescence and visible) were captured using a high-resolution digital camera (Nikon DXM 1200, Nikon Corporation, Tokyo, Japan) attached to the stereoscopic fluorescence microscope and processed using PHOTOSHOP 4.0 software (Adobe Systems).

Since knots generated on *in vitro*-propagated plants were found to differ at the macroscopic level (see *Results* section) depending on the presence or not of PICF7 cells in the inoculation mix, CLSM was used to examine at the microscopic level whether: (i) inner appearance of tumors may differ upon inoculation of Psv alone or Psv and PICF7 together; (ii) localization and/or distribution of NCPPB 3335 in the generated tumor cells may be influenced by the presence of PICF7; and (iii) Psv cells could be found beyond the inoculation point and the hyperplasic area and whether this potential pathogen spread may or not be influenced by the presence of the BCA. Tissue samples used for CLSM were obtained from 'Arbequina' *in vitro*-propagated plants inoculated with a suspension of NCPPB 3335-GFP cells (3.7 x 10^6 cfu ml^{-1}) or a mix containing NCPPB 3335-GFP (7.9 x 10^6 cfu ml^{-1}) and PICF7-RFP (3.0 x 10^7 cfu ml^{-1}) cells. Bacteria were inoculated according to the procedure previously described. Eight plants were used per treatment and two tumors were analyzed each time-point. This bioassay was performed twice and sampling times were 4, 5, 7 and 9

weeks after inoculation for the first assay, and 1, 2, 4 and 6 weeks for the second one. *In vitro* 'Arbequina' plants were kept within controlled-growth chambers as indicated above. Transverse and longitudinal sections (40-60 mm thick) from stems at the inoculation site (initial stages of knot development) or from visible knots were obtained using a Vibratome Series 1000plus (TAAB Laboratories Equipment, Aldermarston, UK) as previously described (Prieto et al., 2007; Prieto and Mercado-Blanco, 2008). Tissue samples were always observed at the moment of sampling with an Axioskop 2 MOT microscope (Carl Zeiss, Jena GmbH, Germany) equipped with a krypton and an argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss). In addition, stem fragments containing the inoculation site (1 cm long) were sectioned longitudinally to assess the possible spread of Psv from the hyperplasic tissue, regardless the presence or absence of strain PICF7. Two plants per treatment were evaluated at 14, 30 and 42 DAI. GFP-tagged bacterial cells were excited with the 488 nm Argon laser line and were detected in the 500-520 nm window. RFP-tagged bacterial cells were excited with the 568 nm laser line and detected in the 580-620 nm window. Data were recorded and transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss). Final figures were processed with PHOTOSHOP 4.0 software (Adobe Systems).

4.3.7. Olive-*Pseudomonas fluorescens-Pseudomonas savastanoi* bioassays using lignified, pot-acclimated plants

Two bioassays (I and II) were carried out to determine whether co-inoculation of strains Psv and PICF7 influenced the onset and/or development of knot disease in 1 and 2-year-old lignified 'Arbequina' plants, already acclimated in pots under greenhouse conditions. In bioassay I, three 2-year-old plants were wounded with a sterile scalpel at five sites along the main stem. Artificial wounds, 0.5 cm long, were made from the surface to the cambial area without removing the tab generated with the cut, and as described by Pérez-Martínez and colleagues (2007). Then, one drop (10 µl) of a bacterial suspension of PICF7 (6.5×10^8 cfu ml^{-1}), NCPPB 3335 (2.8×10^8 cfu ml^{-1}) or a mix of PICF7 (8.1×10^8 cfu ml^{-1}) and Psv (2.4×10^8 cfu ml^{-1}) were applied to each incision. Control plants were inoculated with 10 mM $MgSO_4 \cdot 7H_2O$ and three plants were used per treatment. In bioassay II, four 1-year-old plants per treatment were inoculated as describe above, although only three wounds per plant were generated in this case. As in experiment I, 10 µl drops were applied to the artificial wounds, containing bacterial suspensions of strain PICF7 (4.8×10^8 cfu ml^{-1}), Psv (4.1

x 10^8 cfu ml^{-1}) or a mix of PICF7 (5.0 x 10^8 cfu ml^{-1}) and Psv (3.3 x 10^8 cfu ml^{-1}). Control plants were inoculated with 10 mM $MgSO_4 \cdot 7H_2O$.

Inoculated wounds were covered with their tabs, enveloped with parafilm and plants were bagged in order to increase relative humidity to 100%. Bacterized plants were kept within controlled-growth chambers at 25 ± 1°C with a 16-h photoperiod and a light intensity of 65 µmol m^{-2} s^{-1}. After 7 days plants were remove from bags. Along experiment measures of disease symptoms were recorded according to the scale: 0, no symptoms, to 10, the biggest developed tumor. Tumor necrosis was also held into account. Thus, (-), no necrosis; (+), moderate necrosis; and (++), severe necrosis were scored for each developed knot. At the end of the experiments (92 DAI) all tumors were photographed with a digital camera (Panasonic Fs 42 Lumix) and measures of knots fresh weight and volume, calculated by scoring their length, width and depth (Hosni et al., 2011), were recorded. To examine whether presence of PICF7 produced inner alterations in tumors, four knots per experiment (two per treatment) were sectioned by hand with a blade, photographed and macerated (1 g) in 2 ml of 10 mM $MgSO_4 \cdot 7H_2O$. Serial dilutions of knots macerates were spotted on KBA plates supplemented with ampicillin (50 mg l^{-1}), chloramphenicol (13 mg l^{-1}) and cycloheximide (100 mg l^{-1}) and KBA with nitrofurantoin (100 mg l^{-1}) to score population of Psv.

4.4. Results

4.4.1. *Pseudomonas fluorescens* PICF7 antagonizes *Pseudomonas savastanoi* NCPPB 3335 *in vitro* and colonizes roots and stems of *in vitro*-propagated olive plants

In order to assess whether the indigenous, olive roots inhabitant *P. fluorescens* PICF7 has potential as a BCA against Psv, its effectiveness to antagonize the pathogen *in vitro* and to colonize stems (and roots) of *in vitro*-propagated olive plants were first evaluated. *In vitro* antagonism assays using different culturing media (PDA, KBA, and LBA) showed that strain PICF7 strongly inhibited the growth of Psv NCPPB 3335 in PDA plates (**Figure 4.S1**). However, inhibition haloes in KBA and LBA media were negligible or restricted to the very proximal region surrounding the BCA colony. The relative size of growth inhibition haloes on PDA plates varied depending on the concentration of Psv and on the incubation temperature. For instance, PDA plates harbouring 10^5 cfu ml^{-1} of Psv yielded inhibition haloes with average relative sizes of 0.8 ± 0.03 (at 25°C) and 0.8 ± 0.02 (at 28°C). On the contrary, when the pathogen

population on PDA plates increased up to 10^8 cfu ml^{-1}, relative size of inhibition haloes were 0.6 ± 0.03 (at 25°C) and 0.4 ± 0.08 (at 28°C).

The ability of *P. fluorescens* PICF7 to colonize roots and aboveground tissues of *in vitro*-propagated olive explants was confirmed as well. When the BCA was applied to the root system, colony counts from roots macerates showed that population size of strain PICF7 was stably maintained along the experiment [8.8 ± 0.4 (mean $\log_{10}$ cfu g^{-1} fresh root/stem tissues ± SD) at 0 DAI and 8 ± 1.4 at 60 DAI]. Moreover, GFP-tagged PICF7 colonized endophytically root tissues, and root hairs were found to be important in this process (data not shown). On the contrary, when the BCA was inoculated in the stems, population size of PICF7 increased over time in the segment containing the inoculation point (3.2 ± 0.4 and 5.9 ± 0.6 at 0 and 60 DAI respectively). On the other hand, the possible translocation of PICF7 cells from roots to stems was also examined. Results showed that movement of strain PICF7 from inoculated roots to stems was not evident (12 out of 20 plants examined) or, at most, it remained restricted to the basal segment of the stems (six plants) and with a highly variable population scored (2.4-7.3 at 50 DAI). Only in two plants PICF7 cells were detected in the upper segment of the stems (4.1 and 6 at 50 DAI). Nevertheless, cross-contamination during plant manipulation could not be completely ruled out for these cases. No bacteria were detected in roots and stems of non-inoculated (control) plants at any time.

4.4.2. *Pseudomonas fluorescens* PICF7 applied to roots does not suppress olive knot development

Bioassays designed to assess the ability of *P. fluorescens* PICF7 to control the onset and development of olive knot disease by means of systemic defence response(s) showed that PICF7 was not able to suppress the disease under the experimental conditions used despite its ability to colonize the root system. Thus, when the BCA and the pathogen were spatially separated (roots of *in vitro*-propagated olive explants bacterized with PICF7 1 week prior to Psv inoculation in stems), development of knots and their anatomy did not differ regardless the presence or absence of PICF7 (data not shown). Moreover, population sizes of strain NCPPB 3335 scored in hyperplasic tissues of PICF7-bacterized (3.1 ± 0.5 at 0 DAI and 6.5 ± 1.2 at 60 DAI) and control (3.8 ± 0.6 at 0 DAI and 7.1 ± 0.2 at 60 DAI) plants were not significantly ($P > 0.05$) different along the experiment.

4.4.3. Presence of *Pseudomonas fluorescens* PICF7 in stems affects pathogen population and knot development

Results showed that when *P. fluorescens* PICF7 was inoculated into the stems along with the pathogen, population size of NCPPB 3335 sharply decreased and was significantly ($P < 0.05$) lower during the first 2 weeks after bacterization, compare with that scored in plants only inoculated with Psv (control treatment). However, this fall in the pathogen population was only transitory and Psv counts recovered later on and did not differ significantly ($P > 0.05$) between treatments until the end of the bioassays (64 DAI) (**Figure 4.1**). Population sizes of PICF7 did not significantly differ ($P > 0.05$) regardless the presence (3.3 ± 0.2 at 0 DAI and 6.4 ± 0.4 at 60 DAI around the inoculation points and developed tumors respectively) or absence (segments containing the inoculation point, see above) of the pathogen. Interestingly enough, co-inoculation of PICF7 with Psv significantly altered the macroscopic appearance of the tumors. Thus, less necrotic knots (discolored, whitish tumors) developed when PICF7 was co-inoculated with the pathogen (**Figure 4.2A,C**) in comparison with knots developed in plants inoculated with Psv alone (**Figure 4.2B,D**).

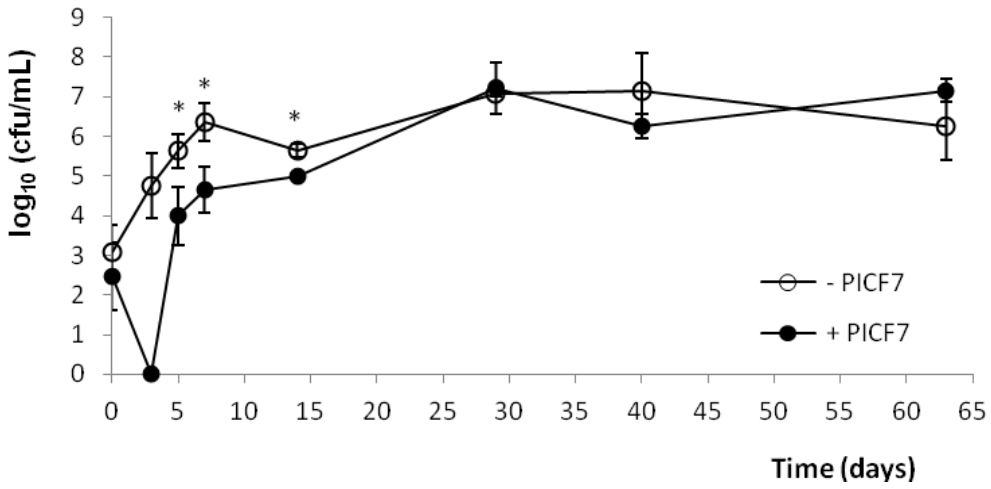


Figure 4.1. Population size of *Pseudomonas savastanoi* NCPPB 3335 recovered from inoculation sites or developed knots from a 63 days bioassay performed with *in vitro*-propagated olive plants co-inoculated (•) or not (o) with *Pseudomonas fluorescens* PICF7 (see text for details). Each score time-point is the mean from three independent samples. Error bars represent standard deviation. Mean values significantly different ($P < 0.05$) according to t-student test are marked by asterisks. Results shown are from a representative bioassay. This experiment was repeated three times with similar results.

Figure 4.2. Tumors produced on *in vitro*-propagated olive plants by *Pseudomonas savastanoi* NCPPB 3335 (Psv) in the presence (**A**) or in the absence (**B**) of *Pseudomonas fluorescens* PICF7 at 58 Days After Inoculation (DAI). Tumors developed in a different experiment upon inoculation with Psv-GFP in the presence (**C**) and in the absence (**D**) of PICF7 were also observed at 27 DAI. Presence of PICF7 in the inoculation mix produced tumors with reduced or no necrosis (**A**,**C**) regardless the presence of Psv or its GFP-tagged derivative (see text for details). Panels **B** and **D** show tumors with symptoms of necrosis.

To further check that transient decrease of Psv population and modification of tumor's macroscopic appearance were due to the presence of strain PICF7, epifluorescence microscopy combined with fluorescent tagging of Psv NCPPB 3335 with GFP (Psv-GFP) was used. In agreement with results from previous bioassays, a decrease of Psv NCPPB 3335 population in tumors developed in plants co-inoculated with the BCA was visualized during the first 14 days. Lower population of the pathogen was revealed as a depletion of the green fluorescence at the inoculation points and within the hyperplasic tissue developed in Psv/PICF7 co-inoculated plants (**Figure 4.3A, 2-13 DAI**), compared with those ones only inoculated with Psv alone

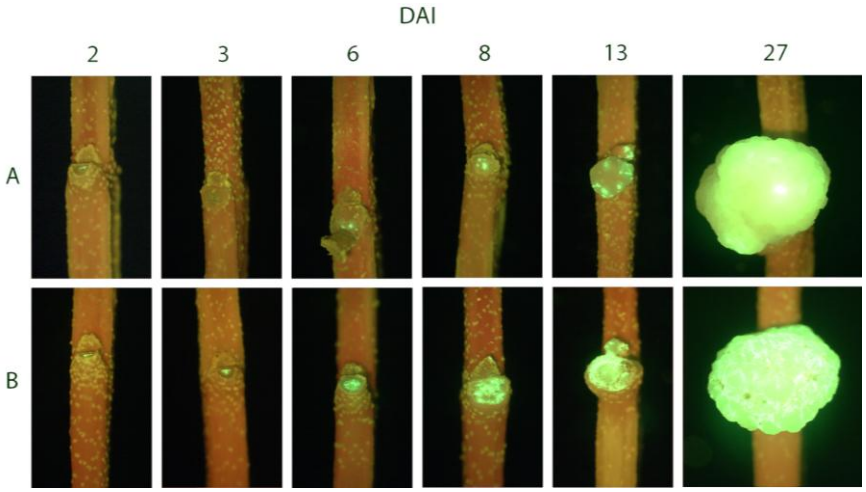


Figure 4.3. Epifluorescence microscopy images showing the presence of GFP-tagged *Pseudomonas savastanoi* NCPPB 3335 (Psv) at the inoculation point and tumors developed on *in vitro*-propagated olive plants during a time-course experiment (27 days). Stems were inoculated with the pathogen alone (**B**) or mixed with *Pseudomonas fluorescens* PICF7 (**A**) (see text for details). Two plants were analyzed per each sampling time-point with similar results. Green fluorescence reveals the presence of living Psv-GFP cells. Plants co-inoculated with Psv-GFP and PICF7 exhibited no detectable fluorescence (3 days after inoculation [DAI]) or less fluorescent areas (6, 8, 13 DAI) compared with plants inoculated with Psv-GFP alone. At the end of the experiment (27 DAI), tumors differ neither in size nor in fluorescence between treatments.

(**Figure 4.3B, 2-13 DAI**). As in previous bioassays, NCPPB 3335 population in Psv/PICF7 co-inoculated plants recovered over time, and tumors from both treatments reached similar levels of fluorescence (**Figure 4.3A,B**, 27 DAI). Population size of Psv recovered from knots along the experiment confirmed that the observed fluorescence fluctuation correlated to a decrease in Psv colony counts (approximately 1.5 order of magnitude lower) at all times except at 27 DAI (6.8 ± 0.3, for Psv alone and 6.5 ± 0.1, for Psv/PICF7 coinoculated plants), as shown in previous bioassays (see above). Finally, macroscopic appearance of tumors developed in this assay differed depending on the presence or not of the BCA (**Figure 4.2C,D**), confirming previous observations.

4.4.4. Co-inoculation of *Pseudomonas fluorescens* PICF7 alters the localization and distribution of Psv in tumors

To assess whether differences observed in the external, macroscopic anatomy between tumors developed in Psv-inoculated and Psv/PICF7 co-inoculated plants could correlate to changes in pathogen distribution mediated by PICF7 presence, fluorescent tagging of bacteria (Psv-GFP and PICF7-RFP), vibratome-sectioning of knot and stem tissues and CLSM were used. By combining these microscopy and biotechnological tools we aimed to explore the inner anatomy of tumors as well as the localization and distribution of the BCA and the pathogen on and within knots *in vivo*, without implementing further tissue manipulation, fixation and/or staining procedures. Overall, sectioning of knots from plants co-inoculated with Psv and PICF7 was more difficult, as they presented spongy consistency compared with tumors generated by single inoculation of Psv. CLSM images showed that, in co-inoculated plants, both bacteria could be found mixed within vascular vessels of the stem at early stages of the knot development (**Figure 4.4A,B**, 7 DAI). However, from 2 weeks after artificial inoculation of bacteria until the end of the experiments (6-9 weeks), each bacterial species tended to be allocated in different regions of the tumors in most of the observations (**Figure 4.4C**). Indeed, while both fluorescently tagged *Pseudomonas* could be found mixed at any place within the knot, particularly at the beginning of the knot development (**Figure 4.4A,B**), PICF7-RFP cells were predominantly visualized at the knot surface or in outer regions of the tumor (**Figure 4.4C,D**). In contrast, Psv-GFP cells were mainly found at the inner regions of the hyperplasic tissue, particularly at later times of the experiment (**Figure 4.4C; Figure 4.5B,D,F**). Remarkably, localization of Psv colonies greatly differed depending on the presence of strain PICF7. Results showed that when strain NCPPB 3335 was

inoculated alone the pathogen predominantly colonized the surface of the tumor (**Figure 4.5A,C,E**).

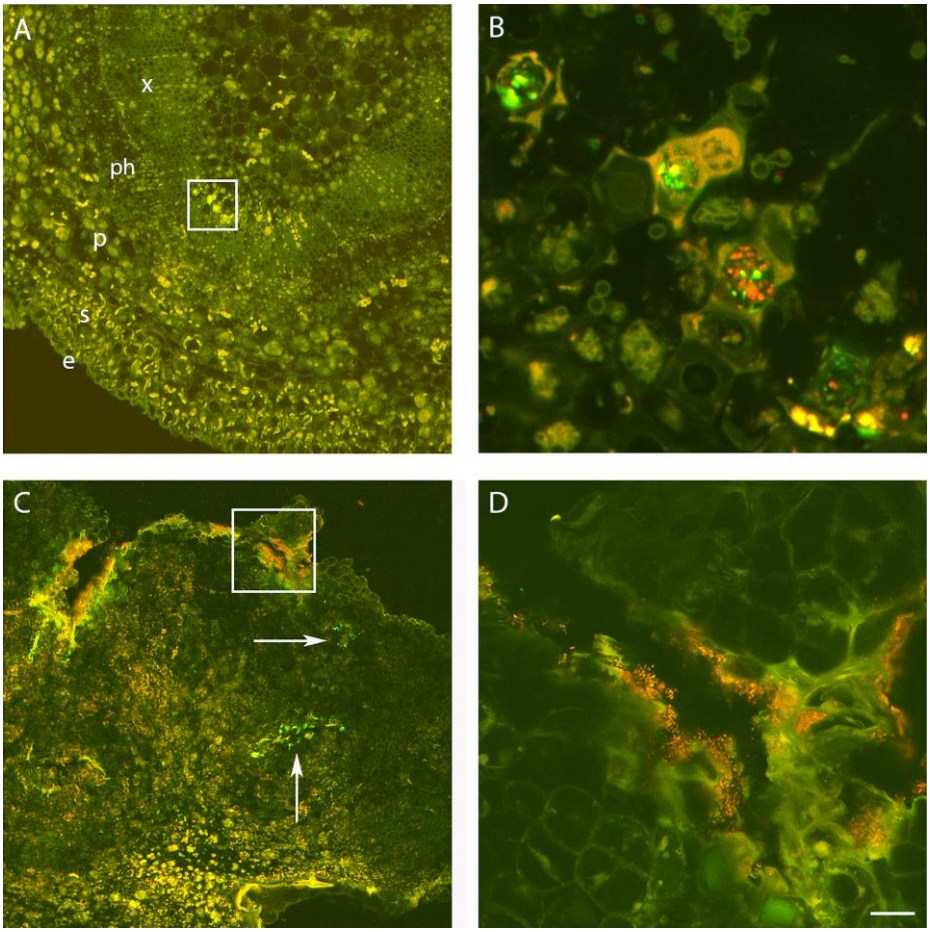


Figure 4.4. Confocal laser scanning microscopy images of transversal vibratome tumor sections (40 µm thick) showing localization of *Pseudomonas savastanoi* (Psv-GFP, green) and *Pseudomonas fluorescens* (PICF7-RFP, red). Images were taken at one (**A**,**B**) and four (**C**,**D**) weeks after inoculation with Psv-GFP and PICF7-RFP.

A. Vibratome transversal section of a representative stem one week after inoculation. Fluorescence located inside xylem vessels is due to the presence of intermixed Psv-GFP and PICF7-RFP cells (inset).

B. Inset in (**A**) showing Psv-GFP and PICF7-RFP cells intermixed inside the vascular vessel cells.

C. Tumor sampled four weeks after inoculation showing events of inner (arrowed) and surface (inset) localization of Psv-GFP and PICF7-RFP cells, respectively.

D. inset in (**C**) showing PICF7-RFP cells at the surface of the knot and a small individual colony of Psv-GFP cells in a different focus plane and not mixed with PICF7-RFP cells (visible as weak green fluorescence at the bottom of the panel).

Scale bar represents 100 µm in **A**, 20 µm in **B**, 150 µm in **C** and 25 µm in **D**. e, epidermis; s, sclereids; p, parenchyma; ph, phloem; x, xylem.

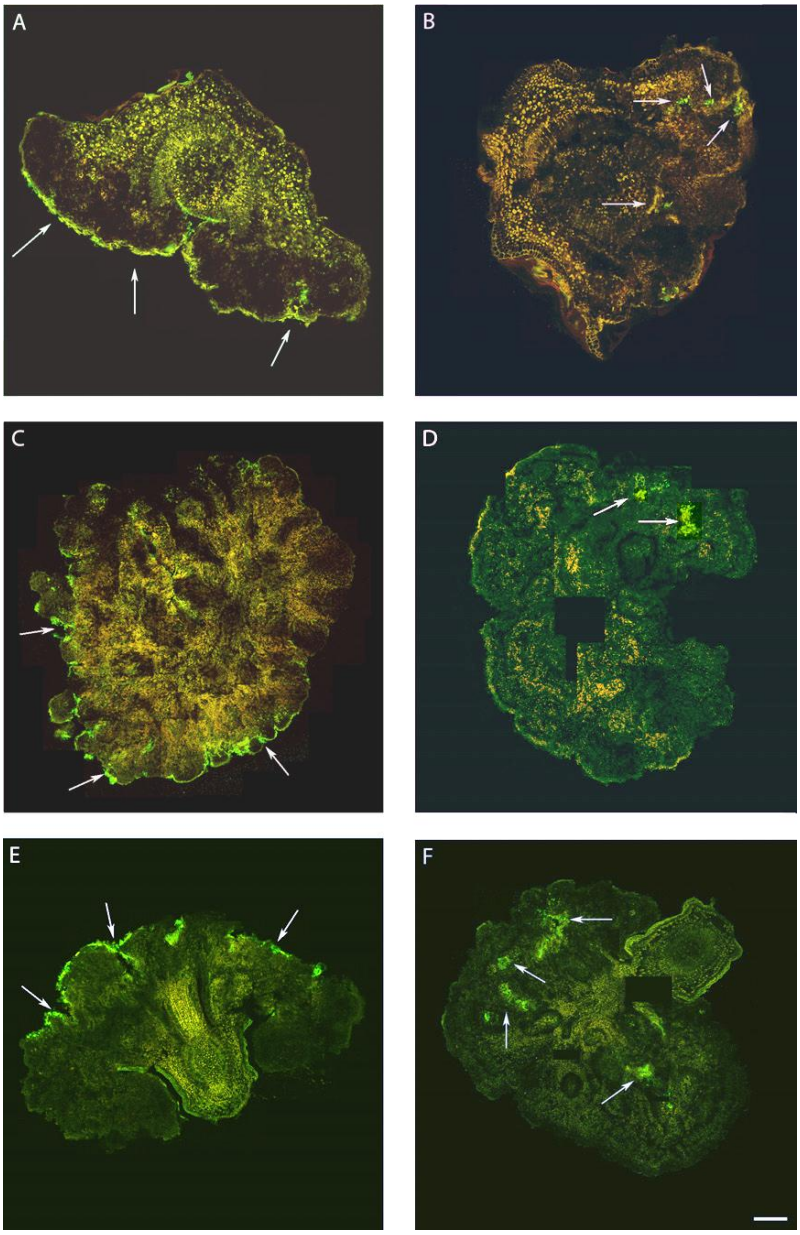


Figure 4.5. Confocal laser scanning microscopy images showing the time course of colonization of *in vitro*-propagated olive tissues by GFP-tagged *Pseudomonas savastanoi* NCPPB3335 (Psv-GFP) in the absence (**A**,**C**,**E**) or in the presence (**B**,**D**,**F**) of *Pseudomonas fluorescens* PICF7. Transversal vibratome tumor sections (40 µm thick) were made to show inner colonization. Each panel is a composition of several images to show the whole knot and from two different bioassays. In the absence of *P. fluorescens* PICF7, Psv-GFP is visualized predominantly and profusely at the knots surface (arrowed) at 2 (**A**), 6 (**C**) and 9 (**E**) weeks after inoculation. In the presence of *Pseudomonas fluorescens* PICF7, Psv-GFP is visualized in inner cavities of the tumor (arrowed) at 2 (**B**), 7 (**D**) and 9 (**F**) weeks after inoculation. Scale bar represents 500 µm in all panels except in (**A** and **B**) where it represents 125 µm.

4.4.5. Systemic movement of *Pseudomonas savastanoi* NCPPB 3335 along olive stems

An interesting finding from CLSM experiments was the repeated observation of Psv-GFP colonies in stem tissues outside the hyperplasic region. Thus, CLSM imagery revealed that the pathogen could move from the inoculation point to healthy areas of the stem, colonizing the xylem vessels (**Figure 4.6**). Psv-GFP cells were first observed 2 weeks after inoculation in xylem vessels close to the tumor (data not shown). At later times after inoculation (4 weeks) Psv-GFP cells were visualized either within the xylem vessels nearby the tumor (node) (**Figure 4.6B,E**) or beyond the hyperplasic tissue (internode) (**Figure 4.6A,D**). Finally, presence of strain PICF7 did not interfere with Psv movement outside the hyperplasic region and throughout the vascular system since the pathogen was also found in stems of Psv/PICF7 co-inoculated plants 2 weeks after inoculation (**Figure 4.6C,F**).

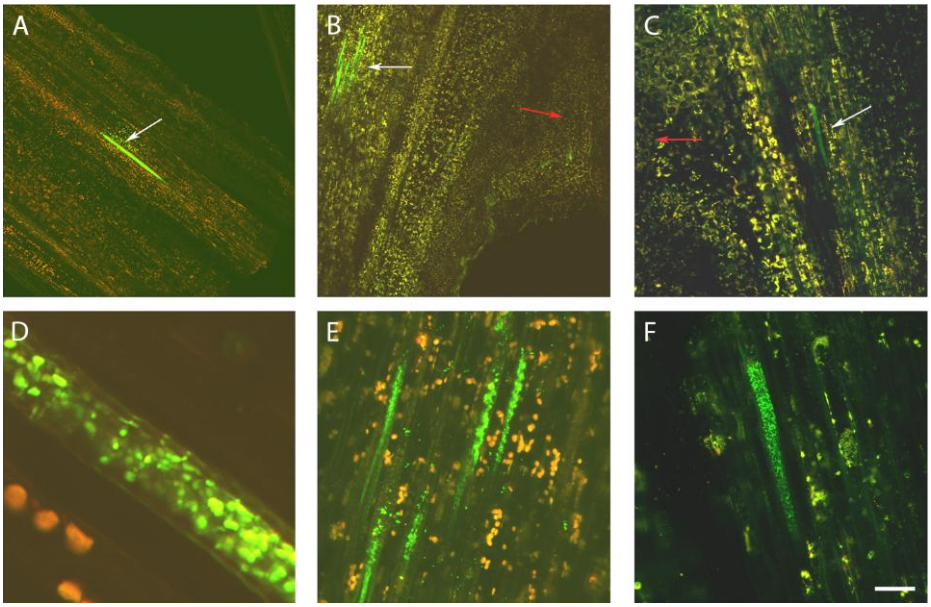


Figure 4.6. Confocal laser scanning microscopy images of *in vitro*-propagated olive plants showing the translocation of GFP-tagged *Pseudomonas savastanoi* NCPPB 3335 (Psv-GFP) from the hyperplasic tissue to the olive stems. Vibratome longitudinal sections of stems (40 µm thick) were made to show Psv-GFP internal colonization of olive vascular vessels (white arrows) away from the knot (**A**) and close to it (**B**, tumor marked by a red arrow) 6 weeks after pathogen inoculation. **C**. Presence of Psv-GFP in olive vascular vessels (white arrow) by the knot (red arrow) in a plant co-inoculated with *Pseudomonas fluorescens* PICF7. **D**, **E** and **F** are magnifications of **A**, **B** and **C**, respectively, showing details of olive stem vascular vessels profusely colonized by the fluorescently-tagged pathogen. Scale bar represents 200 µm in **A** and **B**, 65 µm in **C**, 5 µm in **D** and 20 µm in **E** and **F**.

4.4.6. *Pseudomonas fluorescens* PICF7 decreases *Pseudomonas savastanoi*-induced necrosis of olive knots in woody olive plants

To finally check whether the inoculation of PICF7 induced the same effects in lignified olive plants than those observed on *in vitro* micropropagated explants, bioassays using pot-acclimated olive plants of two ages (1- and 2-year-old) and with lignified stems were carried out. Results showed that Psv/PICF7 co-inoculation did not significantly suppress olive knot disease onset and development in these plants. No significant differences ($P > 0.05$) were observed between plants treated or not with the BCA in volume, weight and density of analyzed tumors in both experiments (**Figure 4.S2**). However, a trend towards an average decrease in volume and fresh weight was observed for Psv/PICF7 co-inoculated olive plants of both ages (**Figure 4.S2**). In addition, transverse section of knots induced by Psv/PICF7-treated plants showed a reduced necrosis of the internal tissues in comparison to control knots induced in plants inoculated with Psv alone, suggesting a delay in the maturation process of knots induced by the presence of the BCA. This effect was more evident in 2-year-old (**Figure 4.S3**) than in 1-year-old (data not shown) plants, probably due to the higher susceptibility to Psv infection of younger plants (Penyalver et al., 2006; Pérez-Martínez et al., 2010).

4.5. Discussion

Successful management of olive knot disease is a complicated undertaking. The most frequent control measure (i.e. continuous application of copper-based bactericides), entails undesirable effects such as high costs, phytotoxicity and increasing risk of pathogen resistance (Krid et al., 2012). On the other hand, breeding for resistance has no current way of successful implementation since Psv-resistant cultivars are not yet available (Penyalver et al., 2006). Therefore, the use of BCAs appears as a promising control tool overcoming the adverse effects of chemical treatments and fitting modern sustainable agriculture criteria. Biological control of olive knot disease has been poorly explored, and only few studies have evaluated the effectiveness of diverse BCAs against Psv with variable results (Lavermicocca et al., 2002; Krid et al., 2012). The present study has examined the *in planta* interaction between *P. fluorescens* PICF7, an olive root endophyte effective against Verticillium wilt of olive (Mercado-Blanco et al., 2004; Prieto et al., 2009), and the causal agent of olive knot disease.

Results have proved that *P. fluorescens* PICF7: (i) inhibits the growth of Psv *in vitro* to a degree; (ii) colonizes and persists in stems of *in vitro*-propagated olive plants when artificially introduced; (iii) induces a transient decrease of Psv population on/in inoculated stem tissues; (iv) modifies the external macroscopic appearance of the tumors produced by the pathogen (less necrotic) on *in vitro*-propagated olive plants; (v) decreases the maturation process of Psv-induced tumors in woody olive plants (less internal necrosis); and (vi) alters the localization of the pathogen on/in tumors, that one being predominantly confined to internal regions of the knots. However, despite these consistent effects, PICF7 was not able to impair knot development under the experimental conditions here reported.

Pseudomonas fluorescens PICF7 is able to colonize and persist in both stems and roots of *in vitro*-propagated 'Arbequina' plants. Successful endophytic root colonization of *in vitro*-propagated olive explants by PICF7 has been corroborated even though plant material utilized in this present work differed in source, phenology and root system morphology than that used in a previous study (Prieto and Mercado-Blanco, 2008). Therefore, this BCA is able to endophytically colonize and persist in root tissues of different olive cultivars under diverse experimental conditions (Prieto et al., 2009, 2011; this study). On the other hand, this is the first study where an indigenous olive root inhabitant has been demonstrated to be successfully established in olive stem tissues after artificial inoculation, maintaining high population levels along time. Therefore, PICF7 can endure in different olive organs, opening new and interesting perspectives for its application as either preventive or palliative BCA in olive. Movement of PICF7 cells from roots to above-ground organs throughout the vascular system could not be faithfully assessed. Previous works using fluorescently tagged PICF7 discarded the presence of this bacterium neither within the xylem vessels of the roots nor in aerial tissues (Prieto and Mercado-Blanco, 2008; Prieto et al., 2011). Therefore, PICF7 cells occasionally found in stem tissue macerates could be explained by stem contamination during the bacterization process.

Strain PICF7 effectively antagonized Psv NCPPB 3335 *in vitro*, although nothing is known on what bacterial trait(s) could be responsible for such inhibitory effect. However, *in vivo* bioassays did not show an effective long-term control of Psv but a transitory drop of the pathogen population size (**Figures 4.1, 4.3**). Nevertheless, a clear modification of the macroscopic appearance of developed tumors (**Figure 4.2**), and a definitive alteration of the pathogen localization in knots (**Figures 4.4, 4.5**)

were observed when the BCA was present. Whether these phenomena are due to effective antibiosis mechanism(s) deployed by PICF7 *in planta* remain to be elucidated. The fall of pathogen population at early times after co-inoculation with the BCA was consistently observed in independent bioassays. This could be related to biosynthesis of inhibitory compounds by PICF7 affecting the pathogen, to a faster growth rate of the BCA compared with that of the pathogen, or to competition for space and nutrients inside olive tissues between the two bacteria. Regardless the mechanism involved, the transient drop of Psv population seemed to have a dramatic influence on the allocation of the pathogen on/in the tumor structure, a situation that could be observed either at macroscopic (**Figure 4.3**) and microscopic (**Figures 4.4, 4.5**) levels. Thus, whereas NCPPB 3335 predominantly colonized the surface of the tumor in the absence of the BCA (**Figure 4.5A,C,E**), the former was predominantly restricted to inner cavities of the tumor (**Figure 4.5B,D,F**). This shift in Psv localization may explain the different external appearance of knots when PICF7 was present (**Figure 4.2B,D**). Moreover, despite the fact that PICF7 could not effectively control knot development, changes observed when the BCA was present may pose important epidemiological consequences. Quesada and colleagues (2010a) demonstrated dissemination of epiphytic Psv cells over short distances within olive orchards, a phenomenon perhaps related to transportation of the pathogen in aerosols (Young, 2004). Indeed, the release of pathogen cells through knot exudates, which could serve as new inoculum source, has been previously related to the localization of Psv cells at the knot surface (Rodríguez-Moreno et al., 2009). Therefore, presence of PICF7 reducing the number of Psv cells located at the surface of the tumor could imply a decrease in the dissemination of the pathogen through knot exudates.

Some beneficial *Pseudomonas* spp., native colonizers of the rhizosphere of diverse plants, can elicit a specific systemic defence response against pathogens in their host plants, a phenomenon known as induced systemic resistance (ISR) (Bakker et al., 2007; Mercado-Blanco and Bakker, 2007). Recent functional genomics studies have demonstrated that colonization by *P. fluorescens* PICF7 induced a broad set of defensive responses in olive root tissues. For instance, the establishment of the BCA on/in roots of 'Arbequina' plants produced the differential expression of genes involved in, among others processes, plant hormones and phenylpropanoids biosynthesis, pathogen-related proteins synthesis and several transcription factor involved in systemic defensive responses, including ISR (Schilirò et al., 2012). Considering this antecedent, the olive-Psv interaction offered an excellent study system to examine whether PICF7 could trigger an effective systemic defence

response against olive knot disease, since the pathogen and the BCA can be applied spatially separated after artificial inoculation (Van Loon et al., 1998). Results showed that despite the fact that strain PICF7 effectively colonized the roots of *in vitro*-propagated plants, even endophytically, knot development on stems was not suppressed or altered, and populations of the pathogen in hyperplasic tissues were similar in both PICF7 root-bacterized and control plants. Therefore, while PICF7 is able to trigger a broad array of defensive responses in olive roots, including genes involved in ISR and systemic acquired resistance (SAR) responses (Schilirò et al., 2012), control of olive knot disease was not observed under experimental conditions assayed. A possible explanation would be that high virulence of the Psv strain used in this study (Rodríguez-Moreno et al., 2008) could overcome any potential systemic response from roots.

Invasion of newly formed xylem vessels inside olive knots induced by Psv strain NCPPB 3335 has been previously reported; however, pathogen cells could not be detected outside the knot area (Rodríguez-Moreno et al., 2009). An additional and interesting finding here performed was the demonstration that strain NCPPB 3335 is able to move from the inoculation site (and from developed tumors) through the xylem vessels. This phenomenon has been hardly evidenced before, particularly using a methodology that does not imply tissue fixation or staining procedure. Systemic invasion of oleander (*Nerium oleander* L.) plants through laticifers and, less frequently, through xylem vessels has been reported for *P. savastanoi* pv. nerii (Wilson and Magie, 1964). On the other hand, movement of Psv cells through the xylem vessels has been related to the formation of secondary knots in olive stems (Penyalver et al., 2006). Visualization of Psv cells within xylem cells in stained stem sections has been earlier reported (Marchi et al., 2009). However, the present study has clearly showed living GFP-tagged Psv cells directly visualized beyond the inoculation point.

In conclusion, by implementing powerful biotechnological and microscopy tools we have been able to uncover phenomena taking place during the interaction between a native bacterial endophyte of olive roots and a pathogen naturally occurring in above-ground tissues of the same plant host. This basic knowledge may have interesting practical information from an epidemiological point of view. Thus, although *P. fluorescens* PICF7 was not able to control olive knot disease, this BCA was demonstrated to colonize and establish in aerial olive tissues and to modify the colonization behaviour of the pathogen in tumors which, in addition, developed

abnormally. Findings here reported can also be of interest to unravel the complex interplay that this pathogen could maintain with the microbiological consortia residing within olive knots (Hosni et al., 2011). Finally, Psv was undoubtedly and *in vivo* visualized migrating from the tumors to areas far beyond the hyperplasic regions, using the xylem vessels to do so.

Acknowledgements

We thank I.M. Aragón, M.P. Castañeda-Ojeda and I.M. Matas for technical support. Authors also want to acknowledge Prof. Antonio Martín for the use of the CLSM.

Supplementary data

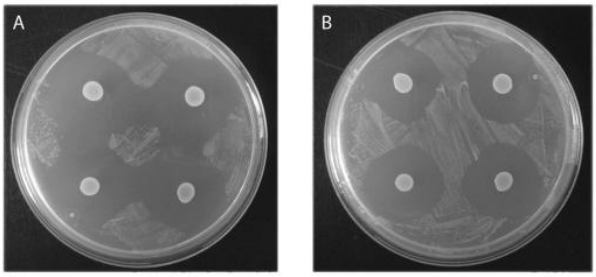


Figure 4.S1. Growth inhibition haloes generated by colonies of *Pseudomonas fluorescens* PICF7 (5 µl droplets, 10^8 cfu ml^{-1}) grown on PDA plates previously inoculated with *Pseudomonas savastanoi* NCPPB 3335 (100 µl, 10^5 cfu ml^{-1}). Plates were incubated at 25°C (**A**) and 28°C (**B**) during 72 h.

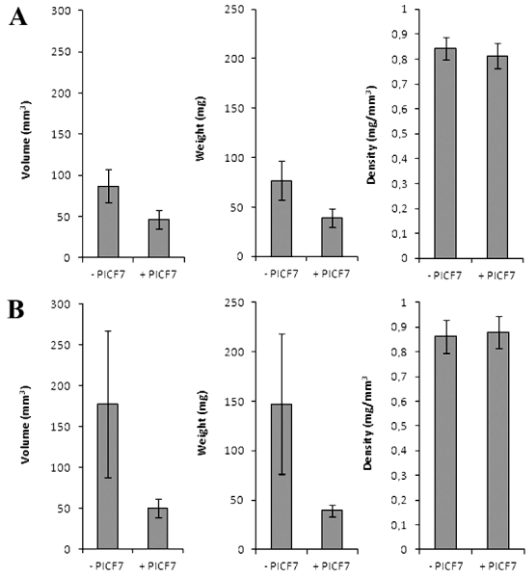


Figure 4.S2. Volume, fresh weight and density of knots developed on 1- (**A**) and 2-year-old (**B**) *in vitro* olive plants when *Pseudomonas savastanoi* NCPPB 3335 was inoculated in the presence (+PICF7) or the absence (−PICF7) of *Pseudomonas fluorescens* PICF7 at 92 days after inoculation. Error bars represent standard error. Mean values [n (tumors analyzed) = 12, in 1-year-old plants, and n = 15, in 2-year-old plants] were not significantly different ($P > 0.05$) according to Student's t-test.

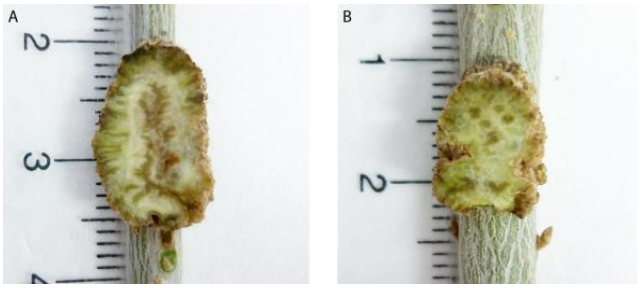
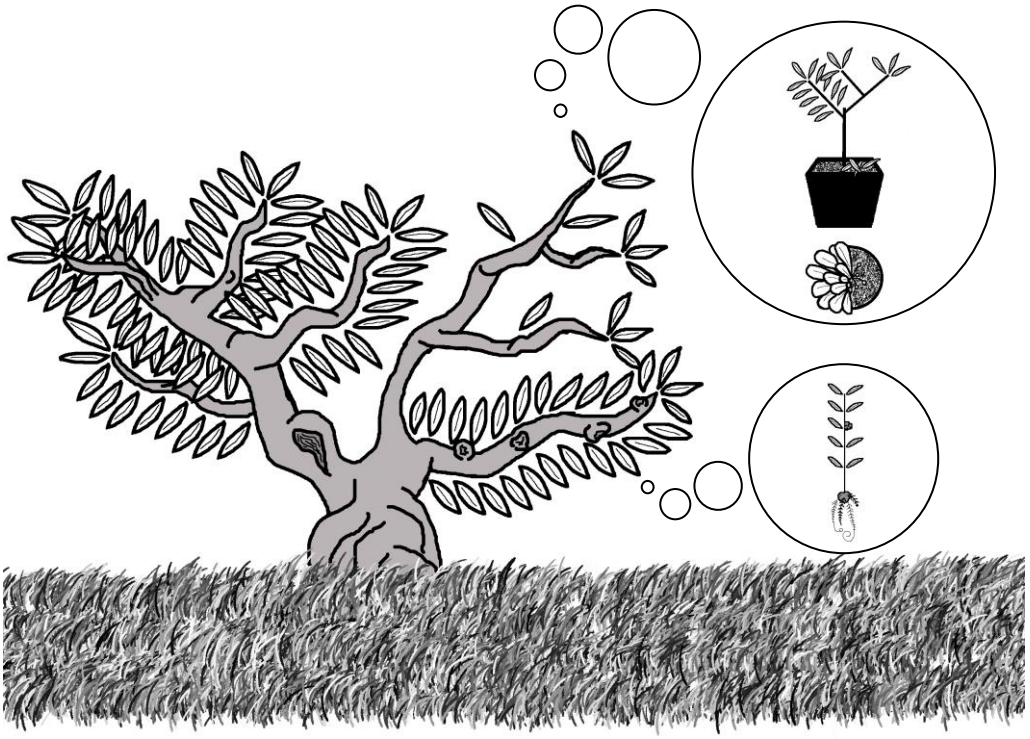


Figure 4.S3. Transversal section of representative knots produced by *Pseudomonas savastanoi* NCPPB 3335 when inoculated in the absence (A) or presence (B) of *Pseudomonas fluorescens* PICF7 (92 days after inoculation).

CHAPTER 5

Conclusions



1. The generation of a random transposon insertion mutant library of *Pseudomonas fluorescens* PICF7 will allow, in combination with other biotechnological tools already available, the search for bacterial phenotypes related with biological control effectiveness, endophytic lifestyle, and/or other relevant ecological, environmental or metabolic traits of this biological control agent (BCA) (Chapter 2).

2. Pyoverdine production and swimming motility are not needed for biocontrol of Verticillium wilt of olive. On the contrary, Cys auxotrophy diminished the ability of strain PICF7 to colonize and establish in the olive rhizosphere and reduced its effectiveness to suppress the disease. Therefore, low availability of Cys in the olive rhizosphere would explain why mutant ME1508 was less efficient in root/rhizosphere colonization and consequently in VWO suppression. However, the ability of this mutant to colonize the root interior remained unaffected even though rhizosphere populations of ME1508 were significantly lower than that of the parental strain. These results encourage future studies on the importance of olive root exudates, and their composition, in both colonization and disease suppression abilities of strain PICF7 (Chapter 2).

3. Current knowledge of bacterial traits implicated in endophytism is scant. Results from this thesis allow to conclude that production of the siderophore pyoverdine and swimming motility are not involved in endophytic colonization of olive roots by strain PICF7. Moreover, mutants altered in metabolic pathways (i.e. *gltA* and Cys auxotrophy) colonized inner tissues of olive roots to the same extent than the wild type, discarding also these phenotypes as needed for the endophytic lifestyle of strain PICF7 (Chapter 2).

4. Strain PICF7 mutants assessed in this thesis behaved similarly in olive and *Arabidopsis*. This outcome encourages the use of *A. thaliana* as a suitable alternative and/or complementary tool for the identification of PICF7 traits involved in the biocontrol of *Verticillium dahliae* (Chapters 2 and 3).

5. Strain PICF7 colonizes and persists on *Arabidopsis thaliana* roots. However, no evidence of endophytic colonization was obtained under the experimental conditions used in this thesis. The inability of PICF7 to colonize the root interior of this model plant in contrast to the endophytic lifestyle displayed in its natural host can help to understand what are the mechanisms underlying inner colonization ability by this bacterium (Chapters 2 and 3).

6. The similar behavior displayed by the defoliating (highly virulent) and non-defoliating (moderately virulent) olive pathotypes of *V. dahliae* in *A. thaliana* and olive support the use of the model plant to assess pathogenicity and virulence of *V. dahliae* isolates infecting olive, thereby saving the time and space usually required for pathogenicity tests performed with this woody host (Chapter 3).

7. The control of the foliar necrotrophic fungus *Botrytis cinerea* upon application of strain PICF7 to *Arabidopsis* roots suggests that this BCA is able to induce systemic resistance in the aerial tissues of the model plant. This result supports previous findings demonstrating that systemic defense responses are triggered in olive aerial tissues after root inoculation with strain PICF7 (Chapter 3).

8. However, the absence of effective suppression of olive knot disease (OKD) after *P. fluorescens* PICF7 colonization of olive roots indicates that this bacterium is unable to mount a systemic defense response successful against *P. savastanoi* pv. savastanoi (Psv), at least under the experimental conditions used in this thesis (Chapter 4).

9. In contrast, strain PICF7 is able to alter the macroscopic appearance of the tumors when co-inoculated with Psv into the stems. This phenomenon could be explained by the transient decrease of the pathogen population size and by the altered localization of Psv cells within the hyperplasic tissue, moving from the knot surface to the inner regions of the tumor when the BCA was present. These changes may pose important epidemiological consequences deserving further studies since the confinement of Psv towards the knot interior as a consequence of the presence of PICF7 may reduce pathogen dissemination from knot exudates. (Chapter 4).

10. Bacterial cells of Psv are able to migrate from the inoculation site and/or developed tumors to areas far beyond the hyperplasic regions through the stem xylem vessels. This finding, hardly evidenced in the past by the use of methodologies such as tissue fixation or staining, has been undoubtedly proved using CLSM and *in vivo* visualization of Psv cells in fresh stem tissues (Chapter 4).

References

Afzal, M., Khan, Q. M., and Sessitsch, A. (2014). Endophytic bacteria: Prospects and applications for the phytoremediation of organic pollutants. *Chemosphere*, *117*, 232-242.

Agrios, G. N. (1997). Plant pathology. *Academic Press, San Diego*, 635.

Ahmad, A. A., Moretti, C., Valentini, F., Hosni, T., Farag, N. S., Galal, A. A., and Buonaurio, R. (2009). Olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* in Egypt. *Journal of Plant Pathology, 91*, 231-240.

Al-Ahmad, M. A., and Mosli, M. N. (1993). Verticillium wilt of olive in Syria. *EPPO Bulletin*, *23*(3), 521-529.

Al-Ahmad, M. A., Moselli, N., and Doksi, A. (1992). Verticillium wilt of olive and the effects of variety, age of trees and other agricultural practices on disease development in middle and northern Syria. *Arab Journal of Plant Protection*, *10*(2), 131-139.

Ali, S., Duan, J., Charles, T. C., and Glick, B. R. (2014). A bioinformatics approach to the determination of genes involved in endophytic behavior in *Burkholderia* spp. *Journal of Theoretical Biology*, *343*, 193-198.

Alit-Susanta, W. G. N., and Takikawa, Y. (2006). Phenotypic characterization of *Pseudomonas fluorescens* PfG32R and its spontaneous *gacS* mutants and biocontrol activity against bacterial wilt disease of tomato. *Journal of General Plant Pathology*, *72*(3), 168-175.

Alizadeh, H., Behboudi, K., Ahmadzadeh, M., Javan-Nikkhah, M., Zamioudis, C., Pieterse, C. M., and Bakker, P. A. H. M. (2013). Induced systemic resistance in cucumber and *Arabidopsis thaliana* by the combination of *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14. *Biological Control*, *65*(1), 14-23.

Alström, S. (2001). Characteristics of bacteria from oilseed rape in relation to their biocontrol activity against *Verticillium dahliae*. *Journal of Phytopathology*, *149*(2), 57-64.

Al-Taae, A. K., and Al-Taae, H. H. (2010). Molecular diagnosis of *Verticillium dahliae* Kleb; the causal agent of Verticillium wilt of olive. *Jordan Journal of Agricultural Sciences*, *6*(1).

Altschul, S. F., Madden, T. L., Schäffer, A. A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D. J. (1997). Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research*, *25*(17), 3389-3402.

Amiot, M. J. (2014). Olive oil and health effects: from epidemiological studies to the molecular mechanisms of phenolic fraction. *OCL*, *21*(5), D512.

Angiolillo, A., Mencuccini, M., and Baldoni, L. (1999). Olive genetic diversity assessed using amplified fragment length polymorphisms. *Theoretical and Applied Genetics*, *98*(3-4), 411-421.

Antoniou, P. P., Markakis, E. A., Tjamos, S. E., Paplomatas, E. J., and Tjamos, E. C. (2008). Novel methodologies in screening and selecting olive varieties and root-stocks for resistance to *Verticillium dahliae*. *European Journal of Plant Pathology*, *122*(4), 549-560.

Antonopoulos, D. F., Tjamos, S. E., Antoniou, P. P., Rafeletos, P., and Tjamos, E. C. (2008). Effect of *Paenibacillus alvei*, strain K165, on the germination of *Verticillium dahliae* microsclerotia in planta. *Biological Control*, *46*(2), 166-170.

Aranda, S., Montes-Borrego, M., Jiménez-Díaz, R. M., and Landa, B. B. (2011). Microbial communities associated with the root system of wild olives (*Olea europaea* L. subsp. *europaea* var. *sylvestris*)

are good reservoirs of bacteria with antagonistic potential against *Verticillium dahliae*. *Plant and Soil*, *343*(1-2), 329-345.

Arbogast, M., Powelson, M. L., Cappaert, M. R., and Watrud, L. S. (1999). Response of six potato cultivars to amount of applied water and *Verticillium dahliae*. *Phytopathology*, *89*(9), 782-788.

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

Ark, P. A., and Thompson, J. P. (1960). Experimental greenhouse control of crown gall and olive knot with antibiotic drenches. *Plant Disease Reporter*, *44*, 197-199.

Arnold, A. E. (2007). Understanding the diversity of foliar endophytic fungi: progress, challenges, and frontiers. *Fungal Biology Reviews*, *21*(2), 51-66.

Atallah, Z. K., Bae, J., Jansky, S. H., Rouse, D. I., and Stevenson, W. R. (2007). Multiplex real-time quantitative PCR to detect and quantify *Verticillium dahliae* colonization in potato lines that differ in response to Verticillium wilt. *Phytopathology, 97*(7), 865-872.

Audenaert, K., Pattery, T., Cornelis, P., and Höfte, M. (2002). Induction of systemic resistance to *Botrytis cinerea* in tomato by *Pseudomonas aeruginosa* 7NSK2: role of salicylic acid, pyochelin, and pyocyanin. *Molecular Plant-Microbe Interactions*, *15*(11), 1147-1156.

Avilés, M., Borrero, C., and Trillas, M. I. (2011). Review on compost as an inducer of disease suppression in plants grown in soilless culture. *Special Issue Compost III—dynamic plant, dynamic soil. Global Science Books*, *5*, 1-11.

Azcón, R., Medina, A., Aroca, R., and Ruiz-Lozano, J. M. (2013). Abiotic stress remediation by the arbuscular mycorrhizal symbiosis and rhizosphere bacteria/yeast interactions. *Molecular microbial ecology of the rhizosphere. Wiley-Blackwell, Hoboken*, 991-1003.

Bacon, C. W., and Hinton, D. M. (2006). Bacterial endophytes: the endophytic niche, its occupants, and its utility. *Plant-associated bacteria. Springer Netherlands*, 155-194.

Baídez, A. G., Gómez, P., del Río, J. A., and Ortuño, A. (2007). Dysfunctionality of the xylem in *Olea europaea* L. plants associated with the infection process by *Verticillium dahliae* Kleb. Role of phenolic compounds in plant defense mechanisms. *Journal of Agricultural and Food Chemistry, 55*(9), 3373-3377.

Bais, H. P., Weir, T. L., Perry, L. G., Gilroy, S., and Vivanco, J. M. (2006). The role of root exudates in rhizosphere interactions with plants and other organisms. *Annual Review of Plant Biology*, *57*, 233-266.

Bakker, P. A. H. M., Pieterse, C. M. J., and Van Loon, L. C. (2007). Induced systemic resistance by fluorescent *Pseudomonas* spp. *Phytopathology*, *97*(2), 239-243.

Bakker, P. A., Ran, L., and Mercado-Blanco, J. (2014). Rhizobacterial salicylate production provokes headaches!. *Plant and Soil*, *382*(1-2), 1-16.

Bakker, P. A. H. M., Ran, L. X., Pieterse, C. M. J., and Van Loon, L. C. (2003). Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. *Canadian Journal of Plant Pathology*, *25*(1), 5-9.

Balestra, G. M., and Varvaro, L. (1997). Influence of nitrogen fertilization on the colonization of olive phylloplane by *Pseudomonas syringae* subsp. *savastanoi*. *Pseudomonas Syringae Pathovars and Related Pathogens*. *Springer Netherlands*, 88-92.

Balestra, G. M., Lamichhane, J. R., Kshetri, M. B., Mazzaglia, A., and Varvaro, L. (2009). First report of olive knot caused by *Pseudomonas savastanoi* pv. savastanoi in Nepal. *Plant Pathology*, *58*(2), 393-393.

Barahona, E., Navazo, A., Martínez-Granero, F., Zea-Bonilla, T., Pérez-Jiménez, R. M., Martín, M., and Rivilla, R. (2011). *Pseudomonas fluorescens* F113 mutant with enhanced competitive colonization ability and improved biocontrol activity against fungal root pathogens. *Applied and Environmental Microbiology*, *77*(15), 5412-5419.

Baratta, B., and Di Marco, L. (1981). Controllo degli attacchi di'rogna'nella cultivar di olivo Nocellara del Belice [in Sicilia]. *Informatore Fitopatologico* (in Italian).

Barbara, D. J., and Clewes, E. (2003). Plant pathogenic *Verticillium* species: how many of them are there?. *Molecular Plant Pathology, 4*(4), 297-305.

Bardaji, L., Pérez-Martínez, I., Rodríguez-Moreno, L., Rodríguez-Palenzuela, P., Sundin, G. W., Ramos, C., and Murillo, J. (2011). Sequence and role in virulence of the three plasmid complement of the model tumor-inducing bacterium *Pseudomonas savastanoi* pv. savastanoi NCPPB 3335. *PloS One*, *6*(10), e25705.

Barranco, D. (1998). Variedades y patrones. *El cultivo del olivo*, *6*, 63-92 (in Spanish).

Basim, H., and Ersoy, A. (2001). Identification of *Pseudomonas savastanoi* pv. savastanoi, olive knot pathogen, by polymerase chain reaction. *Abstracts, Phytopathology Salt Lake*, *91*(S6), 25-29.

Bechtold, N., Ellis, J., and Pelletier, G. (1993). *In planta Agrobacterium* mediated gene transfer by infiltration of adult *Arabidopsis thaliana* plants. *Comptes rendus de l'Académie des sciences. Série 3, Sciences de la Vie*, *316*(10), 1194-1199.

Bejarano-Alcázar, J., Blanco-López, M. A., Melero-Vara, J. M., and Jiménez-Díaz, R. M. (1996). Etiology, importance, and distribution of Verticillium wilt of cotton in southern Spain. *Plant Disease, 80*(11), 1233-1238.

Bella, P., Licciardello, G., Tessitori, M., and Catara, V. (2009). A real-time PCR quantitative detection assay for *Pseudomonas savastanoi* pv. *nerii* in *Nerium oleander*. *Phytopathologia Mediterranea*, *47*(3), 205-213.

Bellahcene, M., Fortas, Z., Geiger, J. P., Matallah, A., and Henni, D. (2000). Verticillium wilt in olive in Algeria: Geographic distribution and extent of the disease. *Olivae*, (82), 41-43.

Benítez, T., Rincón, A. M., Limón, M. C., and Codón, A. C. (2004). Biocontrol mechanisms of *Trichoderma* strains. *International Microbiology*, *7*, 249-260.

Benjama, A. (1994). Étude de la sensibilité variétale de l'olivier au Maroc vis-à-vis de *Pseudomonas syringae* pv. *savastanoi*, agent de la tuberculose. *Cahiers Agricultures, 3*(6), 405-408 (in French).

Bennett, M. D., Leitch, I. J., Price, H. J., and Johnston, J. S. (2003). Comparisons with *Caenorhabditis* (~ 100 Mb) and Drosophila (~ 175 Mb) using flow cytometry show genome size in Arabidopsis to be~ 157 Mb and thus~ 25% larger than the Arabidopsis genome initiative estimate of~ 125 Mb. *Annals of Botany*, *91*(5), 547-557.

Berendsen, R. L., Pieterse, C. M., and Bakker, P. A. (2012). The rhizosphere microbiome and plant health. *Trends in Plant Science*, *17*(8), 478-486.

Berg, G. (2000). Diversity of antifungal and plant-associated *Serratia plymuthica* strains. *Journal of Applied Microbiology*, *88*(6), 952-960.

Berg, G., and Hallmann, J. (2006). Control of plant pathogenic fungi with bacterial endophytes. *Microbial Root Endophytes*, 53-69.

Berg, G., Grube, M., Schloter, M., and Smalla, K. (2014). Unraveling the plant microbiome: looking back and future perspectives. *Frontiers in Microbiology*, *5*, 148.

Berg, G., Opelt, K., Zachow, C., Lottmann, J., Gotz, M., Costa, R., and Smalla, K. (2006). The rhizosphere effect on bacteria antagonistic towards the pathogenic fungus *Verticillium* differs depending on plant species and site. *FEMS Microbiology Ecology*, *56*(2), 250-261.

Bertolini, E., Olmos, A., López, M. M., and Cambra, M. (2003a). Multiplex nested reverse transcription-polymerase chain reaction in a single tube for sensitive and simultaneous detection of four RNA viruses and *Pseudomonas savastanoi* pv. *savastanoi* in olive trees. *Phytopathology*, *93*(3), 286-292.

Bertolini, E., Penyalver, R., García, A., Olmos, A., Quesada, J. M., Cambra, M., and López, M. M. (2003b). Highly sensitive detection of *Pseudomonas savastanoi* pv. *savastanoi* in asymptomatic olive plants by nested-PCR in a single closed tube. *Journal of Microbiological Methods*, *52*(2), 261-266.

Besnard, G., Baradat, P., and Bervillé, A. (2001). Genetic relationships in the olive (*Olea europaea* L.) reflect multilocal selection of cultivars. *Theoretical and Applied Genetics*, *102*(2-3), 251-258.

Bhat, R. G., and Subbarao, K. V. (1999). Host range specificity in *Verticillium dahliae*. *Phytopathology*, *89*(12), 1218-1225.

Bhat, R. G., and Subbarao, K. V. (2001). Reaction of broccoli to isolates of *Verticillium dahliae* from various hosts. *Plant Disease*, *85*(2), 141-146.

Bhat, R. G., Smith, R. F., Koike, S. T., Wu, B. M., and Subbarao, K. V. (2003). Characterization of *Verticillium dahliae* isolates and wilt epidemics of pepper. *Plant Disease*, *87*(7), 789-797.

Binet, M. N., Lemoine, M. C., Martin, C., Chambon, C., and Gianinazzi, S. (2007). Micropropagation of olive (*Olea europaea* L.) and application of mycorrhiza to improve plantlet establishment. *In Vitro Cellular and Developmental Biology-Plant*, *43*(5), 473-478.

Blanco-López, M. A., and DeVay, J. E. (1987). Effect of different irrigation regimes on Verticillium wilt, proline content and Leaf water potential in cotton. In *Proceedings VII Congress of the Mediterranean Phytopathological Union, Granada, Spain*.

Blanco-López, M. A., and Jiménez-Díaz, R. M. (1995). Una propuesta de lucha integrada contra la Verticilosis del olivo. *Fruticultura Profesional*, *70*, 52-58 (in Spanish).

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

Bloemberg, G. V., Wijfjes, A. H., Lamers, G. E., Stuurman, N., and Lugtenberg, B. J. (2000). Simultaneous imaging of *Pseudomonas fluorescens* WCS365 populations expressing three different

autofluorescent proteins in the rhizosphere: new perspectives for studying microbial communities. *Molecular Plant-Microbe Interactions*, *13*(11), 1170-1176.

Böhm, M., Hurek, T., and Reinhold-Hurek, B. (2007). Twitching motility is essential for endophytic rice colonization by the N2-fixing endophyte *Azoarcus* sp. strain BH72. *Molecular Plant-Microbe Interactions*, *20*(5), 526-533.

Boller, T., and Felix, G. (2009). A renaissance of elicitors: perception of microbe-associated molecular patterns and danger signals by pattern-recognition receptors. *Annual Review of Plant Biology*, *60*, 379-406.

Bompadre, M. J., Pérgola, M., Fernández Bidondo, L., Colombo, R. P., Silvani, V. A., Pardo, A. G., Ocampo, J. A., and Godeas, A. M. (2014). Evaluation of Arbuscular Mycorrhizal Fungi Capacity to Alleviate Abiotic Stress of Olive (*Olea europaea* L.) Plants at Different Transplant Conditions. *The Scientific World Journal*, *2014*. DOI:10.1155/2014/378950.

Bubici, G., and Cirulli, M. (2012). Control of Verticillium wilt of olive by resistant rootstocks. *Plant and Soil*, *352*(1-2), 363-376.

Bubici, G., and Cirulli, M. (2014). Natural recovery from Verticillium wilt in olive: can it be exploited in a control strategy?. *Plant and Soil*, *381*(1-2), 85-94.

Bull, C. T., De Boer, S. H., Denny, T. P., Firrao, G., Saux, M. F. L., Saddler, G. S., Scortichini, M., Stead, D. E., and Takikawa, Y. (2010). Comprehensive list of names of plant pathogenic bacteria, 1980-2007. *Journal of Plant Pathology*, *92*, 551-592.

Bultreys, A. (2007). Siderotyping, a tool to characterize, classify and identify fluorescent Pseudomonads. *In Microbial Siderophores. Springer Berlin Heidelberg*, 67-89.

Buysens, S., Heungens, K., Poppe, J., and Hofte, M. (1996). Involvement of pyochelin and pyoverdin in suppression of *Pythium*-induced damping-off of tomato by *Pseudomonas aeruginosa* 7NSK2. *Applied and Environmental Microbiology*, *62*(3), 865-871.

Caballero, P., and Murillo, J. (2003). Protección de cultivos. *Conceptos actuales y fuentes de información*, *Universidad Pública de Navarra*, *Spain* (in Spanish).

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

Caetano-Anollés, G. (1993). Amplifying DNA with arbitrary oligonucleotide primers. *Genome Research*, *3*(2), 85-94.

Calderón, R., Lucena, C., Trapero-Casas, J. L., Zarco-Tejada, P. J., and Navas-Cortés, J. A. (2014). Soil Temperature determines the reaction of olive cultivars to *Verticillium dahliae* pathotypes. *PloS One*, *9*(10), e110664.

Calvente, R., Cano, C., Ferrol, N., Azcón-Aguilar, C., and Barea, J. M. (2004). Analysing natural diversity of arbuscular mycorrhizal fungi in olive tree (*Olea europaea* L.) plantations and assessment of the effectiveness of native fungal isolates as inoculants for commercial cultivars of olive plantlets. *Applied Soil Ecology*, *26*(1), 11-19.

Campbell, C. L., and Madden, L. V. (1990). Introduction to plant disease epidemiology. *John Wiley and Sons, New York.*

Capdevila, S., Martínez-Granero, F. M., Sánchez-Contreras, M., Rivilla, R., and Martín, M. (2004). Analysis of *Pseudomonas fluorescens* F113 genes implicated in flagellar filament synthesis and their role in competitive root colonization. *Microbiology*, *150*(11), 3889-3897.

Carder, J. H., and Barbara, D. J. (1991). Molecular variation and restriction fragment length polymorphisms (RFLPs) within and between six species of *Verticillium*. *Mycological Research*, *95*(8), 935-942.

Carder, J. H., Morton, A., Tabrett, A. M., and Barbara, D. J. (1994). Detection and differentiation by PCR of subspecific groups within two *Verticillium* species causing vascular wilts in herbaceous hosts. *Modern Assays for Plant Pathogenic Fungi: Identification, Detection and Quantification*, 91-97.

Castillo, P., Nico, A. I., Azcón-Aguilar, C., Del Río Rincón, C., Calvet, C., and Jiménez-Díaz, R. M. (2006). Protection of olive planting stocks against parasitism of root-knot nematodes by arbuscular mycorrhizal fungi. *Plant Pathology*, *55*(5), 705-713.

Castillo, P., Nico, A. I., Navas-Cortés, J. A., Landa, B. B., Jiménez-Díaz, R. M., and Vovlas, N. (2010). Plant-parasitic nematodes attacking olive trees and their management. *Plant Disease*, *94*(2), 148-162.

Catara, V., Colina, P., Bella, P., Tessitori, M., and Tirrò, A. (2005). Variabilità di *Pseudomonas savastanoi* pv. *savastanoi* in un'area olivicola della Sicilia e comportamento di alcune varietà di olivo alle inoculazioni [*Olea europaea* L.]. *Tecnica Agricola*, *57*(1-2), 41-52 (in Italian).

Ceccherini, M. T., Luchi, N., Pantani, O. L., Ascher, J., Capretti, P., and Pietramellara, G. (2013). Upward movement of *Verticillium dahliae* from soil to olive plants detected by qPCR. *World Journal of Microbiology and Biotechnology*, *29*(10), 1961-1967.

Chen, C., Bauske, E. M., Musson, G., Rodriguezkabana, R., and Kloepper, J. W. (1995). Biological control of Fusarium wilt on cotton by use of endophytic bacteria. *Biological Control*, *5*(1), 83-91.

Cherrab, M., Bennani, A., Charest, P. M., and Serrhini, M. N. (2002). Pathogenicity and vegetative compatibility of *Verticillium dahliae* Kleb. isolates from olive in Morocco. *Journal of Phytopathology, 150*(11-12), 703-709.

Chin-A-Woeng, T. F., Bloemberg, G. V., Mulders, I. H., Dekkers, L. C., and Lugtenberg, B. J. (2000). Root colonization by phenazine-1-carboxamide-producing bacterium *Pseudomonas chlororaphis* PCL1391 is essential for biocontrol of tomato foot and root rot. *Molecular Plant-Microbe Interactions*, *13*(12), 1340-1345.

Chliyeh, M., Touhami, A. O., Filali-Maltouf, A., El, C., Modafar, A. M., Oukabli, A., Benkirane, R., and Douira, A. (2014). Effect of a composite endomycorrhizal inoculum on the growth of olive trees under nurseries conditions in Morocco. *International Journal of Pure and Applied Bioscience*, *2*(2), 1-14.

Cirulli, M. (1981). Attuali cognizioni sulla verticilliosi dell'olivo. *Informatore Fitopatologico*, *31*(1-2), 101-105 (in Italian).

Civantos, L. (2008). Obtención del aceite de oliva virgen. *Editorial Agrícola Española, Madrid, Spain* (in Spanish).

Clough, S. J., and Bent, A. F. (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *The plant journal*, *16*(6), 735-743.

Collado-Romero, M., Berbegal, M., Jiménez-Díaz, R. M., Armengol, J., Mercado-Blanco, J. (2009). A PCR-based "molecular tool box" for *in planta* differential detection of *Verticillium dahliae* vegetative compatibility groups infecting artichoke. *Plant Pathology*, *58*(3), 515-526.

Collado-Romero, M., Jiménez-Díaz, R. M., and Mercado-Blanco, J. (2010). DNA sequence analysis of conserved genes reveals hybridization events that increase genetic diversity in *Verticillium dahliae*. *Fungal Biology*, *114*(2), 209-218.

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., and Jiménez-Díaz, R. M. (2008). Phylogenetic analysis of *Verticillium dahliae* vegetative compatibility groups. *Phytopathology*, *98*(9), 1019-1028.

Collado-Romero, M., Mercado-Blanco, J., Olivares-García, C., Valverde-Corredor, A., and Jiménez-Díaz, R. M. (2006). Molecular variability within and among *Verticillium dahliae* vegetative compatibility groups determined by fluorescent amplified fragment length polymorphism and polymerase chain reaction markers. *Phytopathology*, *96*(5), 485-495.

Collins, A., Okoli, C. A. N., Morton, A., Parry, D., Edwards, S. G., and Barbara, D. J. (2003). Isolates of *Verticillium dahliae* pathogenic to crucifers are of at least three distinct molecular types. *Phytopathology*, *93*(3), 364-376.

Comai, L., and Kosuge, T. (1980). Involvement of plasmid deoxyribonucleic acid in indoleacetic acid synthesis in *Pseudomonas savastanoi*. *Journal of Bacteriology*, *143*(2), 950-957.

Compant, S., Clément, C., and Sessitsch, A. (2010). Plant growth-promoting bacteria in the rhizo-and endosphere of plants: their role, colonization, mechanisms involved and prospects for utilization. *Soil Biology and Biochemistry*, *42*(5), 669-678.

Compant, S., Duffy, B., Nowak, J., Clément, C., and Barka, E. A. (2005). Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. *Applied and Environmental Microbiology*, *71*(9), 4951-4959.

Conn, V. M., Walker, A. R., and Franco, C. M. M. (2008). Endophytic actinobacteria induce defense pathways in *Arabidopsis thaliana*. *Molecular Plant-Microbe Interactions*, *21*(2), 208-218.

Connor, D. J. (2005). Adaptation of olive (*Olea europaea* L.) to water-limited environments. *Crop and Pasture Science*, *56*(11), 1181-1189.

Connor, D. J., Gómez-del-Campo, M., Rousseaux, M. C., and Searles, P. S. (2014). Structure, management and productivity of hedgerow olive orchards: a review. *Scientia Horticulturae*, *169*, 71-93.

Cornelis, P., and Matthijs, S. (2002). Diversity of siderophore-mediated iron uptake systems in fluorescent pseudomonads: not only pyoverdines. *Environmental Microbiology*, *4*(12), 787-798.

Costa, R., Salles, J. F., Berg, G., and Smalla, K. (2006). Cultivation independent analysis of *Pseudomonas* species in soil and in the rhizosphere of field-grown *Verticillium dahliae* host plants. *Environmental Microbiology*, *8*(12), 2136-2149.

Daayf, F., Nicole, M., and Geiger, J. P. (1995). Differentiation of *Verticillium dahliae* populations on the basis of vegetative compatibility and pathogenicity on cotton. *European Journal of Plant Pathology*, *101*(1), 69-79.

Dag, A., Yermiyahu, U., Ben-Gal, A., Zipori, I., and Kapulnik, Y. (2009). Nursery and post-transplant field response of olive trees to arbuscular mycorrhizal fungi in an arid region. *Crop and Pasture Science*, *60*(5), 427-433.

Debode, J., De Maeyer, K., Perneel, M., Pannecoucque, J., Backer, G. D., and Höfte, M. (2007). Biosurfactants are involved in the biological control of *Verticillium* microsclerotia by *Pseudomonas* spp. *Journal of Applied Microbiology*, *103*(4), 1184-1196.

Debode, J., Van Poucke, K., França, S. C., Maes, M., Höfte, M., and Heungens, K. (2011). Detection of multiple *Verticillium* species in soil using density flotation and real-time polymerase chain reaction. *Plant Disease*, *95*(12), 1571-1580.

Dennis, P. G., Miller, A. J., and Hirsch, P. R. (2010). Are root exudates more important than other sources of rhizodeposits in structuring rhizosphere bacterial communities?. *FEMS microbiology Ecology*, *72*(3), 313-327.

Dervis, S., Erten, L., Soylu, S., Tok, F. M., Kurt, S., Yildiz, M., and Soylu, E. M. (2007). Vegetative compatibility groups in *Verticillium dahliae* isolates from olive in western Turkey. *European journal of Plant Pathology*, *119*(4), 437-447.

Dervis, S., Kurt, S., Soylu, S., Erten, L., Soylu, E. M., Yildiz, M., and Tok, F. M. (2008). Vegetative compatibility groups of *Verticillium dahliae* from cotton in the south-eastern Anatolia region of Turkey. *Phytoparasitica*, *36*(1), 74-83.

Dervis, S., Mercado-Blanco, J., Erten, L., Valverde-Corredor, A., and Pérez-Artés, E. (2010). Verticillium wilt of olive in Turkey: A survey of disease importance, pathogen diversity and susceptibility of relevant olive cultivars. *European Journal of Plant Pathology*, *127*(2), 287-301.

DeVay, J. E., and Pullman, G. S. (1984). Epidemiology and ecology of diseases caused by *Verticillium* species, with emphasis on Verticillium wilt cotton. *Phytopathologia Mediterranea*, *23*, 95-108.

De Vleesschauwer, D., and Höfte, M. (2009). Rhizobacteria-induced systemic resistance. *Advances in Botanical Research*, *51*, 223-281.

Déziel, E., Comeau, Y., and Villemur, R. (2001). Initiation of biofilm formation *by Pseudomonas aeruginosa* 57RP correlates with emergence of hyperpiliated and highly adherent phenotypic variants deficient in swimming, swarming, and twitching motilities. *Journal of Bacteriology*, *183*(4), 1195-1204.

Djavaheri, M. (2007). Iron-regulated metabolites of plant growth promoting *Pseudomonas fluorescens* WCS374: their role in induced systemic resistance. *PhD Thesis, Utrecht university, Utrecht*.

Djavaheri, M., Mercado-Blanco, J., Versluis, C., Meyer, J. M., Loon, L. C., and Bakker, P. A. (2012). Iron-regulated metabolites produced by *Pseudomonas fluorescens* WCS374r are not required for eliciting induced systemic resistance against *Pseudomonas syringae* pv. *tomato* in Arabidopsis. *MicrobiologyOpen*, *1*(3), 311-325.

Dobinson, K. F., Harrington, M. A., Omer, M., and Rowe, R. C. (2000). Molecular characterization of vegetative compatibility group 4A and 4B isolates of *Verticillium dahliae* associated with potato early dying. *Plant Disease*, *84*(11), 1241-1245.

Dobinson, K. F., Patterson, N. A., White, G. J., and Grant, S. (1998). DNA fingerprinting and vegetative compatibility analysis indicate multiple origins for *Verticillium dahliae* race 2 tomato isolates from Ontario, Canada. *Mycological Research*, *102*(9), 1089-1095.

Docampo, D. M., Vázquez, A. A., and Laguna, I. G. (1981). *Verticillium dahliae* kleb, causal de la parálisis parcial del olivo en el área olivera centro oeste argentina. *RIA*, *16*(2), 215-240 (in Spanish).

Dong, X. (2004). NPR1, all things considered. *Current Opinion in Plant Biology*, *7*(5), 547-552.

Driver, J. A., and Kuniyuki, A. H. (1984). *In vitro* propagation of paradox walnut rootstocks. *HortScience: a publication of the American Society for Horticultural Science*, *19*, 507-509.

Dung, J. K., Peever, T. L., and Johnson, D. A. (2013). *Verticillium dahliae* populations from mint and potato are genetically divergent with predominant haplotypes. *Phytopathology*, *103*(5), 445-459.

Duressa, D., Rauscher, G., Koike, S. T., Mou, B., Hayes, R. J., Maruthachalam, K., Subbarao, K. V., and Klosterman, S. J. (2012). A real-time PCR assay for detection and quantification of *Verticillium dahliae* in spinach seed. *Phytopathology*, *102*(4), 443-451.

Durrant, W. E., and Dong, X. (2004). Systemic acquired resistance. *Annual Review of Phytopathology*, *42*, 185-209.

Durland, R. H., and Helinski, D. R. (1987). The sequence encoding the 43-kilodalton trfA protein is required for efficient replication or maintenance of minimal RK2 replicons in *Pseudomonas aeruginosa*. *Plasmid*, *18*(2), 164-169.

Easton, G. M., Nagle, M. E., and Bailey, D. L. (1969). A method for estimating *Verticillium albo-atrum* propagules in field soil and irrigation water. *Phytopathology*, *59*, 1171-1172.

Elena, K. (1999). Genetic relationships among *Verticillium dahliae* isolates from cotton in Greece based on vegetative compatibility. *European Journal of Plant Pathology*, *105*(6), 609-616.

El Hadrami, A., Adam, L. R., and Daayf, F. (2011). Biocontrol treatments confer protection against *Verticillium dahliae* infection of potato by inducing antimicrobial metabolites. *Molecular Plant-Microbe Interactions*, *24*(3), 328-335.

El-Zik, K. M. (1985). Integrated control of Verticillium wilt of cotton. *Plant Disease*, *69*(12), 1025-1032.

Erdogan, O., and Benlioglu, K. (2010). Biological control of Verticillium wilt on cotton by the use of fluorescent *Pseudomonas* spp. under field conditions. *Biological Control, 53*(1), 39-45.

Ercolani, G. L. (1978). *Pseudomonas savastanoi* and other bacteria colonizing the surface of olive leaves in the field. *Journal of General Microbiology*, *109*(2), 245-257.

Estaún, V., Camprubí, A., Calvet, C., and Pinochet, J. (2003). Nursery and field response of olive trees inoculated with two arbuscular mycorrhizal fungi, *Glomus intraradices* and *Glomus mosseae*. *Journal of the American Society for Horticultural Science*, *128*(5), 767-775.

Fahleson, J., Lagercrantz, U., Hu, Q., Steventon, L. A., and Dixelius, C. (2003). Estimation of genetic variation among *Verticillium* isolates using AFLP analysis. *European Journal of Plant Pathology*, *109*(4), 361-371.

FAO. (2013). http://faostat3.fao.org/browse/Q/*/E.

Feldmann, K. A., and Marks, M. D. (1987). *Agrobacterium*-mediated transformation of germinating seeds of *Arabidopsis thaliana*: a non-tissue culture approach. *Molecular and General Genetics MGG*, *208*(1-2), 1-9.

Fernández, J. E., Moreno, F., Cabrera, F., Arrue, J. L., and Martín-Aranda, J. (1991). Drip irrigation, soil characteristics and the root distribution and root activity of olive trees. *Plant and Soil*, *133*(2), 239-251.

Fradin, E. F., and Thomma, B. P. H. J. (2006). Physiology and molecular aspects of *Verticillium* wilt diseases caused by *V. dahliae* and *V. albo-atrum*. *Molecular Plant Pathology*, *7*(2), 71-86.

Frankowski, J., Lorito, M., Scala, F., Schmid, R., Berg, G., and Bahl, H. (2001). Purification and properties of two chitinolytic enzymes of *Serratia plymuthica* HRO-C48. *Archives of Microbiology*, *176*(6), 421-426.

Fravel, D. R. (1988). Role of antibiosis in the biocontrol of plant diseases. *Annual Review of Phytopathology*, *26*(1), 75-91.

Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J. (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. *Science*, *261*(5122), 754-756.

Gayoso, C., de la Ilarduya, O. M., Pomar, F., and de Cáceres, F. M. (2007). Assessment of real-time PCR as a method for determining the presence of *Verticillium dahliae* in different Solanaceae cultivars. *European Journal of Plant Pathology*, *118*(3), 199-209.

Gamliel, A., Austerweil, M., and Kritzman, G. (2000). Non-chemical approach to soilborne pest management-organic amendments. *Crop Protection*, *19*(8), 847-853.

Garber, R. H., and Presley, J. T. (1971). Relation of air temperature to development of Verticillium wilt on cotton in the field. *Phytopathology*, *61*(2), 204.

Garcia-Ruiz, G. M., Trapero, C., Del Rio, C., and Lopez-Escudero, F. J. (2014). Evaluation of resistance of Spanish olive cultivars to *Verticillium dahliae* in inoculations conducted in greenhouse. *Phytoparasitica*, *42*(2), 205-212.

Gardan, L., Bollet, C., Abu Ghorrah, M., Grimont, F., and Grimont, P. A. D. (1992). DNA relatedness among the pathovar strains of *Pseudomonas syringae* subsp. *savastanoi* Janse (1982) and proposal of *Pseudomonas savastanoi* sp. nov. *International Journal of Systematic Bacteriology*, *42*(4), 606-612.

Gardan, L., Shafik, H., Belouin, S., Broch, R., Grimont, F. and Grimont, P. A. D. (1999). DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov.(ex Sutic and Dowson 1959). *International Journal of Systematic Bacteriology*, *49*(2), 469-478.

Garmendia, I., Goicoechea, N., and Aguirreolea, J. (2004). Effectiveness of three *Glomus* species in protecting pepper (*Capsicum annuum* L.) against Verticillium wilt. *Biological Control*, *31*(3), 296-305.

Garmendia, I., Goicoechea, N., and Aguirreolea, J. (2005). Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against *Verticillium*-induced wilt in pepper. *Mycorrhiza*, *15*(5), 345-356.

Gaworzewska, E. T., and Carlile, M. J. (1982). Positive chemotaxis of *Rhizobium leguminosarum* and other bacteria towards root exudates from legumes and other plants. *Journal of general Microbiology*, *128*(6), 1179-1188.

Gharbi, Y., Triki, M. A., Trabelsi, R., Fendri, I., Daayf, F., and Gdoura, R. (2015). Genetic structure of *Verticillium dahliae* isolates infecting olive trees in Tunisia using AFLP, pathogenicity and PCR markers. *Plant Pathology*. DOI: 10.1111/ppa.12323.

Gitte, R. R., Rai, P. V., and Patil, R. B. (1978). Chemotaxis of *Rhizobium* sp. towards root exudate of *Cicer arietinum* L. *Plant and Soil*, *50*(1-3), 553-566.

Guerrero, M. (1999). Detección y cuantificación de *Phytophthora* spp. en olivares afectados de podredumbre radicular. *Msc Thesis, University of Córdoba, Spain* (in Spanish).

Goicoechea, N. (2009). To what extent are soil amendments useful to control Verticillium wilt?. *Pest Management Science*, *65*(8), 831-839.

Gómez, J. A., Guzmán, M. G., Giráldez, J. V., Fereres, E. (2009). The influence of cover crops and tillage on water and sediment yield, and on nutrient, and organic matter losses in an olive orchard on a sandy loam soil. *Soil and Tillage Research*, *106*(1), 137-144.

Gómez-Lama Cabanás, C., Schilirò, E., Valverde-Corredor, A., and Mercado-Blanco, J. (2014). The biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7 induces systemic defense responses in aerial tissues upon colonization of olive roots. *Frontiers in Microbiology*, *5*, 427. DOI:10.3389/fmicb.2014.00427.

Gori, A., Cerboneschi, M., and Tegli, S. (2012). High-resolution melting analysis as a powerful tool to discriminate and genotype *Pseudomonas savastanoi* pathovars and strains. *PloS One*, *7*(1), e30199.

Gouveia, E., and Coelho, V. (2007). Detection of *Verticillium dahliae* kleb. from olives trees with chronic decline and dieback of branches and shoots. *3rd European Meeting of the IOBC/WPRS Working Group Integrated Protection of Olive Crops.*

Gramaje, D., Pérez-Serrano, V., Montes-Borrego, M., Navas-Cortés, J. A., Jiménez-Díaz, R. M., and Landa, B. B. (2013). A comparison of real-time PCR protocols for the quantitative monitoring of asymptomatic olive infections by *Verticillium dahliae* pathotypes. *Phytopathology*, *103*(10), 1058-1068.

Guzmán, P., and Ecker, J. R. (1990). Exploiting the triple response of Arabidopsis to identify ethylene-related mutants. *The Plant Cell Online*, *2*(6), 513-523.

Haas, D., and Défago, G. (2005). Biological control of soil-borne pathogens by fluorescent pseudomonas. *Nature Reviews Microbiology*, *3*(4), 307-319.

Hall, B. H., Cother, E. J., Whattam, M., Noble, D., Luck, J., and Cartwright, D. (2004). First report of olive knot caused by *Pseudomonas savastanoi* pv. *savastanoi* on olives (*Olea europaea*) in Australia. *Australasian Plant Pathology*, *33*(3), 433-436.

Hallmann, J., Quadt-Hallmann, A., Mahaffee, W. F., and Kloepper, J. W. (1997). Bacterial endophytes in agricultural crops. *Canadian Journal of Microbiology*, *43*(10), 895-914.

Hamblin, A. (1995). The concept of agricultural sustainability. *Advances in Plant Pathology*, *11*, 1-19.

Hamdan, H., Weller, D. M., and Thomashow, L. S. (1991). Relative importance of fluorescent siderophores and other factors in biological control of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* 2-79 and M4-80R. *Applied and Environmental Microbiology*, *57*(11), 3270-3277.

Handelsman, J. (2004). Metagenomics: application of genomics to uncultured microorganisms. *Microbiology and Molecular Biology Reviews*, *68*(4), 669-685.

Hao, W., Li, H., Hu, M., Yang, L., and Rizwan-ul-Haq, M. (2011). Integrated control of citrus green and blue mold and sour rot by *Bacillus amyloliquefaciens* in combination with tea saponin. *Postharvest Biology and Technology*, *59*(3), 316-323.

Hardoim, P. R., van Overbeek, L. S., and van Elsas, J. D. (2008). Properties of bacterial endophytes and their proposed role in plant growth. *Trends in Microbiology*, *16*(10), 463-471.

Hartmann, H. T., Schnathorst, W. C., and Whisler, J. E. (1971). Oblonga, a clonal olive root-stock resistant to Verticillium wilt. *California Agriculture*, *25*(6), 12-15.

Hassani, D., Buonaurio, R., and Tombesi, A. (2003). Response of some olive cultivars, hybrid and open pollinated seedling to *Pseudomonas savastanoi* pv. *savastanoi*. *In Pseudomonas syringae and related pathogens. Springer Netherlands*, 489-494.

Hiemstra, J. A. (1998). A compendium of Verticillium wilts in tree species. *Ponsen and Looijen, Wageningen. CPRO, 80*

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

Hiemstra, J. A., and Rataj-Guranowska, M. (2003). Vegetative compatibility groups in *Verticillium dahliae* isolates from the Netherlands as compared to VCG diversity in Europe and the USA. *European Journal of Plant Pathology*, *109*(8), 827-839.

Höfte, M., and Altier, N. (2010). Fluorescent pseudomonads as biocontrol agents for sustainable agricultural systems. *Research in Microbiology*, *161*(6), 464-471.

Horne, W. T., Parker, W. B., and Daines, L. L. (1912). The method of spreading of the olive knot disease. *Phytopathology*, *2*, 101-105.

Hosni, T., Moretti, C., Devescovi, G., Suarez-Moreno, Z. R., Fatmi, M. B., Guarnaccia, C., Pongor, S., Onofri, A., Buonaurio, R., and Venturi, V. (2011). Sharing of quorum-sensing signals and role of interspecies communities in a bacterial plant disease. *The ISME Journal*, *5*(12), 1857-1870.

Howie, W. J. (1987). Effects of soil matric potential and cell motility on wheat root colonization by fluorescent pseudomonads suppressive to take-all. *Phytopathology*, *77*, 286-292.

Huang, J., Li, H., and Yuan, H. (2006). Effect of organic amendments on Verticillium wilt of cotton. *Crop Protection*, *25*(11), 1167-1173.

Huisman, O. C. (1982). Interrelationship of root growth dynamics to epidemiology of root-invading fungi. *Annual Review of Phytopathology, 20*(1), 303-327.

Hynes, M. F., Quandt, J., O'Connell, M. P., and Pühler, A. (1989). Direct selection for curing and deletion of *Rhizobium* plasmids using transposons carrying the *Bacillus subtilis sacB* gene. *Gene*, *78*(1), 111-120.

Iacobellis, N.S. (2001). Olive knot. *Encyclopedia of Plant Pathology*, *1*, 714-715.

Iacobellis, N. S., A. Sisto, G. Surico, A. Evidente, and E. Di Maio. (1994). Pathogenicity of *Pseudomonas syringae* pv. *savastanoi* mutants defective in phytohormone production. *Journal of Phytopathology*, *140*(3), 238-248.

Inderbitzin, P., Bostock, R. M., Davis, R. M., Usami, T., Platt, H. W., and Subbarao, K. V. (2011). Phylogenetics and Taxonomy of the Fungal Vascular Wilt Pathogen *Verticillium*, with the Descriptions of Five New Species. *PLoS One, 6*(12), e28341.

Jetiyanon, K., Fowler, W. D., and Kloepper, J. W. (2003). Broad-spectrum protection against several pathogens by PGPR mixtures under field conditions in Thailand. *Plant Disease*, *87*(11), 1390-1394.

Jiang, J., Fan, L. W., and Wu, W. H. (2005). Evidences for involvement of endogenous cAMP in *Arabidopsis* defense responses to *Verticillium* toxins. *Cell research*, *15*(8), 585-592.

Jiménez-Díaz, R. M., Cirulli, M., Bubici, G., del Mar Jiménez-Gasco, M., Antoniou, P. P., and Tjamos, E. C. (2012). Verticillium wilt, a major threat to olive production: Current status and future prospects for its management. *Plant Disease*, *96*(3), 304-329.

Jiménez-Díaz, R. M., Mercado-Blanco, J., Olivares-García, C., Collado-Romero, M., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Giménez-Jaime, A., García-Jiménez, J., and Armengol, J. (2006). Genetic and virulence diversity in *Verticillium dahliae* populations infecting artichoke in eastern-central Spain. *Phytopathology*, *96*(3), 288-298.

Jiménez-Díaz, R. M., Olivares-García, C., Landa, B. B., del Mar Jiménez-Gasco, M., and Navas-Cortés, J. A. (2011). Region-wide analysis of genetic diversity in *Verticillium dahliae* populations infecting olive in southern Spain and agricultural factors influencing the distribution and prevalence of vegetative compatibility groups and pathotypes. *Phytopathology*, *101*(3), 304-315.

Jiménez-Díaz, R. M., Tjamos, E. C., and Cirulli, M. (1998). Verticillium wilt of major tree hosts: olive. *A compendium of Verticillium wilts in tree species*, 13-16.

Jiménez Díaz, R. M., Trapero Casas, J. L., Boned, J., Landa, B. B., and Navas Cortés, J. A. (2009a). Uso de Bioten para la protección biológica de plantones de olivo contra la Verticilosis causada por el patotipo defoliante de *Verticillium dahliae*. *Boletín de sanidad vegetal. Plagas*, *35*(4), 595-615 (in Spanish).

Jiménez-Díaz, R. M., Trapero-Casas, J. L., Boned, J., Landa del Castillo, B. B., Navas-Cortés, J. A. (2009b). Avances en el control biológico de la verticilosis del olivo. *Vida Rural*, (296), 50-58 (in Spanish).

Jiménez-Gasco, M. D. M., Malcolm, G. M., Berbegal, M., Armengol, J., and Jiménez-Díaz, R. M. (2014). Complex molecular relationship between vegetative compatibility groups (VCGs) in *Verticillium dahliae*: VCGs do not always align with clonal lineages. *Phytopathology*, *104*(6), 650-659.

Joaquim, T. R., and Rowe, R. C. (1990). Reassessment of vegetative compatibility relationships among strains of *Verticillium dahliae* using nitrate-nonutilizing mutants. *Phytopathology*, *80*(11), 1160-1166.

Joaquim, T. R., and Rowe, R. C. (1991). Vegetative compatibility and virulence of strains of *Verticillium dahliae* from soil and potato plant. *Phytopathology*, *81*(5), 552-558.

Kalbe, C., Marten, P., and Berg, G. (1996). Strains of the genus *Serratia* as beneficial rhizobacteria of oilseed rape with antifungal properties. *Microbiological Research*, *151*(4), 433-439.

Kamensky, M., Ovadis, M., Chet, I., and Chernin, L. (2003). Soil-borne strain IC14 of *Serratia plymuthica* with multiple mechanisms of antifungal activity provides biocontrol of *Botrytis cinerea* and *Sclerotinia sclerotiorum* diseases. *Soil Biology and Biochemistry*, *35*(2), 323-331.

Kapulnik, Y., Tsror, L., Zipori, I., Hazanovsky, M., Wininger, S., and Dag, A. (2010). Effect of AMF application on growth, productivity and susceptibility to Verticillium wilt of olives grown under desert conditions. *Symbiosis*, *52*(2-3), 103-111.

Katan, T. (2000). Vegetative compatibility in populations of *Verticillium*-an overview. *Advances in Verticillium Research and Disease Management*, 69-86.

Katan, J. (2015). Soil solarization: the idea, the research and its development. *Phytoparasitica*, *43*(1), 1-4.

Kawaguchi, A., Kondo, K. I., and Inoue, K. (2012). Biological control of apple crown gall by nonpathogenic *Rhizobium vitis* strain VAR03-1. *Journal of General Plant Pathology*, *78*(4), 287-293.

Kennelly, M. M., Cazorla, F. M., de Vicente, A., Ramos, C., and Sundin, G. W. (2007*). Pseudomonas syringae* diseases of fruit trees: progress toward understanding and control. *Plant Disease*, *91*(1), 4-17.

King, E. O., Ward, M. K., and Raney, D. E. (1954). Two simple media for the demonstration of pyocyanin and fluorescin. *Journal of Laboratory and Clinical Medicine*, *44*, 301-307.

Klebahn, H. (1913). Beiträge zur Kenntnis der Fungi Imperfecti I. Eine Verticillium-Krankheit auf Dahliaen. *Mycologisches Zentralblatt*, *3*, 49-66 (in German).

Kloepper, J. W., and Ryu, C. M. (2006). Bacterial endophytes as elicitors of induced systemic resistance. In *Microbial root endophytes*. *Springer Berlin Heidelberg*, 33-52.

Klosterman, S. J., Atallah, Z. K., Vallad, G. E., and Subbarao, K. V. (2009). Diversity, pathogenicity and management of *Verticillium* species. *Annual Review of Phytopathology*, *47*, 39-62.

Kobra, N., Jalil, K., and Toubert, G. (2009). Effects of three *Glomus* species as biocontrol agents against *Verticillium*-induced wilt in cotton. *Journal of Plant Protection Research*, *49*(2), 185-189.

Koike, M., Fujita, M., Nagao, H., and Ohshima, S. (1996). Random amplified polymorphic DNA analysis of Japanese isolates of *Verticillium dahliae* and *V. albo-atrum*. *Plant Disease*, *80*(11), 1224-1227.

Koornneef, A., and Pieterse, C. M. (2008). Cross talk in defense signaling. *Plant Physiology*, *146*(3), 839-844.

Korthals, G. W., Thoden, T. C., van den Berg, W., and Visser, J. H. M. (2014). Long-term effects of eight soil health treatments to control plant-parasitic nematodes and *Verticillium dahliae* in agro-ecosystems. *Applied Soil Ecology*, *76*, 112-123.

Korolev, N., Katan, J., and Katan, T. (2000). Vegetative compatibility groups of *Verticillium dahliae* in Israel: Their distribution and association with pathogenicity. *Phytopathology*, *90*(5), 529-536.

Korolev, N., Pérez-Artés, E., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Katan, J., Katan, T., and Jiménez-Díaz, R. M. (2001). Comparative study of genetic diversity and pathogenicity among populations of *Verticillium dahliae* from cotton in Spain and Israel. *European Journal of Plant Pathology*, *107*(4), 443-456.

Korolev, N., Pérez-Artés, E., Mercado-Blanco, J., Bejarano-Alcázar, J., Rodríguez-Jurado, D., Jiménez-Díaz, R. M., Katan, T., and Katan, J. (2008). Vegetative compatibility of cotton-defoliating *Verticillium dahliae* in Israel and its pathogenicity to various crop plants. *European Journal of Plant Pathology*, *122*(4), 603-617.

Kraus, J., and Loper, J. E. (1992). Lack of evidence for a role of antifungal metabolite production by *Pseudomonas fluorescens* Pf-5 in biological control of *Pythium* damping-off of cucumber. *Phytopathology*, *82*(3), 264-271.

Krid, S., Rhouma, A., Mogou, I., Quesada, J. M., Nesme, X., and Gargouri, A. (2010). *Pseudomonas savastanoi* endophytic bacteria in olive tree knots and antagonistic potential of strains of *Pseudomonas fluorescens* and *Bacillus subtilis*. *Journal of Plant Pathology*, *92*, 335-341.

Krid, S., Triki, M.A., Gargouri, A., and Rhouma, A. (2012). Biocontrol of olive knot disease by *Bacillus subtilis* isolated from olive leaves. *Annals of Microbiology*, *62*(1), 149-154.

Krueger, W. H., Teviotdale, B. L., and Schroth, M. N. (1997). Improvements in the control of olive knot disease. *III International Symposium on Olive Growing*, *474*, 567-572.

Kuklinsky-Sobral, J., Araújo, W. L., Mendes, R., Geraldi, I. O., Pizzirani-Kleiner, A. A., and Azevedo, J. L. (2004). Isolation and characterization of soybean-associated bacteria and their potential for plant growth promotion. *Environmental Microbiology*, *6*(12), 1244-1251.

Kunkel, B. N., and Brooks, D. M. (2002). Cross talk between signaling pathways in pathogen defense. *Current Opinion in Plant Biology*, *5*(4), 325-331.

Kurze, S., Bahl, H., Dahl, R., and Berg, G. (2001). Biological control of fungal strawberry diseases by *Serratia plymuthica* HRO-C48. *Plant Disease*, *85*(5), 529-534.

Ladux, J. L., Jotayan, L., Otero, M. L., González Vera, C., and Ortiz, J. (2012). Incidence of *Verticillium dahlia* in traditional orchards of the olive 'Arauco' in northwest Argentina (La Rioja). *VII International Symposium on Olive Growing. Acta Horticulturae (ISHS)*, *1057*, 127-131.

Lamers, J. G., Schippers, B., and Geels, F. P. (1988). Soil-borne disease of wheat in the Netherlands and results of seed bacterization with pseudomonads against *Gaeumannomyces graminis* var. *tritici*. *Cereal Breeding Related to Integrated Cereal Production. ML Jorna and LAJ Slootmaker, eds. Pudoc, Wageningen, The Netherlands*, 134-139.

Latinovic, J., and Vucinic, Z. (2010). First report of *Verticillium dahliae* on olives in Montenegro. *Petria*, *20*, 253.

Lavermicocca, P., Lonigro, S. L., Valerio, F., Evidente, A., and Visconti, A. (2002). Reduction of olive knot disease by a bacteriocin from *Pseudomonas syringae* pv. ciccaronei. *Applied and Environmental Microbiology*, *68*(3), 1403-1407.

Lavermicocca, P., Valerio, F., Lonigro, S. L., Lazzaroni, S., Evidente, A., and Visconti, A. (2003). Control of olive knot disease with a bacteriocin. *Pseudomonas syringae and related pathogens*. *Springer Netherlands*, 451-457.

Lazarovits, G., Conn, K., and Tenuta, M. (2000). Control of *Verticillium dahliae* with soil amendments: efficacy and mode of action. *Advances in Verticillium Research and Disease Management. APS, St. Paul*, 274-291.

Lemanceau, P., Expert, D., Gaymard, F., Bakker, P. A. H. M., and Briat, J. F. (2009). Role of iron in plant–microbe interactions. *Advances in Botanical Research*, *51*, 491-549.

Leslie, J. F. (1993). Fungal vegetative compatibility. *Annual Review of Phytopathology*, *31*(1), 127-150.

Levin, A. G., Lavee, S., and Tsror, L. (2003a). Epidemiology of *Verticillium dahliae* on olive (cv. Picual) and its effects on yield under saline conditions. *Plant Pathology*, *52*(2), 212-218.

Levin, A. G., Lavee, S., and Tsror, L. (2003b). Epidemiology and effects of Verticillium wilt on yield of olive trees (cvs. Barnea and Souri) irrigated with saline water in Israel. *Phytoparasitica*, *31*(4), 333-343.

Levin, A. G., Lavee, S., Tsror, (Lahkim) L. (2007). The influence of salinity on *Verticillium dahliae* in stem cuttings of five olive cultivars. *Journal of Phytopathology*, *155*(10), 587-592.

Li, W., Estrada-de los Santos, P., Matthijs, S., Xie, G. L., Busson, R., Cornelis, P., Rozenski, J., and De Mot, R. (2011). Promysalin, a salicylate-containing *Pseudomonas putida* antibiotic, promotes surface colonization and selectively targets other *Pseudomonas*. *Chemistry and Biology*, *18*(10), 1320-1330.

Lima, G., De Curtis, F., D Onghia, A. M., and Nigro, F. (2007). Comparison between real-time PCR and semi-selective medium in monitoring *Verticillium dahliae* microsclerotia in the olive rhizosphere and suppression of the pathogen by compost. *IOBC WPRS Bulletin*, *30*(9), 221.

Lindow, S. E. (1988). Lack of correlation of in vitro antibiosis with antagonism of ice nucleation active bacteria on leaf surface by non-ice nucleation active bacteria. *Phytopathology*, *78*(4), 444-450.

Loper, J. E., Hassan, K. A., Mavrodi, D. V., Davis II, E. W., Lim, C. K., Shaffer, B. T., Elbourne, L. D. H., Stockwell, V. O., Hartney, S. L. Breakwell, K. et al. (2012). Comparative genomics of plant-associated *Pseudomonas* spp.: insights into diversity and inheritance of traits involved in multitrophic interactions. *PLoS Genetics*, *8*(7), e1002784.

López-Escudero, F. J., and Blanco-López, M. A. (1999). First report of transmission of *Verticillium dahliae* by infested manure in olive orchards in Andalucía (Southern Spain). *Plant Disease, 83*(12), 1178-1178.

López-Escudero, F. J., and Blanco-López, M. A. (2001). Effect of a single or double soil solarization to control Verticillium wilt in established olive orchards in Spain. *Plant Disease*, *85*(5), 489-496.

López-Escudero, F.J., and Blanco-López, M. A. (2005a). Recovery of young olive trees from *Verticillium dahliae*. *European Journal of Plant Pathology*, *113*(4), 367-375.

López-Escudero, F. J., and Blanco-López, M. A. (2005b). Effects of drip irrigation on population of *Verticillium dahliae* in olive orchards. *Journal of Phytopathology*, *153*(4), 238-239.

López-Escudero, F. J., and Mercado-Blanco, J. (2011). Verticillium wilt of olive: a case study to implement an integrated strategy to control a soil-borne pathogen. *Plant and Soil, 344*(1-2), 1-50.

López-Escudero, F. J., Blanco-López, M. A., and Trapero, A. (2008). Influencia de las cubiertas vegetales en las enfermedades del olivar. *Cubiertas vegetales en olivar. Consejería de Agricultura y Pesca, Junta de Andalucía*, 1001-1114 (in Spanish).

López-Escudero, F. J., Del Río, C., Caballero, J. M., and Blanco-López, M. A. (2004). Evaluation of olive cultivars for resistance to *Verticillium dahliae*. *European Journal of Plant Pathology*, *110*(1), 79-85.

López-Escudero, F. J., García-Cabello, S., Blanco-López, M. A. (2009a). Distribution of *Verticillium dahliae* through watering systems in irrigated olive orchards in Andalucía. *European Journal of Plant Pathology*, *133*(4), 877-885.

López-Escudero, F. J., Roca, L. F., Mercado-Blanco, J., Valverde-Corredor, A., and Blanco-López, M. A. (2011). Verticillium wilt of olive in the Guadalquivir Valley (southern Spain): relations with some agronomical factors and spread of *Verticillium dahliae*. *Phytopathologia Mediterranea*, *49*(3), 370-380.

Lugtenberg, B., and Kamilova, F. (2009). Plant-growth-promoting rhizobacteria. *Annual Review of Microbiology*, *63*, 541-556.

Lugtenberg, B. J. J., and Bloemberg, G. V. (2004). Life in the rhizosphere. In *Pseudomonas*. *Springer US,* 403-430.

Lugtenberg, B. J. J., Dekkers, L. C., and Bloemberg, G. V. (2001). Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology*, *39*(1), 461-490.

Magdelaine, J. and Ottaviani, J.C. (1984). L'occupation pré et proto historique de l'abri de Scaffa Piana près de Saint Florent. *Bulletin de la Societe des Sciences Historiques et Naturelles de la Corse*, *647*, 39-48 (in French).

Magie, A. R., Wilson, E. E., and Kosuge, T. (1963). Indoleacetamide as an intermediate in the synthesis of indoleacetic acid in *Pseudomonas savastanoi*. *Science*, *141*(3587), 1281-1282.

MAGRAMA, Ministerio de Agricultura, Alimentación y Medio Ambiente. (2014). http://www.magrama.gob.es/es/estadistica/temas/estadisticas-agrarias/agricultura/esyrce/

Makhlouf, J., and Geagea, L. (2005). Prevalence and survey of *Verticillium dahliae* on stone fruits and olive trees in two regions of lebanon. *Annales de Recherche Scientifique*, (6), 301-317.

Maldonado-González, M. M., García-Carneros, A., Prieto, P., Lucena-Lucena, N., Molinero-Ruiz, L., and Mercado-Blanco, J. Evaluation of *Pseudomonas fluorescens* PICF7 as potential biocontrol agent against *Plasmopara halstedii* in sunflower. *18th International Sunflower Conference, Asociación Argentina de Girasol, Buenos Aires.*

Maldonado-González, M. M., Prieto, P., Ramos, C., and Mercado-Blanco, J. (2013). From the root to the stem: interaction between the biocontrol root endophyte *Pseudomonas fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB 3335 in olive knots. *Microbial Biotechnology*, *6*(3), 275-287.

Maldonado-González, M. M., Schilirò, E., Prieto, P., and Mercado-Blanco, J. (2015). Endophytic colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming motility. *Environmental Microbiology*. DOI: 10.1111/1462-2920.12725.

Malfanova, N., Lugtenberg, B., and Berg, G. (2013). Bacterial endophytes: who and where, and what are they doing there?. *PhD Thesis, Endophytic bacteria with plant growth promoting and biocontrol abilities*, 15.

Mamluk, O. F. (1984). *A* checklist of plant diseases in Jordan. *University of Jordan, Faculty of Agriculture, Amman, Jordan,* 56.

Marcelo, A., Fernández, M., Fatima Potes, M., and Serrano, J.F. (1999). Reactions of some cultivars of *Olea europaea* L. to experimental inoculation with *Pseudomonas syringae* pv. *savastanoi*. *III International Symposium on Olive Growing*, *474*, 581-584.

Marchi, G., Mori, B., Pollacci, P., Mencuccini, M., and Surico, G. (2009). Systemic spread of *Pseudomonas savastanoi* pv. *savastanoi* in olive explants. *Plant Pathology*, *58*(1), 152-158.

Markakis, E. A., Ligoxigakis, E. K., Avramidou, E. V., and Tzanidakis, N. (2014). Survival, persistence, and infection efficiency of *Verticillium dahliae* passed through the digestive system of sheep. *Plant Disease*, *98*(9), 1235-1240.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Paplomatas, E. J., and Tjamos, E. C. (2009). Symptom development, pathogen isolation and Real-Time QPCR quantification as factors for evaluating the resistance of olive cultivars to *Verticillium* pathotypes. *European Journal of Plant Pathology*, *124*(4), 603-611.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Paplomatas, E. J., and Tjamos, E. C. (2015). Biological control of Verticillium wilt of olive by *Paenibacillus alvei*, strain K165. *BioControl*. DOI: 10.1007/s10526-015-9669-0.

Markakis, E. A., Tjamos, S. E., Antoniou, P. P., Roussos, P. A., Paplomatas, E. J., and Tjamos, E. C. (2010). Phenolic responses of resistant and susceptible olive cultivars induced by defoliating and nondefoliating *Verticillium dahliae* pathotypes, *Plant Disease*, *94*(9), 1156-1162.

Martínez-García, P. M., Ruano-Rosa, D., Schilirò, E., Prieto, P., Ramos, C., Rodríguez-Palenzuela, P., and Mercado-Blanco, J. (2015). Complete genome sequence of *Pseudomonas fluorescens* strain PICF7, an indigenous root endophyte from olive (*Olea europaea* L.) and effective biocontrol agent against *Verticillium dahliae*. *Standards in Genomic Sciences*, *10*(1), 10.

Martínez-Granero, F., Rivilla, R., and Martín, M. (2006). Rhizosphere selection of highly motile phenotypic variants of *Pseudomonas fluorescens* with enhanced competitive colonization ability. *Applied and Environmental Microbiology*, *72*(5), 3429-3434.

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

Martos-Moreno, C., Caballero, J. M., del Río, C., and Blanco-López, M. A. (2001). Epidemiological behavior of olive cultivars in orchard infested with mixtures of defoliating and nondefoliating isolates of *Verticillium dahliae*. *8th Int Verticillium Symposium, Book of Abstracts, Córdoba, Spain*, 67.

Martos-Moreno, C., López-Escudero, F. J., and Blanco-López, M. A. (2006). Resistance of olive cultivars to the defoliating pathotype of *Verticillium dahliae*. *HortScience*, *41*(5), 1313-1316.

Matta, A., and Garibaldi, A. (1977). Control of Verticillium wilt of tomato by preinoculation with avirulent fungi. *Netherlands Journal of Plant Pathology*, *83*(1), 457-462.

Mavrodi, O. V., Mavrodi, D. V., Park, A. A., Weller, D. M., and Thomashow, L. S. (2006). The role of *dsbA* in colonization of the wheat rhizosphere by *Pseudomonas fluorescens* Q8r1-96. *Microbiology*, *152*(3), 863-872.

Meddad-Hamza, A., Beddiar, A., Gollotte, A., Lemoine, M. C., Kuszala, C., and Gianinazzi, S. (2010). Arbuscular mycorrhizal fungi improve the growth of olive trees and their resistance to transplantation stress. *African Journal of Biotechnology*, *9*(8).

Mejía, L. C., Rojas, E. I., Maynard, Z., Van Bael, S., Arnold, A. E., Hebbar, P., Samuels, G. J., Robbins, N. and Herre, E. A. (2008). Endophytic fungi as biocontrol agents of *Theobroma cacao* pathogens. *Biological Control*, *46*(1), 4-14.

Mercado-Blanco, J. (2015a). Life of Microbes Inside the Plant. In *Principles of Plant-Microbe Interactions*. *Springer International Publishing*, 25-32.

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

Mercado-Blanco, J., and Bakker, P. A. H. M. (2007). Interactions between plants and beneficial *Pseudomonas* spp.: exploiting bacterial traits for crop protection. *Antonie van Leeuwenhoek, 92*(4), 367-389.

Mercado-Blanco, J., and López-Escudero, F. J. (2012). Verticillium wilt of olive and its control: The heat is on. *Plant and Soil*, *355*(1-2), 17-21.

Mercado-Blanco, J., and Lugtenberg, B. J. J. (2014). Biotechnological applications of bacterial endophytes. *Current Biotechnology*, *3*(1), 60-75.

Mercado-Blanco, J., Collado-Romero, M., Parrilla-Araujo, S., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2003a) Quantitative monitoring of colonization of olive genotypes by *Verticillium dahliae* pathotypes with real-time polymerase chain reaction. *Physiological and Molecular Plant Pathology*, *63*(2), 91-105.

Mercado-Blanco, J., Rodríguez-Jurado, D., Hervás, A., and Jiménez-Díaz, R. M. (2004). Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp. *Biological Control*, *30*(2), 474-486.

Mercado-Blanco, J., Rodríguez-Jurado, D., Parrilla-Araujo, S., and Jiménez-Díaz, R. M. (2003b). Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae* pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Plant Disease*, *87*(12), 1487-1494.

Mercado-Blanco, J., Rodríguez-Jurado, D., Pérez-Artés, E., and Jiménez-Díaz, R. M. (2001a). Detection of the nondefoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. *Plant Pathology, 50*(5), 609-619.

Mercado-Blanco, J., Rodríguez-Jurado, D., Pérez-Artés, E., and Jiménez-Díaz, R. M. (2002). Detection of the defoliating pathotype of *Verticillium dahliae* in infected olive plants by nested PCR. *European Journal of Plant Pathology*, *108*(1), 1-13.

Mercado-Blanco, J., Van der Drift, K. M., Olsson, P. E., Thomas-Oates, J. E., Van Loon, L. C., and Bakker, P. A. H. M.(2001b). Analysis of the *pmsCEAB* gene cluster involved in biosynthesis of salicylic acid and the siderophore pseudomonine in the biocontrol strain *Pseudomonas fluorescens* WCS374. *Journal of bacteriology*, *183*(6), 1909-1920.

Meschke, H., Walter, S., and Schrempf, H. (2012). Characterization and localization of prodiginines from *Streptomyces lividans* suppressing *Verticillium dahliae* in the absence or presence of *Arabidopsis thaliana*. *Environmental microbiology*, *14*(4), 940-952.

Meschke, H., and Schrempf, H. (2010). *Streptomyces lividans* inhibits the proliferation of the fungus *Verticillium dahliae* on seeds and roots of *Arabidopsis thaliana*. *Microbial Biotechnology, 3*(4), 428-443.

Messner, R., Schweigrofler, W., Ibl, M., Berg, G., and Prillinger, H. (1996). Molecular characterization of the plant pathogen *Verticillium dahliae* Kleb. using RAPD-PCR and sequencing of the 18SrRNA-Gene. *Journal of Phytopathology*, *144*(7-8), 347-354.

Meyer, J. A., and Abdallah, M. A. (1978). The fluorescent pigment of *Pseudomonas fluorescens*: biosynthesis, purification and physicochemical properties. *Journal of General Microbiology*, *107*(2), 319-328.

Meyer, J. M. (2000). Pyoverdines: pigments, siderophores and potential taxonomic markers of fluorescent *Pseudomonas* species. *Archives of Microbiology*, *174*(3), 135-142.

Miller, J. H. (1972) Experiments in molecular genetics. *Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.*

Milgroom, M. G., del Mar Jiménez-Gasco, M., García, C. O., Drott, M. T., and Jiménez-Díaz, R. M. (2014). Recombination between clonal lineages of the asexual fungus *Verticillium dahliae* detected by genotyping by sequencing. *PloS One*, *9*(9), e106740.

Mitter, B., Petric, A., Shin, M. W., Chain, P. S., Hauberg-Lotte, L., Reinhold-Hurek, B., Nowak, J., and Sessitsch, A. (2013). Comparative genome analysis of *Burkholderia phytofirmans* PsJN reveals a wide spectrum of endophytic lifestyles based on interaction strategies with host plants. *Frontiers in Plant Science*, *4*, 120.

Moazzo, G. P. (1994). Les plantes d'Homere et de quelques autres poetes de l'Antiquite. V. L'olivier (Elaie). *In Annales du Musei Goulandris*, *9*, 185-223 (in French).

Mol, L., and Scholte, K. (1995). Formation of microsclerotia of *Verticillium dahliae* Kleb. on various plant parts of two potato cultivars. *Potato Research*, *38*(2), 143-150.

Moradi, A., Almasi, M. A., Jafary, H., and Mercado-Blanco, J. (2014). A novel and rapid loop-mediated isothermal amplification assay for the specific detection of *Verticillium dahliae*. *Journal of Applied Microbiology*, *116*(4), 942-954.

Morera, B., Páez, J. I., Vega, J. M., and Montes, F. (2005). Comparación de métodos de diagnóstico de *Verticillium dahliae* en olivo: aislamiento en medio de cultivo y PCR. *Boletín de sanidad vegetal. Plagas*, *31*(2), 267-276 (in Spanish).

Morschel, J. R., and Morschel, J. R. (1961). Recorded plant diseases in and outside Australia. *Department of Health, Division of Plant Quarantine, Canberra*, 113.

Morton, A., Carder, J. H., and Barbara, D. J. (1995a). Sequences of the internal transcribed spacers of the ribosomal RNA genes and relationships between isolates of *Verticillium albo-atrum* and *V. dahliae*. *Plant Pathology*, *44*(1), 183-190.

Morton A., Tabrett, A. M., Carder, J. H., and Barbara, D. J. (1995b). Subrepeat sequences in the ribosomal RNA intergenic regions of *Verticillium albo-atrum* and *V. dahliae*. *Mycological Research*, *99*(3), 257-266.

Müller, H., and Berg, G. (2008). Impact of formulation procedures on the effect of the biocontrol agent *Serratia plymuthica* HRO-C48 on Verticillium wilt in oilseed rape. *BioControl*, *53*(6), 905-916.

Müller, H., Tejedor-González, E., Mercado-Blanco, J., Rodríguez-Jurado, D., Jiménez-Díaz, R., and Berg, G. (2007). Effect of the biological control strain *Serratia plymuthica* HRO C48 on Verticillium wilt of olive trees cv. *Arbequina. IOBC WPRS Bulletin, 30*(6-1), 173.

Muñoz-Mérida, A., González-Plaza, J. J., Blanco, A. M., del Carmen García-López, M., Rodríguez, J. M., Pedrola, L., et al. (2013). De *novo* assembly and functional annotation of the olive (*Olea europaea*) transcriptome. *DNA Research*, dss036.

Nakaew, N., Rangjaroen, C., and Sungthong, R. (2015). Utilization of rhizospheric *Streptomyces* for biological control of *Rigidoporus* sp. causing white root disease in rubber tree. *European Journal of Plant Pathology*, 1-13.

Narisawa, K., Kawamata, H., Currah, R., and Hashiba, T. (2002). Suppression of Verticillium wilt in eggplant by some fungal root endophytes. *European Journal of Plant Pathology*, *108*(2), 103-109.

Narisawa, K., Usuk, F., and Hashiba, T. (2004) Control of Verticillium yellows in Chinese cabbage by the dark septate endophytic fungus LtVB3. *Phytopathology*, *94*(5), 412-418.

Navas-Cortés, J. A., Landa, B. B., Mercado-Blanco, J., Trapero-Casas, J. L., Rodríguez-Jurado, D., and Jiménez-Díaz, R. M. (2008). Spatiotemporal analysis of spread of *Verticillium dahliae* pathotypes within a high tree density olive orchard in southern Spain. *Phytopathology*, *98*(2), 167-180.

Navazo, A., Barahona, E., Redondo-Nieto, M., Martínez-Granero, F., Rivilla, R., and Martín, M. (2009). Three independent signalling pathways repress motility in *Pseudomonas fluorescens* F113. *Microbial Biotechnology*, *2*(4), 489-498.

Nawrath, C., and Métraux, J. P. (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express PR-2 and PR-5 and accumulate high levels of camalexin after pathogen inoculation. *The Plant Cell Online*, *11*(8), 1393-1404.

Nejad, P., and Johnson, P. A. (2000). Endophytic bacteria induce growth promotion and wilt disease suppression in oilseed rape and tomato. *Biological Control*, *18*(3), 208-215.

Neubauer, C., Heitmann, B., and Müller, C. (2014). Biofumigation potential of Brassicaceae cultivars to *Verticillium dahliae*. *European Journal of Plant Pathology*, *140*(2), 341-352.

Okoli, C. A. N., Carder, J. H., and Barbara, D. J. (1994). Restriction fragment length polymorphisms (RFLPs) and the relationships of some host-adapted isolates of *Verticillium dahliae*. *Plant Pathology*, *43*(1), 33-40.

Omer, M. A., Johnson, D. A., Douhan, L. I., Hamm, P. B., and Rowe, R. C. (2008). Detection, quantification, and vegetative compatibility of *Verticillium dahliae* in potato and mint production soils in the Columbia Basin of Oregon and Washington. *Plant Disease*, *92*(7), 1127-1131.

Ongena, M. A. R. C., Daayf, F., Jacques, P., Thonart, P., Benhamou, N., Paulitz, T. C., Cornélis, P., Koedam, N., and Belanger, R. R. (1999). Protection of cucumber against Pythium root rot by fluorescent pseudomonads: predominant role of induced resistance over siderophores and antibiosis. *Plant Pathology*, *48*(1), 66-76.

Ormeño-Orrillo, E., Rosenblueth, M., Luyten, E., Vanderleyden, J., and Martínez-Romero, E. (2008). Mutations in lipopolysaccharide biosynthetic genes impair maize rhizosphere and root colonization of *Rhizobium tropici* CIAT899. *Environmental Microbiology*, *10*(5), 1271-1284.

Otero, M. L., Roca, M., Zapata, R., Ladux, J. L., Ortiz, J., Zanelli, M., Matía, A. C., and Pérez, B. A. (2012). Effect of solarization, organic matter, and *Trichoderma* on the severity of Verticillium Wilt in olive trees (*Olea europaea* L.) and soil inoculum density. *Acta Horticulturae (ISHS), 1057*, 121-126.

O'Sullivan, D. J., and O'Gara, F. (1992). Traits of fluorescent *Pseudomonas* spp. involved in suppression of plant root pathogens. *Microbiological Reviews*, *56*(4), 662-676.

Overhage, J., Lewenza, S., Marr, A. K., and Hancock, R. E. (2007). Identification of genes involved in swarming motility using a *Pseudomonas aeruginosa* PAO1 mini-Tn*5-lux* mutant library. *Journal of Bacteriology*, *189*(5), 2164-2169.

Owen, R. W., Giacosa, A., Hull, W. E., Haubner, R., Wurtele, G., Spiegelhalder, B., and Bartsch, H. (2000). Olive-oil consumption and health: The possible role of antioxidants. *The Lancet Oncology*, *1*(2), 107-112.

Panagopoulos, C. G. (1993). Olive knot disease in Greece. *EPPO Bulletin*, *23*(3), 417-422.

Paoletti, V. (1933). Osservazioni ed esperimenti orientativi di lotta contro la rogna dell'Olivo. *Rivista di Patologia Vegetale*, 47-50 (in Italian).

Papaioannou, I. A., Ligoxigakis, E. K., Vakalounakis, D. J., Markakis, E. A., and Typas, M. A. (2013). Phytopathogenic, morphological, genetic and molecular characterization of a *Verticillium dahliae* population from Crete, Greece. *European Journal of Plant Pathology*, *136*(3), 577-596.

Papaioannou, I. A., and Typas, M. A. (2014). High-throughput assessment and genetic investigation of vegetative compatibility in *Verticillium dahliae*. *Journal of Phytopathology*, *163* (6), 475-485.

Pegg, G. F., and Brady, B. L. (2002). *Verticillium* wilts.*CAB International,Wallingford*, 293-340.

Penyalver, R., García, A., Ferrer, A., Bertolini, E., Quesada, J. M., Salcedo, C. I., Piquer, J., Pérez-Panadés, J., Carbonell, E. A., del Río, C., Caballero, J. M. and López, M. M. (2006). Factors affecting *Pseudomonas savastanoi* pv. *savastanoi* plant inoculations and their use for evaluation of olive cultivar susceptibility. *Phytopathology*, *96*(3), 313-319.

Penyalver, R., García, A., Ferrer, A., Bertolini, E., and López, M. M. (2000). Detection of *Pseudomonas savastanoi* pv. savastanoi in olive plants by enrichment and PCR. *Applied and Environmental Microbiology*, *66*(6), 2673-2677.

Penyalver, R., García, A., Ferrer, A., and López, M. M. (1998). La tuberculosis del olivo: diagnóstico, epidemiología y control. *Phytoma-España*, *102*, 177-179 (in Spanish).

Pérez-Artés, E., García-Pedrajas, M. D., Bejarano-Alcázar, J., and Jiménez-Díaz, R. M. (2000). Differentiation of cotton-defoliating and nondefoliating pathotypes of *Verticillium dahliae* by RAPD and specific PCR analyses. *European Journal of Plant Pathology*, *106*(6), 507-517.

Pérez-Martínez, I., Rodriguez-Moreno, L., Matas, I. M., and Ramos, C. (2007). Strain selection and improvement of gene transfer for genetic manipulation of *Pseudomonas savastanoi* isolated from olive knots. *Research in Microbiology*, *158*(1), 60-69.

Pérez-Martínez, I., Rodríguez-Moreno, L., Lambertsen, L., Matas, I. M., Murillo, J., Tegli, S., Jiménez, A. J., and Ramos, C. (2010). Fate of a *Pseudomonas savastanoi* pv. savastanoi type III secretion system mutant in olive plants (*Olea europaea* L.). *Applied and Environmental Microbiology*, *76*(11), 3611-3619.

Pérez-Rodríguez, M., Alcántara, E., Amaro, M., Serrano, N., Lorite, I. J., Arquero, O., Orgaz, F., and López-Escudero, F. J. (2013). The influence of irrigation frequency on the onset and development of Verticillium Wilt of olive. *Plant Disease*, *99*(4), 488-495.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., and Van Wees, S. C. (2009). Networking by small-molecule hormones in plant immunity. *Nature chemical biology*, *5*(5), 308-316.

Pieterse, C. M., Van Wees, S. C., Hoffland, E., Van Pelt, J. A., and Van Loon, L. C. (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. *The Plant Cell Online*, *8*(8), 1225-1237.

Pieterse, C. M., Van Wees, S. C., Van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Wisbeek, P. J., and Van Loon, L. C. (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. *The Plant Cell Online*, *10*(9), 1571-1580.

Pieterse, C. M. J., Zamioudis, C., Berendsen, R. L., Weller, D. M., Van Wees, S. C. M. and Bakker, P. A. H. M. (2014). Induced systemic resistance by beneficial microbes. *Annual Review of Phytopathology*, *52*, 347-375.

Podolich, O., Ardanov, P., Zaets, I., Pirttilä, A. M., and Kozyrovska, N. (2015). Reviving of the endophytic bacterial community as a putative mechanism of plant resistance. *Plant and Soil*, 1-11.

Porras-Soriano, A., Marcilla-Goldaracena, I., Soriano-Martin, M. L., and Porras-Piedra, A. (2006). Development and resistance to *Verticillium dahliae* of olive plantlets inoculated with mycorrhizal fungi during the nursery period. *The Journal of Agricultural Science*, *144*(2), 151-157.

Porras-Soriano, A., Soriano-Martin, M. L., and Porras-Piedra, A. (2003). Grafting olive cv. Cornicabra on rootstocks tolerant to *Verticillium dahliae* reduces their susceptibility. *Crop Protection*, *22*(2), 369-374.

Porta-Puglia, A., and Mifsud, D. (2005). First record of *Verticillium dahliae* on olive in Malta. *Journal of Plant Pathology*, *87*(2), 149.

Pramateftaki, P. V., Antoniou, P. P., and Typas, M. A. (2000). The complete DNA sequence of the nuclear ribosomal RNA gene complex of *Verticillium dahliae*: intraspecific heterogeneity within the intergenic spacer region. *Fungal Genetics and Biology*, *29*(1), 19-27.

Prasad, R. D., and Naik, M. K. (2008). Biological control of soil borne plant pathogens using *Trichoderma* species. *Advances in Soil Borne Plant Diseases*, 311.

Prieto, P., and Mercado-Blanco, J. (2008). Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7. *FEMS Microbiology Ecology*, *64*(2), 297-306.

Prieto, P., Moore, G., and Shaw, P. (2007). Fluorescence *in situ* hybridization on vibratome sections of plant tissues. *Nature Protocols*, *2*(7), 1831-1838.

Prieto, P., Navarro-Raya, C., Valverde-Corredor, A., Amyotte, S. G., Dobinson, K. F., and Mercado-Blanco, J. (2009). Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7. *Microbial Biotechnology, 2*(4), 499-511.

Prieto, P., Schilirò, E., Maldonado-González, M. M., Valderrama, R., Barroso-Albarracín, J. B., and Mercado-Blanco, J. (2011). Root hairs play a key role in the endophytic colonization of olive roots by *Pseudomonas* spp. with biocontrol activity. *Microbial Ecology*, *62*(2), 435-445.

Quesada, J. M., García, A., Bertolini, E., López, M. M., and Penyalver, R. (2007). Recovery of *Pseudomonas savastanoi* pv. *savastanoi* from symptomless shoots of naturally infected olive trees. *International Microbiology*, *10*(2), 77-84.

Quesada, J. M., López, M. M., and Penyalver, R. (2012). Epidemiology and control of plant diseases caused by phytopathogenic bacteria: The case of olive knot disease caused by *Pseudomonas savastanoi* pv. *savastanoi*. *INTECH Open Access Publisher*, 299-326.

Quesada, J. M., Penyalver, R., Pérez-Panadés, J., Salcedo, C. I., Carbonell, E. A. and López, M. M. (2010a). Dissemination of *Pseudomonas savastanoi* pv. *savastanoi* populations and subsequent appearance of olive knot disease. *Plant Pathology*, *59*(2), 262-269.

Quesada, J. M., Penyalver, R., Pérez-Panadés, J., Salcedo, C. I., Carbonell, E. A., and López, M. M. (2010b). Comparison of chemical treatments for reducing epiphytic *Pseudomonas savastanoi* pv. *savastanoi* populations and for improving subsequent control of olive knot disease. *Crop Protection, 29*(12), 1413-1420.

Raaijmakers, J. M., Paulitz, T. C., Steinberg, C., Alabouvette, C., and Moënne-Loccoz, Y. (2009). The rhizosphere: a playground and battlefield for soilborne pathogens and beneficial microorganisms. *Plant and Soil*, *321*(1-2), 341-361.

Radišek, S., Jakše, J., Simoncic, A., and Javornik, B. (2003). Characterization of *Verticillium albo-atrum* field isolates using pathogenicity data and AFLP analysis. *Plant Disease*, *87*(6), 633-638.

Ramos, C., Matas, I. M., Bardaji, L., Aragón, I. M., and Murillo, J. (2012). *Pseudomonas savastanoi* pv. *savastanoi*: some like it knot. *Molecular Plant Pathology*, *13*(9), 998-1009.

Reinhold-Hurek, B., and Hurek, T. (1998). Interactions of gramineous plants with *Azoarcus* spp. and other diazotrophs: identification, localization, and perspectives to study their function. *Critical Reviews in Plant Sciences*, *17*(1), 29-54.

Reinhold-Hurek, B., and Hurek, T. (2011). Living inside plants: bacterial endophytes. *Current Opinion in Plant Biology*, *14*(4), 435-443.

Robb, J. (2007). *Verticillium* tolerance: resistance, susceptibility, or mutualism?. *Botany*, *85*(10), 903-910.

Rodríguez, E., García-Garrido, J. M., García, P. A., and Campos, M. (2008). Agricultural factors affecting Verticillium wilt in olive orchards in Spain. *European Journal of Plant Pathology*, *122*(2), 287-295.

Rodríguez, E., García-Garrido, J. M., García, P. A., and Campos, M. (2011). Implications of plant material origin, land use history and soil properties in the incidence of verticillium wilt in olive groves. *Journal of Plant Pathology*, *93*(1), 111-118.

Rodríguez, R. J., White, J. E. Jr, Arnold, A. E., and Redman, R. S. (2009). Fungal endophytes: diversity and functional roles. *New Phytologist*, *182*(2), 314-330.

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

Rodríguez-Jurado, D., Blanco-López, M. A., Rapoport, H. E., and Jiménez-Díaz, R. M. (1993). Present status of verticillium wilt of olive in Andalucía (southern Spain). *EPPO Bulletin*, *23*(3), 513-516.

Rodríguez-Jurado, D., Porras-Alonso, R., Trapero-Casas, J. L., and Jiménez-Díaz, R. M. (2002). Potencial de las hojas de olivo caídas tras la infección por el patotipo defoliante de *Verticillium dahliae* como fuente de inóculo en las epidemias de Verticilosis. *XI Congreso de la Sociedad Española de Fitopatología, SEF-IFAPA, Almería, Spain*, 228 (in Spanish).

Rodríguez-Lizana, A., Espejo-Pérez, A. J., González-Fernández, P., Ordóñez-Fernández, R. (2008). Pruning residues as an alternative to traditional tillage to reduce erosion and pollutant dispersion in olive groves. *Water, Air, and Soil Pollution, 193*(1-4), 165-173.

Rodríguez-Moreno, L., Barceló-Muñoz, A., and Ramos, C. (2008). In vitro analysis of the interaction of *Pseudomonas savastanoi* pvs. *savastanoi* and *nerii* with micropropagated olive plants. *Phytopathology*, *98*(7), 815-822.

Rodríguez-Moreno, L., Jiménez, A. J., and Ramos, C. (2009). Endopathogenic lifestyle of *Pseudomonas savastanoi* pv. *savastanoi* in olive knots. *Microbial Biotechnology*, *2*(4), 476-488.

Rodríguez-Palenzuela, P., Matas, I. M., Murillo, J., López-Solanilla, E., Bardaji, L., Pérez-Martínez, I., et al. (2010). Annotation and overview of the *Pseudomonas savastanoi* pv. savastanoi NCPPB 3335 draft genome reveals the virulence gene complement of a tumour-inducing pathogen of woody hosts. *Environmental Microbiology*, *12*(6), 1604-1620.

Rokni-Zadeh. H., Khavazi, K., Asgharzadeh, A., Hosseini-Mazinani, M., and De Mot, R. (2007). Biocontrol of *Pseudomonas savastanoi*, causative agent of olive knot disease: antagonistic potential of non-pathogenic rhizosphere isolates of fluorescent *Pseudomonas*. *Communications in Agricultural and Applied Biological Sciences*, *73*(1), 199-203.

Romeralo, C., Santamaría, O., Pando, V., and Diez, J. J. (2015). Fungal endophytes reduce necrosis length produced by *Gremmeniella abietina* in *Pinus halepensis* seedlings. *Biological Control*, *80*, 30-39.

Rosenblueth, M., and Martínez-Romero, E. (2004). *Rhizobium etli* maize populations and their competitiveness for root colonization. *Archives of Microbiology*, *181*(5), 337-344.

Rosenblueth, M., and Martínez-Romero, E. (2006). Bacterial endophytes and their interactions with hosts. *Molecular Plant-Microbe Interactions*, *19*(8), 827-837.

Ruggieri, G. (1946). Una nuova malatia dell'olivo. *L'Italia Agricola*, *83*, 369-372 (in Italian).

Ruggieri, G. (1948). Ricerche ed esperienze su una tracheoverticilliosi dell'olivo. *Olivicoltora*, *3*, 6-9 (in Italian).

Sanei, S. J., and Razavi, S. E. (2011). Suppression of Verticillium wilt of olive by *Pseudomonas fluorescens*. *American Journal of Experimental Agriculture*, *1*, 294-305.

Sanei, S. J., Okhoavat, S. M., Hedjaroude, G. A., Saremi, H., and Javan-Nikkhah, M. (2004). Olive verticillium wilt or dieback of olive in Iran. *Communications in Agricultural and Applied Biological Sciences*, *69*(4), 433-442.

Sang, M. K., and Kim, K. D. (2014). Biocontrol activity and root colonization by *Pseudomonas corrugata* strains CCR04 and CCR80 against Phytophthora blight of pepper. *BioControl*, *59*(4), 437-448.

Santiago, T. R., Grabowski, C., Rossato, M., Romeiro, R. S., and Mizubuti, E. S. (2015). Biological control of eucalyptus bacterial wilt with rhizobacteria. *Biological Control*, *80*, 14-22.

Savastano, L. (1889). Il bacillo della tubercolosi dell'olivo. *Rend Regia Accad Lincei*, *5*, 92-94 (in Italian).

Saydam, C., and Copcu, M. (1972). Verticillium wilt of olives in Turkey. *Journal of Turkish Phytopathology*, *9*, 235-252.

Scheffer, R. J., Voeten, J. G. W. F., and Guries, R. P. (2008). Biological control of Dutch Elm Disease. *Plant Disease*, *92*(2), 192-200.

Schena, L., Nigro, F., and Ippolito, A. (2004a). Real-time PCR detection and quantification of soilborne fungal pathogens: the case of *Rosellinia necatrix*, *Phytophthora nicotianae*, *P. citrophthora* and *Verticillium dahliae*. *Phytopathologia Mediterranea*, *43*(2), 273-280.

Schena, L., Nigro, F., Ippolito, A., and Gallitelli, D. (2004b). Real-time quantitative PCR: a new technology to detect and study phytopathogenic and antagonistic fungi. *European Journal of Plant Pathology*, *110*(9), 893-908.

Schilirò, E., Ferrara, M., Nigro, F., and Mercado-Blanco, J. (2012). Genetic responses induced in olive roots upon colonization by the biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7. *PloS One*, *7*(11), e48646.

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

Schnathorst, W. C., and Mathre, D. E. (1966). Cross-protection in cotton with strain of *Verticillium albo-atrum*. *Phytopathology*, *56*(10), 1204.

Schnathorst, W. C., and Sibbett, G. S. (1971a). The relation of strains of *Verticillium albo-atrum* to severity of Verticillium wilt in *Gossypium hirsutum* and *Olea europaea* in California. *Plant Disease Reporter*, *9*, 780-782.

Schnathorst, W. C., and Sibbett, G. S. (1971b). T-1 Verticillium strain: A major factor in cotton and olive wilt. *California Agriculture*, *25*(7), 3-5.

Schroth, M. N., and Hildebrand, D. C. (1968). A chemotherapeutic treatment for selectively eradicating crown gall and olive knot neoplasms. *Phytopathology*, *58*(6), 848-854.

Schroth, M. N., Hildebrand, D. C., and O'Reilly, H. J. (1968). Off-flavor of olives from trees with olive knot tumors. *Phytopathology*, *58*(4), 524-525.

Schroth, M. N., Osgood, J. W. and Miller, T. D. (1973). Quantitative assessment of the effect of the olive knot disease on olive yield and quality. *Phytopathology*, *63*(8), 1064-1065.

Schulz, B., and Boyle, C. (2006). What are endophytes*?*. *Springer Berlin, Heidelberg*, 1-13.

Schwyn, B., and Neilands, J. B. (1987). Universal chemical assay for the detection and determination of siderophores. *Analytical Biochemistry*, *160*(1), 47-56.

Scrivani, P., and Bugiani, A. (1955). Messa a punto de un metodo per la riproduzione artificiale della rogna dell'Olivo e risultati dei primi saggi terapeutici a mezo di sostanze antibiotiche. *L'Italia Agrícola*, *92*, 361-369 (in Italian).

Segarra, G., Van der Ent, S., Trillas, I., and Pieterse, C. M. J. (2009). MYB72, a node of convergence in induced systemic resistance triggered by a fungal and a bacterial beneficial microbe. *Plant Biology*, *11*(1), 90-96.

Serrano, M., Wang, B., Aryal, B., Garcion, C., Abou-Mansour, E., Heck, S., Geisler, M., Mauch, F., Nawrath, C., and Métraux, J. P. (2013). Export of salicylic acid from the chloroplast requires the multidrug and toxin extrusion-like transporter EDS5. *Plant Physiology*, *162*(4), 1815-1821.

Serrhini. M. N., and Zeroual, A. (1995). Verticillium wilt in Morocco. *Olivae*, *58*, 58-61.

Shigo, A. L. (1984). Compartmentalization: a conceptual framework for understanding how trees grow and defend themselves. *Annual Review of Phytopathology*, *22*(1), 189-214.

Short, D. P., Gurung, S., Gladieux, P., Inderbitzin, P., Atallah, Z. K., Nigro, F., Guoquing, L., Benlioglu, S., and Subbarao, K. V. (2015). Globally invading populations of the fungal plant pathogen *Verticillium dahliae* are dominated by multiple divergent lineages. *Environmental Microbiology*. DOI: 10.1111/1462-2920.12789.

Silby, M. W., Winstanley, C., Godfrey, S. A., Levy, S. B., and Jackson, R. W. (2011). *Pseudomonas* genomes: diverse and adaptable. *FEMS Microbiology Reviews*, *35*(4), 652-680.

Simon, R. U. P. A. P., Priefer, U., and Pühler, A. (1983). A broad host range mobilization system for *in vivo* genetic engineering: transposon mutagenesis in gram negative bacteria. *Nature Biotechnology*, *1*(9), 784-791.

Simons, M., Permentier, H. P., de Weger, L. A., Wijffelman, C. A., and Lugtenberg, B. J. (1997). Amino acid synthesis is necessary for tomato root colonization by *Pseudomonas fluorescens* strain WCS365. *Molecular Plant-Microbe Interactions*, *10*(1), 102-106.

Sisto, A., and Iacobellis, N. S. (1999). La "Rogna dell' olivo": aspetti patogenetici, epidemiologici e strategie di lotta. *Olivo and Olio*, *2*(12), 32-38 (in Italian).

Sisto, A., Cipriani, M.G., and Morea, M. (2004). Knot formation caused by *Pseudomonas syringae* subsp. *savastanoi* on olive plants is *hrp*-dependent. *Phytopathology*, *94*(5), 484-489.

Sisto, A., Morea, M., Zaccaro, F., Palumbo, G., and Iacobellis, N. S. (1999). Isolation and characterization of *Pseudomonas syringae* subsp. *savastanoi* mutants defective in hypersensitive response elicitation and pathogenicity. *Journal of Phytopathology*, *147*(6), 321-330.

Smidt, M., and Kosuge, T. (1978). The role of indole-3-acetic acid accumulation by alpha-methyl tryptophan-resistant mutants of *Pseudomonas savastanoi* in gall formation on oleanders. *Physiological Plant Pathology, 13*(2), 203-213.

Smith, E.F. (1920). *Bacterial Diseases of Plants*. *Philadelphia, PA: W.B. Saunders Company.*

Snyder, W. C., Hansen, H. N., and Wilhelm, S. (1950). New hosts of *Verticillium albo-atrum*. *Plant Disease Reporter*, *34*, 26-7.

Soesanto, L., and Termorshuizen, A. J. (2001). Effect of temperature on the formation of microsclerotia of *Verticillium dahliae*. *Journal of Phytopathology*, *149*(11-12), 685-691.

Spiers, A. J., Buckling, A., and Rainey, P. B. (2000). The causes of *Pseudomonas* diversity. *Microbiology*, *146*(10), 2345-2350.

Spoel, S. H., and Dong, X. (2012). How do plants achieve immunity? Defence without specialized immune cells. *Nature Reviews Immunology*, *12*(2), 89-100.

Soesanto, L., and Termorshuizen, A. J. (2001). Effect of temperature on the formation of microsclerotia of *Verticillium dahliae*. *Journal of Phytopathology*, *149*(11-12), 685-691.

Sticher, L., Mauch-Mani, B., and Métraux, A. J. (1997). Systemic acquired resistance. *Annual Review of Phytopathology*, *35*(1), 235-270.

Strausbaugh, C. A., Schroth, M. N., Weinhold, A. R., and Hancock, J. G. (1992). Assessment of vegetative compatibility of *Verticillium dahliae* tester strains and isolates from California potatoes. *Phytopathology*, *82*(1), 61-68.

Subbarao, K. V., Chassot, A., Gordon, T. R., Hubbard, J. C., Bonello, P., Mullin, R., Okamoto, D., Davis, M., and Koike, S. T. (1995). Genetic relationships and cross pathogenicities of *Verticillium dahliae* isolates from cauliflower and other crops. *Phytopathology*, *85*(10), 1105-1112.

Subbarao, K., Hu, X., Gurung, S., Short, D., Sandoya, G. V., SHANG, W., et al. (2015). Non-defoliating and defoliating strains from cotton correlate with races 1 and 2 of *Verticillium dahliae*. *Plant Disease*. DOI: 10.1094/PDIS-03-15-0261-RE.

Surico, G., and P. Lavermicocca. (1989). A semiselective medium for the isolation of *Pseudomonas syringae* pv. *savastanoi*. P*hytopathology, 79*(2), 185-190.

Surico, G., Iacobellis, N. S., and Sisto, A. (1985). Studies on the role of indole-3-acetic acid and citokinins in the formation of knots on olive and oleander plants by *Pseudomonas syringae* pv. *savastanoi*. *Physiological Plant Pathology, 26*(3), 309-320.

Tegli, S., Cerboneschi, M., Libelli, I. M., and Santilli, E. (2010). Development of a versatile tool for the simultaneous differential detection of *Pseudomonas savastanoi* pathovars by End Point and Real-Time PCR. *BMC Microbiology*, *10*(1), 156.

Temsah, M., Hanna, L., and Saad, A. T. (2008). Anatomical pathogenesis of *Pseudomonas savastanoi* on olive and genesis of knots. *Journal of Plant Pathology,* 225-232*.*

Tenuta, M., and Lazarovits, G. (2002). Ammonia and nitrous acid from nitrogenous amendments kill the microsclerotia of *Verticillium dahliae*. *Phytopathology*, *92*(3), 255-264.

Teofrasto. (1988). Historia de las plantas. *Credos, Madrid.*

Termorshuizen, A. J., Van Rijn, E., Van Der Gaag, D. J., Alabouvette, C., Chen, Y., Lagerlöf, J., et al. (2006). Suppressiveness of 18 composts against 7 pathosystems: variability in pathogen response. *Soil Biology and Biochemistry*, *38*(8), 2461-2477.

Terral, J. F. (1997). Debuts de la domestication de l'olivier (*Olea europaea* L.) en Mediterranee nordoccidentale, mise en evidence par l'analyse morphometrique appliquee a du materiel anthracologique. *Comptes rendus de l'Académie des sciences. Série 2. Sciences de la Terre et des Planètes*, *324*(5), 417-425 (in French).

Terral, J.F. and Arnold-Simard, G. (1996). Beginnings of olive cultivation in Eastern Spain in relation to Holocene bioclimatic changes. *Quaternary Research*, *46*(2), 176-185.

Teviotdale, B. L., and Krueger, W. H. (2004). Effects of timing of copper sprays, defoliation, rainfall, and inoculum concentration on incidence of olive knot disease. *Plant Disease*, *88*(2), 131-135.

Thanassoulopoulos, C. C., Biris, D. A., and Tjamos, E. C. (1981). Weed hosts as inoculum source of *Verticillium* in olive orchards. *Phytopathologia Mediterranea, 2* (2-3), 164-168.

Tippett, J. T., and Shigo, A. L. (1981). Barrier zone formation: a mechanism of tree defense against vascular pathogens. *IAWA Journal*, *2*(4), 163-168.

Thomashow, L. S., and Weller, D. M. (1996). Current concepts in the use of introduced bacteria for biological disease control: mechanisms and antifungal metabolites. *Plant-microbe interactions*. *Springer US*, *1*, 187-235

Tjamos, E.C. (1993). Prospects and strategies in controlling Verticillium wilt of olive. *EPPO Bulletin*, *23*(3), 505-512.

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

Tjamos, E. C., and Paplomatas, E. J. (1987). Effect of soil solarization on the survival of fungal antagonists of *Verticillium dahliae*. *Eppo Bulletin*, *17*(4), 645-653.

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

Tjamos, E. C., Biris, D. A., and Paplomatas, E. J. (1991). Recovery of olive trees with Verticillium wilt after individual application of soil solarization in established olive orchards. *Plant Disease*, *75*(6), 557-562.

Tjamos, E. C., Graniti, A., Smith, I. M., and Lamberti, F. (1993). Conference on olive diseases. *EPPO Bulletin*, *23*, 365-550.

Tjamos, E. C., Tsitsigiannis, D. I., Tjamos, S. E., Antoniou, P. P., and Katinakis, P. (2004). Selection and screening of endorhizosphere bacteria from solarized soils as biocontrol agents against *Verticillium dahliae* of solanaceous hosts. *European Journal of Plant Pathology*, *110*(1), 35-44.

Tjamos, S. E., Flemetakis, E., Paplomatas, E. J., and Katinakis, P. (2005). Induction of resistance to *Verticillium dahliae* in *Arabidopsis thaliana* by the biocontrol agent K-165 and pathogenesis-related proteins gene expression. *Molecular Plant-Microbe Interactions*, *18*(6), 555-561.

Tous, J., Romero, A., and Hermoso, J. F. (2007). The hedgerow system for olive growing. *Olea (FAO Olive Network)*, *26*, 20-26.

Tous, J., Romero, A., and Hermoso, J. F. (2010). New trends in olive orchard design for continuous mechanical harvesting. *Advances in Horticultural Science*, *24*, 43-52.

Trapero, A., and Blanco, M. A. (1998). Enfermedades. *El cultivo de olivo. Coedición Junta de Andalucía/Mundi-Prensa, Madrid*, 595-656 (in Spanish).

Trapero, A., and Blanco-López, M. A. (2008). Enfermedades. *El cultivo de olivo. Coedición Junta de Andalucía/Mundi-Prensa, Madrid,* 595-656 (in Spanish).

Trapero. A, Roca, L. F., Trapero, C, López-Escudero, F. J, Moral, J. (2010). Culture and knowledge inspired by olive oil. *Olive Diseases*, 89-135.

Trapero. A, Roca. L. F., Moral, J., López-Escudero, F. J., Blanco-López, M. A. (2009). Enfermedades del olivo. *Phytoma España: La Revista Profesional de Sanidad Vegetal*, *209*, 18-28 (in Spanish).

Trapero, C., Rallo, L., López-Escudero, F. J., Barranco, D., and Díez, C. M. (2015). Variability and selection of verticillium wilt resistant genotypes in cultivated olive and in the *Olea* genus. *Plant Pathology*. DOI: 10.1111/ppa.12330.

Trapero, C., Serrano, N., Arquero, O., Del Río, C., Trapero, A., and López-Escudero, F. J. (2013). Field resistance to Verticillium wilt in selected olive cultivars grown in two naturally infested soils. *Plant Disease*, *97*(5), 668-674.

Triki, M. A., Hadj-Taieb, S. K., Mellouli, I. H., Rhouma, A., Gdoura, R., and Hassaïri, A. (2012). Identification and screening of bacterial isolates from Saharan weeds for *Verticillium dahliae* control. *Journal of Plant Pathology*, *94*(2), 305-311.

Triki, M. A., Hassaïri, A., and Mahjoub, M. (2006). Premières observations de *Verticillium dahliae* sur olivier en Tunisie. *EPPO Bulletin*, *36*(1), 69-71 (in French).

Triki, M. A., Kridi, S., Hsairi, H., Hammedi, I., Renaud, I. O. O. S., Gdoura, R., and Rhouma, A. (2011). Occurrence of *Verticillium dahliae* defoliating pathotypes on olive trees in Tunisia. *Phytopathologia Mediterranea*, *50*(2), 267-272.

Tsror, L. (2011). Epidemiology and control of *Verticillium* wilt on olive. *Israel Journal of Plant Sciences*, *59*(1), 59-69.

Tsror, L., and Levin, A. G. (2003). Vegetative compatibility and pathogenicity of *Verticillium dahliae* Kleb. isolates from olive in Israel. *Journal of Phytopathology*, *151*(7-8), 451-455.

Tsror, L., Lebiush, S., Meshulam, M., Erlich, O., Hazanovsky, M., Aharon, M., Matan, E., Tregerman, M., and Gamliel, A. (2007). Biofumigation for the control of soilborne diseases. *Acta Horticulturae*, *747*, 389-394

Typas, M. A., Griffen, A. M., Bainbridge, B. W., and Heale, J. B. (1992). Restriction fragment length polymorphisms in mitochondrial DNA and ribosomal RNA gene complexes as an aid to the characterization of species and sub-species populations in the genus *Verticillium*. *FEMS Microbiology Letters*, *95*(2-3), 157-162.

Uppal, A. K., El Hadrami, A., Adam, L. R., Tenuta, M., and Daayf, F. (2008). Biological control of potato *Verticillium* wilt under controlled and field conditions using selected bacterial antagonists and plant extracts. *Biological Control*, *44*(1), 90-100.

Van der Ent, S., Verhagen, B. W., Van Doorn, R., Bakker, D., Verlaan, M. G., Pel, M. J., et al. (2008). *MYB72* is required in early signaling steps of rhizobacteria-induced systemic resistance in Arabidopsis. *Plant Physiology*, *146*(3), 1293-1304.

Van Loon, L. C., Bakker, P. A. H. M., and Pieterse, C. M. J. (1998). Systemic resistance induced by rhizosphere bacteria. *Annual Review of Phytopathology*, *36*(1), 453-483.

Van Wees, S. C., De Swart, E. A., Van Pelt, J. A., Van Loon, L. C., and Pieterse, C. M. (2000). Enhancement of induced disease resistance by simultaneous activation of salicylate-and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences*, *97*(15), 8711-8716.

Varvaro, L., and Surico, G. (1978). Comportamento di diverse cultivars di Olivo (*Olea europaea* L.) alla inoculazione artificiale con *Pseudomonas savastanoi* (EF Smith) Stevens. *Phytopathologia Mediterranea, 17*(3), 174-177 (in Italian).

Veronese, P., Narasimhan, M. L., Stevenson, R. A., Zhu, J. K., Weller, S. C., Subbarao, K. V., and Bressan, R. A. (2003). Identification of a locus controlling *Verticillium disease* symptom response in *Arabidopsis thaliana*. *The Plant Journal*, *35*(5), 574-587.

Vigouroux, A. (1975). *Verticillium dahliae*, agent d'un deperissement de l'olivier en France. In *Annales de Phytopathology*, *7*, 37-44 (in French).

Vitullo, D., Altieri, R., Esposito, A., Nigro, F., Ferrara, M., Alfano, G., Ranalli, G., De Cicco, V., and Lima, G. (2013). Suppressive biomasses and antagonist bacteria for an eco-compatible control of

Verticillium dahliae on nursery-grown olive plants. *International Journal of Environmental Science and Technology*, *10*(2), 209-220.

Vlassak, K., Van Holm, L., Duchateau, L., Vanderleyden, J., and De Mot, R. (1992). Isolation and characterization of fluorescent *Pseudomonas* associated with the roots of rice and banana grown in Sri Lanka. *Plant and Soil*, *145*(1), 51-63.

Vlot, A. C., Dempsey, D. M. A., and Klessig, D. F. (2009). Salicylic acid, a multifaceted hormone to combat disease. *Annual Review of Phytopathology*, *47*, 177-206.

Walters, D. R., Ratsep, J., and Havis, N. D. (2013). Controlling crop diseases using induced resistance: challenges for the future. *Journal of Experimental Botany*, *64*(5), 1263-1280.

Wang, Y., Ohara, Y., Nakayashiki, H., Tosa, Y., and Mayama, S. (2005). Microarray analysis of the gene expression profile induced by the endophytic plant growth-promoting rhizobacteria, *Pseudomonas fluorescens* FPT9601-T5 in *Arabidopsis*. *Molecular Plant-Microbe Interactions*, *18*(5), 385-396.

Wang, Y., Wang, Y., and Tian, C. (2013). Quantitative detection of pathogen DNA of Verticillium Wilt on smoke tree *Cotinus coggygria*. *Plant Disease*, *97*(12), 1645-1651.

Wei, F., Fan, R., Dong, H., Shang, W., Xu, X., Zhu, H., Yang, J., and Hu, X. (2015). Threshold microsclerotial inoculum for cotton Verticillium Wilt determined through wet-sieving and Real-Time Quantitative PCR. *Phytopathology*, *105*(2), 220-229.

Weise, T., Thürmer, A., Brady, S., Kai, M., Daniel, R., Gottschalk, G., and Piechulla, B. (2014). VOC emission of various *Serratia* species and isolates and genome analysis of *Serratia plymuthica* 4Rx13. *FEMS Microbiology Letters*, *352*(1), 45-53.

Weller, D. M. (1988). Biological control of soilborne plant pathogens in the rhizosphere with bacteria. *Annual Review of Phytopathology*, *26*(1), 379-407.

Weller, D. M. (2007). *Pseudomonas* biocontrol agents of soilborne pathogens: looking back over 30 years. *Phytopathology*, *97*(2), 250-256.

Weller, D. M., Raaijmakers, J. M., Gardener, B. B. M., and Thomashow, L. S. (2002). Microbial populations responsible for specific soil suppressiveness to plant pathogens. *Annual Review of Phytopathology*, *40*(1), 309-348.

Whipps, J. M. (2001). Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany*, *52*(1), 487-511.

Wilhelm, S. (1955). Longevity of the *Verticillium* wilt fungus in the laboratory and field. *Phytopathology*, *45*(3), 180-181.

Wilhelm, S., and Taylor, J. B. (1965). Control of Verticillium wilt of olive through natural recovery and resistance. *Phytopathology*, *55*(3), 310.

Wilson, E. (1935). The olive knot disease: its inception, development, and control. *Hilgardia*, *9*(4), 233-264.

Wilson, E. E., and Magie, A. R. (1964). Systemic invasion of the host plant by the tumor-inducing bacterium, *Pseudomonas savastanoi*. *Phytopathology*, *54*(5), 576-579.

Xiao, C. L., Subbarao, K.V., Schulbach, K. F., and Koike, S. T. (1998). Effects of crop rotation and irrigation on *Verticillium dahliae* microsclerotia in soil and wilt in cauliflower. *Phytopathology*, *88*(10), 1046-1055.

Xiao, C. L., and Subbarao, K. V. (2000) Effects of irrigation and *Verticillium dahliae* on Cauliflower root and shoot growth dynamics. *Phytopathology*, *90*(9), 995-1004.

Yadeta, K. A., and Thomma, B. P. (2013). The xylem as battleground for plant hosts and vascular wilt pathogens. *Frontiers in Plant Science*, *4*, 97

Yang, J., Kloepper, J. W., and Ryu, C. M. (2009). Rhizosphere bacteria help plants tolerate abiotic stress. *Trends in Plant Science*, *14*(1), 1-4.

Young, J. M. (1987). Orchard management and bacterial diseases of stone fruit. *New Zealand Journal of Experimental Agriculture*, *15*(2), 257-266.

Young, J. M. (2004). Olive knot and its pathogens. *Australasian Plant Pathology*, *33*(1), 33-39.

Young, J. M. (2010). Taxonomy of *Pseudomonas syringae*. *Journal of Plant Pathology*, *92*, S5-S14.

Young, J. M., and C. M. Triggs. (1994). Evaluation of determinative tests for pathovars of *Pseudomonas syringae* van Hall 1902. *Journal of Applied Bacteriology, 77*(2), 195-207.

Young, J. M., Wilkie, J. P., Fletcher, M. J., Park, D. C., Pennycook, S. R., Riggs, C. M., and Watson, D. R. W. (2004). Relative tolerance of nine olive cultivars to *Pseudomonas savastanoi* causing bacterial knot disease. *Phytopathologia Mediterranea*, *43*(3), 395-402.

Zachos, D. G. (1963). La verticilliose de l'olivier en Greece. *Annales de l'Institut Phytopathologique Benaki, 5*, 105-107 (in French).

Zamioudis, C., and Pieterse, C. M. (2012). Modulation of host immunity by beneficial microbes. *Molecular Plant-Microbe Interactions*, *25*(2), 139-150.

Zhang, N., Wang, D., Liu, Y., Li, S., Shen, Q., and Zhang, R. (2014). Effects of different plant root exudates and their organic acid components on chemotaxis, biofilm formation and colonization by beneficial rhizosphere-associated bacterial strains. *Plant and Soil*, *374*(1-2), 689-700.

Zhao, P., Zhao, Y. L., Jin, Y., Zhang, T., and Guo, H. S. (2014). Colonization process of *Arabidopsis thaliana* roots by a green fluorescent protein-tagged isolate of *Verticillium dahliae*. *Protein and Cell*, *5*(2), 94-98.

Zhengjun, X., Achar, P. N., and Gu, B. (1998). Vegetative compatibility grouping of *Verticillium dahliae* from cotton in mainland China. *European Journal of Plant Pathology*, *104*(9), 871-876.

Zeise, K., and Von Tiedemann, A. (2002). Application of RAPD-PCR for virulence type analysis within *Verticillium dahliae* and *V. longisporum*. *Journal of Phytopathology*, *150*(10), 557-563.

Zohary, D., and Spiegel-Roy, P. (1975). Beginnings of fruit growing in the Old World. *Science*, *187*(4174), 319-327.