ESCUELA TÉCNICA SUPERIOR DE INGENIEROS AGRÓNOMOS Y MONTES

DEPARTAMENTO DE AGRONOMÍA

CONTROL OF VERTICILLIUM WILT OF OLIVE USING NUTRIENTS, BIOSTIMULANTS AND RESISTANCE INDUCERS

CONTROL DE LA VERTICILOSIS DEL OLIVO MEDIANTE NUTRIENTES, BIOESTIMULANTES E INDUCTORES DE RESISTENCIA

Programa de Doctorado: Ingeniería agraria, alimentaria, forestal y de desarrollo rural sostenible

Doctoranda: Ana López Moral

Directores: D. Antonio Trapero Casas

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TITULO: *CONTROL OF VERTICILLIUM WILT OF OLIVE USING NUTRIENTS, BIOSTIMULANTS AND RESISTANCE INDUCERS*

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TÍTULO DE LA TESIS: CONTROL DE LA VERTICILOSIS DEL OLIVO MEDIANTE NUTRIENTES, BIOESTIMULANTES E INDUCTORES DE RESISTENCIA

DOCTORANDO/A: ANA LÓPEZ MORAL

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

La doctoranda **Ana López Moral** ha realizado satisfactoriamente y en los plazos previstos el trabajo presentado en esta Tesis Doctoral. A lo largo de su investigación, la doctoranda ha contribuido con diversas aportaciones de interés para la comunidad científica respecto al control biológico de la Verticilosis del olivo causada por el hongo *Verticillium dahliae*, así como a determinar los mecanismos de acción de los mejores candidatos seleccionados como tratamientos biológicos por su alta eficacia frente al control del patógeno y del desarrollo de la enfermedad. Además de la novedad e importancia científica, este trabajo aporta información aplicable en el control de esta enfermedad siendo a su vez de gran relevancia para la continuidad de la línea de investigación sobre el control biológico de la Verticilosis del olivo en el grupo de Patología Agroforestal de la Universidad de Córdoba.

La doctoranda ha realizado una estancia nacional de 2 meses y otra internacional de 4 meses. La estancia nacional la realizó en el Departamento de Ciencias Agrarias y del Medio Natural de la Universitat Jaume I de Castellón, bajo la supervisión del Dr. Eugenio Llorens y la Prof. Dra. Pilar García Agustín, donde se abordó un estudio para determinar la posible inducción de resistencia de los tratamientos seleccionados. Dicho estudio ha sido publicado recientemente en la revista internacional *Frontiers in Plant Scienc*e, incluída en la presente Tesis. Por su parte, la estancia internacional se realizó en el Grupo 'Evolutionary Microbiology', en el 'Institute for Plant Sciences' de la Universidad de Colonia (Alemania), bajo la supervisión del Prof. Dr. Bart Thomma, donde se abordó un estudio sobre la caracterización del microbioma bacteriano de

plantas de tomate en busca de agentes de control biológico frente a *V. dahliae*. Dicho estudio continúa en la actualidad, por lo que en la presente Tesis se incluye un capítulo que recopila los resultados obtenidos hasta el momento.

El trabajo realizado en el Departamento de Agronomía de la Universidad de Córdoba por Ana López Moral queda reflejado en varias contribuciones relacionadas con el control biológico de la Verticilosis en el olivo en las cuales consta como primera autora. A día de hoy, se han publicado cuatro artículos en revistas de prestigio científico indexadas en la base de datos JCR. Además, un quinto artículo ha sido enviado para su publicación en una revista científicas indexadas en JCR, y se espera que el trabajo derivado de la mencionada estancia internacional también acabe enviándose a una revista científica de alto impacto en un futuro próximo. Así mismo, ha presentado dos comunicaciones a un congreso internacional y cuatro a tres congresos nacionales, y ha participado en otras actividades de extensión y divulgación de los resultados obtenidos. Además, ha participado en 12 publicaciones científicas más, cinco comunicaciones en congresos internacionales y 16 comunicaciones en congresos nacionales, bien como primera autora o como co-autora de trabajos de temáticas diversas en el campo de la fitopatología relacionadas con las líneas de investigación del Grupo Patología Agroforestal.

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

Córdoba, 17 de febrero de 2022

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Mención de Doctorado Internacional

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

1) Estancia internacional predoctoral de 4 meses (27 mayo 2021 - 30 septiembre 2021) en la Universidad de Colonia (Alemania), Institute for Plant Sciences, Evolutionary Microbiology research group. Supervisor: Prof. Dr. Bart Thomma.

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

- Dr. José Alberto Pereira. Full profesor. Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Braganca, Portugal

- Dr. Santa Olga Cacciola. Associate Professor in Plant Pathology, Dipartimento di Agricoltura Alimentazione e Ambiente (Di3A), University of Catania, Catania, Italia.

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

Dr. Paula Baptista. Professor. Centro de Investigação de Montanha (CIMO), Instituto Politécnico de Bragança, Braganca, Portugal.

4) Parte de la Tesis Doctoral se ha redactado y se presentará en dos idiomas, castellano e inglés.

La doctoranda

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Tesis por compendio de artículos

Esta tesis cumple el requisito establecido por la Universidad de Córdoba para su presentación como compendio de artículos, consistente en un mínimo de 3 artículos publicados o aceptados en revistas incluidas en los tres primeros cuartiles de la relación de revistas del ámbito de la especialidad y referenciadas en la última relación publicada por el Journal Citations Report (SCI):

Artículo 1: López-Moral, A., Agustí-Brisach, C., Trapero, A., 2021. Plant biostimulants: new Insights into the biological control of Verticillium Wilt of Olive. *Frontiers in Plant Science*, 12:662178 (IF:5.753; Q1).

Artículo 2: López-Moral, A., Agustí-Brisach, C., Leiva-Egea, F.M., Trapero, A., 2022. Influence of the cultivar and biocontrol treatments on the effect of olive stem extracts on the viability of *Verticillium dahliae* conidia. *Plants*, accepted (IF: 3.935; Q1).

Artículo 3: López-Moral, A., Llorens, E., Scalschi, L., García-Agustín, P., Trapero, A., Agustí, Brisach, C., 2022. Resistance induction in olive tree (*Olea europaea*) against Verticillium Wilt by two beneficial microorganisms and a copper phosphite fertilizer. *Frontiers in Plant Science*, 13:831794 (IF:5.753; Q1).

Artículo 4: López-Moral, A., Agustí-Brisach, C., Ruiz-Blancas, C., Antón-Domínguez, B., Alcántara, E., Trapero, A., 2022. Elucidating the effect of nutritional imbalances of N and K on the infection of *Verticillium dahliae* in olive. *Journal of Fungi*, 8:139 (IF: 5.816; Q1).

La doctoranda

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Summary

Verticillium wilt of olive (*Olea europaea*; VWO), caused by the hemibiotrophic fungus *Verticillium dahliae*, is the most serious biotic challenge to this crop in the Mediterranean basin since it causes high levels of tree mortality and fruit yield reduction. Due to the lack of a truly efficient method to control VWO, an integrated management strategy is needed to reduce both pathogen dispersal and disease incidence in olive groves. Thus, the use of natural or biological compounds arises as a potential complementary and eco-friendly control tool against VWO. In this context, the Agroforestry Pathology Research Group AGR-216 from the University of Cordoba (Spain) has been developing an extensive research line on biocontrol of VWO since 2011, which has contributed markedly in generating knowledge on such topic towards to obtain potential biological solutions against the disease in the near future. To reach this goal, more than 280 compounds including microorganisms (fungi, bacteria), organic amendments (waste from animals and food industry), natural substances (essential oils, plant extracts), nutrients and resistance inducers have been tested through a massive screening under controlled conditions. Thus, the main goal of this PhD Thesis was to continue evaluating a broad diversity of biological treatments against the disease as well as determining the mode of action of the most effective treatments.

To this end, the first objective of this PhD Thesis was to evaluate the effect of 32 compounds including microorganisms, biostimulants, copper fertilizers and resistance inducers against the *V. dahliae in vitro* and the disease progress in olive plants of cv. Picual. Among all the compounds tested, *Phoma* sp. and *Aureobasidium pullulans* fungal strains were the most effective in disease reduction *in planta* with foliar application; whereas two phosphite salts, one with copper and the other with potassium, were the most effective in disease reduction *in planta* when they were applied by irrigation, followed by *A. pullulans* and *Bacillus amyloliquefaciens* strains. This study served to select the best candidates to conduct a set of experiments to determine their possible modes of action.

The objective 2 was to evaluate the influence of the cultivar, biocontrol treatments and their interaction on the effect of olive stem extracts (OSE) on the viability of *V. dahliae* conidia. The results indicated that the influence of biological treatments against the pathogen depends on the genotype, since the higher the resistance

of the cultivar, the lower the effect of the treatments on the ability of the OSE to inhibit the germination of conidia. In 'Picual', the biological control agent *B. amyloliquefaciens* and copper phosphite were the most effective treatments in inhibiting conidia germination by the OSE.

The objective 3 was to set up a collection method for root exudates from young olive plants by testing three collection media (deionized distilled water, 0.01 M $CaSO_4$ solution, Hoagland nutrient solution) and four collection periods (2, 4, 6, 12 h). Once the protocol was set up, the effect of root exudates from different cultivars, biocontrol treatments and their interaction on the viability of conidia and microsclerotia (MS) of *V. dahliae* was evaluated. In addition, the dissolved organic carbon (DOC) exudation level from the root exudates was estimated. A 0.01 M $CaSO_4$ solution and 4 h may be regarded a preferential medium and timing combination to collect root exudates from olive. Root exudates induced the germination of *V. dahliae* conidia and MS, with genotype affecting significantly on this ability. *Aureobasidium pullulans* was the most effective biocontrol treatment to enhance the effectiveness of root exudates decreasing the viability of conidia and MS. DOC in the exudate solutions varied depending on the olive cultivars and/or on the biocontrol treatments.

The objective 4 aimed to determine the effectiveness of *A. pullulans*, *B. amyloliquefaciens* and copper phosphite 3 enhancing the plant defence system in olive plants of cv Picual against *V. dahliae* by means of assessing biochemical parameters involved in plant resistance such as reactive oxygen species (ROS) response (H_2O_2) and plant hormones by relative gene expression. High levels of salicylic acid (SA) were detected on leaves from all treatment combinations, but without significant enhancements compared to the non-treated control. Regarding the gene expression related to SA, only the *WRKY5* gene has shown a strong enhancement in the treatment with *B. amyloliquefaciens*. On the other hand, a high accumulation of jasmonic acid (JA) and JA-isoleucine in plants treated with *A. pullulans* was observed in leaf, stem and root tissues as well as in roots of plants treated with *B. amyloliquefaciens* and the copper phosphite 3.

In parallel, the objective 5 was conducted to evaluate the influences of nutritional imbalances of nitrogen (N) and potassium (K) in *V. dahliae* infection to olive. To this end, adjusted treatments with N excess (↑N+↑Na), K deficiency (↓K) and

their combination (↑N+↑Na+↓K) were evaluated on the viability of *V. dahliae* MS; as well as on disease development in olive plants. In parallel, the potential indirect effect of the treatments through the stimuli of root exudates was evaluated on the viability of conidia and MS of *V. dahliae*. Treatments ↑N+↑Na and ↑N+↑Na+↓K decreases MS germination and disease progress; whereas ↓K significantly increases both parameters. Root exudates from treated plants increased *V. dahliae* conidia germination, but reduced MS germination.

Finally, the objective 6 was a complementary study to characterize the bacterial microbiome of tomato plants to search for new potential biological control agents against *V. dahliae* by evolving the most effective bacterial strains towards Ave1 effector protein tolerance.

All the knowledge generated through this PhD Thesis represents a relevant step towards the biological control of VWO, and opens a variety of new paths to explore to better understand the mechanism of action of biological treatments in olive trees against *V. dahliae*.

Resumen

La Verticilosis del olivo (*Olea europaea*; VO), causada por el hongo hemibiotrofo *Verticillium dahliae*, es el desafío biótico más grave para este cultivo en la cuenca mediterránea, ya que causa altos niveles de mortalidad en los árboles y reducción del rendimiento de la cosecha. Debido a la falta de un método realmente eficaz para el control de la VO, es necesaria una estrategia de gestión integrada para reducir tanto la dispersión del patógeno como la incidencia de la enfermedad en los olivares. De este modo, el uso de compuestos naturales o biológicos surge como una herramienta potencial de control complementaria y ecológica contra la VO. En este contexto, el Grupo de Investigación Patología Agroforestal AGR-216 de la Universidad de Córdoba (España) viene desarrollando desde 2011 una amplia línea de investigación sobre el biocontrol de la VO, que ha contribuido notablemente en la generación de conocimiento en este tema hacia la obtención de soluciones biológicas potenciales contra la enfermedad en un futuro próximo. Para alcanzar este objetivo, se han evaluado más de 280 compuestos que incluyen microorganismos (hongos, bacterias), enmiendas orgánicas (residuos de animales y de la industria alimentaria), sustancias naturales (aceites esenciales, extractos de plantas), nutrientes e inductores de resistencia mediante un cribado masivo en condiciones controladas. Así, el objetivo principal de esta Tesis Doctoral fue seguir evaluando una amplia diversidad de tratamientos biológicos contra la enfermedad, así como determinar el modo de acción de los tratamientos más eficaces.

Para ello, el primer objetivo de esta Tesis Doctoral fue evaluar el efecto de 32 compuestos incluyendo microorganismos, bioestimulantes, fertilizantes cúpricos e inductores de resistencia contra *V. dahliae in vitro*, y el progreso de la enfermedad en plantas de olivo cv. Picual. De entre todos los compuestos evaluados, las cepas fúngicas *Phoma* sp. y *Aureobasidium pullulans* fueron las más eficaces en la reducción de la enfermedad en planta por aplicación foliar; mientras que dos sales de fosfito, una a base cobre y otra de potasio, fueron las más eficaces en la reducción de la enfermedad en planta cuando se aplicaron por riego, seguidas de las cepas de *A. pullulans* y *Bacillus amyloliquefaciens*. Este estudio sirvió para seleccionar los mejores candidatos y realizar un conjunto de experimentos para determinar sus posibles modos de acción.

El objetivo 2 fue evaluar la influencia del cultivar, los tratamientos de biocontrol y su interacción en el efecto de los extractos de tallo de olivo (OSE) sobre la viabilidad

de los conidios de *V. dahliae*. Los resultados indicaron que la influencia de los tratamientos biológicos contra el patógeno depende del genotipo, ya que cuanto mayor es la resistencia del cultivar, menor es el efecto de los tratamientos sobre la capacidad de los OSE para inhibir la germinación de los conidios. En 'Picual', el agente de control biológico *B. amyloliquefaciens* y el fosfito de cobre fueron los tratamientos más efectivos para inhibir la germinación de conidios por parte del OSE.

El objetivo 3 fue establecer un método de recolección de exudados radiculares de plantas jóvenes de olivo, probando tres medios de recolección (agua destilada desionizada, solución de $CaSO_4$ 0,01 M, solución nutritiva Hoagland) y cuatro períodos de recolección (2, 4, 6, 12 h). Una vez establecido el protocolo, se evaluó el efecto de los exudados radiculares de diferentes cultivares, los tratamientos de biocontrol y su interacción sobre la viabilidad de los conidios y miscrosclerocios (MS) de *V. dahliae*. Además, se estimó el nivel de exudación de carbono orgánico disuelto (DOC) de los exudados radiculares. La solución de $CaSO_4$ 0,01 M y 4 h puede considerarse la combinación preferente de medio y tiempo para recoger los exudados de las raíces del olivo. Los exudados radiculares indujeron la germinación de conidios y MS de *V. dahliae*, y el genotipo afectó significativamente a esta capacidad. *Aureobasidium pullulans* fue el tratamiento de biocontrol más efectivo para aumentar la eficacia de los exudados radiculares disminuyendo la viabilidad de los conidios y los MS. El DOC en las soluciones de exudado varió en función de los cultivares de olivo y/o de los tratamientos de biocontrol.

El objetivo 4 pretendía determinar la eficacia de *A. pullulans*, *B. amyloliquefaciens* y fosfito de cobre 3 en la mejora del sistema de defensa de las plantas de olivo cv Picual frente a *V. dahliae* mediante la evaluación de los parámetros bioquímicos implicados en la resistencia de la planta, como la respuesta de las especies reactivas del oxígeno (ROS) (H_2O_2) y las hormonas vegetales mediante expresión de genes relativa. Se detectaron altos niveles de ácido salicílico (SA) en las hojas de todas las combinaciones de tratamiento, pero sin mejoras significativas en comparación con el control no tratado. En cuanto a la expresión de genes relacionados con el SA, sólo el gen *WRKY5* mostró un fuerte aumento en el tratamiento con *B. amyloliquefaciens*. Por otro lado, se observó una alta acumulación de ácido jasmónico (JA) y JA-isoleucina en las plantas tratadas con *A. pullulans* en los tejidos de la hoja, el tallo y la raíz, así como en las raíces de las plantas tratadas con *B. amyloliquefaciens* y el fosfito de cobre 3.

En paralelo, el objetivo 5 se llevó a cabo para evaluar la influencia de los desequilibrios nutricionales de nitrógeno (N) y potasio (K) en la infección de *V. dahliae* en olivo. Para ello, se evaluaron tratamientos ajustados con exceso de N (↑N+↑Na), déficit de K (↓K) y su combinación (↑N+↑Na+↓K) sobre la viabilidad de MS de *V. dahliae*; así como sobre el desarrollo de la enfermedad en lplantas de olivo. Paralelamente, se evaluó el potencial efecto indirecto de los tratamientos a través de los de los exudados radiculares sobre la viabilidad de los conidios y MS de *V. dahliae*. Los tratamientos ↑N+↑Na y ↑N+↑Na+↓K disminuyen la germinación de MS y el progreso de la enfermedad; mientras que ↓K aumenta significativamente ambos parámetros. Los exudados radiculares de las plantas tratadas aumentaron la germinación de conidios de *V. dahliae*, pero redujeron la germinación de MS.

Finalmente, el objetivo 6 fue un estudio complementario para caracterizar el microbioma bacteriano de plantas de tomate en busca de nuevos agentes potenciales de control biológico contra *V. dahliae* mediante la evolución de las cepas bacterianas más eficaces hacia la tolerancia a la proteína efectora Ave1.

Todo el conocimiento generado a través de esta Tesis Doctoral representa un paso relevante hacia el control biológico de la VO, y abre una diversidad de nuevos caminos a explorar para entender mejor el mecanismo de acción de los tratamientos biológicos en olivos contra *V. dahliae.*

Abbreviations

ANOVA: analysis of variance

ACL: acetone cyanohydrin lyase

APDA: Potato dextrose agar acidified with lactic acid

bHLH: basic helix-loop-helix

BCA(s): biological control agent(s)

C: Carbon

cDNA (s): complementary Deoxyribonucleic Acid(s)

CFU: colony forming units

CGe: Conidial germination

CSM: cornmeal-sand mixture

Ct: cycle thresholds

CuPh: Copper phosphite

D: defoliating pathotype

DAB: 3′,3-diaminobenzidine

DAUCO: Department of Agronomy at the University of Córdoba

DI: disease incidence

dhJA: dihydrojasmonic acid

DOC: Dissolved organic carbon

DS: Disease severity

EC_{50}: Effective concentrations (µl ml−1) to inhibit 50% of conidial germination.

ET: ethylene

EU: European Union

JA: Jasmonic acid

JA-lle: Jasmonic acid-Isoleucine

HNS: Hoagland nutrient solution

HPDI: host plant defense inducers

HRI: host resistance induction

HSD: honestly significant difference test

IDM: integrated disease management

ITS: internal transcribed spacer region of the ribosomal DNA

FRAC: Fungicide Resistance Action Committee

LBA: lysogeny broth agar

LOX: lipoxygenase

LSD: least significant difference

MGR: Mycelial growth rate

MGI: Mycelial growth inhibition

MS: microsclerotia

MSC: Microscletoria concentration

MSG: Microsclerotia germination

MSI: Microscletoria inhibition

MSPA: modified sodium polypectate agar medium

NB: Nutrient broth

ND: non-defoliating pathotype

OA(s): Organic amendment (s)

OD_{600}: optical density measured at 600 nm

OPDA: 12-oxo-phyto dienoic acid

OSE: Olive stem extract

P: phosphorus

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

PAL: phenylalanine ammonia-lyase

PAMPs: pathogen-associated molecular patterns

PCR: polymerase chain reaction

PDA: potato dextrose agar

qRT-PCR: quantitative real time polymerase chain reaction

RAUDPC: relative area under the disease progress curve

RE: relative expression

RGI: Relative Germination Inhibition

RH: Relative humidity

ROS: reactive oxygen species

R2A: Reason´s 2A agar

SA: Salicylic acid

SDDW: sterile deionized distilled water

TOC-L: Total Organic Carbon Analyzer

TSA: tryptone soya agar

VdAve1: antimicrobial *V. dahliae* effector protein Ave1

VWO: Verticillium wilt of olive

UPLC: ultrahigh-performance liquid chromatography

Contents

Chapter 1

GENERAL INTRODUCTION

The olive crop

The cultivated olive (*Olea europaea* subsp. *europaea* L.) is one of the six subspecies of the *Olea europaea* species complex in the Oleaceae family. Within this complex, *O. europaea* subsp. *europaea* is the only native from the Mediterranean basin, and is considered as one of the first tree species domesticated and cultivated (Green, 2002, 2004; Connor, 2005). The origin of the cultivated olive dates back to about 6,000 years ago in the north of the eastern coast of the Mediterranean. Since then, it was progressively disseminated, firstly to the rest of the Near East, afterwards to western Mediterranean, and currently, it is spread in diverse regions over the world with Mediterranean climate at latitudes between 30° and 45° in the northern and southern hemispheres (Janick, 2005; Rallo, 2005; Besnard *et al.*, 2013).

Today, the olive tree is the most cultivated non-tropical fruit tree in the world. The olive crop has more than 10 million ha of global cultivated area distributed in more than 20 countries worldwide, with around 97% of this area in the Mediterranean basin, and an average world production of around 20 million tons per year (FAO, 2017). Most of the olive production (90%) is destinated to the olive oil industry, while the rest of the production (10%) is dedicated to table olives (FAO, 2017). Spain is the world leader in olive production, generating about 43% of the world's olives. The Spanish olive industry accounts for 25% of the global area devoted to olive production, occupying 2.5 × 10^6 ha (for both table fruit and oil). Approximately 65% of this land is in the Andalusia region of the southern Iberian Peninsula, itself producing 85% of Spain's total production (Barranco *et al.*, 2017).

The olive crop is undergoing a constant change from traditional cultivation systems to more intensive olive cropping systems in order to increase yields by optimizing the use of inputs (e.g., fertilizers, herbicides, insecticides, fungicides, etc.) that minimize the production costs. However, it must be considered that the intensive olive growing systems have negative environmental consequences such as the loss of genetic variability, environmental contamination due to the greater use of inputs, contamination due to the excessive use of agrochemicals, as well as the increase in the incidence and prevalence of pests and diseases and the re-emergence of secondary ones. In the context of this PhD Thesis, these last aspects have contributed to the increase of

the incidence of Verticillium wilt of olive (VWO), which is currently considered one of the main limiting factors in olive crop, both in traditional olive groves and in the new intensive ones, being its control a serious phytosanitary challenge in our society (Muñoz-Díez *et al.*, 2016; Trapero *et al.*, 2017).

Verticillium wilt of olive

Verticillium wilt of olive (VWO) is currently considered the main soilborne disease threatening olive production worldwide since it causes high levels of tree mortality and fruit yield reduction (Jiménez-Díaz *et al.*, 2012; López-Escudero and Mercado Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020). In southern Spain, the disease results in one of the major concerns for olive growers. Although the global disease incidence in this region is around 0.5%, it can reach values above 20% together with high levels of disease severity and tree mortality in certain areas of the Guadalquivir valley (Jiménez-Díaz *et al.*, 2012; Trapero *et al.*, 2017).

Under Guadalquivir Valley conditions, VWO traditionally comprises two symptom complexes: *i*) apoplexy, characterized by a quick death of branches or of the whole plant that occurs mainly from late winter to early spring; and *ii*) slow decline, which occurs in spring-early summer or autumn, with inflorescence necrosis or fruit mummification being the most common symptoms, respectively, along with intense defoliation of green leaves (Barranco *et al.*, 2017).

The causal agent of VWO is the hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb., from which two populations, defoliating (D) and nondefoliating (ND) pathotypes, have been identified in olive, with D pathotype causing the most severe damage (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012). The pathogen produces microsclerotia (MS), which are dormant structures that not only confers its ability to survive in the soil for a long time, but also, they serve as the primary inoculum in natural infections (Jiménez-Díaz *et al.* 2012; López-Escudero and Mercado-Blanco 2011; Montes-Osuna and Mercado-Blanco 2020).

Regarding the life cycle of *V. dahliae*, MS germination is induced by root exudates from the susceptible hosts, giving rise to infective hyphae that penetrate the plant roots growing towards the vascular system (Pegg and Brady 2002). Subsequently, the pathogen colonizes the xylem vessels of the infected plants by mycelia and conidia,

which contribute to the occlusion of the vascular system together with the production of gels and tyloses in the cells as a consequence of natural plant response against the infection. Altogether cause the reduction of water flow, leads to water stress, and consequently, plants become wilted (Ayres 1978; Pegg and Brady 2002; Trapero *et al*. 2018). Disease severity varies depending on the olive cultivars. Most of them have been described as susceptible or extremely susceptible to the pathogen. Among them, 'Picual' is considered one of the most susceptible, whereas 'Changlot Real', 'Empeltre', and 'Frantoio' have shown high levels of tolerance (López-Escudero *et al.*, 2004; Trapero *et al.* 2013). In addition, the effect of mineral nutrition on Verticillium wilt diseases has already been reported in many herbaceous hosts having resulted in either a decrease or an increase in wilt symptoms (Pegg and Brady, 2002). However, the influence of mineral nutrition in the cycle of life of VWO is still uncertain.

To date, no effective control measures to the disease are available. The ability that MS confers to *V. dahliae* to survive for up to 14 years in the soil, the broad diversity of alternative hosts for the pathogen, added to the absence of effective chemical treatments against the disease, makes it difficult to control VWO (Trapero *et al.*, 2015). Therefore, an integrated disease management (IDM) strategy including both pre- and postplanting treatments must be strongly considered for the control of the disease within the framework of sustainable agriculture (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Ostos *et al.*, 2020).

Starting hypothesis and justification of the PhD Thesis

Based on the advances about the control of VWO conducted over the last two decades, there is no doubt that an integrated management strategy is needed to reduce both pathogen dispersal and disease incidence in olive groves (López-Escudero and Trapero, 2017; Montes-Osuna and Mercado-Blanco, 2020). Within this strategy, the combination of several eco-friendly control methods such as genetic resistance, cultural practices and biological control in both pre- and postplanting must be considered (López-Escudero and Mercado-Blanco, 2011; Trapero *et al.*, 2015; Montes-Osuna and Mercado-Blanco, 2020; Ostos *et al.*, 2020).

With respect to biological control against VWO, which is the aim of this PhD Thesis, a wide diversity of compounds including organic amendments; essential oils; seaweeds, plant or microorganism extracts; plant biostimulants; fertilizers; mycorrhizal

fungi; and antagonist microorganisms, i.e. endophytic bacteria and fungi, have been evaluated worldwide over the last two decades as a potential eco-friendly control measure against VWO (Montes-Osuna and Mercado-Blanco, 2020; Poveda and Baptista, 2021). Among all these previous studies, to our knowledge, only a few compounds have showed high or moderate effectiveness against VWO in the field, such as the bacterium *Paenibacillus alvei* strain K165 (Markakis *et al.*, 2016), the non-pathogenic fungus *Fusarium oxysporum* strain FO12 and a commercial mix of *Trichoderma asperellum* and *T. gamsii* (Mulero-Aparicio *et al.*, 2020), *T. harzianum* (Arici and Demirtas, 2019), and several mycorrhizal fungi including diverse *Glomus* spp. (Porras-Soriano *et al.*, 2006) and *Rhizophagus irregularis* (Arici and Demirtas, 2019). In addition, the results also suggest these biological compounds could be acting against *V. dahliae* by means of several modes of action including soil suppression, endophytism, antagonism, and host resistance induction (HRI) (Tjamos *et al.*, 2005, 2004; Ruano-Rosa *et al.*, 2017; Varo *et al.*, 2017, 2018, 2019; Mulero-Aparicio *et al.*, 2019a,b; Azabou *et al.*, 2020; Montes-Osuna and Mercado-Blanco, 2020; Poveda and Baptista, 2021).

On the other hand, the efficacy of the phosphites in crop protection has also been reported based on the experimental evidence from decades that has shown their positive effects on plant metabolism, not only as a fertilizer by increasing plant growth and productivity, but also as a pesticide against various species of pathogenic bacteria, fungi and oomycetes (Guest and Grant, 1991; Cooke and Little, 2002; Gómez-Merino and Trejo-Téllez, 2015; Abbasi and Lazarovits, 2006a,b; Romero *et al.*, 2019). Therefore, phosphites have emerged also as potential HRI, as it has demonstrated their effectiveness against different biotic and abiotic stress factors improving crop protection, yield, and quality (Navarro-Cerrillo *et al.*, 2004; Gómez-Merino and Trejo-Téllez, 2015; Barrós-Rodríguez *et al.*, 2020).

Because both biological and phosphite compounds could be a novel and potentially eco-friendly alternative control measure for VWO in IDM strategies, evaluating a wide diversity of these compounds against the disease, as well as determining their mode of action against the pathogen, results in the need for studies to be conducted for the biological control of VWO.

OBJECTIVES

Due the background explained above, the general objective of this Thesis is to evaluate a wide variety of biological treatments against the pathogen and the disease under controlled conditions, determining the mode/s of action of the most promising candidates, as well as elucidating the influence of mineral nutrition against the development of the disease.

Thus, the specific objectives are:

1) To evaluate the effect of 32 compounds including microorganisms, biostimulants, copper fertilizers and resistance inducers against the *V. dahliae* in vitro and the development of the disease in olive plants. This objective is addressed in **chapter 2**.

2) To elucidate the possible modes of action of the most effective compounds of the Objective 1 by determining the influence of the endosphere and the root exudates of the treated plants on the structures of the pathogen as well as the possible induction of host resistance of the selected treatments. This objective is addressed in **chapters 3, 4 and 5.**

3) To evaluate the influences of nutritional imbalances of nitrogen (N) and potassium (K), alone or combined, on infection by *V. dahliae* in olive plants under controlled conditions. This objective is addressed in **chapter 6**.

4) To characterize the bacterial microbiome of tomato plants to search for new potential biological control agents against *V. dahliae*. This objective is addressed in **chapter 7**.

OUTLINE OF THE THESIS

In the **Chapter 2** of this PhD Thesis, the effect of the 32 selected compounds on mycelial growth and MS inhibition of *V. dahliae* has been evaluated by dual cultures or by *in vitro* sensitivity tests This first step was necessary to check if the products had any fungicidal effect and interfered with the growth or survival of the pathogen. Subsequently, the potential biocontrol effect of all the compounds on the progress of the disease in olive plants inoculated with the pathogen was also evaluated.

The knowledge of the modes of action of any biocontrol compound is essential to develop an effective control strategy against any pathogen. Therefore, the **Chapters 3, 4 and 5** aims to determine the possible modes of action of the most effective compounds from the Chapter 2 as described below: *i*) **Chapter 3** is a first approach to evaluate the influence of the cultivar, biocontrol treatments and their interaction on the effect of olive stem extracts (OSE) on the viability of *V. dahliae* conidia; *ii*) **Chapter 4** aims to set up a method for collecting root exudates from young olive plants and to evaluate the effect of root exudates from different cultivars, biocontrol treatments and their interaction on the viability of *V. dahliae* conidia and MS; and *iii*) **Chapter 5** aims to determine the effectiveness of the most promising biological controls that improve the plant defense system in olive plants of cv Picual against *V. dahliae* by assessing biochemical parameters involved in plant resistance, such as reactive oxygen species (ROS) response (H_2O_2) and plant hormones.

In addition, the main goal of the **Chapter 6** is to evaluate the influences of nutritional imbalances of N and K, alone or combined, on infection by *V. dahliae* in olive plants under controlled conditions. To this end, adjusted treatments with N excess, K deficiency and their combination are tested on the viability of *V. dahliae* MS *in vitro*; as well as on disease development by bioassays in olive plants of cv. Picual.

Finally, the **Chapter 7** aims to characterize the bacterial microbiome of tomato plants to search for new potential biological control agents against *V. dahliae* by selecting the most effective bacterial strains towards Ave1effector protein tolerance.

Chapters 2, 3, 5 and 6 have been published in peer-reviewed journals, while chapter 4 is still under review (see **Appendix: Scientific production**). The Chapter 7 has not yet been published as it is part of an international stay that will be completed in the near future, but a similar format has been applied to it.

Chapter 2

Plant biostimulants: new insights into the biological control of Verticillium wilt of olive

ABSTRACT

Verticillium wilt of olive (*Olea europaea* subsp. *europaea* L.) (VWO), caused by the hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb., is considered the major limiting factor of this crop in Mediterranean-type climate regions of the world. The absence of effective chemical treatments makes the control of the disease difficult. In this way, the use of biostimulants and host plant defense inducers seems to be one of the most promising biological and eco-friendly alternatives to traditional control measures. Thus, the main goal of this study was to evaluate the effect of 32 products, including amino acids, micronutrients, microorganisms, substances of natural origin, copper complex-based products, and organic and inorganic salts against the disease under controlled conditions. To this end, their effects on mycelial growth and microsclerotia (MS) inhibition of *V. dahliae* were evaluated by means of dual cultures or by sensitivity tests *in vitro* as well as on disease progression *in planta*. Wide ranging responses to the pathogen and disease reduction levels were observed among all the products tested, suggesting multiple modes of action. Copper-based products were among the most effective for mycelial growth and MS inhibition, whereas they did not show an important effect on the reduction of disease severity *in planta*. *Phoma* sp. and *Aureobasidium pullulans* were the most effective in disease reduction *in planta* with foliar application. On the other hand, two phosphite salts, one with copper and the other with potassium, were the most effective in disease reduction *in planta* when they were applied by irrigation, followed by *A. pullulans* and *Bacillus amyloliquefaciens*. This study will be useful to select the best candidates for future studies, contributing significantly to new insights into the current challenge of the biological control of VWO.

Keywords: Biocontrol, Biostimulants, *Olea europaea*, Resistance inductors, *Verticillium dahliae*

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INTRODUCTION

Verticillium wilt of olive (*Olea europaea* subsp. *europaea* L.) (VWO) is considered the major limiting factor of this crop in Mediterranean-type climate regions of the world since it causes high levels of tree mortality and fruit yield reduction (Montes-Osuna and Mercado-Blanco, 2020). The disease is caused by the hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb. Two populations of the pathogen, defoliating (D) and nondefoliating (ND) pathotypes, have been well distinguished in olive, with the D pathotype causing the most severe damage. In any case, the pathogen is characterized by the production of infective propagules known as microsclerotia (MS), which are dormant structures that allow the fungus to survive in the soil for long periods of time (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012; Montes-Osuna and Mercado-Blanco, 2020).

The best strategies for the management of VWO should be focused on reducing the survival of MS in the soil as well as preventing their germination (Antonopoulos *et al.*, 2008). However, the ability of MS to survive for up to 14 years in the soil and to infect a broad diversity of alternative hosts, added to the absence of effective chemical treatments against the disease, makes VWO control difficult (Trapero *et al.*, 2015). Therefore, an integrated disease management (IDM) strategy including both pre- and postplanting treatments must be strongly considered for the control of the disease within the framework of sustainable agriculture (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Ostos *et al.*, 2020). In this framework, the implementation of natural products such as essential oils or organic amendments (OAs) as well as biological control agents (BCAs) against VWO has been studied by several authors over the last two decades as a potential eco-friendly control measure against the disease (Montes-Osuna and Mercado-Blanco, 2020). In fact, the search for novel BCAs, including bacteria [*Pseudomonas* spp. (Ruano-Rosa *et al.*, 2017; Gómez-Lama Cabanás *et al.*, 2018b), strains of the Bacillales order (Tjamos *et al.*, 2004, 2005; Markakis *et al.*, 2016; Gómez-Lama Cabanás *et al.*, 2018a; Azabou *et al.*, 2020), etc.] and fungi [i.e., *Aureobasidium* spp.; *Phoma* sp. (Varo *et al.*, 2016); nonpathogenic *Fusarium oxysporum* strains (Mulero-Aparicio *et al.*, 2019a,b; 2020); *Trichoderma* spp. (Carrero-Carrón *et al.*, 2016, 2018; Ruano-Rosa *et al.*, 2016, Morán-Díez *et al.*, 2019), etc.] have led to promising results against VWO. However, most of these studies have been conducted under controlled experimental conditions, with the exception of those

conducted by Markakis *et al.* (2016) and Mulero-Aparicio *et al.* (2020). These authors evaluated the efficiency of the BCA *Paenibacillus alvei* strain K165 (Markakis *et al.*, 2016) and the nonpathogenic *F. oxysporum* strain FO12 (Mulero-Aparicio *et al.*, 2020) in suppressing VWO in naturally infected fields with promising results. However, further research is needed to develop future biological preformulations for their commercialization.

In addition to the BCAs described above, more than 230 natural products, including OAs, microorganisms, plant extracts, essential oils, and mixtures of them, have also been evaluated in recent years to determine their effectiveness in suppressing *V. dahliae* under controlled conditions (Lozano-Tovar *et al.*, 2013; Varo *et al.*, 2016, 2017, 2018). The most effective products derived from these last studies have also been evaluated under experimental field conditions in naturally infested soils by Mulero-Aparicio *et al.* (2020), who showed that a commercial essential oil from *Thymus* sp. and the grape marc compost CGR03 were able to significantly reduce the disease incidence.

Recently, the use of seaweeds such as alginate, laminarin, carrageenan and ulvan in the biological control of VWO has also been evaluated. Seaweeds are considered potential elicitors of phenylalanine ammonia-lyase (PAL) and lignin contents, which could markedly reduce vascular discolouration in affected olive twigs (Salah *et al.*, 2018). Likewise, it is worth mentioning that the use of biostimulants and host plant defense inducers (HPDI)also seems among the most promising biological and ecofriendly alternatives to traditional control measures (Sharma *et al.*, 2014; Drobek *et al.*, 2019; Barros-Rodríguez *et al.*, 2020). It has been demonstrated that plants are able to activate a battery of defensive responses against biotic or abiotic stresses when they are previously stimulated by means of appropriate natural or chemical products (Conrath, 2009; Llorens *et al.*, 2017b). From a phytopathological point of view, these stimuli are interesting since they not only induce plant innate resistance that could be enough to overcome the attack of the pathogen but also persist in the plant for several months with long-term effects preventing new infections (Bektas and Eulgem, 2015; Llorens *et al.*, 2017b). In addition, these products have low or null toxicity, contributing to a reduction in the number of residues in fruit and vegetables (González-Hernández *et al.*, 2018). For all these reasons, biostimulants and HPDI are advantageous in term of sustainability, and their use is allowed in IDM strategies (González-Hernández *et al.*, 2018; Montes-Osuna and Mercado-Blanco, 2020).

Because this kind of products could be a novel and potentially eco-friendly alternative control measure for VWO in IDM strategies, the main goal of this study was to evaluate the effect of 32 products grouped under the terms BCAs, biostimulants, HPDI, and fungicides against the disease under controlled conditions. These last were included for comparative purposes. Most of the products included in this study (22 out of 32; Table 1) were carefully selected for their potential activity as plant biostimulants according to the most recent European regulatory framework on this topic [European Regulation (EU) 2019/1009], which defines plant biostimulants as '*a product stimulating plant nutrition processes independently of the product's nutrient content with the sole aim of improving one or more of the following characteristics of the plant or the plant rhizosphere as a) nutrient use efficiency, b) tolerance to abiotic stress, c) quality traits and d) availability of confined nutrients in soil or rhizosphere*'. First, the effect of the 32 selected products on mycelial growth and MS inhibition of *V. dahliae* was evaluated by means of dual cultures or by sensitivity tests *in vitro*. This first step was useful to check whether the products had any fungicidal effect and interfered with the growth or survival of the pathogen. Subsequently, the potential biocontrol effects of all the products were also evaluated on the progress of the disease in olive plants inoculated with the pathogen. This study will be useful to select better candidates for future studies that should be conducted under the EU 2019/1009, contributing significantly to new insights into the current challenge of the biological control of VWO.

MATERIALS AND METHODS

Fungal isolate and culture conditions

Verticillium dahliae strain V180 isolated from soil samples collected from a commercial olive orchard affected by VWO in Villanueva de la Reina (Jaen province, southern Spain) was used in all the experiments. This isolate was previously characterized as the D pathotype by PCR. It was highly virulent in inoculated olive plants and was maintained as a single-spore isolate on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants fully filled with sterile paraffin oil at 4°C in darkness in the collection of the Department of Agronomy at the University of Córdoba (DAUCO, Spain). Prior to conducting each experiment described below, fresh colonies of the V180 strain were obtained from the collection by plating small mycelial

fragments of the colonized agar from the tube onto PDA acidified with lactic acid [APDA; 2.5% (vol/vol) at 2.5 ml/litre of medium] and incubated at 24°C in darkness for 10 days. Subsequently, fresh colonies were transferred to PDA, incubated as described before and then used as inoculum sources.

Products

A total of 32 products, including amino acids, micronutrients, microorganisms, substances of natural origin, copper complex-based products, and organic and inorganic salts, were evaluated. Twenty-nine of the 32 products were commercial products, and they were tested at the dose indicated by the respective manufacturers for either foliar or irrigation applications (Table 2.1). The three noncommercial products tested were microorganisms that were selected as potential BCAs from the collection of the Department of Agronomy at the University of Cordoba (DAUCO, Spain). These included two fungi [*Aureobasidium pullulans* isolate AP08 (from a leaf of *O. europaea* cv. Picual) and *Phoma* sp. isolate ColPat-375 (from xylem vessels of *O. europaea* cv. Arbequina)] and one bacterium (*Bacillus amyloliquefaciens* isolate PAB-24; from buds of *Pistacia vera* cv. Kerman). The fungal isolates were conserved as described before, and the bacterial isolate was cryopreserved with 30% glycerol at -80°C. Fungal and bacterial inocula of the BCAs were prepared and adjusted according to Varo *et al.* (2016).

Effect on mycelial growth

Dual culture assays

The three potential BCAs described above were tested for antagonism against the *V. dahliae* isolate V-180 by means of dual culture on PDA. For this purpose, a mycelial plug (7.5 mm in diameter) of the pathogen was taken from the edge of a 7-day-old actively growing colony and plated 2 cm beyond the border of a Petri dish (9 cm in diameter) filled with PDA. In this same position, but on the other side of the Petri dish, one mycelial plug (7.5 cm in diameter) of the fungal isolates AP08 or ColPat-375 obtained from the edge of a 7-day-old actively growing colony or a single straight plug from a 2-day-old colony of the bacterial isolate PAB-24 was placed. Additionally, a mycelial plug of *V. dahliae* isolate V-180 was plated alone on PDA as a control. All Petri dishes were incubated at 24°C for 14 days in darkness.

Table 2.1. Biostimulants, resistance inductors and biological products evaluated against VWO.

Active ingredient(s)	Trade name/ Formulation[a]	Manufacturer	Class (FRAC code)[b]	Mode of action[c]	Dose[d]	
					Foliar	Irrigation
Non-commercial products						
Aureobasidium pulullans	AP08	DAUCO[e]	Fungal (NC)	BCA	10^6 conidia/ml	10^6 conidia/ml
Bacillus amyloliquefaciens	PAB-024	DAUCO[e]	Bacterial (BM02)	BCA	10^8 CFU/ml	10^8 CFU/ml
Phoma spp.	ColPat-375	DAUCO[e]	Fungal (NC)	BCA	10^6 conidia/ml	10^6 conidia/ml
Commercial products						
Bacillus subtilis	Serenade®-WG	Bayer CropScience	Bacterial (BM02)	BCA/FG	4 g/l	4 g/l
Fungal extracts	Cybelion®-EW	Adama	Natural compound (NC)	PB	3 ml/l	3 ml/l
Seaweed extracts (*Laminaria digitata*)	Vacciplant Max®-EW	UPL	Natural compound (P04)	PB	1 ml/l	1 ml/l
Bioassimilable sulfur 1	Naturdai S-System®-EW	Idai Nature	Inorganic (M02)	PB	5 ml/l	3 ml/l
Bioassimilable sulfur 2	Thiopron®-EW	UPL	Inorganic (M02)	PB	7 ml/l	7 ml/l
Copper chloride	Copper (II) chloride-SL	Panreac	Inorganic (M01)	FG	5.36 g/l	5.36 g/l
Copper complexed 1	Bioscrop Acticuper®-EW	Econatur	Inorganic (M01)	PB	2 ml/l	2 ml/l
Copper complexed 2	Disper Cu Max®-WG	Disper	Inorganic (M01)	PB	1.5 g/l	2 g/l
Copper gluconate 1	Glucopper®-EW	Tradecorp	Inorganic (M01)	PB	3 ml/l	12 ml/l
Copper gluconate 2	Idai Cobre®-EW	Idai Nature	Inorganic (M01)	PB	4 ml/l	3 ml/l
Copper sulfate	Copper sulfate-SL	Merck	Inorganic (M01)	FG	8 g/l	8 g/l
Hydrogen peroxide	Huwa-San 50 Agro®-SL	Huwa-San España	Inorganic compound (NC)	FG	0.1 ml/l	0.1 ml/l
Aluminum lignosulfonate	Brotaverd®-EW	Idai Nature	Inorganic salt (NC)	PB	5 ml/l	5 ml/l
Potassium silicate 1	Green Silk®-EW	Agrinova	Inorganic salt (NC)	PB	2.5 ml/l	8 ml/l
Potassium silicate 2	Silicasol®-EW	BC Fertilis	Inorganic salt (NC)	PB	3 ml/l	8 ml/l
Copper phosphite 1	Copper phosphite-EW	Nufol	Phosphorous acid and salts (P07)	PB	3 ml/l	10 ml/l
Copper phosphite 2	Naturfos Cu®-EW	Daymsa	Phosphorous acid and salts (P07)	PB	3 ml/l	10 ml/l
Copper phosphite 3	Phoscuprico®-EW	Agrinova	Phosphorous acid and salts (P07)	PB	3 ml/l	10 ml/l
Fosetyl-Al	Aliette®-WG	Bayer CropScience	Phosphorous acid and salts (P07)	HPDI	3 g/l	10 g/l
Potassium phosphite 1	Fitasio®-EW	Agrinova	Phosphorous acid and salts (P07)	PB	3 ml/l	7 ml/l
Potassium phosphite 2	Long life®-EW	Nufol	Phosphorous acid and salts (P07)	PB	2 ml/l	7 ml/l
Potassium phosphite 3	Naturfos®-EW	Daymsa	Phosphorous acid and salts (P07)	PB	3 ml/l	8 ml/l

Potassium phosphite 4	Alexin 75®-EW	Massó	Phosphorous acid and salts (P07)	PB	4 ml/l	4 ml/l
Amino acids	AminoPhos®-EW	Daymsa	Organic compound (NC)	PB	3 ml/l	8 ml/l
Amino acids + Cu	Nanocrop Cobre®-EW	Agrostock	Organic compound (NC)	PB	3 ml/l	3 ml/l
Amino acids + N, P, K and S	Daluben®-WG	Folgrant S.L.	Organic compound (NC)	PB	5 g/l	5 g/l
Chitosan	Biofender Fusarum®-EW	Econatur	Organic compound (NC)	HPDI	2.5 ml/l	2.5 ml/l
Organic carbon	Organihum Plus®-EW	Econatur	Organic compound (NC)	PB	0.5 ml/l	1.5 ml/l
Salicylic acid	Salicylic acid-SL	Sigma-Aldrich	Organic acid (NC)	HPDI	5 mM (0.69 g/l)	5 mM (0.69 g/l)

[a]EW: emulsion, oil in water; SC: suspension concentrate; SL: soluble concentrate; WG: water dispersible granule.
[b]Group and code numbers are assigned by the Fungicide Resistance Action Committee (FRAC) according to different modes of actions (NC: not classified; for more information, see http://www.frac.info/).
[c]Mode of action known currently for each product, although we cannot discard that they can act by means another mode of action. BCA (Biological Control Agent); PB (Plant Biostimulant; selected according to manufacturer instructions, based on European Regulation 2019/1009); HPDI (Host Plant Defense Inducers; selected according to FRAC); Fungicides (FG; selected according to manufacturer instructions); n/d: non determined.
[d]Maximum foliar or irrigation dosage recommended for the manufacturers of the commercial products was used in this study. Fungal and bacterial inocula from the BCAs (AP08, PAB-024 and ColPat-375) were prepared and adjusted according to Varo *et al*. (2016).
[e]Microorganisms (BCAs) conserved in the collection of the Department of Agronomy at the University of Córdoba (DAUCO, Spain).

The experiment was conducted twice, and a randomized complete block design with four replicated Petri dishes per BCA was used. After 14 days of incubation, the largest and smallest diameters of the colonies of *V. dahliae* were measured, and the mean data were converted to obtain the mycelial growth rate (MGR, mm day^{-1}). The mycelial growth inhibition percentage [mycelial growth inhibition, MGI (%)] was calculated as follows:

$$MGI = [1-(MGR_{dc} / MGR_{control})] \times 100$$

where 'MGR_{dc}' is the MGR of *V. dahliae* in dual cultures with BCAs, and '$MGR_{control}$' is the MGR of *V. dahliae* alone.

***In vitro* sensitivity tests**

The effect of the twenty-nine remaining products on the mycelial growth of *V. dahliae* isolate V-180 was examined by sensitivity tests on PDA. Because the concentration of the main active ingredient was not specified by the manufacturers for most of the products evaluated in this study (mainly biostimulants and resistance inductors), we were not able to calculate specific concentrations according to the active ingredient. Thus, the following three levels of dosage were established: *i*) high: the maximum dose recommended by the manufacturer of each product for its application by irrigation (Table 1); *ii*) medium: ¼ of the high dose; and *iii*) low: 1/16 of the high dose. To this end, the appropriate volume (ml/l) or weight (g/l) of each product was added to sterilized PDA at approximately 45°C to achieve the required dose and poured into 9-cm diameter Petri dishes. After solidification, a mycelial plug (7.5 mm in diameter) of *V. dahliae* isolate V-180 obtained from the edge of a 7-day-old actively growing colony was placed in the centre of the Petri dish. Additionally, a mycelial plug of *V. dahliae* isolate V-180 was also placed in the centre of a nonamended PDA Petri dish as a control. Petri dishes were incubated for 14 days as described before. There were four replicated Petri dishes per product and dose combination. A factorial design with two independent factors (29 products and three doses per product) was used ($29 \times 3 \times 4 = 348$ Petri dishes in total). The experiment was conducted twice. The MGR (mm day^{-1}) was obtained, and the MGI (%) was calculated for each product and dose combination as follows:

$$MGI = [1-(MGR_{treatment} / MGR_{control})] \times 100$$

where '$MGR_{treatment}$' is the MGR of *V. dahliae* on PDA amended with the respective treatment, and '$MGR_{control}$' is the MGR of *V. dahliae* on non-amended PDA.

Effect on microsclerotia viability

Soil samples were collected from a commercial cotton (*Gossypium hirsutum* L.) field naturally infested with *V. dahliae*, located in Villanueva de la Reina (Jaen province, Andalusia region, southern Spain; Geographic coordinates 38°00'10.8"N 3°55'57.5"W). Five soil subsamples (5,000 g each) were randomly collected across the field from the upper 30 cm. Once in the laboratory, the samples were mixed to obtain a single homogenized sample, which was air-dried at room temperature until completely dry and manually sifted through a 0.8 mm-diameter sieve to remove organic debris and large particles (Trapero *et al.*, 2013).

The experiment was conducted using sterile plastic pots (100 ml vol.) with holes previously drilled in the base (5 holes, 2 mm in diameter each) to facilitate percolation. Subsequently, each plastic pot was filled with 60 g of naturally infested air-dried soil and irrigated with 30 ml of the treatment suspension. Treatments were performed using the irrigation dose indicated for each product in Table 1. An additional plastic pot filled with 60 g of naturally infested soil was irrigated with sterile distilled water and used as a positive control. After the treatment percolated, the plastic pots were hermetically closed and incubated for 24 hours at room temperature. After incubation, treated soil samples were removed from the plastic pots, deposited in individual aluminium trays, and air-dried at room temperature for 10-14 days. A completely randomized design was used with three replicated plastic pots for each product or control treatment ($33 \times 3 = 99$ plastic pots in total). The experiment was conducted twice.

The inoculum density of *V. dahliae* expressed as the number of colony forming units (CFU) or microsclerotia per g of soil (MS) in each treated soil sample was estimated by wet sieving (Huisman and Ashworth, 1974) using 10 replicated Petri dishes of modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977) following the protocol described by Varo *et al.* (2016). Subsequently, the percentage of inoculum density reduction was calculated with respect to the control and is expressed as MS inhibition (MSI, %).

Effect on verticillium wilt development in olive plants

Plant material, inoculum preparation and inoculation

Healthy six-month-old rooted olive cuttings of cv. Picual (highly susceptible to *V. dahliae*; López-Escudero *et al.*, 2004) growing in peat moss in plastic pots (0.5 l) were obtained from a commercial nursery. To induce the active growth of the plants, they were maintained in a controlled-growth chamber [22 ± 2°C, with a 14:10-h (light:dark) photoperiod of white fluorescent light (10.000 lux) and 60% relative humidity (RH)] for one month before conducting the experiment, and they were irrigated three times per week.

For inoculum preparation, 2-l Erlenmeyer flasks were filled with 1 kg of a cornmeal-sand mixture (sand, cornmeal and distilled water; 9:1:2, weight:weight:volume) and double-sterilized on two consecutive days at 120°C for 50 min (1st day) and 120°C for 20 min (2nd day). Flasks were manually shaken between the two sterilizations. Subsequently, 50 mycelial plugs (7.5 mm in diameter) of *V. dahliae* isolate V180 growing on PDA as described before were introduced into each flask, and the flasks were incubated at 24°C in darkness for 4 weeks. To favour the homogeneous colonization of the cornmeal-sand mixture by the pathogen, flasks were manually shaken just after inoculation as well as once a week during the incubation period. After four weeks of incubation, the inoculum density of the colonized cornmeal sand was estimated by means of the serial dilution method on PDA and expressed as colony-forming units (CFUs) (Mulero-Aparicio *et al.*, 2019a).

At the inoculation time, olive plants were transplanted to plastic pots (0.8 l) previously disinfested with a commercial sodium hypochlorite solution at 20% for 2 h and filled with a 20% (weight/weight) mixture of colonized corn meal-sand and sterile peat moss (theoretical inoculum density of the final substrate = 10^7 CFU g^{-1}; Mulero-Aparicio *et al.*, 2019a). Additionally, olive plants transplanted into plastic pots filled with a 20% (weight/weight) mixture of sterile corn meal-sand and sterile peat moss were used as negative controls. All plants were incubated in a growth chamber at 20°C in darkness and 100% relative humidity (RH) for 7 days. Subsequently, light and humidity parameters were progressively modified over one week until reaching 23°C, a 12-h photoperiod of fluorescent light [10,000 lux] and 70% RH, which were maintained until the end of the experiment. Plants were irrigated three times per week.

Plant treatment and experimental design

All the products included in this study were evaluated *in planta* by foliar spray and irrigation application. The doses used for each type of application are shown in Table 1. The foliar and irrigation applications were made by spraying 15 ml per plant or by irrigation with 350 ml per plant of the dilutions of each product, respectively. Plant treatments were conducted after one preconditioning month in a growth chamber as follows: *i*) four foliar applications, 14, 7, and 2 days before inoculation and 10 days after inoculation; *ii*) three irrigation applications, 7 and 2 days before inoculation and 10 days after inoculation. These schedules were established according to the manufacturer's instructions. Exceptionally, hydrogen peroxide treatments were conducted three times per week from one week before inoculation to two weeks after inoculation for both foliar and irrigation applications according to the manufacturer's recommendation. Nontreated and inoculated or noninoculated olive plants were also included as positive or negative controls, respectively. For each type of application, a randomized complete block design (three blocks) was used with 32 treatments and two controls (positive and negative) as independent variables and five replicated olive plants per treatment and block ($34 \times 3 \times 5 = 510$ plants per type of application; 1.020 plants in total).

Disease severity assessment

Disease severity (DS) was evaluated weekly for 12 weeks after inoculation using a 0 to 16 rating scale. This scale was designed to estimate the percentage of affected tissue by means four main categories: 0-25, 26-50, 51-75, and 76-100% of affected tissue, with four values per each category (0.25, 0.5, 0.75, 1). Thus, scale values (X) represent the number of sixteenths of affected plant area (4 values per category × 4 categories), and they are linearly related to the percentage of affected tissue (Y) by the equation $Y = 6.25X - 3.125$ (Varo *et al.*, 2018). DS data were used to calculate the relative area under the disease progress curve (RAUDPC) at the end of the experiment by the trapezoidal integration method (Campbell and Madden, 1990). In parallel, disease incidence (DI) and mortality were also assessed at the end of the experiment as the percentage of symptomatic or dead plants, respectively.

In addition, three symptomatic plants per treatment combination were randomly selected at the end of each experiment to confirm the infection of the pathogen by fungal isolation. Basal stems of the plants were washed under running tap water for 2 h.

Subsequently, small fragments of the affected tissue were cut and surface sterilized by dipping them in a 10% solution of commercial bleach (Cl at 50 g L^{-1}) for 1 min, air-dried on sterilized filter paper for 10 min, and plated onto APDA. Petri dishes were incubated as described before.

Data analyses

Data from this study were analysed using Statistix 10.0 software (Analytical Software, Tallahassee, USA). All the experiments were conducted twice, and data from the two repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the *F* test ($P \geq 0.05$). Subsequently, in any cases, data were tested for normality, homogeneity of variances, and residual patterns, and the square root transformation of the data was used when necessary. For the dual culture assays, ANOVA was conducted with 'MGR' or 'MGI' as the dependent variable, 'BCAs' as the independent variable and each replicated Petri dish as a block. For the *in vitro* sensitivity tests, factorial ANOVA was conducted with 'MGR' or 'MGI' as dependent variables and 'product', 'dose' and their interaction as independent variables. Because the interaction 'product' × 'dose' was significant in both cases ($P \leq 0.0001$), the differences in the effect on MGR and MGI among the evaluated products were analysed separately for each dose as a completely randomized design. In any cases, treatments that showed 100% MGI were not included in the analysis. For the effect on MS viability, ANOVA was conducted with 'MS density' or 'MSI' as dependent variables and 'soil treatment' as an independent variable. Soil treatments showing 100% MSI were not included in the analysis. For *in planta* experiments, ANOVA was conducted separately for each type of application (foliar or irrigation) with RAUDPC as the dependent variable and 'treatment' as the independent variable. Treatments that did not show symptoms were not included in the analysis. Treatment means of the MGR or 'MS concentration' were compared using Dunnett's multiple comparison test with a control (MGR = 3.5 mm/day; MS concentration = 54.5 MS/g of soil) at $P = 0.05$. The treatment means of the MGI, MSI, RAUDPC and DS were compared according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985). Data on the final DI (% of affected plants) and mortality (% of dead plants) were analysed by multiple comparisons for proportions tests at $P = 0.05$ (Zar, 2010). Additionally, the Pearson correlation coefficients (*r*) between the MGI and MSI of *V. dahliae*, and the RAUDPC

of inoculated plants, were calculated using the average values of the three variables for each of the products evaluated at the irrigation dose (Table 2.1; $n = 32$).

RESULTS

Effect on mycelial growth

Dual culture assay

Among the three BCAs tested in dual cultures, *B. amyloliquefaciens* PAB-24 and *Phoma* sp. ColPat-375 significantly reduced ($P = 0.0071$) the MGR of *V. dahliae* isolate V-180 in comparison with the control. Likewise, these two BCAs showed significantly ($P = 0.0365$) higher MGI values (47.3 ± 6.84 and 40.8 ± 15.2% for *B. amyloliquefaciens* PAB-24 and *Phoma* sp. ColPat-375, respectively) than that observed for *A. pullulans* AP08 (MGI = 19.5 ± 11.58%), which did not differ from the control (Table 2.2).

Table 2.2. Effect of three BCAs on mycelial growth of *Verticillium dahliae* in dual cultures. Pictures below illustrate the mycelial growth of each BCA in dual cultures against *V. dahliae* isolate V180.

Biological Control Agents	Isolate	MGR (mm day^{-1})[a,b]	MGI (%)[a,c]
Aureobasidium pulullans	AP08	2.8 ± 0.40	19.5 ± 11.58 b
Bacillus amyloliquefaciens	PAB-24	1.8 ± 0.24*	47.3 ± 6.84 a
Phoma sp.	ColPat-375	2.1 ± 0.53*	40.8 ± 15.2 a
Control (*Verticillium dahliae*)	V180	3.5 ± 0.12	-

[a]Mycelial Growth Rate (MGR; mm day^{-1}) and Mycelial Growth Inhibition (MGI; %) of *V. dahliae* were obtained after growing both the BCAs and *V. dahliae* in dual cultures on PDA at 24ºC for 14 days in darkness. In any cases, data represents the average of eight replicated Petri dishes per BCA or control ± the standard error of the means.
[b]Means followed by an asterisk differ significantly from the 'Control' according to Dunnett's multiple comparison test at $P = 0.05$.
[c]Means followed by the same letter do not differ significantly according to Fisher´s protected LSD test at $P = 0.05$.

Control V180 | AP08 vs V180 | PAB-24 vs V180 | ColPat-375 vs V180

In vitro sensitivity test

The different products evaluated varied significantly ($P \leq 0.0001$ in all cases) in their effect on the reduction of the MGR and MGI of *V. dahliae* depending on the dose tested. In general, *Bacillus subtilis*, copper chloride, copper sulfate, copper phosphites and fosetyl-Al inhibited the mycelial growth of *V. dahliae* by 100% at high and medium

doses. In addition, *Bacillus subtilis* inhibited the mycelial growth of *V. dahliae* by 100% at a low dose, whereas copper gluconate-1 only showed a 100% MGI at a high dose. On the other end, several products had no significant effect on the reduction of the MGR in comparison with the control at any dose tested. These products were seaweed extracts from *Laminaria digitate*, copper complex-1, organic carbon, hydrogen peroxide, and potassium silicate-1 and -2. In the remaining products, there was great variability in their effect on the MGR depending on the dose used. In general, the inhibition of mycelial growth by this group of products decreased when reducing the dose of the product, ranging from 77.7% for salicylic acid at the high dose to no significant effects of many products at the medium (n = 8) and low (n = 19) doses. Detailed MGR and MGI data for each product and dose combination are shown in Table 2.3.

Effect on microsclerotia viability

The commercial products and BCAs evaluated showed significant differences among them for the MS viability of *V. dahliae* ($P \leq 0.0001$ for both 'MS concentration' and 'MS inhibition' variables). The MS concentrations ranged from 22.1 ± 2.9 to 53.3 ± 3.8 MS g of soil^{-1} for potassium phosphite-1 and hydrogen peroxide, respectively. The following products showed significant differences in the reduction of MS concentration in comparison with the control (54.5 ± 4.7 MS g of soil^{-1}): potassium phosphite-1, fosetyl-Al, copper complexed-2, copper phosphite-2, *B. amyloliquefaciens* (isolate PAB-034), copper sulfate, copper phosphite-3, copper chloride, aluminium lignosulfonate, chitosan, bioassimilable sulfur-2, and copper gluconate-1, with the 'MS concentration' ranging between 38.5 ± 2.2 and 53.3 ± 3.8 MS g of soil^{-1}. Likewise, the values of MS inhibition ranged from 59.4 ± 5.4 to 5.2 ± 5.2% for potassium phosphite-1 and hydrogen peroxide, respectively. The significant differences between products in terms of their effect on MSI were given by a critical value for means comparison of 16.5% according to Fisher´s protected LSD test at P = 0.05. It is interesting to note that our own BCAs evaluated in this study showed remarkable differences in their effect on MS viability. Only *B. amyloliquefaciens* isolate PAB-024 showed a significant reduction (43.1 ± 7.3%) in the MSI of *V. dahliae*, while the inhibition due to *Phoma* sp. isolate ColPat-375 (20.8 ± 7.7%) and *A. pullulans* isolate AP08 (5.3 ± 3.8%) did not differ significantly from that of the control (Fig. 2.1). Linear correlation analysis showed that there was a low but significant correlation (r = 0.4603; P = 0.0080) between the MGI and MSI when products were evaluated at irrigation doses.

Table 2.3. Effect of the commercial products evaluated in this study on mycelial growth of *Verticillium dahliae* isolate V180.

Products	Dose[a]					
	High		Medium		Low	
	MGR (mm day^{-1})[b,c]	MGI (%)[b,d]	MGR (mm day^{-1})[b,c]	MGI (%)[b,d]	MGR (mm day^{-1})[b,c]	MGI (%)[b,d]
Aluminum lignosulfonate	1.9 ± 0.40*	45.6 ± 11.56	2.3 ± 0.35*	34.9 ± 10.09	1.7 ± 0.09*	51.7 ± 2.57
Amino acids	1.6 ± 0.11*	55.2 ± 3.11	1.7 ± 0.07*	50.7 ± 1.93	2.3 ± 0.14*	33.3 ± 3.88
Amino acids + Cu	2.5 ± 0.04	27.9 ± 1.09	2.6 ± 0.03*	24.6 ± 0.79	2.8 ± 0.07	18.5 ± 2.11
Amino acids + N, P, K and S	1.6 ± 0.11*	54.3 ± 3.17	1.7 ± 0.27*	51.0 ± 7.87	2.4 ± 0.27*	32.3 ± 7.65
Bacillus subtilis	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00
Bioassimilable sulfur 1	1.2 ± 0.06*	66.2 ± 1.86	1.6 ± 0.05*	53.6 ± 1.45	2.7 ± 0.30	21.7 ± 8.71
Bioassimilable sulfur 2	1.6 ± 0.13*	53.5 ± 3.71	2.1 ± 0.06*	40.0 ± 1.80	2.2 ± 0.20*	37.0 ± 5.81
Chitosan	1.9 ± 0.26*	44.5 ± 7.49	2.0 ± 0.06*	43.2 ± 1.67	2.9 ± 0.27	17.5 ± 7.70
Copper chloride	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00	2.9 ± 0.44	16.5 ± 12.61
Copper complexed 1	2.7 ± 0.08	23.8 ± 2.20	3.1 ± 0.32	13.8 ± 8.07	3.3 ± 0.17	5.5 ± 4.73
Copper complexed 2	2.1 ± 0.04*	39.8 ± 1.18	3.0 ± 0.09	13.09 ± 2.47	3.0 ± 0.17	15.0 ± 4.89
Copper gluconate 1	0.0 ± 0.00*	100 ± 0.00	1.8 ± 0.10*	47.8 ± 2.96	2.5 ± 0.13*	29.7 ± 3.77
Copper gluconate 2	1.9 ± 0.04*	46.7 ± 0.99	2.4 ± 0.22*	30.8 ± 6.23	2.9 ± 0.22	16.2 ± 5.63
Copper phosphite 1	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00	2.1 ± 0.17*	41.0 ± 4.97
Copper phosphite 2	1.5 ± 0.10*	57.5 ± 2.68	2.2 ± 0.08*	38.0 ± 2.43	2.5 ± 0.10*	28.7 ± 2.87
Copper phosphite 3	1.2 ± 0.08*	65.4 ± 2.35	3.2 ± 0.07	8.6 ± 1.58	2.9 ± 0.18	18.5 ± 4.53
Copper sulfate	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00	2.6 ± 0.27	25.0 ± 7.65
Fosetyl-Al	0.0 ± 0.00*	100 ± 0.00	0.0 ± 0.00*	100 ± 0.00	2.2 ± 0.10*	37.1 ± 2.78
Fungal extracts	1.1 ± 0.21*	69.8 ± 6.10	2.4 ± 0.13*	31.1 ± 3.69	2.7 ± 0.24	22.3 ± 6.79
Hydrogen peroxide	2.4 ± 0.32	31.2 ± 9.07	3.0 ± 0.25	16.3 ± 6.27	2.9 ± 0.23	16.5 ± 5.76
Organic carbon	2.7 ± 0.09	21.7 ± 2.59	3.1 ± 0.07	11.6 ± 1.94	3.1 ± 0.25	14.0 ± 5.72
Potassium phosphite 1	1.2 ± 0.06*	64.3 ± 1.76	2.5 ± 0.08*	27.0 ± 2.40	3.0 ± 0.06	15.0 ± 1.73
Potassium phosphite 2	1.8 ± 0.09*	48.4 ± 2.56	2.0 ± 0.08*	43.6 ± 2.27	2.7 ± 0.24	27.2 ± 1.48
Potassium phosphite 3	1.1 ± 0.11*	67.4 ± 3.19	2.2 ± 0.08*	37.1 ± 2.20	2.5 ± 0.05	23.7 ± 6.93
Potassium phosphite 4	1.4 ± 0.05*	58.7 ± 1.43	2.3 ± 0.11*	33.0 ± 3.32	2.9 ± 0.09	17.7 ± 2.42
Potassium silicate 1	3.3 ± 0.09	6.4 ± 2.53	3.2 ± 0.11	7.9 ± 3.27	3.2 ± 0.17	8.24 ± 4.34
Potassium silicate 2	3.3 ± 0.46	14.6 ± 8.52	2.9 ± 0.22	17.0 ± 5.84	2.4 ± 0.42*	31.6 ± 10.72
Salicylic acid	0.8 ± 0.27*	77.7 ± 7.64	2.0 ± 0.06*	44.0 ± 1.62	2.6 ± 0.24	24.9 ± 6.93
Seaweed extracts (*Laminaria digitata*)	3.4 ± 0.08	2.72 ± 2.02	3.8 ± 0.08	0.0 ± 0.00	3.5 ± 0.24	4.5 ± 4.04
Control	3.5 ± 0.12	-	3.5 ± 0.12	-	3.5 ± 0.12	-

[a]Dose: high, the maximum dose recommended by the manufacturer of each product for its application by irrigation (Table 1); medium, ¼ of the high dose; low, 1/16 of the high dose.

[b]Mycelial Growth Rate (MGR; mm day^{-1}) and Mycelial Growth Inhibition (MGI; %) were obtained after growing *V. dahliae* isolate V180 onto amended or non-amended PDA at 24ºC for 14 days in darkness. In both cases, data represents the average of eight replicated Petri dishes per product and dose combination or control ± the standard

error of the means. Only for the MGR variable in the high dose experiment, the square root transformation of the data was performed to satisfy the ANOVA requirements for normality, homogeneity of variances and residual patterns.

[c]For each dose, means of MGR followed by an asterisk differ significantly from the 'Control' according to Dunnett's multiple comparison test at $P = 0.05$.

[d]For each dose, significant differences between any treatment means of MGI are given by a critical value for means comparison [$LSD_{0.05}$ = 12.5, 14.0 and 16.8 % for high, medium and low dose, respectively] according to Fisher's protected LSD test at $P = 0.05$.

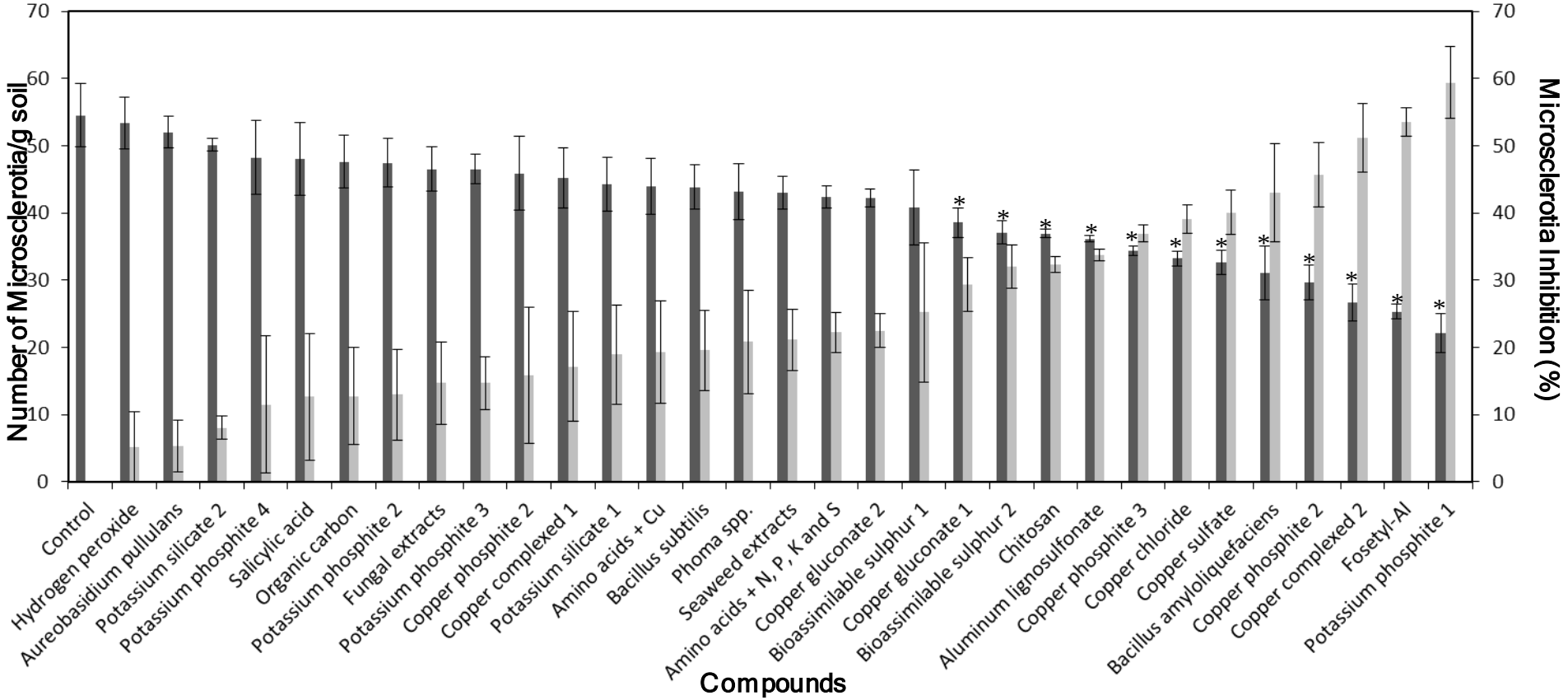

Figure 2.1. Effect of the evaluated products on the microsclerotia (MS) concentration [Number of MS/g of soil (dark grey columns)] and on the viability of MS [MS inhibition (MSI), % (light grey columns)] of *Verticillium dahliae* in naturally infested soil. Treatments were performed using the irrigation dose indicated for each product in Table 1. For each parameter, columns represent the average of six replicated plastic pots. Vertical bars represent the standard error of the means. For MS concentration, columns with an asterisk differ significantly from the control according to Dunnett's multiple comparison test at $P = 0.05$. For MSI, significant differences between treatments were determined by a critical value for means comparison of 16.5% according to Fisher´s protected LSD test at $P = 0.05$.

Effect on Verticillium wilt development in olive plants

The factorial ANOVA for the 32 evaluated products with the two types of applications (foliar and irrigation) showed a non-significant effect of application type ($P = 0.4533$ and $P = 0.9138$ for RAUDPC and DS, respectively), but the variable 'product' and the interaction between product and type of application were significant ($P \leq 0.0001$ in any cases for both dependent variables). Thus, *in planta* experiments were analysed separately for each type of application, foliar and irrigation, and the data are shown in Table 2.4 and Table 2.5, respectively.

Nontreated control plants inoculated with *V. dahliae* isolate V180 showed typical symptoms of VWO approximately 30 days after inoculation, with a progressively increasing DS until reaching a final DI of 100% at 3 months after inoculation. Although the factorial ANOVA did not show a significant effect of the type of application, the irrigation treatments seemed more effective in reducing disease progress (RAUDPC) and DS than foliar treatments. Regarding foliar treatments, a significant effect was observed between products ($P = 0.0014$) on the RAUDPC, with 14 out of the 32 products evaluated showing a significant effect in the reduction of disease progress in comparison with the control. The RAUDPC values of these products ranged from 52.8±11.2 to 35.9±17.6% for potassium silicate-1 and *Phoma* sp. (isolate ColPat-375), respectively. In addition, the BCA *A. pullulans* (isolate AP08) was the second most effective product (RAUDPC = 36.4±2.7) after *Phoma* sp. Potassium silicate-2 showed a remarkably higher RAUDPC (124.6±21.0%) than the control (RAUPDC = 100%), probably due to its phytotoxic effect *in planta* with spray application. Considering the final DS, copper phosphite-1 (104.6±15.0%) and potassium phosphite-4 (99.2±11.8%) were the least effective products, while amino acids and bioassimilable sulfur-2 were the most effective products, with a final DS of 29.0±14.5 and 29.0±4.5%, respectively. Values of DI among the evaluated products ranged from 100% for *B. amyloliquefaciens*, chitosan, copper complex-1, copper phosphite-1 and hydrogen peroxide to 53.8% for bioassimilable sulfur-2. Finally, values of plant mortality among the evaluated products ranged from 80.0% for copper phosphite-1 to 0.0% for aluminium lignosulfonate, amino acids+N,P,K,S, copper chloride, hydrogen peroxide and *Phoma* sp. (Table 2.4)

Table 2.4. Disease-related parameters for olive plants grown in artificially infested substrate with the defoliating *Verticillium dahliae* isolate V180 and treated with the evaluated products by foliar application[a].

Products	Incidence (%)[b]	Mortality (%)[b]	Disease severity (%)[c]	RAUDPC (%)[d]
Negative control	0.0	0.0	0.0 ± 0.00	0.0 ± 0.00
Positive control	100.0	100.0	100.0 ± 0.00	100.0 ± 0.00
Aluminum lignosulfonate	93.3	0.0	60.8 ± 11.49	66.5 ± 13.55
Amino acids	64.3	9.5	29.0 ± 4.92	43.3 ± 13.81
Amino acids + Cu	78.6	19.1	50.6 ± 16.18	66.5 ± 24.52
Amino acids + N, P, K and S	69.2	0.0	34.7 ± 12.65	38.0 ± 18.86
Aureobasidium pulullans	80.4	14.0	42.7 ± 2.91	36.4 ± 2.72
Bacillus amyloliquefaciens	100.0	44.4	83.5 ± 6.89	65.9 ± 6.39
Bacillus subtilis	75.0	22.3	68.9 ± 26.31	108.2 ± 47.01
Bioassimilable sulfur 1	77.3	42.9	44.5 ± 10.46	79.0 ± 19.61
Bioassimilable sulfur 2	53.8	20.5	29.0 ± 4.51	49.7 ± 17.84
Chitosan	100.0	53.3	86.4 ± 4.55	102.1 ± 7.11
Copper chloride	93.3	0.0	42.6 ± 9.49	50.9 ± 5.40
Copper complexed 1	100.0	35.6	76.1 ± 8.93	60.9 ± 11.91
Copper complexed 2	87.9	35.6	91.8 ± 12.39	72.2 ± 13.90
Copper gluconate 1	86.7	8.9	32.4 ± 5.11	48.5 ± 6.70
Copper gluconate 2	91.7	15.6	61.4 ± 6.28	66.5 ± 13.55
Copper phosphite 1	100.0	80.0	104.6 ± 15.03	99.4 ± 18.05
Copper phosphite 2	64.3	38.1	52.5 ± 8.71	80.8 ± 9.17
Copper phosphite 3	87.9	41.1	81.3 ± 9.81	64.6 ± 8.48
Copper sulfate	86.7	19.1	65.8 ± 6.39	67.6 ± 12.07
Fosetyl-Al	93.3	8.9	32.4 ± 16.20	42.8 ± 17.88
Fungal extracts	91.7	44.4	51.4 ± 12.87	60.3 ± 19.60
Hydrogen peroxide	100.0	0.0	42.1 ± 4.65	47.1 ± 4.67
Organic carbon	86.0	13.7	48.0 ± 12.31	46.7 ± 16.3
Phoma spp.	75.0	0.0	34.1 ± 13.31	35.6 ± 17.56
Potassium phosphite 1	75.0	22.3	55.4 ± 20.33	62.3 ± 28.66
Potassium phosphite 2	78.6	9.5	39.2 ± 9.69	48.5 ± 15.25
Potassium phosphite 3	71.7	18.4	46.0 ± 14.54	65.1 ± 16.25
Potassium phosphite 4	78.6	35.6	99.2 ± 11.80	81.5 ± 14.88
Potassium silicate 1	93.3	17.8	48.3 ± 9.14	52.8 ± 11.24
Potassium silicate 2	83.3	22.3	70.3 ± 11.73	124.6 ± 20.98
Salicylic acid	72.4	14.0	36.4 ± 8.44	46.3 ± 11.11
Seaweed extracts (*L. digitata*)	78.6	9.5	45.5 ± 2.56	49.3 ± 4.26
$LSD^{*}_{0.05}$	19.7[e]	21.6[e]	30.4[f]	45.1[f]

[a]Olive plants were treated several times before and after inoculation and disease parameters were assessed weekly for 12 weeks after inoculation with *V. dahliae*.

[b]Percentage of symptomatic plants (Incidence) or dead plants (Mortality) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180 ($n = 30$).

[c]Final disease severity ± Standard Error of the means (SE) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180. Disease severity was assessed using a rating scale of 0 to 16 (0 = no lesions, 16 = 94–100% of affected plant tissues).

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

[e]Critical value for means comparison according to Zar's multiple comparisons for proportions test at $P = 0.05$ (Zar, 2010).

[f]Critical value for means comparison according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

Table 2.5. Disease-related parameters for olive plants grown in artificially infested substrate with the defoliating *Verticillium dahliae* isolate V180 and treated with the evaluated products by irrigation[a].

Products	Incidence (%)[b]	Mortality (%)[b]	Disease severity (%)[c]	RAUDPC (%)[c]
Negative control	0.0	0.0	0.0 ± 0.00	0.0 ± 0.00
Positive control	100.0	100.0	100.0 ± 0.00	100.0 ± 0.00
Aluminum lignosulfonate	80.0	0.0	36.9 ± 7.26	28.7 ± 3.09
Amino acids	71.4	19.1	49.4 ± 9.39	54.8 ± 22.12
Amino acids + Cu	91.7	0.0	46.9 ± 12.84	53.4 ± 15.58
Amino acids + N, P, K and S	92.3	0.0	51.9 ± 7.52	55.7 ± 14.35
Aureobasidium pulullans	77.3	4.6	32.1 ± 1.64	25.6 ± 2.62
Bacillus amyloliquefaciens	80.0	17.8	41.5 ± 3.28	27.3 ± 6.55
Bacillus subtilis	83.3	44.4	64.6 ± 17.67	89.4 ± 14.35
Bioassimilable sulfur 1	100.0	66.7	69.7 ± 14.35	96.3 ± 22.72
Bioassimilable sulfur 2	78.6	0.0	45.5 ± 8.93	46.5 ± 6.47
Chitosan	93.3	17.8	76.7 ± 12.80	77.8 ± 16.96
Copper chloride	100.0	100.0	136.4 ± 0.01	196.1 ± 0.45
Copper complexed 1	100.0	35.6	71.6 ± 30.13	50.8 ± 23.60
Copper complexed 2	91.7	77.8	65.8 ± 9.78	100.0 ± 12.95
Copper gluconate 1	86.7	8.9	42.0 ± 8.48	40.1 ± 15.08
Copper gluconate 2	85.9	4.5	41.5 ± 3.28	35.8 ± 6.41
Copper phosphite 1	100.0	53.3	102.9 ± 8.60	155.1 ± 12.59
Copper phosphite 2	78.6	19.1	43.8 ± 10.43	71.7 ± 38.3
Copper phosphite 3	51.5	2.8	22.7 ± 2.87	20.6 ± 5.17
Copper sulfate	100.0	44.4	77.8 ± 11.91	67.8 ± 7.11
Fosetyl-Al	100.0	93.3	88.6 ± 4.92	176.0 ± 14.26
Fungal extracts	93.3	80.0	70.7 ± 12.85	107.2 ± 44.35
Hydrogen peroxide	100.0	0.0	58.0 ± 9.49	53.3 ± 13.11
Organic carbon	62.6	14.7	37.8 ± 7.65	42.1 ± 8.48
Phoma spp.	100.0	33.3	71.0 ± 27.41	71.9 ± 33.87
Potassium phosphite 1	66.7	11.1	43.3 ± 17.93	48.5 ± 22.33
Potassium phosphite 2	42.9	19.1	21.0 ± 16.77	46.5 ± 39.13
Potassium phosphite 3	70.9	10.5	37.8 ± 7.17	22.3 ± 11.58
Potassium phosphite 4	100.0	38.1	76.1 ± 11.34	79.9 ± 22.68
Potassium silicate 1	93.3	0.0	40.9 ± 7.43	37.1 ± 6.55f
Potassium silicate 2	50.0	0.0	26.3 ± 3.76	60.4 ± 18.11
Salicylic acid	85.1	17.6	55.7 ± 6.42	67.0 ± 9.63
Seaweed extracts (*L. digitata*)	78.6	19.1	29.8 ± 9.29	45.5 ± 14.26
$LSD^{*}_{0.05}$	19.5[e]	22.7[e]	33.2[f]	54.0[f]

[a]Olive plants were treated several times before and after inoculation and disease parameters were assessed weekly for 12 weeks after inoculation with *V. dahliae*.

[b] Percentage of symptomatic plants (Incidence) or dead plants (Mortality) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180 ($n = 30$).

[c]Final disease severity ± Standard Error of the means (SE) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180. Disease severity was assessed using a rating scale of 0 to 16 (0 = no lesions, 16 = 94–100% of affected plant tissues).

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

[e]Critical value for means comparison according to Zar's multiple comparisons for proportions test at $P = 0.05$ (Zar, 2010).

[f]Critical value for means comparison according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

On the other hand, a significant effect was also observed on the RAUDPC between products ($P \leq 0.0001$) when they were applied by irrigation, with 10 out of the 32 products evaluated showing a significant effect in the reduction of disease progress in comparison with the control. Among the most effective products, we found RAUDPC values ranging from 45.5±14.3 to 20.6±5.2% for seaweed extracts (*L. digitata*) and copper phosphite-3, respectively. However, copper chloride, fosetyl-Al and copper phosphite-1 showed a significantly higher RAUDPC in comparison with the control, ranging between 196.1±0.5, 176.0±14.3 and 155.1±12.6, respectively. These higher RAUDPC values could also be attributed to their phytotoxic effects in potted plants. Based on the final DS, copper chloride (166.4<±0.01%), fosetyl-Al (88.6±4.9%) and copper phosphite-1 (102.9±8.65%) were also the least effective products, whereas copper phophite-3 (22.7±2.9%) and potassium phosphite-2 (21.02±16.8%) were the most effective products. Nine out of the 32 evaluated products resulted in 100% DI, but copper phosphite-3, potassium phosphite-2 and potassium silicate-2 resulted in DI values of approximately 50%. Finally, seven out of the 32 products evaluated resulted in null mortality, whereas fosetyl-Al (93.3%) and copper chloride (100%) resulted in the highest values (Table 5). Linear correlation analysis showed that there was not significant correlation between the MGI and RAUDPC (r = 0.2424; P = 0.2139), and between MSI and RAUDPC (r = 0.0608; P = 0.7586) when products were evaluated at irrigation doses (Table 2.5).

The pathogen was successfully reisolated from the basal stem tissues of all selected symptomatic plants (consistency of isolation = 60-90%), confirming the infection by *V. dahliae* in the inoculated plants.

DISCUSSION

Plant biostimulants are characterized by a wide and non-precise definition, but they never can be defined as fertilizers since they do not provide nutrients directly to the plant. Likewise, a broad range of substances or mixtures of substances, including enzymes, proteins, amino acids, nutrients, phenols, humic and fulvic acid, protein hydrolases and microorganisms (fungi and bacteria), are included under the term biostimulants. Despite the diversity of products grouped as biostimulants, they must all improve the condition of treated plants but not cause adverse side effects in any case (Drobek *et al.*, 2019). Therefore, considering all these aspects and according to the

recent European Regulation (EU) 2019/1009 on biostimulants, most of the products (22 out of 32) evaluated in this study were selected to determine their effect against VWO. In addition, chemicals such as fosetyl-Al were also included in this study since they have been grouped as HPDI by Fungicide Resistance Action Committee (FRAC), as well as chitosan and salicylic acid. Altogether, this study represents novel and relevant information on the biological control of VWO using BCAs, plant biostimulants and HPDIs.

Concerning the BCAs from our own collection (non-commercial products), the bacterium *B. amyloliquefaciens* isolate PAB-024 and the fungus *Phoma* sp. isolate ColPat-375 significantly reduced the MGR, whereas only *B. amyloliquefaciens* reduced significantly the MS concentration of *V. dahliae* in comparison with the controls. However, the fungus *A. pullulans* (isolate AP08) showed no effect on the pathogen. Recently, Varo *et al.* (2016) evaluated a wide diversity of microorganisms against *V. dahliae*, showing similar results for the MGI of the pathogen for another *Phoma* sp. isolate. However, our results on the effect of *B. amyloliquefaciens* are in contrast with those obtained by these same authors since our isolate of *B. amyloliquefaciens* showed a remarkably higher MGI of *V. dahliae* in comparison with that obtained by Varo *et al.* (2016). It is interesting to note that the isolate of *B. amyloliquefaciens* used in this study also showed a high effect on disease reduction *in planta* when it was applied by irrigation. Thus, *B. amyloliquefaciens* isolate PAB-024 was highly effective in reducing pathogen development (MGR and MSI) as well as in reducing the disease *in planta*. These results suggest that this BCA could act as a direct antagonist of the pathogen. In contrast, *A. pullulans* isolate AP08 was highly effective in the inhibition of the disease in olive plants with both foliar and irrigation applications, but it showed no effect on either the MGI or MSI of *V. dahliae*. The differences obtained for the effect of *A. pullulans* between *in vitro* and *in planta* experiments conducted in the present study suggest that this BCA could act as a HPDI instead of as an antagonist of *V. dahliae*. In addition, the significant effect on the reduction of disease severity observed with foliar applications of *Phoma* sp. agrees again with that obtained by Varo *et al.* (2016), who also showed an important effect on the reduction of disease severity *in planta* using another *Phoma* sp. isolate.

The effect of the remaining 29 selected products (commercial products) on mycelial growth and the MSI of *V. dahliae* was evaluated by *in vitro* sensitivity tests to

check whether the commercial products had any fungicidal effect interfering with the growth of the pathogen. Seventy-six percent of the commercial products significantly reduced the MGR of *V. dahliae* in comparison with the control. However, only 12 out of the 32 products evaluated were able to significantly reduce the MS concentration of the pathogen in naturally infested soil. In this case, the lack of direct toxicity of some products is not a reason *a priori* to discard them as potential effective products against the disease since they could act as HPDIs (Llorens *et al.* 2017b). Likewise, all the products were evaluated *in planta* not only to determine their effect on disease development but also to evaluate their possible phytotoxic effects. It is worth mentioning that no correlation was observed in the effectiveness of the products between *in vitro* and *in planta* experiments. This lack of correlation could be due to their effect as HPDIs.

In general, irrigation treatments were more effective than foliar treatments. This result is in concordance with that obtained in previous studies evaluating the effect of plant biostimulants on different pathosystems, where irrigation treatments were always more effective in enhancing plant innate defences than foliar treatments (Llorens *et al.*, 2017a, 2019; González-Hernández *et al.*, 2019).

Copper-based products, including copper chloride, copper sulfate, complexed copper and copper gluconates, were among the most effective products in the reduction of MGR and MS concentrations of *V. dahliae*. However, they did not show an important effect on the reduction of disease severity *in planta* with any type of application (foliar or irrigation). Only copper chloride showed high levels of phytotoxicity among the copper-based products tested. These differences in their effect between *in vitro* and *in planta* experiments could be attributed to the protective mode of action of copper (Roca *et al.*, 2007). Copper-based products do not penetrate plant tissues but prevent pathogen growth by forming a protective surface sheet, thus preventing the infection from spreading to healthy tissues. For this reason, although copper-based products have been traditionally used to prevent the main olive foliar diseases due to their high effectiveness (Roca *et al.*, 2007), their use as fungicides against VWO is limited because they are not able to penetrate into the vascular tissues of the affected plants. However, the current formulations of complexed copper has been mentioned as a potential alternative to prevent plant infections in other pathosystems since this kind of formulation could enhance plant innate defences even before the pathogen infection

event (González-Hernández *et al.*, 2018). Phytotoxicity was shown in many plants treated with copper chloride, probably because the product tested was not a commercially formulated fungicide. Thus, its higher solubility in comparison with commercial phytosanitary formulations may have led to the development of severe phytotoxicity.

On the other hand, phosphite salts are emerging as novel and relevant plant biostimulants. They have not been proven to have a direct effect on plant nutrition, but they are able to markedly improve crop yield and quality and stimulate both biotic and abiotic stress responses in crops (Gómez-Merino and Trejo-Téllez, 2015). In fact, two out of the six phosphites evaluated in this study (copper phosphite-3 and potassium phosphite-1) were the most effective products against VWO *in planta* when they were applied by irrigation. Their high effectiveness in reducing the DS and mortality of olive plants infected by *V. dahliae* make them promising candidates preventing VWO. It is worth mentioning that important differences between products belonging to the same chemical group (i.e., complexed copper, copper phosphites, potassium phosphites or silicates, etc.) were observed on the effect of fungal or disease development in *in vitro* or *in planta* experiments, respectively. In this sense, it is remarkable that irrigation treatments with copper phosphite-3 showed the highest levels of disease reduction *in planta*, whereas copper phosphite-1 resulted in high levels of phytotoxicity. This aspect could be attributed to the different formulations of the products made by the respective manufacturers, and it should always be considered in the selection of the appropriate products.

A wide diversity in the response to the pathogen and disease reduction was observed for the rest of the commercial products tested. Some products, such as fosetyl-Al or fungal extracts or potassium silicate-2, showed phytotoxicity when they were applied by irrigation or foliar treatments, respectively. In addition to the BCAs tested in this study, the only commercial BCA evaluated (commercial strain of *B. subtilis*) was the most effective biological treatment against the pathogen *in vitro* but had a markedly minor effect on reducing DS *in planta*. The results suggest that this BCA acts directly on the pathogen, although we cannot discard its potential effect as a HPDI in other pathosystems. Salicylic acid showed an intermediate disease reduction *in planta* when it was sprayed, but no significant difference was observed in comparison with the control when it was applied by irrigation. Similar results regarding foliar treatments were

obtained by Gharbi *et al.* (2016), who showed that foliar applications with salicylic acid before inoculation were able to significantly reduce the progression of VWO in potted olive plants. Finally, seaweed extract showed intermediate effectiveness in disease reduction for both foliar and irrigation applications *in planta*, but it had a null effect on the MGI and MSI of *V. dahliae*. Although it was not among the most effective products of this study against VWO, the null toxicity of seaweed to the fungi makes it a potential plant biostimulant against wilt diseases. In fact, the use of seaweeds in the biological control of VWO has been previously investigated because they can act as elicitors of phenylalanine ammonia-lyase (PAL) and lignin in olive (Montes-Osuna and Mercado-Blanco, 2020). In this way, Salah *et al.* (2018) demonstrated that applications of seaweed extracts including alginate, carrageenan, laminarin or ulvan in olive twigs increased the PAL activity, which was correlated with the lignin content in the treated twigs. In addition, treated twigs showed a significant reduction in vascular discolouration caused by *V. dahliae* (Salah *et al.*, 2018)

In summary, our results suggest that microorganisms from our own collection are among the most effective treatments for the reduction of VWO *in planta*. *Phoma* sp. isolate ColPat-375 and *A. pullulans* isolate AP08 were most effective when applied with foliar application. On the other hand, *B. amyloliquefaciens* isolate PAB-024 and *A. pullulans* isolate AP08 were among the most effective irrigation treatments after potassium phosphite-3 and copper phosphite-3. As we mentioned throughout the discussion, these BCAs or plant biostimulants could present different modes of action, such as antagonism or host resistance induction. However, the methodology used in this study was not sufficient to determine the modes of action of the different products. Therefore, further research is needed to select the most effective products and determine their mode of action by means of biochemical tools. In this way, monitoring the main parameters involved in plant resistance, such as quantification of H_2O_2 and callose deposition, and evaluation of hormones related to plant defence in olive tissues after treatments will be necessary to elucidate their role as HPDIs. Therefore, the present work will be useful to select better candidates for future studies on biocontrol, contributing significantly to new insights into the current challenge of the biological control of VWO.

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Chapter 3

Influence of the cultivar and biocontrol treatments on the effect of olive stem extracts on the viability of *Verticillium dahliae* conidia

ABSTRACT

The effect of olive (*Olea europaea*) stem extracts (OSE) on the viability of conidia of *Verticillium dahliae*, the causal agent of Verticillium wilt of olive (VWO), is not yet well understood. Thus, the aim of this study was to determine the influence of the olive genotype (cultivar resistance), and the interaction between olive cultivars and biocontrol treatments on the effect of OSE on conidial germination of *V. dahliae* by *in vitro* sensitivity tests. To this end, OSE from cultivars Frantoio, Arbequina, and Picual, respectively tolerant, moderately susceptible, and highly susceptible to *V. dahliae* were tested alone or after treatments with Biological Control Agents (BCA) and commercial products efficient to reduce the progress of VWO. As BCA, *Aureobasidium pullulans* strain AP08, *Phoma* sp., and *Bacillus amyloliquefaciens* strain PAB- 24 were considered. Aluminium lignosulfonate (IDAI Brotaverd®), copper phosphite (Phoscuprico®), potassium phosphite (Naturfos®) and salicylic acid were selected as commercial products. Our results indicated that the influence of biological treatments against the pathogen depends on the genotype, since the higher the resistance of the cultivar, the lower the effect of the treatments on the ability of the OSE to inhibit the germination of conidia. In 'Picual', the biological control agent *Bacillus amyloliquefaciens* PAB024 and copper phosphite were the most effective treatments in inhibiting conidia germination by the OSE. This work represents a first approach to elucidate the role of the cultivar and biological treatments in modifying the effect on the pathogen of the endosphere content of olive plants.

Keywords: biocontrol; endosphere; *Olea europaea*; plant-pathogen interactions; vascular pathogen; Verticillium wilt

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INTRODUCTION

Verticillium wilt of olive (*Olea europaea* subsp. *europaea*; VWO) causes high levels of tree mortality and reduces fruit yield in most olive growing areas worldwide, this disease being considered the main limiting factor of this crop in Mediterranean-type climate regions (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; López-Moral *et al.*, 2021b). In southern Spain, the disease results in one of the major concerns for olive growers. Although the global disease incidence in this region is around 0.5%, it can reach values higher than 20% together with high levels of disease severity and tree mortality in certain areas across the Guadalquivir valley. The causal agent of VWO is the hemibiotrophic soil-borne fungus *Verticillium dahliae*, from which two populations, defoliating (D) and nondefoliating (ND) pathotypes, have been identified in olive, with D pathotype causing the most severe damage (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012). The pathogen develops microsclerotia (MS), which are dormant structures that not only confers its ability to survive in the soil for a long time, but also, they serve as the primary inoculum source in natural infections (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Jiménez-Díaz *et al.*, 2012). Regarding the life cycle of *V. dahliae*, MS germinate by the stimuli of the root exudates from the susceptible hosts, giving rise to infectious hyphae that penetrate the plant roots and grow until they reach the vascular system. Then, the xylem vessels of the infected plants are colonized by the pathogen through mycelia and conidia development, contributing to the occlusion of the vascular system besides to the production of gels and tyloses in the xylem vessels. Altogether cause the reduction of water flow, leading to water stress, and consequently, plants become wilted and eventually death (Ayres, 1978; Pegg and Brady, 2002; López-Moral *et al.*, 2021a).

The innate biology of the pathogen, besides of the agronomical factors related to the intensification of olive crop, have favoured an increase of disease incidence year by year and have made it difficult to control VWO, making this disease one of the largest threats to the olive grove worldwide (López-Escudero and Mercado-Blanco, 2011; Alström, 2001; Pérez-Rodríguez *et al.*, 2015). Likewise, not truly efficient method to control VWO has already reported anywhere. Thus, there is no doubt that the use of an integrated disease management (IDM) strategy is needed to prevent *V. dahliae*

infections by both pre- and post-planting control measures in olive groves (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; Ostos *et al.*, 2020).

Considering the IDM against VWO, both genetic resistance and biological control methods must be combined seeking their synergistic effectiveness to reduce both pathogen dispersal and disease incidence in the field. In this way, it is well known that disease severity varies depending on the olive cultivars, with the selection of the cultivar being essential to avoid serious infections in the field. For instance, 'Picual' is considered one of the most susceptible olive cultivars, whereas 'Arbequina' or 'Hojiblanca' have shown moderate susceptibility; and 'Changlot Real', 'Empeltre', or 'Frantoio' high levels of tolerance (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013). On the other hand, important advances on biological control against VWO have been conducted during the last two decades (Montes-Osuna and Mercado-Blanco, 2020; Poveda and Baptista, 2021). With this regard, a broad diversity of essential oils, organic amendments, biological control agents (BCAs) including endophytic bacteria and fungi, plant biostimulants and host resistance inducers have been evaluated both under controlled and natural field conditions towards select the best candidates for the control of VWO (López-Moral *et al.*, 2021b; Varo *et al.*, 2016, 2017, 2018; Mulero-Aparicio *et al.*, 2019, 2020). Foliar or root applications with two beneficial microorganisms, the fungus *Aureobasidium pullulans* AP08 and the bacterium *Bacillus amyloliquefaciens* PAB-024; and two phosphonate salts, one of copper and the other of potassium, were effective in reducing disease progression in artificially inoculated olive plants (López-Moral *et al.*, 2021b).

Due to the different behaviour patterns previously observed on the effect of all these treatments against VWO, their mechanisms of action have been explored, elucidating both direct such as dual culture assays, *in vitro* sensitivity tests (López-Moral *et al.*, 2021b) and indirect effects such as effect on the root exudates inhibiting MS and conidia germination, enhancing the natural plant defence response (López-Moral *et al.*, 2021a; 2022) on the viability of the infectious structures of *V. dahliae* and on the VWO progress. These previous studies were useful to better understanding how these biocontrol products can act against the pathogen, but the knowledge generated here open news paths to be explored on such topic. In this way, determining whether these products can modulate the effect of the endosphere contents of the treated olive

plants on *V. dahliae* infection would suppose another relevant knowledge towards to better elucidating their mode of action.

The xylem vessels are an ideal niche for microbial endophytes such as the Verticillium wilt pathogen by providing an effective internal pathway for whole plant colonization, and by acquiring the scarce nutrients available in the xylem sap, either by enzymatic digestion of host cell walls, or by invading neighbouring cells, or by inducing leakage of nutrients from surrounding tissues (McCully, 2001; Agrios, 2005). Indeed, xylem sap contains a wide range of compounds beyond water and minerals, such as amino acids (Sauter, 1981), organic acids (Tatar *et al.*,1998) and vitamins (García *et al.*, 2011). The xylem sap composition of woody plant species, including olive tree has been characterized in several previous studies (Anguita-Maeso *et al.*, 2021a). But this composition can be influenced by multiple factors, such as the water content of the soil (Álvarez *et al.*, 2008), the cultivar, and type and age of organs selected, and the incidence of microbial interactions including infection by plant pathogens, among others (Anguita-Maeso *et al.*, 2021a). Related to this aspect, recently, Anguita-Maeso *et al.* (2021b) determined that the xylem microbiome of olive plants inoculated with *V. dalhliae* increases the diversity of bacterial communities compared to non-inoculated plants. In addition, these same authors also showed a breakdown of resistance to *V. dahliae* in wild olive trees related to a modification of their xylem microbiome (Anguita-Maeso *et al.*, 2021b). However, to our knowledge, there are no scientific studies that address the effect of the content of the olive endosphere, including xylem sap, on the viability of infectious structures of *V. dahliae*. Thus, the aim of this study was to determine the influence of the olive genotype (cultivar resistance), and the interaction between cultivars and biocontrol treatments on the effect of olive stem extracts (OSE) on the viability of conidia of *V. dahliae in vitro*.

MATERIALS AND METHODS

Plant material

Healthy 6-month-old rooted cuttings of three olive cultivars representative of different degrees of susceptibility to *V. dahliae* were used: 'Frantoio' (tolerant), 'Arbequina' (moderately susceptible) and 'Picual' (highly susceptible) (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013). The plants were obtained from a commercial nursery

and were growing in peat moss in plastic pots (0.5 l). They were pre-conditioned in a controlled-growth chamber [22 ± 2 °C, with a 14:10-h (light:dark) photoperiod of white fluorescent light (10,000 lux) and 60% relative humidity (RH)] for 1 month to induce the active growth. During this month, plants were irrigated three times per week with 350 ml of water per plant.

Fungal strain and inoculum preparation

The *V. dahliae* strain V180 was used in all the experiments of this study (López-Moral *et al.*, 2021b). It was stored as single-spore isolate on potato dextrose agar (PDA; Difco® Laboratories, MD, United States) slants full-filled with sterile paraffin oil at 4°C in darkness in the collection of the Department of Agronomy at the University of Córdoba (DAUCO, Spain). Before conducting each experiment, small mycelial fragments of the colonized agar from the tube were plated onto PDA acidified with 2.5 ml l^{-1} lactic acid (APDA) and incubated at 24°C in darkness for 10 days in order to obtain fresh colonies. Then, they were transferred to PDA, and incubated as described before.

Effect of olive stem extracts (OSE) on conidial viability of *Verticillium dahliae*

Obtaining stem extracts

For obtaining the olive stem extract (OSE), the main stem of the plants was cut at its base and the entire main stem and shoots were used. Leaves and roots were discarded. Subsequently, most of the cortical tissue of stems and shoots was removed manually using sandpaper, and the peeled stems were sprayed with distilled water and kept at 4°C in the dark to avoid desiccation until further processing. The OSE was obtained by the analytical laboratory 'C+E Analítica' (San José de la Rinconada, Seville, Spain) following the protocol described by Cadahía (2008). To conduct this protocol, once in the analytical laboratory, the shoots and stems were cut into 0.5 cm fractions, immersed in ethyl ether and kept at -20°C for 2 h. As a consequence of the freezer step, the water contained in the plant tissues crystallises, breaking the cell walls, which later allows the obtaining of a sap-like extract. At the same time, the ether extracts the chlorophyll that could interfere with the analytical process. After this step,

the plant material was defrosted and the aqueous phase (endosphere contents) was separated from the ether to obtain the OSE by means of a hydraulic press (Cadahía, 2008).

Experiment I: Effect of olive cultivars

For evaluating the effect of OSE from different olive cultivars on conidia viability, plants of cvs. Frantoio, Arbequina and Picual were used. They were maintained for one month in growth chambers as described before. For this period, they were arranged in a randomized complete block design with three blocks and four replicated plants per cultivar each ($3 \times 4 = 12$ plants per cultivar; 36 plants in total). The OSE was obtained joining all the plants of each block, so there were three experimental units of OSE (≈20 ml) per cultivar.

Experiment II: Influence of treatments

For evaluating the influence of treatments with different treatments on the effect of the OSE on *V. dahliae*, olive plants of cv. Picual were treated with seven treatments, including three biological control agents (BCAs) and four commercial products (Table 3.1). These treatments and the type of application (foliar and/or irrigation) were selected for this study because their significant efficacy against *V. dahliae in vitro* as well as against the progress of VWO *in planta* showed in previous studies (López-Moral *et al.*, 2021b).

Plants were treated with the respective water solution or suspension of each commercial product or BCA (Table 3.1), respectively, at 6-, 5-, and 3-weeks before obtaining the OSE. Additionally, non-treated but inoculated plants by cornmeal-sand mixture (CSM; sand, cornmeal and distilled water; 9:1:2, weight: weight volume) colonized by *V. dahliae* strain V180 (theoretical inoculum density of the final substrate = 10^7 CFU g^{-1}) were included as positive control; and non-treated and non-inoculated plants as negative control. Plant inoculation was conducted 4 weeks before obtaining the OSE. Treatments or plant inoculation were conducted following the protocols described by López-Moral *et al.* (2021b). A randomized complete block design with three blocks and four replicated olive plants per treatment ($n = 10$; eight treatments and two controls) was used (120 plants in total).

This experiment was maintained for six weeks after the first treatment was applied. Subsequently, the OSE was obtained from plants of each treatment as well as from plants of both positive and negative control, joining all the plants of each block i.e., there were three experimental units of OSE (≈20 ml) per treatment or control.

Experiment III: Interaction between olive cultivars and treatments

For evaluating the interaction between olive cultivars and treatments on the effect of the OSE on *V. dahliae*, olive plants of the three cvs. described above were treated with two BCAs (the fungus *Aureobasidium pullulans* strain AP08; and the bacterium *Bacillus amyloliquefaciens* strain PAB-24) and two commercial products [copper phosphite (Phoscuprico®, and potassium phosphite (Naturfos®)] (Table 3.1). These treatments were selected to conduct this experiment for their efficacy to inhibit conidial germination of *V. dahliae* in the *Experiment II*.

Plants were treated 6-, 5-, and 3-weeks before obtaining the OSE, and inoculated 4-weeks before obtaining the OSE. The treatments, OSE extraction and plant inoculation were conducted as described before. For each olive cultivar, a positive and a negative control were included as described in the *Experiment II*. A randomized complete block design with three blocks with four replicated olive plants per treatment ($n = 6$, four treatments and two controls) and cultivar ($n = 3$) combination was used (72 plants per olive cultivar; 216 plants in total).

This experiment was maintained for six weeks after the first treatment was applied. Subsequently, OSE was obtained from plants of each treatment and olive cultivar combination as well as from plants of positive and negative control, joining all the plants of each block, so there were three experimental units of OSE (≈20 ml) per treatment or control and cultivar combination.

Conidia viability *in vitro*

For each set of experiments, conidial suspensions were obtained from 14 days-old colonies of *V. dahliae* strain V180 growing on PDA as described before, and adjusted at 8×10^5 conidia ml^{-1} using a haematocytometer. In parallel, OSE solutions were adjusted at 0, 1, 10, 20, 30, 40, 50 and 100% in sterile deionized distilled water (SDDW). Subsequently, a 5-µl drop of the conidial suspension was placed in the centre

Table 3.1. Biological and chemical products evaluated in this study[a].

Active ingredient(s)	Trade name/ Formulation[b]	Manufacturer	Class (FRAC code)[c]	Dose[d]	
				Foliar	Root
Biological control agents (BCAs)[e]					
Aureobasidium pullulans	AP08	DAUCO[d]	Fungal (NC)	10^6 conidia ml^{-1}	10^6 conidia ml^{-1}
Bacillus amyloliquefaciens	PAB-024	DAUCO	Bacterial (NC)	n/e	10^8 CFU ml^{-1}
Phoma sp.	ColPat-375	DAUCO	Fungal (NC)	10^6 conidia ml^{-1}	n/e
Chemical products					
Aluminum lignosulfonate	IDAI Brotaverd®-EW	IDAI Nature	Inorganic salt (NC)	n/e[f]	5 ml l^{-1}
Copper phosphite	Phoscuprico®-EW	Agri nova Science	Phosphorous acid and salts (P07)	n/e	10 ml l^{-1}
Potassium phosphite	Naturfos®-EW	Daymsa	Phosphorous acid and salts (P07)	n/e	8 ml l^{-1}
Salicylic acid	Salicylic acid-SL	Sigma-Aldrich	Organic acid (NC)	5 mM (0.69 g l^{-1})	n/e

[a]Products and type of application evaluated in this present study were selected for their efficacy against *V. dahliae* observed in the previous study conducted by López-Moral *et al.* (2021b).
[b]EW: emulsion, oil in water; SL: soluble concentrate.
[c]Group and code numbers are assigned by the Fungicide Resistance Action Committee (FRAC) according to different modes of actions (NC: not classified; for more information, see http://www.frac.info/).
[d]Maximum dose for foliar or root applications recommended for the manufacturers of the commercial compounds evaluated in this study. Fungal and bacterial inocula from the BCAs (AP08, PAB-024 and ColPat-375) were prepared and adjusted according to Varo *et al.* (2016).
[e]All the BCAs used in this study are maintained in the collection of the Agroforestry Pathology Research Group at the Department of Agronomy, University of Córdoba (DAUCO), Spain.
[f]n/e: non-evaluated products and dose combinations in this study.

of a microscope coverslip (20 × 20 mm); and then, a 5-µl drop of the OSE solution was mixed. Thus, the OSE was evaluated at the following final concentrations: 0, 0.5, 5, 10, 15, 20, 25 and 50%, where concentration 0 consisting of a 5-µl drop of the conidial suspension mixed with a 5-µl drop of sterile SDDW as a control. The coverslips were placed inside Petri dishes containing water agar, which were used as humid chambers, and were incubated at 23 ± 2°C in the dark for 24 h. After incubation period, a 5-µl drop of 0.01% acid fuchsine in lactoglycerol (1:2:1 lactic acid:glycerol:water) was added to each coverslip to stop conidial germination, and they were mounted in a slide. For each experiment (*I*, *II* and *III*), there were three replicated coverslips per concentration of OSE obtained from each block and from each treatment or control (OSE at 0%, i.e., only SDDW). All the experiments were conducted twice.

In all cases, a total of 120 randomly selected conidia per replicated coverslip were observed at a ×400 magnification by means of a Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan), and the germinated and non-germinated conidia were counted. Conidia were considered germinated when the germ tube was at least one-half of the longitudinal axis of the conidia. Conidial viability was estimated as percentage (%) of conidial germination for each OSE concentration, and then, the inhibition of conidial germination (RGI; %) was estimated with respect to the control according to the formula:

$$RGI\ (\%) = [(Ge_{control} \times Ge_{OSEsolution})/Ge_{control}]$$

where $Ge_{control}$ = percentage of germinated conidia after incubation in the SDDW and $Ge_{OSEsolution}$ = percentage of germinated conidia after incubation in the OSE solution from treated plants (Moral *et al.*, 2018). The RGI data were linearly regressed over the OSE concentration, and the predicted values of the effective OSE concentrations (µg ml^{-1}) inhibiting 50% (EC_{50}) of conidial germination were obtained from the fitted regressions

Data analysis

Data of EC_{50} from the two repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test ($P \geq 0.05$). Subsequently, in any cases, data were tested for normality, homogeneity of variances, and residual patterns. For the *Experiment I* and *II*, a one-way ANOVA was conducted

with the 'EC_{50}' as dependent variable, and 'cultivar' or 'treatment', as independent variables, respectively. For *Experiment III*, a factorial ANOVA was conducted with 'EC_{50}' as dependent variable, and 'cultivar', 'treatment' and their interaction as independent variables. Since interaction was significant ($P = 0.0001$), one-way ANOVAs were conducted for each olive cultivar. Treatment means were compared according to Fisher's protected LSD test or to Tukey´s HSD test (both at $P = 0.05$) for the *Experiment I* ($n = 3$) and *III* ($n = 6$), or for the *Experiment II* ($n = 10$), respectively (Steel and Torrie, 1985). Data of this study were analyzed using the software Statistix 10.0 (Anonimous, 2013).

RESULTS

Effect of olive stem extracts on conidial viability of *Verticillium dahliae*

Experiment I: Effect of olive cultivars

The results on the effect of OSE from plants of cvs. Frantoio (tolerant), Arbequina (moderately susceptible) and Picual (highly susceptible) at 5, 10, 15, 20, 25 and 50% concentration on the conidial viability of *V. dahliae* showed that the higher OSE concentration the higher was the RGI. Marked differences between the three olive cultivars were detected from OSE at 10%, where that from 'Frantoio' showed higher RGI values than that from 'Arbequina' and 'Picual', but in these last two cases the inhibition did not differ significantly. At the highest concentration evaluated (OSE at 50%), RGI values ranged from 68.9 ± 2.9 to 80.5 ± 1.9% for 'Picual' and 'Frantoio', respectively (Fig. 3.1).

In addition, significant differences for the EC_{50} between cultivars ($P = 0.0239$) were observed. The OSE from 'Frantoio' showed the lowest EC_{50} value ($EC_{50} = 16.2 \pm 0.88$) compared to the OSE from the rest of cultivars; while OSE from 'Arbequina' ($EC_{50} = 21.7 \pm 1.15$) and 'Picual' ($EC_{50} = 20.4 \pm 1.08$) did not differ significantly from each other (Table 3.2).

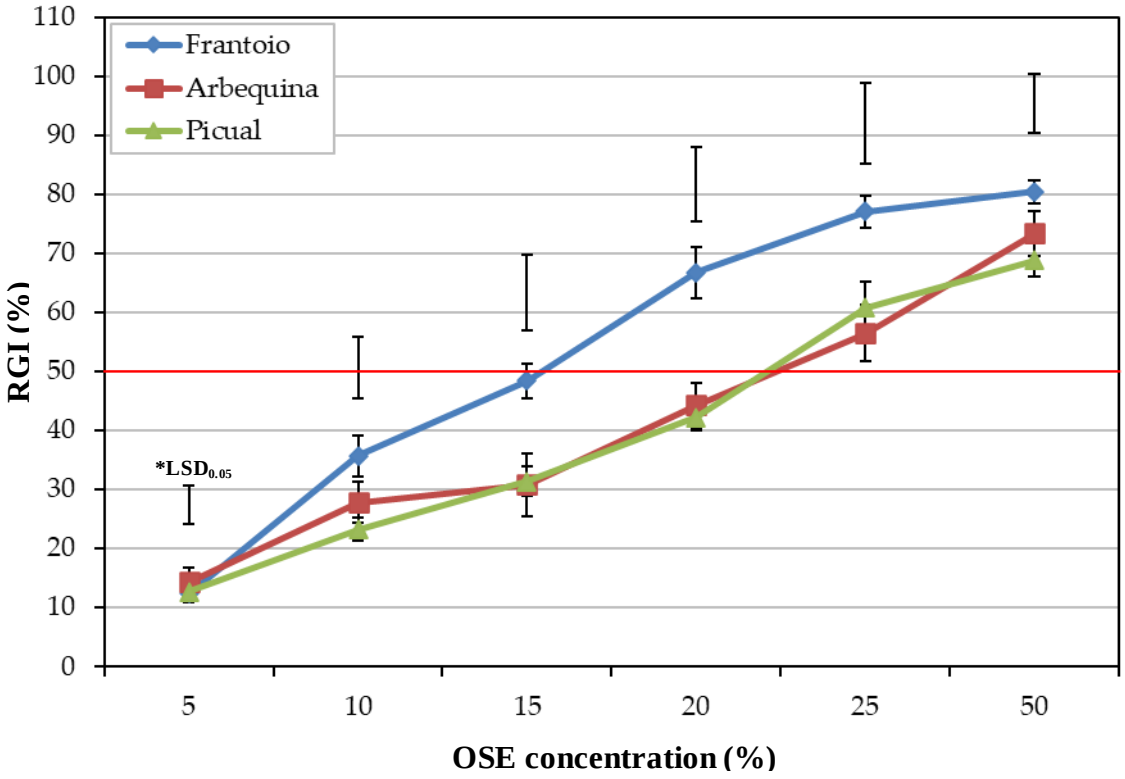

Figure 3.1. Effect of olive stem extracts (OSE) from healthy olive plants of cvs. 'Frantoio' (tolerant), 'Arbequina' (moderately susceptible) and 'Picual' (highly susceptible) on relative conidial germination inhibition (RGI) of *Verticillium dahliae* isolate V180. Data are the means of 240 conidia (6 replicates) per each combination of olive cultivar and OSE concentration. Vertical bars represent the standard error of the means. *LSD bars represent the critical values for comparison at $P = 0.05$.

Table 3.2. Effective concentrations of olive stem extracts (OSE) from cvs. Frantoio, Arbequina and Picual to inhibit 50% of conidial germination (EC_{50}; µl ml^{-1}) of the *Verticillium dahliae* isolate V180.

Cultivar	EC_{50} (µl ml^{-1})[a]
Frantoio	16.2 ± 0.88 b
Arbequina	21.7 ± 1.15 a
Picual	20.4 ± 1.08 a

[a]EC_{50} of conidial germination was calculated as predicted value of the linear regression of the relative germination inhibition (%) over OSE concentration. Values represent the average of 240 conidia (6 coverslips) per extract (cultivar) and OSE concentration. Means followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

Experiment II: Influence of treatments

The influence of the OSE from olive plants of cv. Picual treated with different treatments on the conidial viability showed great differences between treatments (Fig. 3.2). In general, the OSE from plants treated with commercial products showed higher RGI values than those from OSE obtained from plants treated with BCAs. Only the

OSE from plants treated by root applications with *A. pullulans* AP08, aluminium lignosulfonate and copper phosphite, showed RGI values higher than 80% when they were tested at the highest concentration.

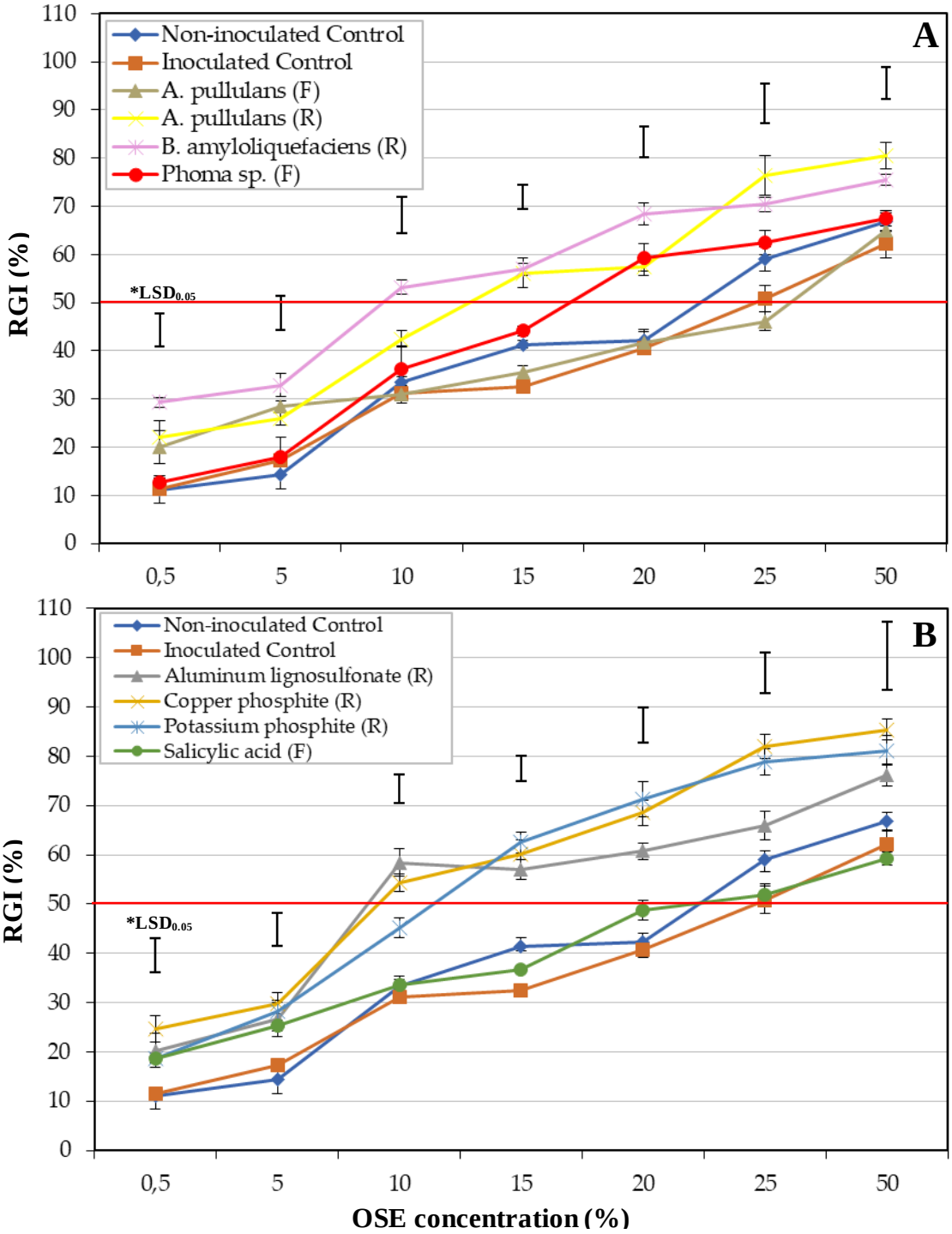

Figure 3.2. Effect of olive stem extracts (OSE) from healthy olive plants of cv. Picual treated by foliar (F) or root (R) applications with **A**, microorganisms (Biological control agents) and **B**, commercial products on the relative conidial germination inhibition (RGI) of *Verticillium dahliae* isolate V180. In each graph, data are the means of 240 conidia (6 replicates) per each combination of compound and OSE concentration. Vertical bars represent the standard error of the means. *LSD bars represent the critical values for comparison at $P = 0.05$.

There were significant differences for EC_{50} between treatments ($P < 0.0001$). In general, OSE from treated plants showed significantly lower EC_{50} values than that from both negative and positive control (EC_{50} = 21.5 ± 1.43 and 24.9 ± 0.69, respectively), with the exception of OSE from plants treated by spraying with *A. pullulans* AP08 (EC_{50} = 24.7 ± 1.61), *Phoma* sp. (EC_{50} = 17.3 ± 0.55) and salicyclic acid (EC_{50} = 22.1 ± 2.05) which did not differ from the controls (Table 3.3).

Table 3.3. Effective concentrations of olive stem extracts (OSE) from cv. Picual treated with several compounds to inhibit 50% of conidial germination ($EC_{50;}$ µl ml−1) of the *Verticillium dahliae* isolate V180.

Treatment[a]	**Application**	**EC_{50} (µl ml^{-1})**[b]
Control (-)	-	21.5 ± 1.43 ab
Control (+)	-	24.6 ± 0.69 a
Aluminum lignosulfonate	Root	12.1 ± 1.38 cd
Aureobasidium pullulans	Foliar	24.7 ± 1.61 a
A. pullulans	Root	13.1 ± 1.01 cd
Bacillus amyloliquefaciens	Root	10.3 ± 1.26 d
Copper phosphite	Root	10.1 ± 1.34 d
Phoma sp.	Foliar	17.3 ± 0.55 bc
Potassium phosphite	Root	11.8 ± 1.52 cd
Salicylic acid	Foliar	22.1 ± 2.05 ab

[a]All the compounds were applied by foliar or root applications in non-inoculated plants. Control (-): non-treated and non-inoculated plants; Control (+): non-treated and inoculated plants with *V. dahliae* isolate V-180.
[b]EC_{50} of conidial germination was calculated as predicted value of the linear regression of the relative germination inhibition (%) over OSE concentration. Values represent the average of 240 conidia (6 coverslips) per extract (cultivar) and OSE concentration. Means followed by a common letter do not differ significantly according to Tukey´s HSD Test (P = 0.05).

Experiment III: Interaction between olive cultivar and treatments

The OSE from plants of cv. Frantoio did not show marked differences on the RGI between treatments. A moderate effect on RGI was observed for the OSE from treated plants of cv. Arbequina, with RGI slightly increasing for the OSE from plants treated with *A. pullulans* and copper phosphite. On the other hand, great differences on the RGI were observed in 'Picual' between the OSE from treated and control plants as well as between the OSE from plants treated with the different BCAs and chemical products (Fig. 3.3).

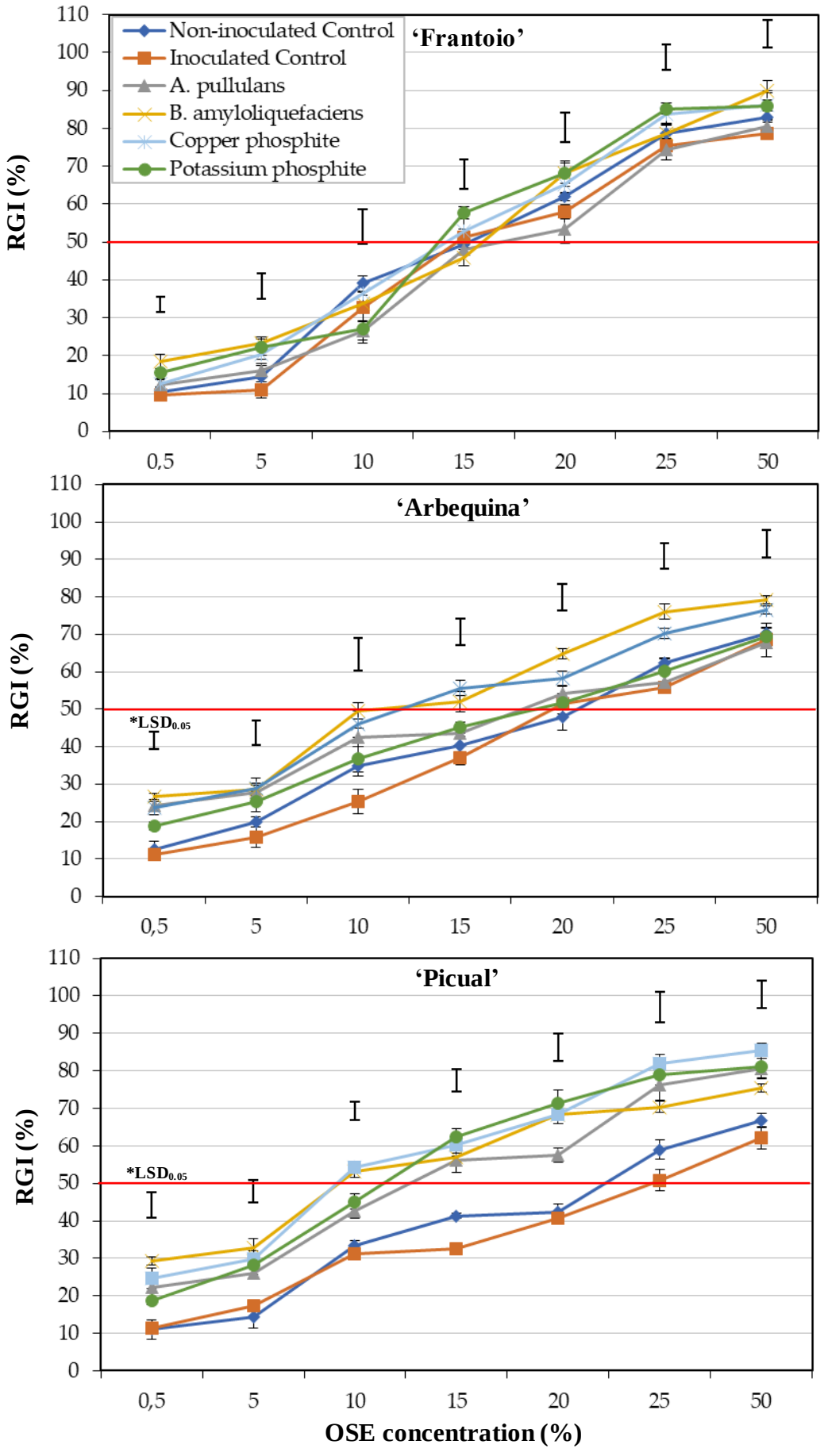
Non-inoculated Control
Inoculated Control
A. pullulans
B. amyloliquefaciens
Copper phosphite
Potassium phosphite
'Frantoio'
'Arbequina'
'Picual'
RGI (%)
*LSD0.05
OSE concentration (%)
0,5
5
10
15
20
25
50

Figure 3.3. Effect of olive stem extracts (OSE) from healthy olive plants of cvs. Frantoio, Arbequina and Picual treated by root applications with the most effective compounds from the *Experiment II* (*Aureobasidium pullulans*, *Bacillus amyloliquefaciens*, Copper phosphite or Potassium phosphite) and from non-treated control plants, both non-inoculated and inoculated with *Verticillium dahliae* isolate V-180 on the relative conidial germination inhibition (RGI) of *V. dahliae* isolate V180. In each graph, data are the means of 240 conidia (6 replicates) per each combination of olive cultivar and OSE concentration. Vertical bars represent the standard error of the means. *LSD bars represent the critical values for comparison at $P = 0.05$.

ANOVA confirmed that there were no significant differences for EC_{50} between treated and non-treated plants of cv. Frantoio ($P = 0.9510$). In 'Arbequina', significant differences for EC_{50} were only observed between the OSE from plants treated with *B. amyloliquefaciens* (EC_{50} = 13.0 ± 1.57) and copper phosphite (EC_{50} = 14.2 ± 1.19) compared to that obtained for OSE from the controls. Finally, in 'Picual', the OSE from treated plants showed significantly lower EC_{50} values than those of the OSE from both negative and positive controls, but the EC_{50} did not differ significantly between the different products evaluated (Table 3.4).

Table 3.4. Effective concentrations of olive stem extracts (OSE) from cvs. Frantoio, Arbequina and Picual and treated with the most effective compounds to inhibit 50% of conidial germination ($EC_{50;}$ µl ml−1) of the *Verticillium dahliae* isolate V180.

Treatment[a]	EC_{50} (µl ml^{-1})[b]		
	Frantoio	**Arbequina**	**Picual**
Control (-)	15.2 ± 2.07 a	20.1 ± 0.90 a	21.5 ± 0.85 a
Control (+)	16.1 ± 1.39 a	20.0 ± 2.44 a	24.6 ± 0.69 a
Aureobasidium pullulans	16.4 ± 1.36 a	17.9 ± 0.88 ab	13.1 ± 1.01 b
Bacillus amyloliquefaciens	14.8 ± 1.87 a	13.0 ± 1.57 c	10.3 ± 1.26 b
Copper phosphite	14.9 ± 0.83 a	14.2 ± 1.19 bc	10.1 ± 1.34 b
Potassium phosphite	14.7 ± 1.23 a	18.3 ± 0.92 ab	11.8 ± 1.52 b

[a]All the evaluated treatments were applied by root applications in non-inoculated plants. Control (-): non-treated and non-inoculated plants; Control (+): non-treated and inoculated plants with *V. dahliae* isolate V-180.

[b]EC_{50} of conidial germination was calculated as predicted value of the regression: relative germination inhibition (%) over OSE concentration. Values represent the average of 240 conidia (6 coverslips) per extract (cultivar) and OSE concentration. Means followed by a common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

DISCUSSION

Although recent studies have revealed that *V. dahliae* modulates the xylem microbiome in olive plants by increasing the diversity of bacterial communities when the pathogen is present in the soil (Anguita-Maeso *et al.*, 2021b), the influence that olive cultivar, biological treatments and their interaction could have on the endosphere

against the viability of the infectious structures of *V. dahliae* has not yet been reported. Thus, this work represents a first approach to elucidate the role of the cultivar and biological treatments in modifying the effect of the endosphere contents on the pathogen in olive plants.

In this study, 6-month-old potted olive plants were used because, based on our extensive experience working with the olive tree and *V. dahliae* pathosystem, it is not only the age limit to reproduce the symptoms of the disease by artificial inoculation of plants in small pots (Varo *et al.*, 2016; López-Moral *et al.*, 2021b), but also considering the limitations that the size and physiological structure of plant tissues could have in the extraction of contents from the endosphere by the Cahahía's method (Cahahía, 2008). Regarding this last aspect, we have to consider that this author call 'sap' to the total liquid extract from the endosphere (called OSE in this study) that comes from both xylem and phloem of the plant. Probably, the ideal would be to obtain raw sap (xylem) and elaborated sap (phloem) separately to evaluate only the effect of the raw sap, but despite this limitation, we choose this method instead of the sap extraction method using the Scholander chamber (Alexou and Peuke, 2013) for the following reasons: *i*) because it has been demonstrated that the extract of pure sap from the xylem of olive plants contains a wide diversity of bacterial communities (Anguita-Maeso *et al.*, 2021b), which could mask the effect of the OSE on the germination of conidia in the *in vitro* sensitivity tests; *ii*) consequently to this first reason, the extraction of OSE using the Cadahía's method guaranties the absence of living microorganisms as well as cellular debris in the extract, since the plant material is subjected to consecutive immersion and freezing treatments in diethyl-ether during the extraction process; and *iii*) because it has been recognized as reliable method to determine the nutritional levels of the plants, since the results using 'sap-like' extracts (OSE) have contrasted well with those of the yield and quality of the harvest of several fruit crops, including the olive tree, for more than 40 years of experience (Cadahía, 2008).

Notice also that the effect of OSE was only evaluated on the germination of conidia in this study because conidia are the infectious structures of *V. dahliae* directly affected for the sap considering the life cycle of the pathogen, i.e., the pathogen infects plants through the root by the germinated MS, and then systemically colonizes the infected plants by producing conidia in the xylem vessels (López-Escudero and Mercado-Blanco, 2011; Pegg and Brady, 2002). In addition, the treatment combinations

(treatments and/or mode of application) evaluated in this study were selected because they all resulted in high effectiveness in inhibiting the viability of the infectious structures of *V. dahliae* as well as reducing the progress of the disease in previous studies by López-Moral *et al.* (2021b).

Our results revealed that the OSE from 'Frantoio' (tolerant) showed higher RGI values than that from 'Arbequina' (moderately susceptible) and 'Picual' (highly susceptible), whereas the inhibition did not differ markedly between the last two cultivars (*Experiment I*). In 'Picual', the influence of the OSE from the treated plants on the inhibition of conidia germination varied significantly between the treatments evaluated. In this cultivar, the BCA *B. amyloliquefaciens* PAB024, and a phosphonate salt [copper phosphite (Phoscuprico®)] were the most effective in inhibiting conidia germination by the OSE (*Experiment II*). In addition, when the four selected treatments were applied to the three olive cultivars, their influence on the effect of OSE on the inhibition of conidia germination was not significant between treatments for 'Frantoio', whereas moderate and markedly significant differences between treatments were observed for 'Arbequina' and 'Picual', respectively (*Experiment III*). Finally, although some differences can be observed between both positive and negative controls in RGI as the OSE concentration increases (Fig. 2 and 3), the EC_{50} data did not show significant differences between both controls in any case. Therefore, these data would suggest that the biotic stress caused by the infection of the pathogen in the plant does not influence the effect of OSE on the conidial germination of *V. dahliae*.

As a first conclusion, our results indicated that the influence of biological treatments against the pathogen depends on the genotype, since the greater the resistance of the cultivar the lower the influence of the treatments on the ability of the OSE to inhibit conidia germination. Thus, the results suggest that the highly tolerance to *V. dahliae* conferred by the 'Frantoio' genotype prevails over the treatments, even those that were more effective against the pathogen in susceptible cultivars. These results are in agreement with those obtained recently by López-Moral *et al.* (2021a), who determined the influence of cultivars and biological treatments on the effect of root exudates from olive plants on the viability of MS and conidia of *V. dahliae*. These authors demonstrated that root exudates induced germination of conidia and MS of *V. dahliae*, and that the genotype significantly affected this ability: the root exudates from 'Frantoio' did not show a significant effect on the induction of MS and conidia

germination compared to the control, while those from 'Arbequina' and 'Picual' showed a moderate and marked effect inducing the viability of both MS and conidia of *V. dahliae*, respectively (López-Moral *et al.*, (2021a).

Regarding genetic resistance, our study reveals new knowledge about the relationship that the sap and the olive cultivar could have favouring or interfering with the colonization of the xylem by the pathogen. But, in order to elucidate how the cultivar factor could influence the effect of its sap on the colonization of the pathogen, further interaction studies with the xylem anatomy of each cultivar should be conducted in the future. In fact, previous studies that evaluated the anatomy of the xylem of healthy olive trees of cvs. 'Frantoio' and 'Picual' showed significant differences not only in the parameters related to water transport, but also in the density of vessels associated with a larger or smaller conduction area in the xylem tissue, both parameters being significantly higher in 'Frantoio' than in 'Picual' (Molina, 2010).

Regarding the influence of the treatments on the effect of the OSE on the viability of the conidia, our results are also in agreement with those obtained by López-Moral *et al.* (2021a). These authors revealed that the root exudates from plants of the three cultivars ('Frantoio', 'Arbequina', and 'Picual') treated with the same four treatments evaluated in the *Experiment III* of the present study showed significant differences in their effect on MS and conidia germination, and the genotype also significantly affected this ability. In this case, the treatment with *A. pullulans* AP08 resulted in the most effective showing a significant effect inhibiting conidia germination in 'Arbequina' and MS germination in 'Arbequina' and 'Picual', but non-signicant effect in these two parameters was observed in 'Frantoio'. Regarding the rest of treatments evaluated, the root exudates from plants treated with copper phosphite, potassium phosphite and *B. amyloliquefaciens* PAB-24 gave rise to a significant inhibition in the germination of conidia or MS, but only in cv. Arbequina (López-Moral *et al.*, 2021a). In addition, previous studies conducted by López-Moral *et al.* (2022) to evaluate these treatments as potential inducers of host resistance against VWO showed that both BCAs, *A. pullulans* AP08 and *B. amyloliquefaciens* PAB-024, as well as the phosphonate salt Phoscuprico® had the ability to accumulate jasmonic acid (JA) and JA-isoleucine in leaves, stem or roots of treated olive plants of cv. Picual. These results suggest also an implication of the JA in the host resistance induced by these treatments. This last aspect could be directly related to our results, since it is well known that xylem

infections by vascular pathogens cause drastic metabolic changes in the cells of the xylem parenchyma adjacent to the infected vessels. These metabolic changes lead to the accumulation of different proteins and secondary metabolites in the xylem sap during pathogen colonization, including pathogenesis-related proteins (PR-proteins), enzymes (e.g. peroxidases, proteases, xyloglucan-endotransglycosylase, xyloglucan specific endoglucanase protein inhibitor), phenols, phytoalexins, and lignin, which help to enhance the natural defence mechanisms of the plant (Hilaire *et al.*, 2001; Rep *et al.*, 2002; Basha *et al.*, 2010; Gayoso *et al.*, 2010; Llorens *et al.*, 2017b). On the other hand, due to the OSE did not have alive microbiome as a consequence of the extraction method used, studies to determine whether the treatments applied in this work could influence the modification of the xylem microbiome of olive plants must be conducted. In this way, recent studies performed by Anguita-Maeso *et al.* (2021b) determined that the xylem microbiome of olive plants inoculated with *V. dahliae* increases the diversity of bacterial communities compared to non-inoculated plants. However, how the xylem microbiome could be modified by the biological treatments favouring *V. dahliae* inhition is still uncertain.

In summary, the method used in this study to obtain endosphere contents of olive plants, called as OSE or 'sap-like', for further analysis in the laboratory against *V. dahliae* can be considered valid and useful since all our results agree with those obtained in previous studies (López-Moral *et al.*, 2021a,b; 2022). The knowledge generated here results in a first approach to go ahead studying how the genotype and/or biological treatments can influence the extracts of olive plants by inhibiting the germination of conidia or of MS of *V. dahliae*. All this knowledge could be useful in the future to prevent infections or mitigate the progression of the disease within the framework of the current 'From farm to fork' strategy towards to obtain safe and healthy fruits.

CONCLUSIONS

Our results indicated that the influence of biological treatments against the pathogen depends on the genotype, since the greater the resistance of the cultivar the lower the influence of the treatments on the ability of the OSE to inhibit conidia germination. In 'Picual', the most susceptible cultivar to VWO, the BCA *B. amyloliquefaciens* PAB024, and a phosphonate salt [copper phosphite (Phoscuprico®)]

were the most effective treatments in inhibiting conidia germination by the OSE. Thus, the results suggest that the highly tolerance to *V. dahliae* conferred by the 'Frantoio' genotype prevails over the treatments, even those that were more effective against the pathogen in susceptible cultivars. The method used in this study to obtain endosphere contents of olive plants, called as OSE or 'sap-like', for further analysis in the laboratory against *V. dahliae* can be considered valid and useful since all our results agree with those obtained in previous studies. Thus, this work represents a first approach to elucidate the role of the cultivar and biological treatments in modifying the effect on the pathogen of the endosphere content of olive plants.

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Chapter 4

Elucidating the role of root exudates of olive plants against *Verticillium dahliae*: effect of the cultivar and biocontrol treatments

ABSTRACT

Aims The present study aimed to set up a collection method for root exudates from young olive plants and evaluate the effect of root exudates from different cultivars and biocontrol treatments combinations on the viability of conidia and microsclerotia (MS) of the soil-borne pathogen *Verticillum dahliae*.

Methods In the first experiment, three collection media (deionized distilled water, 0.01 M $CaSO_4$ solution, Hoagland nutrient solution) and four collection periods (2, 4, 6, 12 h) were assessed to identify the most appropriate combination for root exudates collection in olive plants of cv. Picual. In another set of experiments, root exudates from olive cultivars with different susceptibility to *V. dahliae* ('Arbequina', 'Frantoio', 'Picual') and treated or not treated with several biocontrol compounds were also obtained. The effect of root exudates on germination of conidia and MS of *V. dahliae* was evaluated *in vitro*. Additionally, the Dissolved Organic Carbon in the root exudates was estimated.

Results A 0.01 M $CaSO_4$ solution and 4 h may be regarded a preferential medium and timing combination to collect root exudates from olive. Root exudates induced conidial and MS germination of *V. dahliae*, with genotype affecting significantly on this ability. *Aureobasium pullulans* AP08 was the most effective biocontrol treatment to enhance the effectiveness of root exudates decreasing the viability of conidia and MS. Organic carbon in the exudate solutions varied depending on the olive cultivars and/or on the biocontrol treatments.

Conclusions The collection method for root exudates proposed in our study was useful to assess the effect of root exudates from olive plants and the olive-*V. dahliae* interaction.

Keywords Biocontrol, *Olea europaea*, Plant-Pathogen interactions, Rhizosphere, Soilborne pathogen, Verticillium wilt

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INTRODUCTION

The hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb., the causal agent of Verticillium wilt of olive (*Olea europaea* subsp. *europaea* L.) (VWO), is able to survive in the soil for a long time by means of microsclerotia (MS). They are dormant structures which act as the primary inoculum of the pathogen in natural infections as well as the main dispersal propagule (Jiménez-Díaz *et al.* 2012; López-Escudero and Mercado-Blanco 2011; Montes-Osuna and Mercado-Blanco 2020). MS germination is induced by root exudates from the susceptible hosts, giving rise to infective hyphae that penetrate the plant roots growing towards the vascular system (Pegg and Brady 2002). Subsequently, the pathogen colonizes the xylem vessels of the infected plants by developed mycelia and conidia, which contribute to the occlusion of the vascular system together with the production of gels and tyloses in the cells as a consequence of natural plant response against the infection. Altogether cause the reduction of water flow, leads to water stress, and consequently, plants become wilted (Ayres 1978; Trapero *et al.* 2018). Disease severity varies depending on the olive cultivars. Most of them have been described as susceptible or extremely susceptible to the pathogen. Among them, 'Picual' is considered one of the most susceptible, whereas 'Changlot Real', 'Empeltre', and 'Frantoio' have shown high levels of resistance (López-Escudero *et al.*, 2004; Trapero *et al.* 2013).

As we mentioned above, it has been described that root exudates from olive plants play a key role in the life cycle of *V. dahliae* (Pegg and Brady 2002). However, specific knowledge about how collect root exudates from olive plants to be used for further studies to elucidating how they mediate the plant-pathogen interaction in the infection of *V. dahliae* to olive into the rhizosphere is still uncertain. For this reason, the present study is designed mainly to know how to obtain root exudates from olive plants using a capable method, and to demonstrate whether the methodology preserves the ability of root exudates to induce the germination of conidia and MS of *V. dahliae in vitro*. Within this context, it is well known that plants are constantly releasing a broad diversity of compounds to their surrounding media (Vives-Peris *et al.* 2020). Among them, root exudates are the main compounds secreted into the rhizosphere, which are a complex mixture of primary (e.g., carbohydrates, amino acids, and organic acids) and secondary metabolites (e.g., fatty acids, nucleotides, tannins, phenolic acids, terpenoids, steroids,

alkaloids, polyacetylenes, glucosinolates, auxins, vitamins, etc.) (Pantigoso *et al.* 2021; Vives-Peris *et al.* 2020). Likewise, it has been demonstrated that root exudates are involved in numerous interactions within the rhizosphere, attracting beneficial or parasitic microorganisms, enhancing nutrient absorption, carbon and nitrogen circulation, or resource competition, along with many other interactions (Pantigoso *et al.* 2021; Vives-Peris *et al.* 2020).

In relation to the interaction between plants and microorganism in the soil, root exudates are considered the main form of communication of the plant with the surrounding microbial communities (Pantigoso *et al.* 2021). Several studies have demonstrated the positive effect of root exudates over beneficial microorganisms, recruiting and promoting the colonization of plant growth-promoting rhizobacteria such as *Bacillus amyloliquefaciens* (Weng *et al.* 2013; Yuan *et al.* 2015) or *Pseudomonas putida* (Neal *et al.* 2012; Vives-Peris *et al.* 2018), as well as symbiotic arbuscular mycorrhizal fungi (Yoneyama *et al.* 2012). Concerning plant pathogenic fungi, several studies have reported that root exudates are able to modulate the rhizosphere, inducing or inhibiting the activity of soil-borne pathogens such as *Fusarium oxysporum* (Huang *et al.* 2020; Vives-Peris *et al.* 2020; Xu *et al.* 2015).

It is also worth mentioning that roots are able to release organic substances that not only influences nutrient availability in the rhizosphere, but also they can act as messengers to initiate physiological interactions between the roots and the soil system (Pantigoso *et al.* 2021). Therefore, root exudates increase the availability of phosphorus (P) in the rhizosphere (Hinsiger 2001) and stimulate the atmospheric dinitrogen fixation (N_2) (Coskun *et al.* 2017). Concerning carbon (C) circulation in soil, it has been estimated that from 5 to 21% of all photosynthetically C fixed is transferred to the rhizosphere through root exudates, and they serve as the preferred C source of beneficial rhizosphere bacteria (Pantigoso *et al.* 2021). All this suggests that many processes related to soil nutrition, i.e. C management, biochemical cycling of N and P, need the communication between microorganisms and root exudates (Pantigoso *et al.* 2021; Vives-Peris *et al.* 2020). Likewise, in this study, we also pretend to know if the quantity of the Dissolved Organic Carbon (DOC) released by the roots of olive plants could be related to the stimulation of the germination of conidia and MS of *V. dahliae*.

Finally, the effect of root exudates into the rhizosphere can be also affected by other biological factors, intrinsic to the plant, such as the genotype (cultivar resistance).

Studies conducted in *Arabidopsis thaliana*, as well as in other herbaceous crops, such as *Solanum tuberosum* and *Zea mays*, have shown that genetic alterations affect the production of root exudates and markedly modulate changes into the microbial community where the host grows (Vives-Peris *et al.* 2020). In addition, Zhang *et al.* (2020) demonstrated that root exudates from resistant cultivars of *Nicotiana tabacum* had an inhibitory effect on the growth of *Phythopthora nicotianae*, which causes black shank disease, whereas those from susceptible cultivars stimulated its growth.

Despite much basic information about the effect of root exudates mediating the interaction between plants and microorganisms into the rhizosphere is available in the literature (Pantigoso *et al.* 2021; Vives-Peris *et al.* 2020), most of the studies on such topic have been conducted in herbaceous plants, but not in woody plants such as olive. The difficulty to collect root exudates from plants grown under field conditions, or from plants that have greater difficulty in reproducing their grow conditions in the laboratory, such as woody crops, could explain the lack of knowledge about the methodology for collecting root exudates from these hosts. Consequently, this also explains the little information available on how woody plant root exudates act in the biotic and abiotic interactions in the surrounding rhizosphere. Thus, due to the important role that olive root exudates play in the plant root infection by *V. dahliae*, together with the lack of validated methodology to collect root exudates from olive plants, the main goal of this study was to set-up a root exudate collection method from olive plants using different collection media and periods. In parallel, the effect of root exudates on the viability of conidia and MS of *V. dahliae* was evaluated *in vitro*. Once the root collection method was validated, two additional experiments were conducted to determine the effect of the olive genotype (cultivar resistance), and the interaction between cultivars and biocontrol treatments on the root exudates and their activity on the viability of conidia and MS of *V. dahliae*. Additionally, in all the experiments the DOC content in the root exudates was estimated to know if its concentration could be related to the stimulation of infectious structures of *V. dahliae*. We hypothesized that root exudates from tolerant cultivars could show a different effect on the viability of conidia and MS of *V. dahliae* than those from susceptible cultivars; and biocontrol treatments could influence the effect of root exudates on the viability of *V. dahliae* structures. Moreover, we also hypothesized that the concentration of DOC in the root exudates could have some

relationship with the susceptibility of the olive varieties or with the biocontrol treatments and their effects on the infectious structures of *V. dahliae.*

MATERIAL AND METHODS

Plant material

All the experiments were conducted using healthy six-months-old olive potted plants growing in peat moss into plastic pots (0.5 l) that were obtained from a commercial nursery. The root exudate collection method (*Experiment I*) was set up using olive plants of cv. Picual. In subsequent sets of experiments (*Experiments II, III*), three olive cultivars representative of three degrees of susceptibility to VWO were used ['Frantoio' (tolerant); 'Arbequina' (moderately susceptible), and 'Picual' (highly susceptible); (López-Escudero *et al.* 2004; Trapero *et al.* 2013)]. In all cases, plants were pre-conditioned in a controlled-growth chamber [22 ± 2 °C, with a 14:10-h (Light: Darkness) photoperiod of white fluorescent light (10,000 lux) and 60% relative humidity (RH)] along one month before to conduct each experiment, and irrigated three times per week to keep moisture near field capacity.

Fungal strain and inoculum preparation

The *Verticillium dahliae* strain V180 was used in all the experiments. It was recovered from soil samples collected from a commercial olive orchard affected by VWO in Villanueva de la Reina (Jaen province, Andalusia region, southern Spain; UTM coordinates X: 38.012845, Y: 3.909219). This strain was previously characterized as defoliating (D) pathotype by PCR, and acted as highly virulent in inoculated olive plants (López-Moral *et al.* 2021b). It is maintained as single-spore strain on potato dextrose agar (PDA; Difco® Laboratories, MD, USA) slants full-filled with sterile paraffin oil at 4 ºC in darkness in the collection of the Department of Agronomy at the University of Córdoba (DAUCO, Spain). Before conducting each experiment described below, small mycelial fragments of the colonized agar from the tube were plated onto PDA acidified with lactic acid [APDA; 25% (vol/vol) at 2.5 ml liter^{-1} of medium] and incubated at 24 ºC in darkness for 10 days to obtain fresh colonies of the V180 strain. These fresh colonies were transferred to PDA and incubated as described before. Then, they were used as the initial inoculum source for further experiments.

Effect of root exudates on viability of conidia and microsclerotia of *Verticillium dahliae*

Experiment I: Influence of media and time periods.

To set up the root exudate collection method from olive plants, a first set of experiments using olive plants of cv. Picual (highly susceptible to VWO) was conducted to evaluate the combination of three collection media and four collection periods. The three collection media evaluated were: i) Hoagland nutrient solution (HNS) [containing 5 mM $Ca(NO_3)_2 \cdot 4H_2O$, 5 mM KNO_3, 2 mM $MgSO_4$, 0.1 µM KCl, 0.3 µM $Ca(H_2PO_4)_2 \cdot H_2O$, 50 µM H_3BO_3, 4 µM $MnSO_4 \cdot H_2O$, 4 µM $ZnSO_4 \cdot 7H_2O$, 0.1 µM $CuSO_4 \cdot 5H_2O$ and 6 µM Na_2MoO_4; adapted from Hoagland (1920)], ii) deionized distilled water (DDW) and, iii) calcium sulphate solution ($CaSO_4$ solution; 0.01 M $CaSO_4 \cdot 2H_2O$; adapted from Aulakh *et al.* 2001)]. The four collection periods tested were 2, 4, 6, and 12 h. The HNS and $CaSO_4$ solutions were prepared in DDW just before the beginning of the experiment. There were three replicated plants per collection media (n = 3) and collection period (n = 4) combination in a completely randomized design. There were 36 plants, with each plant being considered an experimental unit. Additionally, a control without plant was included for each collection media (three experimental units per collection media of 350 ml each) as a negative control for further analysis. The experiment was conducted twice.

Experiment II: Effect of olive cultivars

Based on the results obtained in the first experiment, root exudates were collected from plants of the olive cvs. Frantoio (tolerant), Arbequina (moderately susceptible) and Picual (highly susceptible) using two collection media [DDW, and $CaSO_4$ solution (Aulakh *et al.* 2001)], and 4 h as collection period. The $CaSO_4$ solution was prepared in DDW just before use. There were three replicated plants per collection media (n = 2) and cultivar combination (n = 3) in a completely randomized design. There were 18 plants, with each plant being considered an experimental unit.

A control without plant was also included for each collection media (three experimental units per collection media of 350 ml each) as a negative control for further analysis. The experiment was conducted twice.

Experiment III: Interaction between olive cultivars and biocontrol treatments

To evaluate the interaction between olive cultivars and biocontrol treatments on the effect of the root exudates on *V. dalhiae*, olive plants of the three cvs. described above were treated with two commercial biostimulant phosphite salts [copper phosphite (Phoscuprico®), and potassium phosphite (Naturfos®)], and two Biological Control Agents (BCAs) from the collection of DAUCO (the fungus *Aureobasidium pullulans* strain AP08, and the bacterium *Bacillus amyloliquefaciens* strain PAB-24) (López-Moral *et al.* 2021b). The commercial phosphite salts were applied at the maximum irrigation doses recommended by the manufacturers (10 and 8 ml l^{-1} for copper phosphite and potassium phosphite, respectively). For the BCAs, *A. pullulans* and *B. amyloliquefaciens* suspensions were prepared and adjusted at 10^6 conidia ml^{-1} and 10^8 CFU ml^{-1}, respectively (López-Moral *et al.* 2021b; Varo *et al.* 2016). These commercial compounds and BCAs, and the type of application (by irrigation) were selected to conduct this study due to their greater effectiveness against *V. dahliae* and the progress of VWO *in planta* shown in previous studies (López-Moral *et al.*, 2021b).

Plants were treated by watering 300 ml per plant of the respective water solution or suspension of each commercial compound or BCA, respectively, 6, 5, and 3 weeks before root exudates collection, following the same protocols described by López-Moral *et al.* (2021b) for irrigation applications. Additionally, a non-treated group of plants for each of the three cvs. described above was inoculated by a cornmeal-sand mixture (CSM; sand, cornmeal and distilled water; 9:1:2, weight:weight:volume), colonized by *V. dahliae* strain V180 (theoretical inoculum density of the final substrate = 10^7 CFU g^{-1}) as described by López-Moral *et al.* (2021b). Plant inoculation was conducted 4 weeks before root exudates collection. A non-treated and non-inoculated lot of plants for each of the three cvs. was also included. A randomized complete block design (three blocks) was used with treatments including biocontrol compounds (n = 4), non-treated and inoculated, and non-treated and non-inoculated lot of plants as independent variable. There were five replicated olive plants per treatment and block (90 plants of each cultivar, 270 plants in total).

This experiment was maintained for six weeks after the first biocontrol treatment was applied. Subsequently, root exudates were collected from three plants of each biocontrol treatment and cultivar combination as well as from three plants per cultivar of both non-treated and non-inoculated or non-treated and inoculate lots, using the $CaSO_4$ solution

and 4 h as collection media and period, respectively. An additional control of $CaSO_4$ solution without plant (three experimental units of 350 ml each) was also included as a negative control for further analysis. The experiment was conducted twice.

Root exudate collection

To collect root exudates, the olive plants were taken out of the plastic pots (0.5 l) together with the intact substrate. To avoid damage to the roots in the process to removing the surrounding substrate, the roots were carefully washed by spraying tap water until most of the substrate was removed, and finally they were washed again with DDW. Immediately after washing, intact plants were placed in cylindrical PVC pots (9.5 cm in diameter × 11 cm in height; ≈ 0.5 l vol.) without drainage hole and containing 350 ml of collection media with the complete root system of the plant submerged. For the fixation of the individual plants in each pot, polyethylene discs of 10 mm in diameter were designed, making a small hole (4 mm in diameter) in the centre to fix the basal end of the stem. Then, the pot was closed with the polyethylene disc with the fixed plant, keeping the entire root system of the plant in the collection media at 23 ± 2°C in the dark. After the time of root exposure in the collection media (see above for each experiment), the effluent containing the root exudates was collected individually from each pot. Subsequently, it was double filtered, firstly through filter paper (43-48 µm pore size; Filter-Lab®, Filtros Anoia, Barcelona, Spain) to remove root detritus; and secondly, through a 0.45 µm membrane filter to remove microbial cell debris. The filtrate was immediately frozen in plastic vials at -20 ºC until the subsequent analysis of this study was conducted. Prior to use for each set of experiments, vials were defrosted overnight at 4ºC in the dark. The root exudate collection method from olive plants was adapted from Aulakh *et al.* (2001).

Assessment, processing and analyses

Conidial viability *in vitro*

For each set of experiments, conidial suspensions were obtained from 14 days-old colonies of *V. dahliae* strain V-180 growing on PDA as described before, and adjusted at 8×10^5 conidia ml^{-1} using a haematocytometer. A 5-µl drop of the conidial suspension was placed in the centre of a microscope coverslip (20 × 20 mm); and

subsequently, a 5-µl drop of the root exudate solution was mixed. Coverslips were placed inside Petri dishes containing water agar, which were used as humid chambers, and incubated at 23 ± 2°C in darkness for 24 h. After the incubation period, a 5-µl drop of 0.01% acid fuchsine in lactoglycerol (1:2:1 lactic acid:glycerol:water) was added to each coverslip in order to stop conidial germination, and they were mounted in a slide. A total of 100 randomly selected conidia per coverslip were observed at a ×400 magnification by means of a Nikon Eclipse 80i microscope (Nikon Corp., Tokyo, Japan), and germinated and non-germinated conidia were counted. Conidia were considered germinated when the germ tube was at least one-half of the longitudinal axis of the conidia. Conidial viability was estimated as percentage (%) of conidial germination (CGe) for each exudate solution or control (collection media without plant); and the induction or reduction of conidia germination was estimated as percentage (%) of increased or reduced germination respect to the control. For each experiment (*I, II* and *III*), there were three replicate coverslips per exudate solution collected from each of the three selected plants per treatment combination or control (collection media without plant). The experiment was conducted twice.

Microsclerotia viability in naturally infested soils

Soil samples naturally infested with *V. dahliae* were collected from a commercial cotton (*Gossypium hirsutum* L.) field located in Villanueva de la Reina (Jaen province, Andalusia region, southern Spain; UTM coordinates X: 38.012845, Y: 3.909219). This soil is characterized as Vertisol (USDA 2014) with a pH of 8.0, a low content in organic matter (around 10 g kg-1) and a clay loam texture. Five soil sub-samples (5,000 g each) from the upper 30 cm of the soil surface were randomly collected across de field. Then, they were mixed obtaining a single homogenized sample, which was air-dried at room temperature until completely dry. Finally, organic debris and large particles were removed from the soil samples by manually sifting (0.8 mm in diameter sieve) (Trapero *et al.* 2013).

For each set of experiments, sterile plastic pots (100-ml vol.) were previously drilled in the base (5 holes, 2-mm in diameter each) to make easier the percolation, filled with 60 g of the naturally infested air-dried soil, and irrigated with 30 ml of the respective exudate solution. Additional plastic pots (100-ml vol.) filled with 60 g of the naturally infested soil were irrigated with the collection media without plant (Hoagland

solution, DDW, and/or $CaSO_4$solution), and they were used as control. The plastic pots were hermetically closed after treatment percolation, and incubated for 72 hours at room temperature. Then, treated-soil samples were removed from the plastic pots, and air-dried at room temperature into individual aluminum trays for 10-14 days. For each set of experiments (*I*, *II* and *III*), a completely randomized design was used with three replicated plastic pots per exudate solution collected from each one of the three selected plants per treatment combination or control (collection media without plant). The experiment was conducted twice.

In all cases, the wet sieving (Huisman and Ashworth 1974) method using modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay 1977) was conducted to estimate the inoculum density of *V. dahliae* [colony forming units (CFU) or microsclerotia per g of soil (MS)] following the protocol described by Varo *et al.* (2016). Then, the inoculum density was expressed as MS concentration (MSC; MS per g of soil) for each exudate solution and control (collection media without plant); and the induction of MS germination was estimated as percentage (%) respect to the control.

Carbon exudation per plant: Dissolved organic Carbon analyses

Dissolved organic C (DOC, also known as non-purgeable organic carbon or NPOC) in the exudate solutions was analysed in a Total Organic Carbon Analyzer (TOC-L, Shimadzu Corporation) through the 680 °C combustion catalytic oxidation method/NDIR detection, after the root exudates samples were defrosted overnight at 4 °C. For that, an aliquot of 4 ml of each exudate solution was mixed with 16 ml of deionized water. Two calibration curves (0–10 mg C l^{-1} and 10–100 mg C l^{-1}) were prepared and blanks (deionized water) and a solution of a known concentration in C were inserted every 12 samples (root exudate solutions). The samples were automatically acidified in the TOC-L analyzer with 1 M HCl to bring the pH of the samples to 2–3. Root exudate rates of C were estimated according to the following equation, adapted from Aulakh *et al.* (2001):

$$\text{C exudation per plant}\left(\frac{\text{mg C}}{\text{plant} \times \text{h}}\right) = \left[\text{DOC in a sample with a plant}\left(\frac{\text{mg C}}{\text{l}}\right) - \text{DOC in a control without a plant}\left(\frac{\text{mg C}}{\text{l}}\right)\right] \times \frac{0.35\ l}{collection\ period\ (h)}$$

where 0.35 l means de total volume of the collection media used per plant in all the experiments of this study.

Data analyses

Data from the two repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test ($P \geq 0.05$). Subsequently, in all cases, data were tested for normality, homogeneity of variances, and residual patterns; and they were logarithmically transformed when necessary. For all the experiments, factorial ANOVA were previously conducted with 'CGe', 'MSC', induction of 'CGe' and 'MSC', or 'C exudation per plant' as dependent variables, and 'collection media' and 'collection period', 'collection media' and 'cultivar', and 'cultivars' and 'biological treatments', and their respective interactions, as independent variables for *Experiment I, II,* and *III*, respectively. Because the interactions were significant ($P < 0.05$), independent ANOVA were conducted in each experiment as follows. For *Experiment I*, the 'collection period' was used as the independent variable to determine its effect for each collection media; for the *Experiment II*, the 'cultivar' was used as the independent variable to determine their effect in each collection media; and for the *Experiment III*, the 'biological treatment' was used as the independent variable to determine their effect in each olive cultivar. In all cases, treatment means of 'CGe', 'MSC', induction or reduction of 'CGe' and 'MSC' or 'C exudation per plant' were compared according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie 1985). Data of this study were analyzed using the software Statistix 10.0 (Analytical Software, Tallahassee, USA).

RESULTS

Effect of root exudates on viability of conidia and microsclerotia of *Verticillium dahliae*

Experiment I: Influence of media and time periods

The results on the effect of root exudates from olive plants collected with DDW, $CaSO_4$ solution or HNS at 0, 2, 4, 6 or 12 h of collection period on the conidial and microsclerotia viability of *V. dahliae* are shown in Table 4.1.

The root exudates obtained using DDW as a collection medium showed significant differences in their effect both on the germination of conidia ($P = 0.0422$) and MS ($P = 0.0453$) for the collection periods tested, observing the highest germination percentage at 4 h of the collection period, both for germination of conidia (86.6 ± 3.6%) and MS (50.9 ± 1.2%). However, non-significant differences were observed on the induction of conidia and MS germination with respect to the control between the collection periods tested, although the highest values of induction of germination were observed for 4 h. The root exudates collected in the $CaSO_4$ solution showed significant differences on their effect on germination of conidia and MS, and on the induction of conidial and MS germination with respect to the control between the collection periods tested. The highest germination values for conidia and MS were observed in root exudates collected at 4 and 6 h after dipping. Conidia germination was 86.2 ± 1.4 and 86.5 ± 1.1% in root exudates collected at 4 and 6 h after dipping, with 7.3 ± 2.0 and 7.9 ± 3.6% increase in conidial germination with respect to the control, respectively. The MS germination was significantly higher in root exudates collected at 4 h after dipping (54.4 ± 1.2 MS/g of soil) in comparison with the control (46.9 ± 3.8 MS/g of soil), showing an increase of 17.7 ± 11.5% of MS with respect to the control. However, the conidia and MS were significantly reduced when they were treated with root exudates collected from a $CaSO_4$ solution at a sampling time of 12 h. Finally, the root exudates obtained using the HNS medium did not show any significant effect on the germination of conidia and MS of *V. dahliae* at any collection period in comparison with the control.

Based on this preliminary experiment, the HNS was discarded, and the combination of DDW or CaSO4 and 4 h of collection period was selected for further experiments.

Experiment II: Effect of olive cultivars

The results about the effect of root exudates from olive plants of cvs. Arbequina, Frantoio and Picual, collected by dipping the roots in DDW or in a $CaSO_4$ solution for 4 h, on the conidia and MS viability of *V. dahliae* are shown in Table 4.2.

In general, the root exudates from 'Frantoio', independently of the collection media used, showed similar effects on the germination of conidia and MS than that

Table 4.1. Effect of root exudates from olive plants of cv. Picual on the viability of conidia and microsclerotia of *Verticillium dahliae*[a].

Conidia viability (Germination, %; Induction, %)[b,d]						
Collection	**DDW**		**$CaSO_4$**		**HNS**	
period (h)	**Germination**	**Induction**	**Germination**	**Induction**	**Germination**	**Induction**
0 (control)	71.8 ± 2.9 b	-	80.3 ± 1.8 bc	-	85.0 ± 1.5 a	-
2	78.7 ± 2.2 ab	10.2 ± 7.8 a	82.9 ± 3.5 ab	3.1 ± 3.0 a	85.2 ± 3.3 a	0.24 ± 3.6 a
4	86.6 ± 3.6 a	20.7 ± 2.2 a	86.2 ± 1.4 a	7.3 ± 2.0 a	84.4 ± 2.7 a	-0.58 ± 4.3 a
6	82.8 ± 0.1 a	15.7 ± 5.1 a	86.5 ± 1.1 a	7.9 ± 3.6 a	82.1 ± 0.3 a	-3.4 ± 1.6 a
12	85.1 ± 4.6 a	19.4 ± 11.0 a	76.2 ± 1.9 c	-5.2 ± 0.3 b	85.1 ± 1.8 a	0.15 ± 2.6 a
$P_{\alpha = 0.05}$	0.0422	0.4611	0.0106	0.0242	0.8421	0.7950
Microsclerotia viability (Germination, MS/g soil; Induction, %)[c,d]						
Collection	**DDW**		**$CaSO_4$**		**HNS**	
period (h)	**Germination**	**Induction**	**Germination**	**Induction**	**Germination**	**Induction**
0 (control)	40.7 ± 2.3 b	-	46.9 ± 3.8 bc	-	51.7 ± 3.9 a	-
2	48.4 ± 0.2 ab	19.9 ± 7.8 a	47.5 ± 1.7 ab	2.8 ± 10.4 b	45.0 ± 0.7 a	-10.0 ± 8.4 a
4	50.9 ± 1.2 a	26.2 ± 8.4 a	54.4 ± 1.2 a	17.7 ± 11.5 a	50.7 ± 2.0 a	-1.5 ± 4.0 a
6	41.5 ± 3.9 b	2.5 ± 10.1 a	48.7 ± 1.1 ab	5.0 ± 8.5 b	52.7 ± 1.5 a	3.6 ± 11.5 a
12	45.9 ± 3.3 ab	14.6 ± 15.6 a	40.3 ± 1.7 c	-13.1 ± 7.6 c	43.7 ± 2.7 a	-15.2 ± 1.6 a
$P_{\alpha = 0.05}$	0.0453	0.1948	0.0223	0.0015	0.0886	0.0921

[a]Root exudates were collected after dipping the roots in deionized distilled water (DDW), in a 0.01 M $CaSO_4$ solution ($CaSO_4$) or in a Hoagland nutrient solution (HNS) for 0, 2, 4, 6 or 12 h periods.

[b]Conidia viability: Percentage (%) of germinated conidia of *Verticillium dahliae* strain V180 after incubation in the root exudates at 23 ± 2 ºC for 24 h in the dark; and induction effect of root exudates on conidial germination (%) with respect to the control (collection media not exposed to the roots; collection period = 0 h). For both parameters, and for each collection medium and time combination, values represent the mean of two independent experiments with three replicated cover slides each ± standard error of the means.

[c]Microsclerotia (MS) viability: Number of germinated MS per g of naturally infested soil by *V. dahliae* after 72 h in contact with the root exudates; and induction effect of root exudates on MS germination (%) with respect to the control (collection media not exposed to the roots; collection period = 0 h). For both parameters, and for each collection media and time combination, values represent the mean of two independent experiments with three replicated soil-treated subsamples each ± standard error of the means.

[d]In each column, means followed by the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

observed for the controls; while those from 'Arbequina' or 'Picual' showed the highest values of conidia and MS germination. The root exudates obtained using DDW as collection media only showed significant differences in their effect on both conidial ($P = 0.0354$) and MS germination ($P = 0.0372$) between cultivars and control. The highest conidial germination percentages were observed for root exudates obtained from 'Arbequina' (90.3 ± 2.2%) and 'Picual' (89.4 ± 1.2%); whereas the root exudates collected from 'Frantoio' had a lower germination (87.5 ± 2.0%), similar to that observed for the control (82.5 ± 1.5%). Although a significant effect on the induction of conidial germination was not observed between cultivars, marked differences were shown since the induction of conidial germination with respect to the control was higher for the root exudates from 'Arbequina' and 'Picual' in comparison with those from 'Frantoio'. A similar pattern was observed for the MS germination and the induction of

MS germination with respect to the control. The highest MS germination percentages were also observed for root exudates obtained from 'Arbequina' (54.1 ± 3.0%) followed by 'Picual' (52.5 ± 1.3%); whereas a similar effect on MS germination was observed for root exudates collected from 'Frantoio' (45.7 ± 2.6%) and the control (44.9 ± 1.3%). In spite of non-significant effect was observed on the induction of MS germination between cultivars, marked differences were shown since the induction of MS germination with respect to the control was higher for the root exudates from 'Arbequina' and 'Picual' in comparison with those from 'Frantoio'. Concerning the root exudates obtained in $CaSO_4$ collection media, no significant differences were observed in the effect on conidial germination among root exudates from different cultivars. However, root exudates showed a significant effect on MS germination between cultivars and control (P = 0.0388) as well as on the induction of MS germination between cultivars with respect to the control (P = 0.0495). The MS germination was significantly higher for root exudates collected from 'Arbequina' (53.5 ± 2.1 MS/g of soil) and 'Picual' (52.4 ± 1.1 MS/g of soil) in comparison with those from 'Frantoio' (42.0 ± 4.3 MS/g of soil) and the control (42.8 ± 2.4 MS/g of soil). An increase of 25.5 ± 7.0 and 23.1 ± 6.2% of induction on MS germination with respect to the control were observed for the treatments with root exudates from 'Arbequina' and 'Picual', respectively. Finally, treatments with root exudates from 'Frantoio' did not produce any significant effect on the induction of MS germination with respect to the control.

Experiment III: Interaction between olive cultivars and biocontrol treatments

The results related to the effect of root exudates from olive plants of cvs. Arbequina, Frantoio and Picual that were subjected to biocontrol treatments on the viability of conidia and MS of *V. dahliae* are shown in Table 4.3.

Since the general factorial ANOVA showed a significant interaction between cultivars and treatments, the data were analyzed separately for each cultivar, performing two factorial ANOVAs, one for the three controls of the experiment and the other for the plants that received the biocontrol treatments. This last group of plants was

Table 4.2. Effect of root exudates from the olive cvs. Arbequina, Frantoio and Picual on the the viability of conidia and microsclerotia of *Verticillium dahliae*[a].

Conidia viability (Germination, %; Induction, %)[b,d]				
Olive Cultivar	**DDW**		**$CaSO_4$**	
	Germination	**Induction**	**Germination**	**Induction**
Control	82.5 ± 1.0 b	-	92.5 ± 1.7 a	-
Arbequina	90.3 ± 2.2 a	9.4 ± 2.7 a	90.2 ± 2.2 a	-2.4 ± 4.0 a
Frantoio	87.5 ± 2.0 ab	6.1 ± 4.0 a	88.0 ± 1.5 a	-4.9 ± 0.5 a
Picual	89.4 ± 1.2 a	8.5 ± 2.5 a	88.2 ± 0.8 a	-4.6 ± 2.5 a
$P_{\alpha = 0.05}$	0.0354	0.3012	0.3438	0.6556
Microsclerotia viability (Germination, MS/g soil; Induction, %)[c,d]				
Olive cultivar	**DDW**		**$CaSO_4$**	
	Germination	**Induction**	**Germination**	**Induction**
Control	44.9 ± 1.3 c	-	42.8 ± 2.4 b	-
Arbequina	54.1 ± 3.0 a	20.9 ± 9.6 a	53.5 ± 2.1 a	25.5 ± 7.0 a
Frantoio	45.7 ± 2.6 bc	2.2 ± 8.3 a	42.0 ± 4.3 b	-1.1 ± 12.8 b
Picual	52.5 ± 1.3 ab	16.9 ± 0.5 a	52.4 ± 1.1 a	23.1 ± 6.2 ab
$P_{\alpha = 0.05}$	0.0372	0.1133	0.0388	0.0495

[a]Root exudates were collected after dipping the roots of young olive plants in deionized distilled water (DDW) or in a 0.01 M $CaSO_4$ solution ($CaSO_4$) for 4 h.
[b]Conidial viability: Percentage (%) of germinated conidia of *Verticillium dahliae* strain V180 after incubation in the root exudates at 25ºC for 24 h in the dark; and induction effect of root exudates on conidial germination (%) with respect to the control (collection media not exposed to the roots). For both parameters, and for each collection medium and cultivar combination, values represent the mean of two independent experiments with three replicated cover slides each ± standard error of the means.
[c]Microsclerotia (MS) viability: Number of germinated MS per g of naturally infested soil by *V. dahliae* after 72 h in contact with the root exudates; and induction effect of root exudates on MS germination (%) with respect to the control (collection media not exposed to the roots). For both parameters, and for each collection media and cultivar combination, values represent the mean of two independent experiments with three replicated soil-treated subsamples each ± standard error of the means.
[d]In each column, means followed by the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

compared with non-treated and non-inoculated plants as the only control to check the inhibition of conidia and MS germination.

The comparison between the three controls of this experiment showed two general patterns although there was an exception. First, the root exudates stimulated the germination of conidia and MS (average 93.3% and 44.2 UFC/g, respectively) with respect to the collection medium without root exudates (average 78.0% and 39.4 UFG/g, respectively), and there were no differences between exudates from non-inoculated or inoculated plants. Second, the root exudates from cv. Frantoio did not show a stimulating effect on the germination of conidia and MS compared to those from

'Arbequina' or 'Picual'. The only exception was the root exudates from inoculated plants, which increased conidia germination but not MS germination.

Regarding the effect of root exudates from biocontrol treated plants on the germination of conidia and MS, the treatment with *A. pullulans* AP08 showed a significant effect inhibiting conidia germination in 'Arbequina' plants and MS germination in 'Arbequina' and 'Picual' plants. In addition, the root exudates from 'Frantoio' plants treated with *A. pullulans* AP08 also showed the highest values of inhibition of conidia and MS germination, although they were not significant. Other biocontrol treatments whose root exudates gave rise to a significant inhibition in the germination of conidia or DM only occurred in the Arbequina cultivar and were potassium phosphite and copper phosphite, inhibiting conidia germination, and potassium phosphite and *B. amyloliquefaciens*, inhibiting MS germination.

Effect of root exudates on carbon exudation: *Dissolved organic Carbon analyses*

Experiment I: Influence of collection media and period

The results on the effect of root exudates from olive plants collected with DDW, $CaSO_4$ solution or HNS at 0, 2, 4, 6 or 12 h of collection period on C exudation per plant are shown in Fig. 4.1.

In general, for the three collection media evaluated, the highest values of C exudation per plant (mg C $plant^{-1}$ h^{-1}) was observed in root exudates collected at 4 h. This was also the only evaluated time in which C was observed from exudates collected with HNS (6.2 ± 3.1 mg C $plant^{-1}$ h^{-1}).

Significant differences in plant C exudation were observed between collection periods when root exudates were collected with DDW (P = 0.0258). The highest values of C exudation were observed in the root exudates collected at 4 h (4.7 ± 1.4 mg C $plant^{-1}$ h^{-1}), followed by those collected at 2 h (2.4 ± 0.7 mg C $plant^{-1}$ h^{-1}). However, C exudation per plant was significantly reduced for collection periods over 4 h, with that ranging from 1.0 ± 0.2 to 0.4 ± 0.1 mg C $plant^{-1}$ $h^{-1}$for root exudates collected at 6 and 12 h, respectively.

Table 4.3. Effect of root exudates from the olive plants of cvs. Arbequina, Frantoio and Picual subjected to biological or biostimulants treatments on the viability of conidia and microsclerotia of *Verticillium dahliae*[a].

Conidia viability (Germination, %; Inhibition, %)[b,d]						
	'Arbequina'		**'Frantoio'**		**'Picual'**	
Treatment	**Germination**	**Inhibition**	**Germination**	**Inhibition**	**Germination**	**Inhibition**
Control (CaSO4 solution)	78.0 ± 0.4 d	-	78.0 ± 0.4 c	-	78.0 ± 0.4 c	-
Non-inoculated control*	91.8 ± 0.9 a	0.0 c	81.1 ± 1.6 bc	0.0 a	92.1 ± 1.5 a	0.0 a
Inoculated control	88.5 ± 1.3 abc	-	87.7 ± 0.9 a	-	91.7 ± 2.4 a	-
Aureobasidium pullulans	85.0 ± 1.0 c	7.5 ± 1.5 a	77.7 ± 3.3 c	4.3 ± 2.5 a	89.5 ± 1.7 ab	2.8 ± 2.9 a
Bacillus amyloliquefaciens	89.7 ± 1.0 ab	2.3 ± 0.5 bc	84.6 ± 2.4 ab	-4.4 ± 3.3 a	88.4 ± 1.3 ab	4.0 ± 1.6 a
Copper phosphite	85.5 ± 0.8 c	6.9 ± 0.9 ab	85.3 ± 1.4 ab	-5.2 ± 0.3 a	90.3 ± 1.6 a	1.9 ± 2.9 a
Potassium phosphite	86.7 ± 1.9 bc	5.6 ± 2.9 ab	85.6 ± 1.7 ab	-5.7 ± 3.9 a	84.9 ± 1.0 b	7.8 ± 0.5 a
$P_{\alpha = 0.05}$	0.0001	0.0261	0.0167	0.1155	0.0004	0.0878
Microsclerotia viability (Germination, MS/g soil; Reduction, %)[c,d]						
	'Arbequina'		**'Frantoio'**		**'Picual'**	
Treatment	**Germination**	**Inhibition**	**Germination**	**Inhibition**	**Germination**	**Inhibition**
Control (CaSO4 solution)	39.5 ± 2.0 cd	-	39.5 ± 2.0 a	-	39.4 ± 2.0 cd	-
Non-inoculated control*	48.7 ± 0.7 a	0.0 b	37.6 ± 2.3 a	0.0 a	47.5 ± 1.8 ab	0.0 b
Inoculated control	45.6 ± 0.5 ab	-	37.6 ± 1.4 a	-	48.0 ± 1.5 ab	-
Aureobasidium pullulans	38.9 ± 2.9 cd	19.9 ± 6.6 a	35.7 ± 1.1 a	4.6 ± 3.1 a	35.1 ± 2.0 d	26.0 ± 3.9 a
Bacillus amyloliquefaciens	40.3 ± 1.4 bcd	17.3 ± 1.9 a	40.0 ± 1.4 a	-6.9 ± 5.4 a	42.3 ± 1.7 bc	10.9 ± 2.9 ab
Copper phosphite	43.9 ± 1.3 abc	9.9 ± 2.1 ab	37.7 ± 0.6 a	-0.9 ± 4.7 a	49.7 ± 2.7 a	-5.4 ± 9.1 b
Potassium phosphite	38.0 ± 2.1 d	22.0 ± 3.2 a	40.0 ± 0.8 a	-8.4 ± 7.9 a	41.7 ± 3.4 bcd	11.5 ± 9.9 ab
$P_{\alpha = 0.05}$	0.0081	0.0195	0.3341	0.1260	0.0065	0.0255

[a]Root exudates were collected after dipping the roots of young olive plants in a 0.01 M CaSO4 solution (CaSO4) for 4 h.

[b]Conidia viability: Percentage (%) of germinated conidia of *Verticillium dahliae* strain V180 after incubation in the root exudates at 25ºC for 24 h in the dark; and reduction effect of root exudates from treated plants on conidial germination (%) with respect to that of root exudates from non-inoculated and non-treated control plants. For both parameters, and for each cultivar and biological treatment combination, values represent the mean of two independent experiments with three replicated cover slides each ± standard error of the means.

[c]Microsclerotia (MS) viability: Number of germinated MS per g of naturally infested soil by *V. dahliae* after 72 h in contact with the root exudates; and reduction effect of root exudates on MS germination (%) from treated plants with respect to that of root exudates from non-inoculated and non-treated control plants. For both parameters, and for each cultivar and biological treatment combination, values represent the mean of two independent experiments with three replicated soil-treated subsamples each ± standard error of the means.

[d]In each column, means followed by the same letter do not differ significantly according to Fisher's protected LSD test ($P < 0.05$).

*Non-inoculated control refers to non-inoculated and non-treated olive plants from which root exudates were collected. Then, exudates were evaluated *in vitro* to determine their effect on conidia and MS germination of *V. dahliae*.

Regarding the presence of C in the root exudates collected in the $CaSO_4$ solution, no significant differences were observed between collection periods (P = 0.3476). Despite this fact, some differences in C exudation were observed between collection periods following a similar pattern to that described for root exudates collected with DDW. The highest values of C exudation were also observed in the root exudates collected at 4 h (4.4 ± 2.5 mg C plant^{-1} h^{-1}), followed by those collected at 2 h (2.2 ± 0.1 mg C plant^{-1} h^{-1}) or at 6 h (2.2 ± 0.5 mg C plant^{-1} h^{-1}). Finally, the lowest values of C exudation were observed in root exudates collected at 12 h (0.5 ± 0.1 mg C plant^{-1} h^{-1}).

Experiment II: Effect of olive cultivars.

The results on the effect of root exudates from olive plants of cvs. Arbequina, Frantoio and Picual, collected by dipping the roots in DDW or in a $CaSO_4$ solution during 4 h on C exudation per plant are shown in Fig. 4.2.

There were no significant differences in C exudation per plant between olive cultivars when the root exudates were collected in DDW (P = 0.5021). Minor differences on C exudation per plant were observed in the root exudates collected from the three cultivars tested ('Arbequina' = 5.9 ± 1.2 mg C plant^{-1} h^{-1}; 'Frantoio' = 4.7 ± 1.4 mg C plant^{-1} h^{-1}; 'Picual' = 6.2 ± 1.3 mg C plant^{-1} h^{-1})

On the other hand, significant differences in C exudation were observed between olive cultivars when root exudates were collected in the $CaSO_4$ solution (P = 0.0073). The highest values of C exudation were observed in the root exudates from 'Picual' (16.3 ± 0.8 mg C plant^{-1} h^{-1}), followed by those from 'Arbequina' (8.7 ± 1.4 mg C plant^{-1} h^{-1}) and 'Frantoio' (6.4 ± 0.3 mg C plant^{-1} h^{-1}).

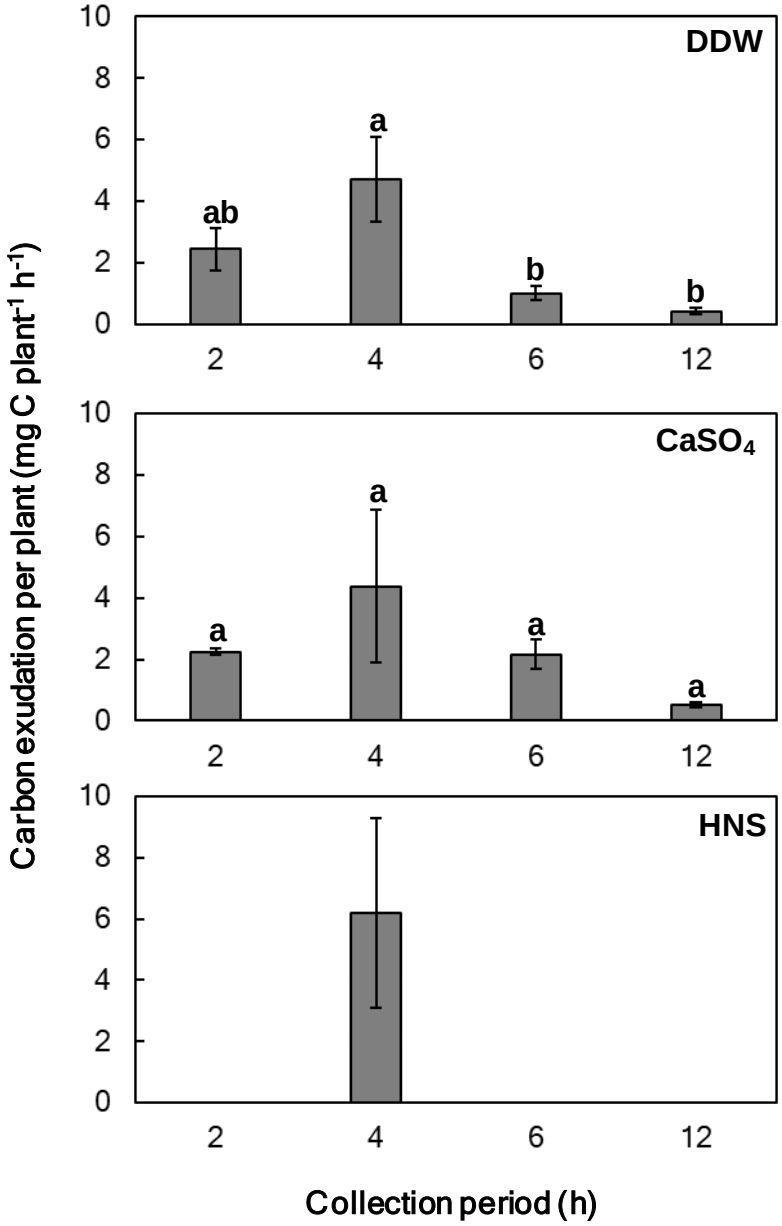

Figure 4.1 Carbon exudation per plant (mg C plant^{-1} h^{-1}) obtained from root exudates of olive plants of cv. Picual collected with deionized distilled water (DDW), 0.01 M $CaSO_4$ solution ($CaSO_4$) or Hoagland nutrient solution (HNS) at 2, 4, 6 or 12 h of collection period. For each collection media and period combination, columns represent the mean of two independent experiments with three replicated plants. Vertical bars are the standard error of the means. In each graph, columns with common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

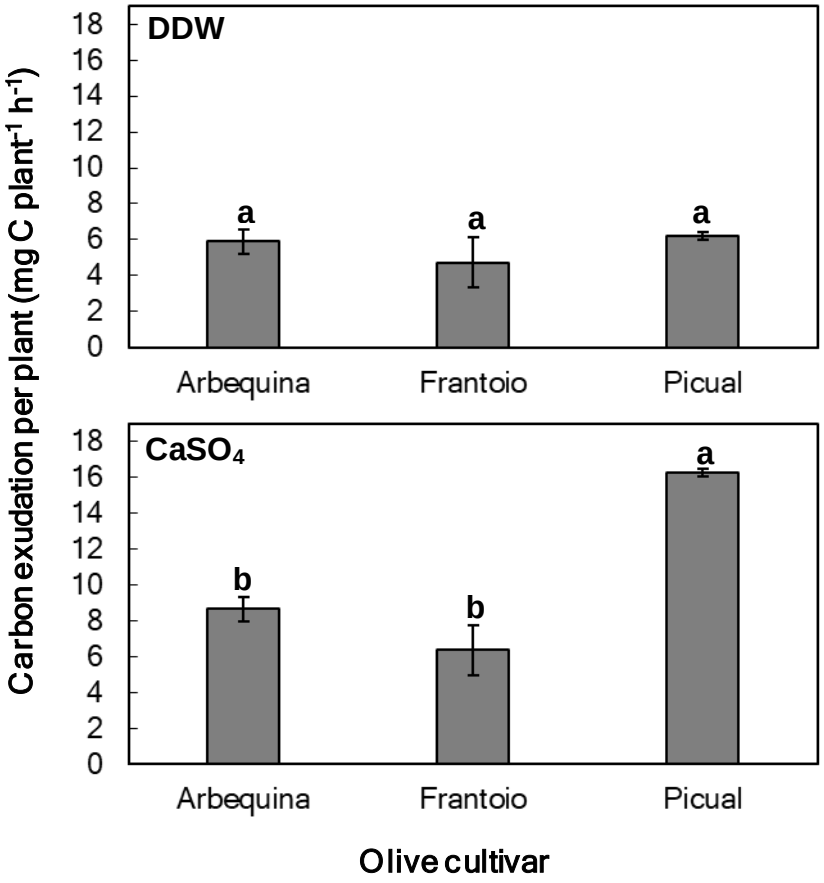

Figure 4.2 Carbon exudation per plant (mg C plant^{-1} h^{-1}) obtained from root exudates of olive plants of cvs. Arbequina, Frantoio or Picual collected with deionized distilled water (DDW) or 0.01 M $CaSO_4$ solution ($CaSO_4$) at 4 h of collection period. For each collection media and cultivar combination, columns represent the mean of two independent experiments with three replicated plants. Vertical bars are the standard error of the means. In each graph, columns with common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

Experiment III: Interaction between olive cultivars and biocontrol treatments.

The results on the effect of root exudates from olive plants of cvs. Arbequina, Frantoio and Picual, subjected to biocontrol treatments, collected by dipping the roots in a $CaSO_4$ solution during 4 h on C exudation per plant are shown in Fig. 4.3.

In general, for the three olive cultivars evaluated, root exudates from plants inoculated with *V. dahliae* always showed higher values of C exudation per plant compared to the rest of treatments; whereas the effect of the different biocontrol treatments on C exudation per plant varied depending on the olive cultivars

The root exudates from 'Arbequina' showed significant differences on C exudation between biocontrol treatments and controls ($P = 0.0186$). Root exudates from plants inoculated with *V. dahliae* showed the highest values of C exudation per plant (2.4 ± 0.4 mg C plant^{-1} h^{-1}), but no differences in the effect of root exudates on C exudation were observed between biocontrol treatments (ranging from 1.5 ± 0.2 to 1.0 ± 0.3 mg C plant^{-1} h^{-1} for *B. amyloliquefaciens* PAB-024 and potassium phosphite, respectively) or for non-treated and non-inoculated plants (1.4 ± 0.3 mg C plant^{-1} h^{-1}). Although there were no significant differences between treatments ($P = 0.4996$), a similar pattern to that described for 'Arbequina' was observed for the root exudates collected from 'Picual'. In this case, root exudates collected from inoculated plants with *V. dahliae* or from plants treated with *B. amyloliquefaciens* PAB-024 also showed the highest values of C exudation per plant (1.8 ± 0.2 and 2.1 ± 0.3 mg C plant^{-1} h^{-1}, respectively).

Finally, root exudates collected from 'Frantoio' showed a significant effect on C exudation depending on the biocontrol treatments and controls ($P = 0.0136$). Once again, root exudates from plants treated with *B. amyloliquefaciens* PAB-024 and those inoculated with *V. dahliae* showed the highest values of C exudation per plant (3.2 ± 0.7 and 2.7 ± 0.3 mg C plant^{-1} h^{-1}, respectively). Among the remaining biocontrol treatments, the C exudation per plant ranged between 2.1 ± 0.2 and 0.8 ± 0.3 mg C plant^{-1} h^{-1} for potassium- and copper phosphite, respectively.

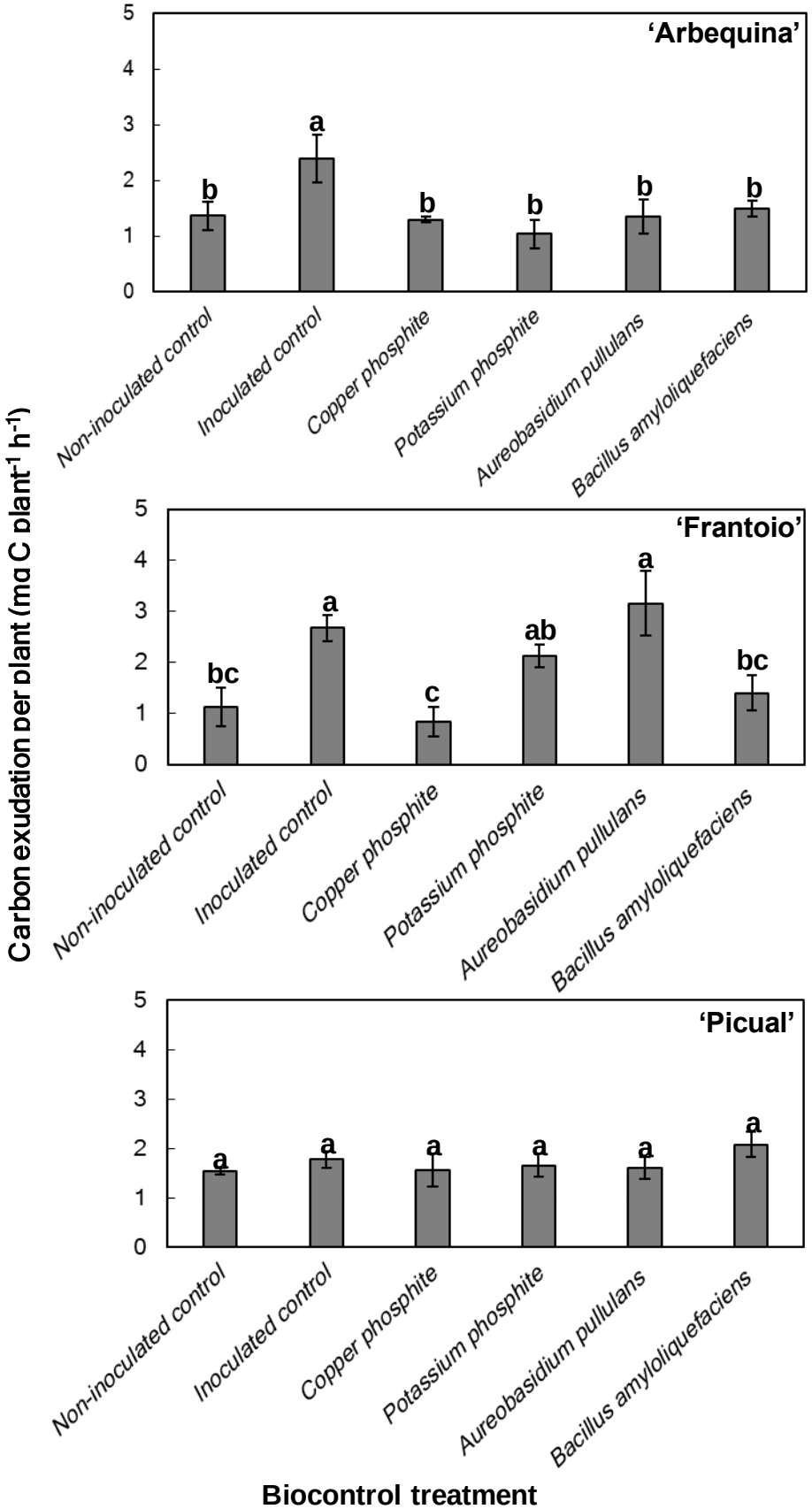

Figure 4.3. Carbon exudation per plant (mg C plant^{-1} h^{-1}) obtained from root exudates of olive plants of cvs. Arbequina, Frantoio or Picual collected with 0.01 M $CaSO_4$ solution at 4 h of collection period, at 6 weeks after initiate the biocontrol treatments. For each cultivar and biocontrol treatment combination, columns represent the mean of two independent experiments with three replicated plants. Vertical bars are

the standard error of the means. In each graph, columns with common letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$.

DISCUSSION

Root exudates are involved in a wide diversity of interactions within the rhizosphere such as the attraction of beneficial or parasitic microorganisms (Pantigoso *et al.* 2021; Vives-Peris *et al.* 2020), playing an important role in the olive infection process by the fungus *V. dahliae* (Pegg and Brady 2002). Despite the importance that the knowledge about how root exudates affect the infection of *V. dahliae* to olive could be useful to better understand its life cycle, no information on such topic is available in the literature. Therefore, the importance of technologies based on controlled environment and sterile conditions to collect root exudates providing the roots with physical particles, chemical gradients, and microbial populations that interact to mimic the soil (Pantigoso *et al.* 2021), as well as the need of implementing an *in vitro* method for the collection of root exudates in olive trees, reveal the relevance of this study to be able to advance in the clarification of the role of root exudates in the cycle of life of *V. dahlie* in olive plants in future studies. Indeed, although protocols to collect root exudates have been quite well developed in many plant species since the early 1900s, there are a broad range of limitations regarding root exudate collection that must been resolved to improve and adapt these protocols to other plants (Pantigoso *et al.* 2021). For all these reasons, the present study aimed to set-up a root exudate collection method from olive plants by *in vitro* technologies, besides to evaluated the effect of root exudates from different cultivars or, cultivars and biocontrol treatment combinations, on the viability of conidia and MS of *V. dahliae*. In addition, through this study, we have also determined the DOC content in the root exudates to give a first approach on wether its concentration could be related to the stimulation of infectious structures of *V. dahliae*. To our knowledge, this study represents the first report not only to shed light on how obtain root exudates from olive plants by means of a suitable *in vitro* method, but also to show that the methodology preserves the ability of root exudates to affect the germination of conidia and MS of *V. dahliae*.

Based on our longstanding experience working with the olive and *V. dahliae* pathosystem and considering that we focus this study on the development of a method to collect root exudates in vitro under controlled conditions, 6-months-old olive potted plants were used in this study. Young plants not only favour the management in the

laboratory to collect root exudates, but also, we need to use potted olive plants of no more than 6 months because it is the age limit to reproduce the symptoms of *V. dahliae* by artificial inoculation in small plastic pots (Varo *et al.* 2016; López-Moral *et al.* 2021b). The method has the limitation that we can not determine wether root exudates of mature olive trees could have a different effect on the pathogen structures than those from young trees. Nevertheless, the method is useful in determining the effect of root exudates from young olive trees, which are the most susceptible to infection when planted in a naturally infested soil.

The preliminary experiment evaluating different collection media and collection periods combination (*Experiment I*) revealed that 4 h was the optimum collection period in all cases. For all the collection media tested, collection periods over 4 h decreased the effect of root exudates on the different parameters evaluated, i.e. MS and conidial germination, quantity of DDC. It may be due to microbial degradation of these compounds that could occur faster with long periods of exposure to the collection media (Pantigoso *et al.* 2021). Indeed, some examples on such topic has been previously described by several authors. Likewise, studies analysing root exudates from herbaceous hosts for 2- to 7.5 h in both $CaSO_4$ and DDW showed that the longer the collection period the lower was the carbohydrate C and organic acid C, probably due to the decomposition or degradable C compounds by microorganism present on the rizosphere (Aulakh *et al.* 2001). On the other hand, Kirk *et al.* (1999) estimate a half-life of <5 h for citrate in exudate suspensions due to its fast degradation by microbes. Finally, a selective re-absorption of specific compounds exuded by the roots (e.g. ^{14}C labelled glucose, mannose and citric acid) after long collection periods that cause a loss and alteration of the exudate spectrum was also reported by Jones and Darrah (1992).

Concerning collection media, the HNS was the first collection media discarded since we observed that it masked the effect on the viability of conidia and MS of *V. dahliae* because it showed an intrinsic effect inducing conidia and MS germination, probably for its nutrient-based composition. In fact, it has been described that exudation obtained in hydroponic cultures with liquid media supplemented with nutrients can negatively affect the root exudates analysis (Vives-Peris *et al.* 2020). In this same way, HNS was also unsuccessful to determine the C exudation per plant because C exudation was only detected in root exudates for 4 h of collection period. This fact could be in concordance with those previously described by several authors who indicated that analysis of root exudates in HNS for organic C revealed difficulties for the

interpretation from the composition of the nutrient solution, with this media being not suitable for this kind of analysis probably due to the interference of choride or orher ions present (Nelson and Sommers, 1996; Aulakh *et al.*, 2021). On the other hand, *Experiments I* and *II* showed similar patterns on the effect of root exudates collected with DDW or with $CaSO_4$ solutions on the different variables evaluated. After checking these last results, we decided to select 0.01 M $CaSO_4$ solution as collection media for further analysis, according to the recommendations of Aulak *et al.* (2001). These authors indicated that using 0.01 M $CaSO_4$ solution avoids a possible turgor effect of root cells, which could occur when other collection media such as nutrient solutions or distilled water are used. In addition, these same authors also reported the collection media can greatly affect the composition of root exudates enhancing the release of sugars when they are collected in deionized water, while the exudation or organic acids is lower in deionized water than in $CaSO_4$. Notice that our experiment was not designed to analysys organic acids and other secondary metabolites, but we want to consider all these aspects so the protocol can also be useful in future studies. For all these reasons the use of 0.01 M $CaSO_4$ solution for 4 h may be regarded a preferential medium and timing combination for collecting root exudates from olive plants. We show that root exudates collected using this protocol are viable and may preserve to evaluate their effect on the viability of conidia and MS of *V. dahliae*, among other variables.

Regarding the influence of the olive genotype (cultivar resistance) on the effect of root exudates, it is interesting to note that the root exudates from 'Frantoio' showed a similar effect on the germination of conidia and MS than that observed for the control; while those from 'Arbequina' or 'Picual' favoured the germination of conidia and MS of *V. dahliae*. These results are in concordance with those described by Vives-Peris *et al.* (2020), who reported that genotype can modulate the effect of root exudates mediating the interaction between plants and microorganisms within the rhizosphere. Furthermore, root exudates from 'Frantoio' showed the lowest values of C exudation per plant in comparison with those from 'Arbequina' or 'Picual'. This result is also relevant in the context of this experiment since it is well known that the process of C management and recycling in the rhizosphere requires communication between root exudates (plant) and microorganisms (Pantigoso *et al.* 2021). Thus, all these results make sense because 'Frantoio' has been described as one of the most tolerant olive cultivars against *V. dahliae*, whereas 'Arbequina' and 'Picual' have been characterized

as susceptible or highly susceptible (López-Escudero *et al.*, 2004; Trapero *et al.* 2013). It suggests that the high resistance of 'Frantoio' against the disease could be related to the composition of their root exudates, which probably act as plant defence mechanism. Likewise, further research to characterize the primary and secondary metabolites of the root exudates excreted from olive cultivars with different levels of resistance against this disease are of major importance to determine which metabolites could be involved in the pathogenesis of *V. dahliae*.

When the interaction of cultivar resistance and biocontrol treatments on the effect of root exudates on *V. dahliae* was evaluated, the root exudates from 'Frantoio' showed again the lowest effect on the germination of conidia and MS of *V. dahliae* compared to those from 'Arbequina' and 'Picual' or in comparison with the control, independently of the biocontrol treatments. In addition, root exudates from non-treated plants of 'Arbequina' and 'Picual' induced conidial and MS germination of *V. dahliae* in comparison with the control, but this effect did not occur for the root exudates from 'Frantoio'. Once again, it suggests that root exudates from 'Frantoio' play an important role making difficult the infection process of the pathogen. In relation to the effect of biocontrol treatments, *A. pullulans* AP08 resulted in the most effective treatment since root exudates collected from treated plants of 'Arbequina' and 'Picual' were able to significantly reduce the conidia and MS germination of the pathogen. This result is in concordance with those described by López-Moral *et al.* (2021a,b), who suggested that *A. pullulans* AP08 is a promising biological control agent probably as a host resistance inducer against *V. dahliae* due to its high effectiveness reducing the disease progress *in planta*. In addition, it is worth mentioning that our results also suggest that *A. pullulans* AP08 could have an indirect effect on the control of the disease since they could also mitigate the pathogen infection, modulated by reducing the effectiveness of the root exudates from treated plants on the induction of MS and conidia germination of *V. dahliae*. Moreover, the remaining biocontrol treatments showed less evident effect modulating root exudates on the behaviour of *V. dahliae*, probably because they act against the pathogen by using other mechanism of action, i.e. antagonism, induction of host resistance, etc. In any case, further research is needed to unravel in how biocontrol treatments can modulate the root exudates from olive plants in reducing their effect on the induction of germination of infetious structures of *V. dahliae.*

Concerning the the potential effect of C sources in the root exudates modulating the infection of *V. dahliae*, interestingly, the C exudation of the root exudates from treated plants of 'Frantoio' showed significant differences between treatments, whereas important differences in the C exudation from root exudates of 'Arbequina' and 'Picual' were not observed between treatments. In this case, root exudates from plants of 'Frantoio' treated with *A. pullulans* AP08 showed the highest levels of C exudation per plant in the whole of the experiment, followed by potassium phosphite. Once again, it suggests that root exudates from 'Frantoio' may have specific metabolites that not only reduce the viability of propagules of *V. dahliae*, but also are able to mediate the plant-pathogen interactions making difficult the pathogen infection as well as attracting beneficial microorganisms within the surrounding rhizosphere soil. Thus, these results represent a first approach to know if root exudates could modulate the activity of the beneficial microorganisms of the rhizosphere through the exudation of C (Pantigoso *et al.* 2021). It makes sense if we consider that species belonging to *Verticillium* genus can metabolize a wide range of C sources such as arabinose, glucose, fructose, galactose, maltose, ribose, sucrose, etc. For instance, arabinose, galactose or ribose can delay growth of *V. dahliae*; D-manitol, D-glucitol or erythritol reduce or inhibit the production of MS by *V. dahliae*; while trehalose and glycerol incite the growth of *V. dahliae* (Pegg and Brady, 2002). Thus, there is no doubt that further research is needed to characterize the carbohydrates and organic acid by metabolomic techniques to better understanding how the root exudates could modulate the infection of *V. dahliae* to olive plants.

In summary, the method developed here using *in vitro* technologies may be really useful to obtain root exudates from young olive plants, h olive plants, which are used to establish new orchards in naturally infested soils (frequent scenarios in southern Spain); and ii) to generate knowledge as how the genotype and/or the biological treatments can interact in reducing the effectiveness of root exudates stimulating the germination of MS of *V. dahliae* in the soil. All these knowledges could be useful in the furture preventing infections or mitigating the disease progress. Nevertheless, further studies to dilucidated the effect of root exudates of different olive cultivars as well as of different ages of olive plants on the pathogenicity of *V. dahliae* are also need to better determining the role of root exudates on pathogen infection in the different natural olive scenarios that we can find across the Mediterranean basin. In addition, the interaction

between genotype or age of olive pants and bicontrol treatments on the effect of root exudates in the infection of *V. dahliae* should be also of major interest to improve the integrated management strategies of the disease.

CONCLUSIONS

A 0.01 M $CaSO_4$ solution and a root inmersion time of 4 h may be regarded a preferential medium and timing combination to collect root exudates from young olive plants. The collected root exudates can be preserved to evaluate their effect on the viability of conidia and MS of *V. dahliae*, among other variables such as DOC. We also demonstrated that root exudates from olive plants are able to induce conidial and MS germination of *V. dahliae*. Furthermore, the genotype plays a key role in the capacity of root exudates to induce germination of the propagules of *V. dahliae*, since root-exudates from 'Frantoio' did not show any effect on the germination of conidia and MS; while those from 'Arbequina' or 'Picual' induced conidia and MS germination of *V. dahliae*. *Aureobasium pullulans* AP08 resulted in the most effective treatment since root exudates collected from treated plants of 'Arbequina' and 'Picual' were able to significantly reduce the conidia and MS germination of the pathogen. Finally, the C content in the root exudates from treated plants of 'Frantoio' showed significant differences between biocontrol treatments, whereas no important differences were observed detween treatments on the C content in root exudates of 'Arbequina' and 'Picual'. This study generates important knowledge on the effect of root exudates on the biology of *V. dahliae* to olive infection, together with the set-up of the root exudate collection method from olive plants.

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Chapter 5

Resistance induction in olive tree (*Olea europaea*) against Verticillium wilt by two beneficial microorganisms and a copper phosphite fertilizer

ABSTRACT

Enhancement of the plant's natural defenses by beneficial microorganisms, i.e., endophytic bacteria and fungi or fertilizers such as copper phosphonates, could result in a potential alternative strategy against Verticillium Wilt of Olive tree (*Olea europaea*; VWO). In this study, two beneficial microorganisms (the fungus *Aureobasidium pullulans* AP08 and the bacterium *Bacillus amyloliquefaciens* PAB-024) and a phosphonate salt copper phosphite (CuPh) were evaluated for their effectiveness as host resistance inducers against *Verticillium dahliae* in olive. To this end, 6-month-old healthy olive plants of the susceptible cultivar Picual were treated by foliar or root applications by spraying 15 ml per plant or by irrigation with 350 ml per plant of the dilutions of each product (CuPh: 3 or 10 ml/l^{-1}, respectively; PAB-024: 10^8 UFC ml^{-1}; AP08: 10^6 UFC ml^{-1}). Treatments were conducted weekly from two weeks before inoculation to 10 days after inoculation. A cornmeal-water-sand mixture (1:2:9; w:v:w) colonized by *V. dahliae* was used for plant inoculation. Additionally, treated and non-inoculated, non-treated and inoculated, and non-treated and non-inoculated plants were included for comparative purposes. Disease severity progress and shoot fresh weight were assessed. Parameters involved in plant resistance were monitored through determination and quantification of reactive oxygen species (ROS) response (H_2O_2), and evaluation of hormones by gene expression analysis. *A. pullulans* and CuPh were the most effective in disease reduction *in planta* by foliar or root application, respectively. Plants treated with CuPh showed significantly higher shoot fresh weight compared to the other treatments. ROS was significantly enhanced in plants treated with *B. amyloliquefaciens* PAB-024 compared to the rest of treatments and control. Concerning hormones evaluation, high levels of salicylic acid were detected on leaves from all treatment combinations, but without significant enhancements compared to the non-treated control. Regarding the gene expression related to salicylic acid, only the *WRKY5* gene has shown a strong enhancement in the treatment with *B. amyloliquefaciens*. On the other hand, a strong accumulation of jasmonic acid and jasmonic acid-isoleucine in plants treated with *A. pullulans* was observed in all the tissues analyzed as well as in roots of plants treated with *B. amyloliquefaciens* and CuPh.

Keywords: biological control agents, biostimulation, induced resistance, priming, *Verticillium dahliae*

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INTRODUCTION

Verticillium wilt of olive (*Olea europaea* subsp. *europaea* L.) (VWO), caused by the hemibiotrophic soil-borne fungus *Verticillium dahliae* Kleb., is considered the most important disease of this crop worldwide due to the high levels of tree mortality and fruit yield reduction that it causes (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al*., 2012; Montes-Osuna and Mercado-Blanco, 2020). This fact is not only because of the difficulty to control this pathogen, which can survive for up to 14 years in the soil, forming microsclerotia (MS) (Pegg and Brady, 2002) or as endophyte in the xylem vessels of the infected plants (Alström, 2001), but also because of the lack of effective chemical treatments against the disease (Trapero *et al*., 2015; Mulero-Aparicio *et al*., 2020). Thus, based on the advances about the control of VWO conducted over the last two decades, there is no doubt that an integrated management strategy is needed to reduce both pathogen dispersal and disease incidence in olive orchards (López-Escudero and Trapero, 2017; Montes-Osuna and Mercado-Blanco, 2020). Within this strategy, the combination of several eco-friendly control methods such as genetic resistance, cultural practices and biological control in both pre- and postplanting must be considered (López-Escudero and Mercado-Blanco, 2011; Trapero *et al*., 2015; Montes-Osuna and Mercado-Blanco, 2020; Ostos *et al*., 2020; López-Moral *et al*., 2021b).

With respect to biological control, a wide diversity of compounds including organic amendments; essential oils; seaweeds, plant or microorganism extracts; plant biostimulants; fertilizers; mycorrhizal fungi; and antagonist microorganisms, i.e., endophytic bacteria and fungi, have been evaluated worldwide over the last two decades as a potential eco-friendly control measure against VWO (Montes-Osuna and Mercado-Blanco, 2020; López-Moral *et al*., 2021b; Poveda and Baptista, 2021). Most of these studies have been conducted under controlled experimental conditions both *in vitro* and *in planta*. Nevertheless, the efficacy of most of these compounds has not yet been demonstrated against VWO under natural conditions in olive groves. To our knowledge, only a few studies reveal high or moderate effectiveness of some of these compounds against VWO in the field, e.g. the bacterium *Paenibacillus alvei* strain K165 (Markakis *et al*., 2016), the non-pathogenic fungus *Fusarium oxysporum* strain FO12 and a commercial mix of *Trichoderma asperellum* and *T. gamsii* (Mulero-Aparicio *et al*., 2020), *T. harzianum* (Arici and Demirtas, 2019), and several mycorrhizal fungi

including diverse *Glomus* spp. (Porras-Soriano *et al.*, 2006) and *Rhizophagus irregularis* (Arici and Demirtas, 2019). In addition, the results obtained from studies on biocontrol of VWO suggest that all these evaluated compounds could be acting against the pathogen by means of several modes of action including soil suppression, endophytism, antagonism, and host resistance induction (HRI) (Tjamos *et al.*, 2005, 2004; Ruano-Rosa *et al.*, 2017; Varo *et al.*, 2018; Mulero-Aparicio *et al.*, 2019a,b; Azabou *et al.*, 2020; Montes-Osuna and Mercado-Blanco, 2020; López-Moral *et al.*, 2021b; Poveda and Baptista, 2021).

Regarding the efficacy of the phosphites in crop protection, experimental evidence from decades has shown positive effects of phosphites on plant metabolism, which are more evident when applied to the roots in hydroponic systems or to the leaves by spraying. Their efficacy has been demonstrated not only as a fertilizer by increasing plant growth, production, and productivity, but also as a pesticide against various species of pathogenic bacteria, fungi and oomycetes (Guest and Grant, 1991; Cooke and Little, 2002; Gómez-Merino and Trejo-Téllez, 2015; Abbasi and Lazarovits, 2006a,b; Romero *et al.*, 2019). Moreover, phosphites have emerged also as potential HRI, as it has demonstrated their effectiveness against different biotic and abiotic stress factors improving crop protection, yield, and quality (Navarro-Cerrillo *et al.*, 2004; Gómez-Merino and Trejo-Téllez, 2015; Barrós-Rodríguez *et al.*, 2020).Among the diversity of modes of action that all these compounds may perform preventing plant pathogen infection, their capability to induce the natural plant defences by triggering a systemic resistance through jasmonic acid (JA)/ethylene (ET)- and/or salicylic acid (SA)-dependent signaling pathways has been currently considered as one of the most promising eco-friendly strategies against plant diseases (Llorens *et al.*, 2017a,b; Poveda and Baptista, 2021). In this way, HRI consists in stimulating the defensive plant response by means of appropriate natural or chemical compounds to plants become tolerant against both biotic and abiotic stresses (Conrath, 2009; Llorens *et al.*, 2017b; Poveda and Baptista, 2021). The stimuli not only induce the plant innate resistance for overcoming the attack of the pathogen, but also can persist in the plant with long-term effects preventing new infections (Bektas and Eulgem, 2015; Llorens *et al.*, 2017b). For these reasons, besides the low or null toxicity that the host resistance inducers have for the environment (González-Hernández *et al.*, 2018), HRI deserves to be explored as a potential biocontrol tool against VWO. Indeed, the effect of several compounds such as

SA (Gharbi *et al.*, 2016) or seaweed extracts (Salah *et al.*, 2018) which are able to enhance the olive plant resistance against *V. dahliae* has been already demonstrated *in planta* with promising results. Moreover, the ability of the endophytic bacteria and fungi acting as HRIs against plant infections by the major olive pathogens has been already demonstrated in several plant models such as *Arabidopsis thaliana*, *Brassica napus* or *Solanum lycopersicum* (Poveda *et al.*, 2019; Montes-Osuna *et al.*, 2020; Poveda and Baptista, 2021). In the particular case of VWO, Tjamos *et al.* (2005) demonstrated that the bacterium *Paenibacillus alvei* K165 mediates the HRI genes related to the components of the isochorismate pathway and a functional nonexpressing NPR1 protein since they were expressed in *Arabidopsis* mutants and transgenic plants impaired in defense signaling pathways. In addition, Shilirò *et al.* (2012) showed that many genes related to plant defence and response to different stresses were expressed by suppression subtractive hybridization in olive plants after root colonization by *Pseudomonas fluorescens* PICF7. In this same study, the induction of lipoxygenase, phenylpropanoid, terpenoids, and plant hormones biosynthesis transcripts in PICF7-colonized olive roots was confirmed by quantitative real-time PCR (qRT-PCR) analyses (Shilirò *et al.*, 2012). Nevertheless, a much in-depth knowledge of the mechanisms related to HRI to VWO based on molecular and physiological analyses is needed to better determine the effectiveness of natural compounds as resistance inducers of olive plants against *V. dahliae*.

In spite of the literature cited above, the regulation of SA-, JA-, or ET-related genes enhancing innate olive resistance to *V. dahliae* through treatments by beneficial microorganisms and plant biostimulants is still poorly understood. Within this context, previous studies evaluating 32 potential HRIs against VWO showed that two beneficial microorganisms (the fungus *Aureobasidium pullulans* AP08; and the bacterium *Bacillus amyloliquefaciens* PAB-24) and a phosphonate salt copper phosphite (CuPh) were highly effective in reducing the disease progress *in planta* (López-Moral *et al.*, 2021b). It is worth mentioning that *A. pullulans* AP08 did not show a direct effect on the pathogen since it did not inhibit mycelial growth and MS germination of *V. dahliae*; while it resulted in the most effective treatment in reducing the disease progress (López-Moral *et al.*, 2021b). This latter result suggested that *A. pullulans* AP08 could act against the pathogen through different modes of action such as antagonism or HRI. However, the methodology used in this previous study was not enough to determine

their mode of action. Therefore, the main goal of this study was to elucidate the effectiveness of *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024 and CuPh enhancing the plant defence system in olive plants ('Picual') against *V. dahliae*. To this end, inoculated- or non-inoculated, and treated- and non-treated plants combinations per each compound were monitored for disease severity and plant growth. In parallel, biochemical parameters involved in plant resistance i.e., reactive oxygen species (ROS) response (H_2O_2) and plant hormones were validated by gene expression qRT-PCR analysis.

MATERIALS AND METHODS

Plant material and growth conditions

Healthy six-month-old olive potted plants of cv. Picual (highly susceptible to *V. dahliae*; López-Escudero *et al.*, 2004) growing in peat moss into plastic pots (0.5 l) from a commercial nursery were used in this study. Prior to start the experiments, plants were maintained in a controlled-growth chamber at 22 ± 2°C in a 14:10-h (light:dark) photoperiod of white fluorescent light (10.000 lux) and 60% relative humidity (RH) for one month, and irrigated three times per week with 300 ml of water per plant.

Plant treatments, inoculation, and experimental design

After one month under the preconditioning conditions described above, plants were treated with the BCAs *Aureobasidium pullulans* AP08, and *Bacillus amyloliquefaciens* (PAB-024), and with the commercial phosphonate salt copper phosphite (CuPh; Phoscuprico®, AGRI nova Science, Almería, Spain). The two microorganisms belong to the collection of the Department of Agronomy of the University of Córdoba (Spain). The fungus *A. pullulans* AP08 is maintained as single-spore culture on potato dextrose agar (PDA; Difco Laboratories®, MD, USA) slants with sterile paraffin oil (Panreac Química SA, Barcelona, Spain) at 4°C in the dark; and the bacterium *B. amyloliquefaciens* PAB-024 is cryopreserved with 30% glycerol at -80°C. An aqueous inoculum was prepared for the two microorganisms. For AP08, fresh colonies were obtained from the stock growing on PDA at 23 ± 2ºC for 7 days in a 12:12-h (light:dark) photoperiod. Then, the surface of the colonized Petri dishes was covered with sterile distilled water, scraped from the medium with a sterile glass rod and filtered through a sterile gauze. For PAB-024, 20-µl of the stock was introduced in

a 250-ml Erlenmeyer flask with 100 ml of nutrient broth (NB; Difco Laboratories®, MD, USA), and incubated at 23 ± 2ºC for 2 days in a 12:12-h (light:dark) photoperiod in an orbital shaker. Subsequently, 1-ml of the microorganisms solutions was introduced in a 2-l Erlenmeyer flask containing 1 l of sterile potato dextrose broth (PDB; Difco Laboratories®) or 1 l of sterile NB for AP08 and PAB-024, respectively, and incubated at 23 ± 2ºC for 3 days in a 12:12-h (light:dark) photoperiod in an orbital shaker. Finally, *A. pullulans* AP08 and *B. amyloliquefaciens* PAB-024 suspensions were adjusted at 10^6 conidia ml^{-1} and 10^8 CFU ml^{-1}, respectively, being used as the final inoculum solutions to conduct the plant treatments. Regarding the commercial product, CuPh was applied at the maximum irrigation doses recommended by the manufacturer (3- and 10 ml l of $water^{-1}$ for foliar and root applications, respectively; *see below*) (López-Moral *et al.*, 2021b).

Plants were treated by spraying 15 ml (foliar application) or by irrigation, adding 300 ml (root application) per plant of the respective solution or suspension of each compound described above or water. Treatments were conducted as follows: *i*) foliar applications, 14, 13, and 11 weeks before plant sampling (*see below*); *ii*) irrigation applications, 13 and 11 weeks before plant sampling (*see below*). There were two independent plant lots per treatment and type of application (foliar or root) combination: *i*) non-inoculated plants; and ii) plants that were inoculated with *V. dahliae* 8 weeks before sampling. Additionally, lots of non-treated and non-inoculated, and non-treated and inoculated plants were also included as negative and positive controls, respectively.

The *V. dahliae* inoculum was prepared using 2-l Erlenmeyer flasks filled with 1 kg of a cornmeal-sand mixture (750 g of sand, 83 g of cornmeal and 167 ml of distilled water). Then, they were double-sterilized on two consecutive days at 120°C for 50 min (1st day) and 120°C for 20 min (2nd day), with the flasks being manually shaken between each sterilization step. Subsequently, 50 mycelial plugs (7.5 mm in diameter) of *V. dahliae* isolate V180 growing on PDA at 23 ± 2ºC for 14 days in the dark were introduced into each flask, and the flasks were incubated at 24°C in darkness for 4 weeks. To favour the homogeneous colonization of the cornmeal-sand mixture by the pathogen, flasks were manually shaken just after inoculation as well as once a week during the incubation period. After four weeks of incubation, the inoculum density of the colonized cornmeal sand was estimated by means of the serial dilution method on

PDA, and expressed as colony-forming units (CFUs). Then, a mix of the colonized cornmeal-sand mixture *V. dahliae* and sterile peat most was adjusted to 10^7 CFU g^{-1} of the final substrate (López-Moral *et al.*, 2021b), which was used to conduct plant inoculation.

For inoculation, plants were taken out of the plastic pots, the original peat moss was carefully removed from the roots, and plants were transplanted in 0.7 l plastic pots filled with the adjusted final substrate described above. Just after inoculation, plants were immediately irrigated with distilled water and incubated in a growth chamber at 20 ± 2ºC at 100 RH in the dark for 4 days. Subsequently, light and humidity parameters were progressively modified over 1 week until reaching 23 °C, a 12-h photoperiod of fluorescent light [10,000 lux], and 70% RH, which were maintained until the end of the experiment. Moreover, after the last root treatment application, plants were irrigated three times per week with 300 ml of water per plant until the end of the experiment.

A factorial randomized complete block design (three blocks) was used with treatments (n = 4; Water treated control, AP08, PAB-024 or CuPh), type of application (n = 2; spray or irrigation), and inoculation with *V. dahliae* (n = 2; inoculated or non-inoculated plants) as independent variables and five replicated olive plants per treatment and block (4×2×2×3×5 = 240 plants in total). The experiment was maintained for 12 weeks after inoculation.

Disease severity and plant growth evaluation

Disease severity (DS) was periodically assessed from symptoms onset to 12 weeks after inoculation when the disease progress stopped. A 0-4 rating scale of 17 values was used to estimate the percentage of affected tissue of the aerial part of the plants by means of five main categories of severity: 0 = no symptoms; 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100% (dead plant) of affected tissue, with three intermediate values (0.25, 0.50 and 0.75) between main categories (Varo *et al.*, 2018). Therefore, the scale values (X) were related to the percentage of affected plant (Y) according to the equation: $Y = 25X$. DS data were used to calculate the relative area under the disease progress curve (RAUDPC) at the end of the experiment by the trapezoidal integration method (Campbell and Madden, 1990). Additionally, disease incidence (DI) and

mortality were also estimated as the percentage of symptomatic or dead plants, respectively, at the end of the experiment.

In parallel, the fresh weight of leaves and stems of each plant was measured at the end of the experiment to estimate the effect of biological treatment on the plant growth development. For each type of tissue (leaf or stem), data of the five plants of each block were averaged.

Assessment of the biochemical parameters involved in plant resistance

Sampling plant material

For both foliar and root lots of treated plants of each compound tested, one replicated plant per block and treatment combination (treated and non-inoculated; treated and inoculated) was randomly selected at the end of the experiment (3 months after inoculation). One random plant per block of the respective controls (non-treated and inoculated; non-treated and inoculated) was also sampled. A total of three plants per treatment or control combination were used. In each plant, leaves were carefully separated from the shoots and stem, and sampled as separated tissues (leaves and stems) for further analysis. Roots of the same plants were also sampled. The plant material was subjected to different protocols as described below depending on the biochemical parameters evaluated in this study.

Determination and quantification of hydrogen peroxide (H_2O_2)

For each compound, and treatment and control combinations, samples of 10 leaves and 10 stem segments (≈ 5 cm in length) were collected at 11 weeks after the last spray or irrigation application. Tissues colleted from the two sampled plants were immersed immediately in independent recipients in 1 mg ml^{-1} of 3′,3-diaminobenzidine (DAB) at pH <3 for 24 h in darkness. Subsequently, they were discolored in 96% ethanol and rehydrated in distilled water for 30 min. To determine the reactive oxygen species (ROS), the formation of brown precipitates were quantified in micrographic as described by Llorens *et al.* (2017), and expressed as the number of dark-brown DAB pixels with respect to the total pixels corresponding to plant material using the GIMP program (version 2.6.12).

Evaluation of hormones related to plant defence

For each compound, and treatment and control combinations, a third lot of samples of 10 leaves and 10 shoot-stem segments (≈ 5 cm in length) was collected at 11 weeks after the last spray or irrigation application. Additionally, the root system of each plant was also sampled in its integrity at the same time. All the fresh plant material was immediately frozen in liquid N, maintained at -20°C for at least 24 h in independent plastic bags, and then lyophilized at -43°C, ≈0.344 mBar (Telstar® Cryodos, Azbil Group, Terrassa, Spain) for 10 to 14 days. Subsequently, dry tissue (0.05 g) of each sample was homogenized in 1.5 ml of ultrapure water, and 100µL of internal standard at 100 ng ml^{-1} {deuterated SA ($[_2H_4]$), dihydrojasmonic acid (dhJA) and propylparaben} were added prior to extraction in order to quantify the level of hormones JA, JA-isoleucine (JAIle), 12-oxo-phyto dienoic acid [OPDA], and SA). The samples were centrifuged at 5000 rpm for 45 min. at 4°C. The supernatant was partitioned against diethylether, dried in a speed vacuum, and then resuspended in 90:10 H_2O:MeOH. After extraction, a 20 µl aliquot of the homogenized suspension of each sample was injected directly into the ultrahigh-performance liquid chromatography (UPLC) system; and analysed by means of a Waters Alliance 2690 HPLC system (Milford, MA, USA) with a nucleosil ODS reversed-phase column (100 mm × 2mm, i.d. 5 µm; Scharlab, Barcelona, Spain; http://www.scharlab.com). The chromatographic system was interfaced to a Quatro LC (quadrupole–hexapole–quadrupole) mass spectrometer (Micromass; http://www.micromass.co.uk). Finally, the quantitative data from calibration standards and plant samples was conducted using the MASSLYNX NT software version 4.1 (Micromass) (Llorens *et al.* 2017a).

We can consider that the absence of hormones for certain treatment combinations is due to the fact that they are at a concentration below the UPLC limit of quantification. Despite the hormones being present, and we can observe a signal in the chromatogram, the method of peak integration and calculation based on the peak of internal standard and curve of calibration makes it not possible to obtain realistic values below 1ppb.

Data Validation and Expression Profile Analysis by Quantitative Real-Time PCR

Leaf, stem and root tissue samples were obtained as described above, immediately frozen in liquid nitrogen and kept at -80ºC until RNA extraction. Total

RNA was extracted using E.Z.N.A Plant RNA kit (OMEGA biotek, http://www.omegabiotek.com), according to the manufacturer's instructions. 1 µg of total RNA after DNase treatment (Promega, http://www.promega.com) was reverse transcribed into cDNA using an oligodT primer and primescript RT enzyme mix 1 (Primescript RT reagent kit, TaKaRa). Finally, the mRNA purity and quality were checked by spectrophotometry using a NanoDrop 2000 Spectrophotometer (Thermo Scientific (Thermo Scientific, Waltham, MA, USA).

Quantitative Real-Time PCR (qRT-PCR) experiments were conducted to validate the expression of hormones related to plant defence identified in the previous biochemical analysis. To determine whether plant treated by CuPh or treated and colonized by AP08 or PAB-024 strains triggers defense responses in olive tissues, several genes identified as induced and putatively involved in the synthesis of acetone cyanohydrin lyase (*ACL*) and lipoxygenase (*LOX*), as well as the transcription factors involved in systemic defensive responses *WRKY5*, i.e. SA signal transduction and basic helix-loop-helix (*bHLH*) or factors responsive to jasmonic acid, were selected for validation by qRT-PCR analysis (Schilirò *et al.*, 2012). The specific primer pairs used to amplify each gene are shown in Table 5.1. The range of concentrations at which target cDNA and cycle thresholds (Ct) values were determined by linear correlation; and specific qRT-PCR assays were conducted using cDNAs synthesized from 10-fold serially diluted (1 mg, 100 ng, 10 ng, 1 ng, 100 pg) RNA simples to estimate PCR efficiencies. For each selected gene, relative standard curves were constructed using reverse transcribed cDNA from serial dilutions of total RNA of a ''Reference Sample'' of the same pooled total RNA used for the subtraction experiment. Ct values and the logarithm of cDNA concentrations were linearly correlated for each of the examined genes. For all transcripts, reactions were repeated twice (independent qRT-PCR experiments) included three biological replicates per transcript and per plate in all cases.

Quantitative real-time PCR was performed with Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher) in a StepOneTM Real-Time PCR System (Thermo Fisher). The following thermal cycling profile was used for all PCR reactions: 94ºC for 5 min, 50 cycles of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 40 s. Linear equations, determination coefficients (R^2) and reaction efficiencies were calculated in all cases. Melting curves of qRT-PCR products were assessed from 55ºC to 95ºC to confirm the amplification of single PCR products. The *Olea europaea b-actin* gene (*olive act1*) was

used as housekeeping gene and amplified in the same conditions in order to normalize the data resulting from qRT-PCR and to calculate the relative expression (RE) levels (Schilirò *et al.*, 2012).

Table 5.1. Transcripts used in this study.

Clone ID[a]	Putative Gene	Process	Primer pair[c]	Amplicon length
ARBRI-C140	Acetone cyanohydrin Lyase (*ACL*)	Salicylic acid-binding protein 2	Fw: GAAAGAGATGGAAGCGGAAA; Fv: ACACAGGGAAATGCATCAAA	246 b
ARBRI-2_T7_F05	Lipoxygenase (*LOX*)	Jasmonic acid biosynthesis	Fw: TATCTCCCGGTTCGTCCTAC Rv: CCTCAACTTCGTCCCAAATC	264 bp
ARBRI-C83	*WRKY5*	SA signal transduction	Fw: GCATGGTGCAAGAAGTAGGA Rv: CAGCAACAAACGCTACACCT	213 bp
ARBRI-C74	*bHLH*	JA-responsive transcription	Fw: TTACAGCGCAGAATCCCTAA Rv: TGTGCGGGGGAATATAGAAT	213 bp
AF545569[b]	*Olea europaea beta-actin* (*olive act1*)	Citoskeletal integrity	Fw: GCTTGCTTATGTTGCTCTCGAC Rv: TGATTTCCTTGCTCATACGGTC	308 bp

[a]All transcripts were selected and analysed according to Schilirò *et al.* (2012).
[b]*olive act-1* gene used as reference to normalize relative expression (Gene Bank Accession Number).
[c]Fw: Forward; Rv: Reverse.

Data analyses

The experiment was conducted twice, and data from the two repetitions of the experiment were combined after checking for homogeneity of the experimental error variances by the *F* test ($P \geq 0.05$). Then, data were tested for normality, homogeneity of variances, and residual patterns, and they were logarithmically transformed when necessary. To evaluate the effect of treatments on disease progression and plant growth in inoculated plants, factorial ANOVAs were conducted with RAUDPC (%) or DS (%) as dependent variables, and treatments [n = 5; 3 products + 2 controls (positive and negative)], type of application (n = 2; foliar and irrigation) and their interaction as independent variables. Since interaction was significant in both cases ($P < 0.05$), two separate one-way ANOVAs were conducted for each type of application (foliar or irrigation). Data on the final DI (% of affected plants) and mortality (% of dead plants) were analysed by multiple comparisons for proportions tests at $P = 0.05$ (Zar, 2010). Regarding the fresh weight (g) data from leaf and stem tissues, two separate factorial ANOVAs, which included inoculated or non-inoculated plants and treatments, for each type of application were conducted since the interactions between the type of

application and treatments were significant ($P < 0.05$). To evaluate the effect of compounds on the expression of parameters related to HRI (ROS, hormone levels and gene expression), a factorial ANOVA was conducted for each olive tissue (leaf, stem or root) combination, with ROS, SA, JA, JA-Ile, OPDA, *LOX*, *ACL*, *WRKY5*, or *bHLH* as dependant variables and the compound (AP08, PAB-024, or CuPh), the type of application (foliar or root) and inoculation (inoculated or non-inoculated plants), and their respective interactions as independent variables. When the interactions were significant, they were used to compare the treatment means. Mean values were compared using Fisher's protected LSD test for experiments with independent variables with less than six levels or Tukey's HSD tests for experiments with independent variables with six or more levels, both at $P = 0.05$ (Steel and Torrie 1985). Data from this study were analysed using Statistix 10.0 software (Analytical Software, Tallahassee, USA).

RESULTS

Effect of treatments on Verticillium wilt development in olive plants

Symptoms of verticillium wilt disease were only observed in inoculated plants, so non-inoculated plants were excluded from this analysis. In inoculated plants, disease symptoms developed from 4 weeks to 12 weeks after inoculation, when disease progress was stopped. The results of the effect of the foliar or root applications on the disease parameters are summarized in Table 2. When the treatments were applied by spraying, *A. pullulans* AP08 was the most effective treatment showing significantly lower values, both for DS (42.7 ± 2.9%) and for RAUDPC (36.4 ± 2.7%), compared to the rest of the treatments. However, *B. amyloliquefaciens* PAB-024 and CuPh showed a similar effect between them for DS and RAUDPC, but only for RAUDPC they showed a significant effect compared with the untreated and inoculated control. Concerning the DI, plants treated with *B. amyloliquefaciens* PAB-024 showed 100% of DI, whereas the mortality (46.8%) was significantly lower in comparison with the untreated and inoculated control (100%). *Aureobasidium pullulans* AP08 and CuPh treatments showed a similar reduction on DI (80.4 and 87.9%, respectively), but *A. pullulans* AP08 treated plants showed the lowest mortality (14.0%) (Table 5.2).

On the other hand, when treatments were applied by irrigation, there was a significant reduction in RAUDPC and DS for the three products compared with the

untreated and inoculated control, but no differences were observed between them. Although a similar effect was observed among the three products, CuPh showed the lowest values of RAUDPC (20.6 ± 5.2%) and DS (22.7 ± 2.9%). Regarding the DI, CuPh was also the most effective treatment (DI = 51.5%), whereas *A. pullulans* AP08 (DI = 77.3%) and *B. amyloliquefaciens* PAB-024 (DI = 80.0%) showed a lower effect between them. Despite the fact that the DI for the plants treated with two microorganisms was high, the mortality was significantly lower for all the tested products compared to the untreated and inoculated control, with a range between 17.8 and 2.8 % for *B. amyloliquefaciens* PAB-024 and CuPh, respectively (Table 5.2).

Table 5.2. Disease-related parameters for olive plants grown in artificially infested substrate with the defoliating *Verticillium dahliae* isolate V180 and treated with several products by spraying (foliar application) or irrigation (root application)[a].

[a]Olive plants were treated several times before and after inoculation and disease parameters were assessed weekly from 4 to 12 weeks after inoculation with *V. dahliae*. Water treatments in inoculated or in non-inoculated plants were used as positive or negative controls, respectively.

Foliar application				
Treatment	**Incidence (%)[b]**	**Mortality (%)[b]**	**Disease severity (%)[c]**	**RAUDPC (%)[d]**
Aureobasidium pullulans	80.4 b	14.0 c	42.7 ± 2.91 b	36.4 ± 2.7 c
Bacillus amyloliquefaciens	100.0 a	46.8 b	83.5 ± 6.9 a	65.9 ± 6.4 b
Copper phosphite	87.9 b	41.1 b	81.3 ± 9.8 a	64.6 ± 8.5 b
Water (inoculated)	100.0 a	100.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Water (non-inoculated)	0.0 c	0.0 d	0.0 ± 0.0 c	0.0 ± 0.0 d
Root application				
Treatment	**Incidence (%)[b]**	**Mortality (%)[b]**	**Disease severity (%)[c]**	**RAUDPC (%)[d]**
Aureobasidium pullulans	77.3 b	4.6 c	32.1 ± 1.6 b	25.6 ± 2.6 b
Bacillus amyloliquefaciens	80.0 b	17.8 b	41.5 ± 3.3 b	27.3 ± 6.6 b
Copper phosphite	51.5 c	2.8 c	22.7 ± 2.9 b	20.6 ± 5.2 b
Water (inoculated)	100.0 a	100.0 a	100.0 ± 0.0 a	100.0 ± 0.0 a
Water (non-inoculated)	0.0 d	0.0 c	0.0 ± 0.0 c	0.0 ± 0.00 c

[b]Percentage of symptomatic plants (Incidence) or dead plants (Mortality) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180 ($n = 30$). For each parameter and type of application, means in a column followed by the same letter do not differ significantly according to Zar's multiple comparisons for proportions test at $P = 0.05$ (Zar, 2010).

[c]Final disease severity ± Standard Error of the means (SE) 12 weeks after planting in the infested substrate with *V. dahliae* isolate V180. Disease severity was assessed using a 0-4 rating scale of 17 values, in which the scale values (X) were related to the percentage of affected plant (Y) according to the equation: $Y = 25X$. For each type of application, means in a column followed by the same letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

[d]Relative area under the disease progress curve (RAUDPC) ± SE developed over the assessment period. For each type of application, means in a column followed by the same letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985).

Effect of treatments on plant growth

In general, the non-inoculated plants showed higher plant biomass than the inoculated plants for all treatments and for the two types of application, as demonstrated by the lack of interaction between the variable inoculation and the other two variables evaluated. The global mean reduction in weight due to inoculation was 16.4%, with 14.5% corresponding to leaves and 18.8% to stems. Likewise, root applications showed a higher general effect on plant biomass than foliar applications for both stem and leaf tissues, but in this case the effect of the treatments depended on the type of application. Since the general ANOVA showed that interactions between the inoculation and the treatments or the type of application were not significant, but the interaction between treatments and type of application was significant, the data on the weight of the plant tissues are presented separately, but averaging the inoculation data for analysis. The main differences in the effect of the treatments on the plant biomass were observed for the plants treated with CuPh, which showed the highest leaf and stem fresh weight compared with the rest of the treatments for both types of application. The two microorganisms did not show any effect on the plant biomass, except for the foliar treatment with AP08, which significantly increased the fresh weight of the leaves compared to the control (Fig. 5.1).

Effect of treatments enhancing the biochemical parameters involved in plant resistance

Determination and quantification of hydrogen peroxide (H_2O_2)

The ROS accumulation (H_2O_2 staining) in leaves of olive plants of cv. Picual treated with *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024, and CuPh is shown in Fig. 5.2. In general, it can be observed that the ROS is significantly enhanced in the plants treated with *B. amyloliquefaciens* PAB-024; whereas the plants treated with *A. pullulans* AP08 and CuPh did not show significant ROS accumulation in comparison with the control (4,163 ± 438 and 5,920 ± 417 pixels image^{-1} for inoculated and non-inoculated treated control plants, respectively).

Results relative to the plants treated with *B. amyloliquefaciens* PAB-024 showed a significant strong accumulation of ROS both in foliar and root applications, as well as in inoculated (17,581 ± 2,748 and 24,972 ± 3,039 pixels image^{-1} for foliar and root

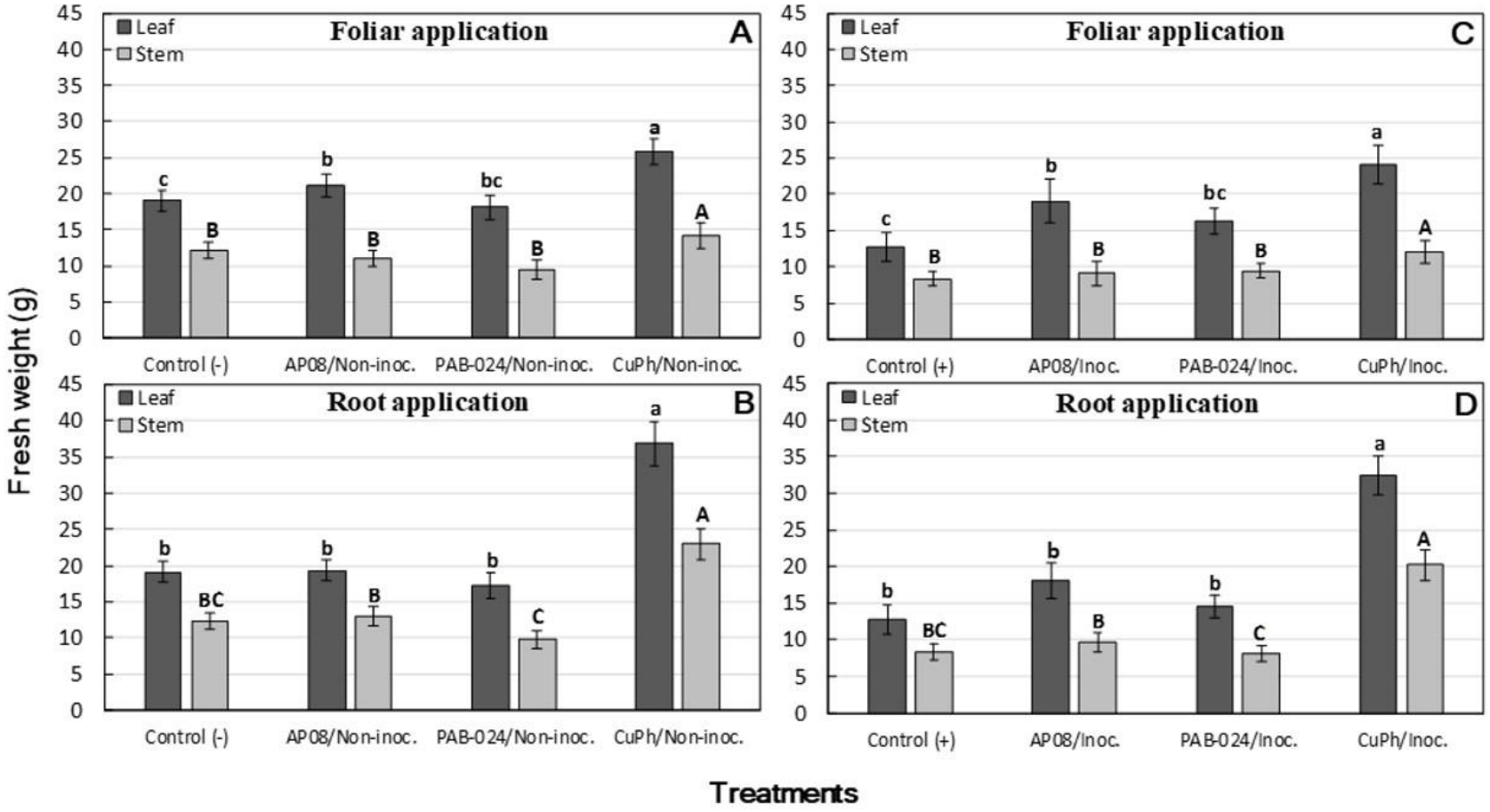

Figure 5.1. Effect on plant biomass [fresh weight (g) of leaves and stem] in olive plants of cv. Picual, non-inoculated (**A**, **B**) or inoculated (**C**, **D**) with *Verticillium dahliae*, 3 months after treatment by spray (foliar application) or irrigation (root application) with the following products: water [Control: (-) = non-inoculated control; (+) = inoculated control), *Aureobasidium pullulans* (AP08), *Bacillus amyloliquefaciens* (PAB-024) or Copper Phosphite (CuPh). In each graph, columns represent the mean of 30 plants [two experiments with three replicated blocks and five plants per block]. Columns with a common uppercase or lowercase letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ for fresh weight of stem and leaf tissues, respectively. Vertical bars are the standard error of the means.

applications, respectively) and non-inoculated plants (11,690 ± 1,443 and 9,740 ± 916 pixels image^{-1} for foliar and root applications, respectively) compared to the control, with ROS levels to be always higher in inoculated plants. In the plants treated with *A. pullulans* AP08, only the foliar application notably increased the accumulation of ROS, but in this case, it was higher in inoculated plants (10,684 ± 1,450 pixels image^{-1}) than in non-inoculated ones (7,280 ± 998 pixels image^{-1}). Finally, plants treated with PhCu did not show an increase of ROS for any combination of treatments, showing ROS values similar to those observed for the control plants (ROS in inoculated plants = 8,070 ± 1,235 and 4,615 ± 456 pixels image^{-1} for foliar and root applications, respectively; ROS in non-inoculated plants = 5,931 ± 693 and 5,617 ± 496 pixels image^{-1} for foliar and root applications, respectively).

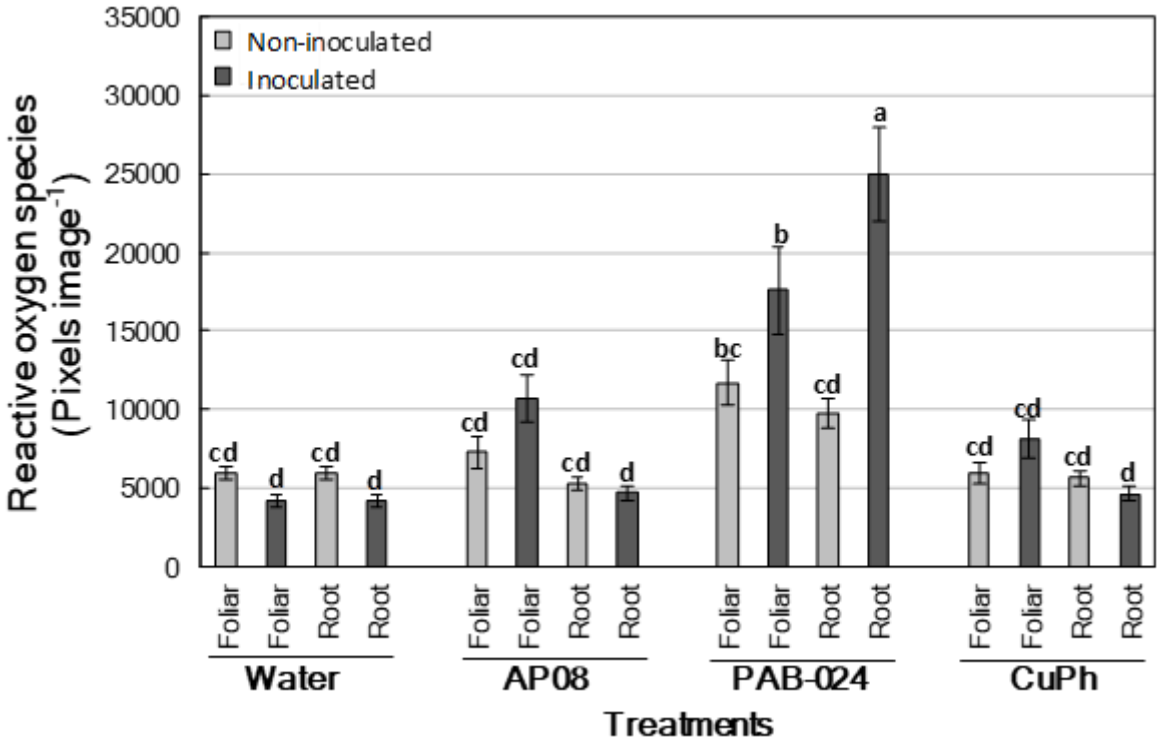

Figure 5.2. H_2O_2 staining [reactive oxygen species (ROS); pixels image^{-1}] estimated by using DAB staining in the leaves of olive plants of cv. Picual, which were non-inoculated (light grey colums) or inoculated (dark grey columns) with *Verticillium dahliae*, three months after treatment by spray (foliar application) or irrigation (root application) with the following products: water (Control), *Aureobasidium pullulans* (AP08), *Bacillus amyloliquefaciens* (PAB-024) and Copper Phosphite (CuPh). Columns represent the mean of 30 plants (two experiments with three replicated blocks and five plants per block each). Columns with common letters do not differ significantly according to Tukey's HSD test at P = 0.05. Vertical bars are the standard error of the means.

The ROS accumulation (H_2O_2 staining) was also estimated in roots of olive plants of cv. Picual treated with *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024, and CuPh, but non-significant differences were observed between compounds and control for any combination of treatments (*data not shown*).

Evaluation of hormones related to plant defence

The levels of hormones SA, JA, OPDA, and JA-Ile in leaf, stem and root samples from olive plants of cv. Picual treated with *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024, and CuPh are shown in Table 5.3.

Treatments with *A. pullulans* AP08 were able to stimulate the levels of SA mainly in the leaves, with the higher values being observed in the non-inoculated plants treated by root application. However, none of the values observed were significantly different from those observed in the positive controls as well as in the rest of the treatments. The treatment with *B. amyloliquefaciens* PAB-024 only showed an enhancement of the SA content mainly in leaves from inoculated plants, whereas those non-inoculated showed low levels of SA. Finally, the CuPh increased the levels of SA in all the treatments, regardless of the plant tissue, the type of application or the presence of *V. dahliae*.

The levels of JA were significantly stimulated in comparison to the controls only in certain tissues and treatment combinations with *A. pullulans* AP08 or *B. amyloliquefaciens* PAB-024. Interestingly, the plants treated with *A. pullulans* AP08 showed a significant strong enhancement of JA both in foliar and root applications as well as in inoculated and non-inoculated plants. In detail, it can be observed that *A. pullulans* AP08 is able to induce a systemic enhancement of JA, regardless of the plant tissue, but especially in leaves. In the same way, the treatment with *B. amyloliquefaciens* PAB-024 was also able to increase the levels of JA; however, this enhancement is important only in the roots of treated and inoculated plants. The treatment with CuPh increases the levels of JA in inoculated plants treated by spraying, regardless of the plant tissue, but it did not show significant differences compared to the control.

Regarding the metabolites present in the JA pathway, in general, an increase of OPDA in roots of inoculated plants was observed compared with control plants in all

cases. This increase can also be observed in the plants treated with *A. pullulans* AP08 both by foliar or root applications, in which the inoculated plants showed significantly higher levels than the non-inoculated ones. In general, the treatment with *B. amyloliquefaciens* PAB-024 showed similar levels of OPDA to those observed in positive control plants. However, inoculated plants treated by spraying showed significantly higher levels than the rest of the bacteria treated plants. Minor or non-significant differences were observed in the levels of OPDA in plants treated with CuPh compared with the positive controls.

Finally, the treatment with *A. pullulans* AP08 strongly increases the presence of JA-ile, which is the bioactive metabolite from JA pathway, in all the plant tissues evaluated. According to the results observed with the JA, the treatment with the *B. amyloliquefaciens* PAB-024 was also able to induce the JA-ile, but this enhancement is only present in the stem and roots of both inoculated and inoculated plants, but without significant differences with the controls. In the same way, minor or non-significant differences were observed in the levels of JA-ile in plants treated with CuPh in comparison with the positive controls.

Validation and Expression Profile Analysis by Quantitative Real-Time PCR

The relative expression of *ACL*, *LOX*, *WRKY5*, and *bHLH* genes in leaf, stem and root samples from olive plants of cv. Picual treated with *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024, and CuPh are shown in Table 5.3.

The expression of genes related to the SA (*ACL* and *WRKY5*) showed a discrete enhancement in all the treatments. Exceptionally, a remarkable enhancement of the transcription factor *WRKY5* was observed in the roots of the inoculated plants treated by root application, and on the non-inoculated plants treated by foliar application with *B. amyloliquefaciens* PAB-024. On the other hand, *ACL* expression shows similar levels to those observed in negative controls for all treatment combinations and tissues.

Regarding the gene expression related to the JA (*LOX* and *bHLH*), it can be observed that the *LOX* gene is strongly enhanced in the plants treated with *B. amyloliquefaciens* PAB-024 whereas in the rest of the treatments the expression of this

gene at this timepoint is low. However, the expression of the transcription factor *bHLH* is enhanced in the different treatments. Results showed in plants treated with *A. pullulans* AP08 indicate that the treatment enhances high levels of *LOX* and *bHLH* expression in the leaves from the non-inoculated and foliar treated plant, as well as high levels of *bHLH* expression in the roots from non-inoculated and foliar treated plants. On the other hand, plants treated with *B. amyloliquefaciens* PAB-024 showed an increase of *bHLH* levels only in the stem of foliar treated and non-inoculated plants, and in the roots of inoculated plants treated by root applications. Finally, plants treated with PhCu showed an increase of this gene mainly in non-inoculated plants.

DISCUSSION

This study was conceived to shed light on the biochemical and genetic responses associated with HRI that could take place during the interaction between biocontrol treatments, a woody host (olive) and a vascular pathogen (*V. dahliae*). In this work, two microorganisms (*A. pullulans* AP08 and *B. amyloliquefaciens* PAB-024) and a phosphonate salt copper phosphite (CuPh) were selected for their high effectiveness previously observed against *V. dahliae* in olive plants treated by foliar or root applications before pathogen infection (López-Moral *et al.*, 2021b). From this preliminary study, we had the hypothesis that these compounds could be acting by inducing the resistance of the plant rather than a direct effect on the pathogen. Since the induction of defense is usually a systemic response of the plant, is not necessary to apply the treatment in the same tissue as the pathogen. Whit this rationale, it is interesting to ascertain if the foliar treatments are effective in extensive crops like olive which the soil treatments are much more difficult due to the lack of an irrigation system. Thus, the present study is useful to clarify the mechanism of action of these compounds and to determine their role in enhancing the olive immune system against infections by *V. dahliae*.

The effect of the different compounds in the disease reduction was in concordance with those previously described by López-Moral *et al.* (2021b). Thus, in general, root applications resulted in higher effectiveness than foliar applications. Considering the type of application, *A. pullulans* AP08 was the most effective compound when it was applied by foliar applications, whereas CuPh was the most effective by root application. In addition, treatments with CuPh resulted in a

Table 5.3. Hormone levels (ng per g of dry weight) related to salicylic acid (SA), jasmonic acid (JA), precursor of JA (OPDA), and JA-Isoleucine (JA-Ile) and relative expression of genes involved in the synthesis of acetone cyanohydrin lyase (*ACL*) and lipoxygenase (*LOX*), and the transcription factors involved in SA signal transduction (*WRKY5*) and factors responsive to JA (basic helix-loop-helix; *bHLH*) in leaf, stem and root samples from olive plants of cv. Picual treated with *Aureobasidium pullulans* AP08 *B. Amyloliquefaciens* PAB-024 and copper phosphite (CuPh) by foliar or root applications, and inoculated or non-inoculated with *Verticillium dahliae* strain V-180.

Leaf samples

			Hormone levels (ng/g of dry weight)				Relative gene expression[b]			
Treatment combination			**SA**	**JA**	**JA-Ile**	**OPDA**	***LOX***	***ACL***	***WRKY5***	***bHLH***
A. pullulans AP08	Foliar	Non-inoculated	1714.6 ± 615.8 ab	24.5 ± 1.1 ab	70.6 ± 11.7 ab	5.0 ± 0.6 bc	18.4 ± 1.7 a	0.31 ± 0.12 b	6.3 ± 1.5 a	80.7 ± 6.5 a
		Inoculated	1978.5 ± 615.5 ab	10.7 ± 0.7 b	83.5 ± 45.3 ab	30.1 ± 4.0 a	1.6 ± 0.6 b	0.01 ± 0.0 b	0.3 ± 0.1 b	3.4 ± 0.8 b
	Root	Non-inoculated	3818.2 ± 1092.2 ab	23.2 ± 3.3 ab	45.9 ± 12.5 b	0.0 ± 0.0 c	1.0 ± 0.1 b	0.04 ± 0.04 b	0.02 ± 0.01 b	0.8 ± 0.6 b
		Inoculated	2263.9 ± 414.57 ab	44.7 ± 10.0 a	122.2 ± 11.04 a	11.3 ± 5.8 b	2.1 ± 0.9 b	0.01 ± 0.01 b	0.2 ± 0.2 b	0.8 ± 0.6 b
B. amyloliquefaciens	Foliar	Non-inoculated	651.8 ± 170.4 b	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	5.0 ± 1.2 b	0.1 ± 0.1 b	0.0 ± 0.0 b	0.7 ± 0.4 b
PAB-024		Inoculated	3111.3 ± 672.1 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	2.5 ± 0.6 b	0.02 ± 0.0 b	0.2 ± 0.1 b	0.2 ± 0.1 b
	Root	Non-inoculated	526.8 ± 37.8 b	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	4.0 ± 1.2 b	0.1 ± 0.1 b	0.02 ± 0.01 b	0.4 ± 0.3 b
		Inoculated	2323.0 ± 656.0 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	1.1 ± 0.5 b	0.3 ± 0.2 b	0.2 ± 0.04 b	0.3 ± 0.2 b
Copper Phosphite (CuPh)	Foliar	Non-inoculated	2519.6 ± 323.8 a	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	3.3 ± 2.2 b	1.7 ± 0.3 a	1.6 ± 1.1 b	1.1 ± 0.3 b
		Inoculated	2104.8 ± 112.8 ab	0.0 ± 0.0 c	9.1 ± 1.3 c	0.0 ± 0.0 d	0.9 ± 0.4 b	0.02 ± 0.01 b	0.4 ± 0.2 b	0.2 ± 0.1 b
	Root	Non-inoculated	3484.4 ± 748.3 a	0.0 ± 0.0 c	0.0 ± 0.0 d	9.3 ± 2.5 b	0.2 ± 0.1 b	0.1 ± 0.01 b	0.1 ± 0.02 b	0.1 ± 0.1 b
		Inoculated	2275.6 ± 577.1 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	2.1 ± 1.3 b	0.1 ± 0.1 b	1.2 ± 0.5 b	1.0 ± 0.2 b
Water (Control)	Foliar	Non-inoculated	1217.4 ± 430.4 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 c	1.8 ± 0.6 b	0.41 ± 0.2 b	0.4 ± 0.3 b	0.4 ± 0.1 b
		Inoculated	2667.5 ± 1310.6 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	1.1 ± 0.3 c	5.5 ± 0.9 b	0.03 ± 0.02 b	2.6 ± 1.6 b	0.2 ± 0.0 b
	Root	Non-inoculated	1217.4 ± 430.4 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	0.0 ± 0.0 d	1.8 ± 0.6 b	0.41 ± 0.2 b	0.4 ± 0.3 b	0.4 ± 0.1 b
		Inoculated	2667.5 ± 1310.6 ab	0.0 ± 0.0 c	0.0 ± 0.0 d	1.1 ± 0.3 c	5.5 ± 0.9 b	0.03 ± 0.02 b	2.6 ± 1.6 b	0.2 ± 0.0 b

Stem samples

			Hormone levels (ng/g of dry weight)				Relative gene expression			
Treatment combination			**SA**	**JA**	**JA-Ile**	**OPDA**	***LOX***	***ACL***	***WRKY5***	***bHLH***
A. pullulans AP08	Foliar	Non-inoculated	103.2 ± 18.5 b	26.6 ± 18.3 a	43.7 ± 18.9 b	18.6 ± 3.4 b	0.3 ± 0.02 a	0.0 ± 0.0 a	0.2 ± 0.1 a	0.03 ± 0.0 a
		Inoculated	99.8 ± 7.6 b	6.2 ± 3.1 a	53.1 ± 10.7 ab	11.0 ± 1.8 b	0.7 ± 0.3 a	0.02 ± 0.01 a	2.3 ± 0.8 a	1.5 ± 0.3 a
	Root	Non-inoculated	84.0 ± 4.4 b	19.7 ± 9.6 a	23.9 ± 8.5 b	21.0 ± 1.3 b	0.5 ± 0.1 a	0.0 ± 0.0 a	0.3 ± 0.2 a	0.2 ± 0.1 a
		Inoculated	86.0 ± 21.2b	20.9 ± 2.6 a	97.0 ± 17.3 a	8.8 ± 2.8 b	0.8 ± 0.1 a	0.0 ± 0.0 a	0.5 ± 0.2 a	0.2 ± 0.1 a
B. amyloliquefaciens	Foliar	Non-inoculated	81.2 ± 7.2 b	3.9 ± 2.1 ab	2.4 ± 0.6 c	10.6 ± 1.5 b	0.6 ± 0.3 a	0.4 ± 0.3 a	0.9 ± 0.4 a	12.6 ± 2.7 a
PAB-024		Inoculated	94.6 ± 5.7 b	0.2 ± 0.03 b	0.2 ± 0.1 cd	11.3 ± 2.2 b	1.3 ± 0.7 a	0.04 ± 0.03 a	0.9 ± 0.2 a	0.2 ± 0.1 a

	Root	Non-inoculated	75.3 ± 8.6 b	0.0 ± 0.0 c	0.0 ± 0.0 d	6.5 ± 1.8 b	0.8 ± 0.2 a	0.02 ± 0.01 a	0.1 ± 0.01 a	0.0 ± 0.0 a
		Inoculated	97.8 ± 12.9 b	5.6 ± 0.8 ab	3.7 ± 0.3 c	17.3 ± 1.2 b	1.8 ± 0.1 a	0.1 ± 0.0 a	2.7 ± 0.3 a	0.1 ± 0.03 a
Copper Phosphite (CuPh)	Foliar	Non-inoculated	65.8 ± 6.0 b	0.0 ± 0.0 c	0.4 ± 0.1 c	12.6 ± 4.0 b	0.4 ± 0.3 a	0.1 ± 0.1 a	0.1 ± 0.1 a	17.9 ± 4.0 a
		Inoculated	115.7 ± 13.3 ab	3.0 ± 0.9 b	2.1 ± 0.9 c	79.6 ± 14.2 a	1.2 ± 0.5 a	0.04 ± 0.02 a	1.1 ± 0.4 a	0.1 ± 0.1 a
	Root	Non-inoculated	95.4 ± 11.6 b	0.0 ± 0.0 c	0.0 ± 0.0 d	15.8 ± 2.3 b	1.0 ± 0.1 a	0.2 ± 0.2 a	0.7 ± 0.6 a	0.7 ± 0.4 a
		Inoculated	124.6 ± 18.1 ab	0.2 ± 0.1 b	1.9 ± 0.2 c	7.8 ± 0.2 b	1.0 ± 0.4 a	0.1 ± 0.03 a	1.7 ± 0.8 a	0.1 ± 0.04 a
Water (Control)	Foliar	Non-inoculated	85.2 ± 25.1 b	2.8 ± 0.9 b	2.3 ± 0.4 c	18.7 ± 3.1 b	1.4 ± 0.3 a	0.1 ± 0.02 a	1.4 ± 0.6 a	0.1 ± 0.1 a
		Inoculated	178.4 ± 32.6 a	4.8 ± 1.4 ab	6.2 ± 2.1 c	12.5 ± 3.9 b	1.9 ± 0.1 a	0.3 ± 0.3 a	0.5 ± 0.2 a	0.03 ± 0.0 a
	Root	Non-inoculated	85.2 ± 25.1 b	2.8 ± 0.9 b	2.3 ± 0.4 c	18.7 ± 3.1 b	1.4 ± 0.3 a	0.1 ± 0.02 a	1.4 ± 0.6 a	0.1 ± 0.1 a
		Inoculated	178.4 ± 32.6 a	4.8 ± 1.4 b	6.2 ± 2.1 c	12.5 ± 3.9 b	1.9 ± 0.1 a	0.3 ± 0.3 a	0.5 ± 0.2 a	0.03 ± 0.0 a

Root samples

			Hormone levels (ng/g of dry weight)				Relative gene expression			
Treatment combination			**SA**	**JA**	**JA-Ile**	**OPDA**	***LOX***	***ACL***	***WRKY5***	***bHLH***
A. pullulans AP08	Foliar	Non-inoculated	29.4 ± 7.8 ef	7.7 ± 3.5 bc	17.1 ± 2.2 a	35.6 ± 6.6 b	0.1 ± 0.1 c	0.3 ± 0.1 a	15.4 ± 6.5 e	14.7 ± 6.9 b
		Inoculated	48.2 ± 9.2 def	26.2 ± 1.3 a	47.6 ± 5.9 a	75.1 ± 19.0 a	1.3 ± 1.0 c	0.4 ± 0.4 a	320.0 ± 54.5 c	3.7 ± 1.2 b
	Irrigation	Non-inoculated	29.6 ± 7.4 ef	11.8 ± 0.7 bc	28.2 ± 5.5 a	40.4 ± 11.6 b	0.3 ± 0.3 c	0.1 ± 0.03 a	9.0 ± 3.4 e	0.7 ± 0.4 b
		Inoculated	25.9 ± 1.8 ef	21.3 ± 6.3 a	29.8 ± 5.5 a	67.7 ± 16.12 a	2.3 ± 2.0 c	0.1 ± 0.04 a	93.4 ± 30.3 cde	6.3 ± 2.5 b
B. amyloliquefaciens	Foliar	Non-inoculated	23.6 ± 2.1 ef	6.5 ± 0.3 c	11.7 ± 2.1 a	32.2 ± 6.3 b	368.5 ± 31.0 a	0.7 ± 0.1 a	1951.4 ± 171.5 a	0.04 ± 0.02 b
PAB-024		Inoculated	31.0 ± 9.2 ef	34.4 ± 10.3 ab	25.3 ± 5.5 a	81.6 ± 13.9 a	47.0 ± 14.2 bc	0.6 ± 0.5 a	293.8 ± 86.7 c	0.3 ± 0.2 b
	Root	Non-inoculated	17.4 ± 2.3 f	3.3 ± 0.6 c	10.1 ± 2.6 a	33.6 ± 13.7 b	8.2 ± 0.6 bc	0.1 ± 0.1 a	154.3 ± 42.9 cde	0.1 ± 0.1 b
		Inoculated	78.1 ± 38.4 cde	12.2 ± 3.1 ab	25.6 ± 10.8 a	37.6 ± 3.7 a	134.8 ± 28.4 ab	1.1 ± 0.5 a	1624.0 ± 228.7 b	5.2 ± 1.1 b
Copper Phosphite (CuPh)	Foliar	Non-inoculated	128.7 ± 13.2 abcd	13.4 ± 5.6 c	16.1 ± 5.5 a	43.2 ± 16.6 b	0.5 ± 0.5 c	0.6 ± 0.2 a	62.5 ± 27.7 de	3.2 ± 2.5 b
		Inoculated	162.8 ± 8.8 abc	14.3 ± 3.7 abc	27.1 ± 9.9 a	40.1 ± 19.8 ab	0.01 ± 0.0 c	0.1 ± 0.1 a	65.3 ± 20.1 de	0.5 ± 0.3 b
	Root	Non-inoculated	123.2 ± 14.5 abcd	3.6 ± 2.7 c	7.0 ± 2.6 a	27.5 ± 6.8 b	0.3 ± 0.03 c	0.6 ± 0.2 a	2.4 ± 1.2 e	36.5 ± 21.7 a
		Inoculated	116.7 ± 8.6 bcd	13.3 ± 6.7 abc	20.1 ± 6.6 a	43.1 ± 5.9 ab	0.01 ± 0.0 c	1.1 ± 0.6 a	55.3 ± 13.7 de	0.5 ± 0.3 b
Water (Control)	Foliar	Non-inoculated	267.9 ± 46.0 a	2.0 ± 0.8 c	15.7 ± 2.1 a	23.5 ± 4.5 b	0.2 ± 0.03 c	0.1 ± 0.3 a	119.2 ± 44.4 cde	1.3 ± 0.3 b
		Inoculated	318.6 ± 61.8 a	4.4 ± 2.2 c	13.3 ± 1.6 a	50.3 ± 12.8 a	0.01 ± 0.0 c	0.1 ± 0.0 a	261.9 ± 46.5 cd	0.1 ± 0.1 b
	Root	Non-inoculated	267.9 ± 46.0 a	2.0 ± 0.8 c	15.7 ± 2.1 a	23.5 ± 4.5 b	0.2 ± 0.03 c	0.1 ± 0.3 a	119.2 ± 44.4 cde	1.3 ± 0.3 b
		Inoculated	318.6 ± 61.8 a	4.4 ± 2.2 c	13.3 ± 1.6 a	50.3 ± 12.8 a	0.01 ± 0.0 c	0.1 ± 0.0 a	261.9 ± 46.5 cd	0.1 ± 0.1 b

[a] For each treatment combination and plant tissue, the data represent the mean of three replicated plants (biological repetitions) and three measurements per simples plant (technical repetitions) ± the standard error of the means. Different letters in the same column represent statistically significant differences according to Tuhey's HSD test at $P \leq 0.05$.

[b] Relative gene expression in comparison with the *Olive act-1* gene (Schilirò *et al*., 2012).

significantly higher fresh weight of the plants compared with the rest of the treatments, which suggests their potential effect by biostimulation as previously supported by Gómez-Merino and Trejo-Téllez (2015). Subsequently, the hormonal pathways and the related gene expression were analyzed to ascertain the implication of the defensive pathways in the enhancement of the HRI by the evaluated compounds. Interestingly, different results were obtained, depending on the compound, type of application or inoculation treatment combinations.

It is well known that plant defense against pathogen infection is regulated by the hormones SA, JA and ethylene (Llorens *et al.*, 2017b; Montes-Osuna and Mercado-Blanco, 2020). The SA pathway is the responsible to control the defense against biotrophic pathogens, however, its activation is also required for effective plant defense against hemibiotrophic pathogens such as *V. dahliae*. In this work, high levels of SA were detected on leaves from all treatment combinations, but without significant enhancements compared to the non-treated controls. Considering the gene expression linked to salicylic acid, only the relative *WRKY5* gene expression showed a strong enhancement in olive plants treated with *B. amyloliquefaciens* PAB-024. Our results are not at all in concordance with those shown by Gharbi *et al.* (2016), who linked the early activation of SA with an effective resistance to *V. dahliae* reducing wilt symptoms. These authors monitored the expression of SA related genes PR10, PR1, PR5, PLD and *WRKY5* at early time points (from 1 to 30 days) showing that the higher expression of this pathway is around 8 to 15 days after infection, and a progressive reduction since then (Gharbi *et al.*, 2016). These results are interesting since it has been previously described that the constitutive overexpression of *GbMPK3* (Long *et al.*, 2020) or *GhWRKY70* (Xiong *et al.*, 2019) enhance the SA-mediated defense pathway genes which produced an increased susceptibility to *V. dahliae*, probably due to the negative crosstalk between the JA/SA pathways. Under our conditions, the implication of SA is not so evident since the evaluation of hormones and gene expression was performed three months after inoculation, which could skip the biotrophic phase of the fungi. Nevertheless, it is worth mentioning that plants treated with *B. amyloliquefaciens* PAB-024 showed high levels of ROS accumulation, which simultaneously accompany the up-regulation of SA-related genes (Llorens *et al.*, 2017a). It has been widely studied that against pathogens infection, there is an increase in SA levels preceded by apoplastic H_2O_2 bursts. This mechanism suggests that H_2O_2 originated in chloroplasts and

peroxisomes triggers SA biosynthesis, which is essential for main defense responses: transcriptional reprogramming, cell death, and stomatal closure (Herrera-Vásquez *et al.*, 2015)

Regarding the JA, which is the hormone related to the defense against necrotrophic pathogens, our results show a strong accumulation of Ja and Ja-ile in plants treated with *A. pullulans* AP08 in all tissues analyzed, as well as in plants treated with *B. amyloliquefaciens* PAB-024 and CuPh in roots. These findings could suggest an implication of the JA in the induced resistance by the tested compounds. Our results are in concordance with those previously shown by Gharbi *et al.* (2017), who reported that the JA, as well as the expression of the genes responsive to this hormone, could be related to the higher resistance of olive trees to *V. dahliae*. In addition, these authors also observed that the activation of JA pathway is produced later than the SA response, probably when the necrotrophic phase of the pathogen starts. Our results agree with this hypothesis since at 3 months after inoculation there is a strong accumulation of JA and JA-related metabolites. The relation of JA with the resistance against *V. dahliae* has been thoroughly reported in several plant species. Xiong *et al.* (2020) demonstrated that the downregulation of *GhWRKY70D13* in cotton plants increased the accumulation of JA and JA-ile which enhanced resistance to *V. dahliae*. In the same way, Luo *et al.* (2021) observed that phosphate deficiency in cotton induced the activation of JA biosynthesis and accumulates a series of anti-fungal flavonoids which, in turn, confer protection against this fungus. Moreover, it is interesting to note that the olive resistance against *V. dahliae* mediated by the JA pathway could be also linked with the cultivar resistance. Likewise, studies conducted by Gharbi *et al.* (2017) demonstrated the expresión of *JZIM* (jasmonate ZIM domain) and *bHLH* genes were strongly induced in olive of cv Sayali but not in 'Chemlali' (susceptible) (Gharbi *et al.*, 2017). Altogether suggest that the JA pathway could play an important role in inducing the plant defences in olive against *V. dahliae*, which could be modulated by the genotype, the application of certain biocontrol treatments, and probably, also by the combined effect of both genotype and biocontrol treatments.

In addition, callose accumulation was also evaluated in this study following the protocol described by Llorens *et al.* (2017a), but no levels of callose deposition could be observed for any treatment and control (*data not shown*). This fact makes sense in our experimental conditions for two main reasons: *i*) we evaluated all the parameters related

to HRI at 3 months after inoculation, but callose accumulation is a cellular response of early post-invasive defences based on the generation of physical barriers in the side of infection to prevent the pathogen colonization and typically triggered by conserved pathogen-associated molecular patterns (PAMPs) (Luna *et al.*, 2011; Voigt, 2014; Llorens *et al.*, 2017a); and *ii*) the biology of *V. dahliae*, that infects olive through the roots by microsclerotia germination mediated by root exudation, did not match with the biotrophic pathogens i.e. powdery mildew that usually enhances the plant responses to induce callose deposition (Ellinger *et al.* 2013; Llorens *et al.*, 2017a)

Summarizing, several transcription factors were previously shown to play-role in plant defence in olive against *V. dahliae* (Schilirò *et al.*, 2012) such as *LOX*, *ACL*, *bHLH*, and *WRKY5* have been shown in this present study to be induced in olive plants after different biological treatments with the presence or absence of the pathogen. Up-to-date, their role in the modulation of HRI in olive is still uncertain. Therefore, this study helps us to reinforce the hypothesis that the three compounds tested here can act as HRI in olive against *V. dahliae*. This fact can be concluded mainly in the case of *A. pullulans* AP08, which resulted in low effectiveness inhibiting mycelial growth and MS germination of *V. dahliae in vitro*, but in higher effectiveness reducing the disease progress in previous studies conducted by López-Moral *et al.* (2021b), showing also high levels of JA and metabolites and genes present in the JA pathway in this study. Thus, these findings could suggest an implication of the JA in the HRI by some of the tested compounds. The knowledge generated in this work results in an important step towards selecting potential candidates for effective biocontrol of VWO. To continue this study, new insights into the effect of these three compounds as HRI over time should be conducted to determine the durability of their effect inarching the olive defences against *V. dahliae*.

CONCLUSIONS

In general, root applications resulted in higher effectiveness in reducing the disease progress than foliar applications. *Aureobasidium pullulans* AP08 was the most effective compound when it was applied by foliar applications, whereas CuPh was the most effective by root application. Treatments with CuPh resulted in a significantly higher fresh weight of the inoculated and non-inoculated plants compared with the rest of the treatments, suggesting their potential fertilizer effect *in planta*. Concerning the

effect of compounds enhancing HRI in olive, high levels of SA were detected on leaves from all treatment combinations, but without significant enhancements compared to the non-treated control. ROS was significantly enhanced in plants treated with *B. amyloliquefaciens* PAB-024 compared to the remaining treatments and control. Considering the gene expression linked to salicylic acid, only the relative *WRKY5* gene expression showed a strong enhancement in olive plants treated with *B. amyloliquefaciens* PAB-024. Interestingly, a strong accumulation of JA and JA-ile was observed in plants treated with *A. pullulans* AP08 in all the tissues analyzed as well as in plants treated with *B. amyloliquefaciens* PAB-024 and CuPh in roots. These findings could suggest an implication of the JA in the HRI by the tested compounds. Finally, callose accumulation was not observed in any tissue for any treatment and control, probably for the experimental conditions as well as for the pathosystems used in this study. The knowledge generated in this work results in an important step towards selecting potential candidates for an effective biocontrol of VWO.

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Chapter 6

Elucidating the effect of nutritional imbalances of N and K on the infection of *Verticillium dahliae* in olive

ABSTRACT

The effect of mineral nutrition on wilt diseases has been previously reported in many herbaceous hosts, though such an effect on Verticillium wilt in olive (*Olea europaea* L; VWO), caused by *Verticillium dahliae*, is still uncertain. Field observations reveal that nitrogen (N) excess or imbalances of N-potassium (K) favour VWO epidemics. However, this has yet to be demonstrated. Thus, the aim of this study was to evaluate the influences of nutritional imbalances of N and K in *V. dahliae* infection of olive. To this end, adjusted treatments with N excess (↑N+↑Na), K deficiency (↓K) and their combination (↑N+↑Na+↓K) were evaluated on the viability of *V. dahliae* microsclerotia (MS), as well as on disease development in olive plants. In parallel, the potential indirect effect of the treatments on the viability of conidia and MS of *V. dahliae* was evaluated through the stimuli of root exudates. Treatments ↑N+↑Na and ↑N+↑Na+↓K decreased MS germination and disease progress; whereas ↓K significantly increased both parameters. Root exudates from treated plants increased the conidia germination of *V. dahliae* but reduced the MS germination. The results of this study will be the basis for planning further research towards a better understanding of the effect of mineral nutrition on VWO.

Keywords: Mineral nutrition; nitrogen; *Olea europaea*; potassium; Verticillium wilt.

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INTRODUCTION

The effect of mineral nutrition on Verticillium wilt diseases has already been reported in many herbaceous hosts, resulting in either a decrease or an increase in wilt symptoms (Pegg and Brady, 2002). However, there is a substantial lack of knowledge on the influence of the mineral nutrition in the development of Verticillium wilt of olive (VWO; *Olea europaea* L.), which is considered the most serious disease of this woody crop in the Mediterranean basin (López-Escudero and Mercado-Blanco, 2011; Jiménez-Díaz *et al.*, 2012; Montes-Osuna and Mercado-Blanco, 2020). It is well known that VWO is caused by the soil-borne fungus *Verticillium dahliae* Kleb., which is able to survive for a long time in the soil through the formation of microsclerotia (MS). MS not only serve as resistance structures, but are also the primary inoculum of the pathogen. They germinate due to the biochemical stimuli from host root exudates, giving rise to infectious hyphae that penetrate the roots of the plants and grow into the xylem vessels (Pegg and Brady, 2002; López-Moral *et al.*, 2021b). The biology of the pathogen suggests that important attention should be paid to elucidate the effect of soil mineral nutrition into its life cycle in olive tree, and particularly in the infection process in this woody host.

Regarding our knowledge of the effect of mineral nutrition on VWO, in a first approach based on field observations in the Guadalquivir valley (Andalusia region, southern Spain), it has been observed that excess nitrogen (N) or imbalances of N-potassium (K) favour VWO epidemics in affected olive groves (López-Escudero *et al.*, 2011; Trapero *et al.*, 2017). However, despite this evidence, studies on the effect of plant mineral nutrition on the occurrence and development of VWO are almost non-existent. To the best of our knowledge, only Pérez-Rodríguez *et al.* (2016a) evaluated the interaction between irrigation and mineral nutrition on VWO in the susceptible olive cv. 'Picual' under semi-controlled conditions. These authors demonstrated that increased frequency of irrigation combined with fertilization of N-P-K (15-15-15) favoured the development of the disease in soils naturally infested by *V. dahliae*. However, in this study, no conclusions could be drawn regarding the effects of certain nutrients, such as N excess, K deficiency, or the imbalances of N/K in VWO.

With regard to plant nutrition, it is generally understood that N influences a wide range of pathogens and their hosts, with the form of N available to the pathogen being

essential for disease development, regardless of the rate of N available. The influence of N in plant diseases is due both to direct effects, such as alterations in the growth or virulence of the pathogen, and indirect effects, such as changes in the physiology of the plant or in the biotic and abiotic environments, mainly in the rhizosphere since the pathogen is soil-borne (Pegg and Brady, 2002; Huber and Thompson, 2007; Roca *et al.*, 2018). The effect of N on *V. dahliae* infection has been previously reported in many herbaceous hosts, but with contradictory results in the literature. For instance, although the general understanding is that N excess increases disease incidence (Pegg and Brady, 2002; Huber and Thompson, 2007), direct effects of NO_3^- and NH_4^+ have been reported by reducing the number of propagules of *V. dahliae* in the soil and consequently mitigating the disease (Pegg and Brady, 2002).

Concerning the effect of K on plant diseases, it has also been reported that K alone, or in combination with N or other nutrients, influences the severity of diseases caused by many soil-borne pathogens, including Verticillium wilt diseases (Pegg and Brady, 2002; Prabhu *et al.*, 2007; Pettigrew, 2008; Hafsi *et al.*, 2014; Fontana *et al.*, 2020). Indeed, applications of K in K-deficient soils increase host resistance against diseases such as Verticillium wilt of cotton (Pegg and Brady, 2002); nevertheless, the mechanisms implicated are still poorly understood (Prabhu *et al.*, 2007; Hafsi *et al.*, 2014). Moreover, although there is a vast amount of literature on the relationship between K and plant diseases, the understanding of the relationship between K and other nutrients with plant diseases in agroecological ecosystems has been very little studied (Prabhu *et al.*, 2007).

Therefore, determining the influence of nutritional imbalances of one or several nutrients on the incidence and severity of VWO is essential to establish an adequate nutrient program preventing major *V. dahliae* infections. This issue is even more relevant in the case of VWO since adequate nutrition management must be taken into account within an integrated disease management strategy (López-Escudero and Mercado-Blanco, 2011; Montes-Osuna and Mercado-Blanco, 2020; López-Moral *et al.*, 2021b). In addition, this last aspect is of major importance to be considered in olive groves under fertigation systems, where not only the imbalanced mineral nutrition, but also the water regimes, can affect the severity of VWO (Pérez-Rodríguez *et al.*, 2015, 2016b) while the interaction between irrigation and fertilization can act in favour of VWO epidemics (Pérez-Rodríguez *et al.*, 2016a).

Therefore, as little attention has been previously given to understanding the relationship between N and K and other nutrients on VWO, the aim of this study was to evaluate the influences of nutritional imbalances of N and K, alone or combined, in *V. dahliae* infection to olive under laboratory-controlled conditions. To this end, adjusted treatments with N excess, K deficiency and their combination were tested on the viability of *V. dahliae* microsclerotia (MS) *in vitro* and on disease development by means of bioassays in olive plants of cv. Picual. In parallel, root exudates from treated plants were collected, evaluating the potential indirect effect of the treatments through root exudate stimuli on the viability of conidia and the MS of *V. dahliae in vitro*.

MATERIALS AND METHODS

Fungal isolate and inoculum preparation

The *V. dahliae* isolate V323, recovered from soil samples collected from a commercial olive orchard affected by VWO in Villanueva de la Reina (Jaen province, southern Spain), was used in all of the experiments in this study. Previously, it was characterized as the defoliating (D) pathotype by PCR following the protocol described by Mercado-Blanco *et al.* (2007) and as highly virulent in olive plants by pathogenicity tests. It is maintained as a single-spore isolate on potato dextrose agar (PDA; Difco® Laboratories, MD, United States) slants fully filled with sterile paraffin oil at 4°C in darkness in the collection of the Department of Agronomy at the University of Córdoba (DAUCO, Spain).

Prior to conducting the experiments, fresh colonies of the isolate V323 were obtained from the collection by plating small mycelial fragments of the colonized agar from the tube onto PDA acidified with lactic acid [APDA; 2.5 mL L^{-1} of medium] and incubated at 24°C in the dark for 7 days. Subsequently, fresh colonies were transferred to PDA and incubated as described before.

For inoculum preparation, 1 kg of cornmeal-sand mixture (CMS; sand, cornmeal and distilled water; 9:1:2, weight:weight:volume) was placed in 2-L Erlenmeyer flasks. Subsequently, flasks were sterilized following the protocol described by López-Moral *et al.* (2021b). For CMS pathogen colonization, 50 mycelial plugs (7.5 mm in diameter) of *V. dahliae* isolate V323 growing on PDA were introduced into each flask. The flasks

were incubated at 24°C in the dark for 4 weeks and manually shaken once a week to favour a homogeneous colonization. Finally, the inoculum density of the colonized CMS was estimated by means of the serial dilution method on PDA and is expressed as colony-forming units (CFUs) (Mulero-Aparicio *et al.*, 2019a; López-Moral *et al.* 2021b).

Treatments

Five treatments were designed, containing different concentrations of N, K and Na (Table 6.1). The first treatment (Control N-K) contained moderate concentrations of N and K and was used as reference for the following treatments. The second treatment (↑N+↑Na) was designed to contain a high concentration of N, and because $NaNO_3$ was used, it also contained a high concentration of Na. The third treatment (↑NaCl) contained a high concentration of Na as NaCl but no high N, in contrast with the second treatment. The fourth treatment (↓K) was similar to the Control N-K treatment but without K. The fifth treatment (↑N+↑Na+↓K) was similar to the second treatment but without K. All of the treatments were prepared by diluting the mineral nutrients (Merck KGaA, Darmstadt, Germany) in deionized distilled water (DDW). Thus, an additional treatment (Water) containing only DDW was included for comparative purposes.

Table 1. Composition of the treatments used in this study.

Treatment	Compound [mM]	Element [mM]
Control N-K	$Ca(NO_3)_2$ [1.5] KNO_3 [2.0]	N [5.0] K [2.0]
N and Na excess (↑N+↑Na)	$Ca(NO_3)_2$ [1.5] KNO_3 [2.0] $NaNO_3$ [45.0]	N [50.0] K [2.0] Na [45.0]
NaCl excess (↑NaCl)	$Ca(NO_3)_2$ [1.5] KNO_3[2.0] NaCl [45.0]	N [5.0] K [2.0] Na [45.0]
K deficiency (↓K)	$Ca(NO_3)_2$ [1.5] $NaNO_3$ [2.0]	N [5.0] Na [2.0]
N and Na excess and K deficiency (↑N+↑Na+↓K)	$Ca(NO_3)_2$ [1.5] $NaNO_3$ [47.0]	N [50.0] Na [47.0]

Viability of *Verticillium dahliae* microsclerotia

Preparation of artificially pathogen-colonized substrate

The CMS colonized by *V. dahliae* isolate V323 was removed from the flasks, deposited in aluminium trays and air-dried on a laboratory bench at 22 ± 2°C for 10-14 days. Subsequently, it was sieved with a 0.8-mm sieve, and the inoculum density of *V. dahliae*, expressed as the number microsclerotia (MS) per g of substrate, was estimated by the wet sieving method (Huisman and Ashworth, 1974) using 10 replicated Petri dishes of modified sodium polypectate agar medium (MSPA) (Butterfield and DeVay, 1977) following the protocol described by Varo *et al.* (2016). Due to the high MS concentration obtained (≈1,300 MS per g of substrate), an adjusted amount of sterile sand was added to the colonized CMS to reduce the MS concentration to 200 MS per g of substrate (CMS-200). This step was necessary to homogenize the amount of inoculum and to make it possible to count the germinated MS in downstream analyses.

Application of treatments

The experiment was conducted using sterile transparent PVC pots (100 mL vol.) with holes in the base (5 holes, each 2 mm in diameter) to facilitate percolation. Subsequently, each pot was filled with 60 g of the CMS-200 and irrigated with 30 mL of the corresponding treatment. After the percolation, the pots were hermetically closed and incubated in an environmental controlled chamber at 22 ± 2°C at 60% relative humidity (RH) for 1, 7 or 14 days (exposure periods). A completely randomized design was used with three replicated pots per treatment and different period of exposure combinations (6 treatments × 3 exposure periods × 3 replicated pots = 54 pots in total). The experiment was conducted twice.

Assessment

After each exposure period, the pot contents were deposited in individual aluminium trays and air-dried in a vertical laminar flow cabinet at 22 ± 2°C for 10-14 days. The inoculum density was estimated by wet sieving as described above, and it was expressed as the number of MS per g of CMS-200 (MSG).

Verticillium wilt development

Plant material

Healthy 6-month-old rooted olive cuttings of cv. Picual (highly susceptible to *V. dahliae*; López-Escudero *et al.*, 2004) growing in peat moss in opaque PVC pots (0.5 L) were obtained from a commercial nursery. To induce the active growth of the plants, they were pre-conditioned in a controlled-growth chamber at 22 ± 2°C, with a 14:10-h (light:dark) photoperiod of white fluorescent light (10,000 lux) and 60% RH for 1 month. During this month, plants were irrigated three times per week using 350 mL of DDW per plant.

Application of treatments and plant inoculation

After the preconditioning period of the plants, the last well-developed leaf on the stem was marked to estimate the effect of the treatments on plant growth. From this moment, plants were irrigated with the different treatments using 350 mL per plant once a week throughout the experiment. Moreover, plants were irrigated with 350 mL of DDW twice a week.

The plant inoculation was carried out one month after the first application of treatments. For this purpose, plants were transplanted to opaque PVC pots (0.8 L) previously disinfested with a commercial sodium hypochlorite solution at 20% for 2 h and filled with a 20% (weight:weight) mixture of colonized CMS and sterile sand (theoretical inoculum density of the final substrate = 10^7 CFU g^{-1}; Mulero-Aparicio *et al.*, 2019a; López-Moral *et al.* 2021b). Non-treated and inoculated plants were also included as a positive control. Additionally, plants that were transplanted in a similar way but with sterile CMS served as negative controls (non-inoculated plants). Just after inoculation, all plants were irrigated and incubated in a controlled-growth chamber at 20°C in the dark and 100% RH for 7 days. Subsequently, light and humidity parameters were progressively modified over 1 week until they reached 23°C, a 12-h photoperiod of fluorescent light [10,000 lux] and 70% RH, which were maintained until the end of the experiment (20 weeks after the first treatment application; 16 weeks after inoculation).

A randomized complete block design (three blocks) was used with five treatments, with inoculated or non-inoculated plants as the independent variable and four replicated olive plants per treatment and block [6 treatments or control × 2 inoculation conditions (inoculated and non-inoculated plants) × 3 blocks × 4 replicated plants = 144 plants in total]. The experiment was conducted twice.

Assessment

Disease severity (DS) was evaluated weekly for 16 weeks after inoculation using a 0 to 4 rating scale with 17 values, which estimates the percentage of affected tissue by means of five main categories (0 = no symptoms; 1 = 25%, 2 = 50%, 3 = 75%, and 4 = 100%, dead plant) of affected tissue, with three intermediate values (0.25, 0.50 and 0.75) between the main categories (Varo *et al.*, 2018; López-Moral *et al.* 2021b). Therefore, the scale values (*X*) were related to the percentage of affected plant (*Y*) according to the equation: $Y = 25X$. At the end of the experiment, DS data were used to calculate the area under the disease progress curve (AUDPC) using the following formula:

$$AUDPC = \sum_{i=1}^{n} [(DS_{i+1} + DS_i)/2](t_{i+1} \times t_i)]$$

where DS is the scale value described above at each evaluation moment (*i*), t_i is the time (days) at the evaluation moment, and *n* is the total of number of evaluations (Campbell and Madden, 1990). Subsequently, both the final DS and AUDPC were expressed as relative percentage (RDS, RAUDPC; %) to disease parameter values of the inoculated plants treated with only DDW [Water (+)] at the end of the experiment. In addition, disease incidence (DI) and mortality were assessed as the percentage (%) of symptomatic or dead plants, respectively. At the end of the experiment, the apical shoot growth was determined by the length increase and its fresh weight after the 20 weeks of treatments.

Finally, one plant per block and treatment of inoculated and non-inoculated plants was randomly selected to establish the infection by means of fungal re-isolation from the stem. For this purpose, basal stems of the selected plants were washed under running tap water for 2 h. Subsequently, small fragments of the asymptomatic or affected tissue were cut and surface-sterilized by dipping them in a 10% solution of

commercial bleach (Cl at 50 g L^{-1}) for 1 min, air-dried on sterilized filter paper for 10 min, and plated onto APDA. There were three Petri dishes per plant, with seven attempts at isolation (fragments of stem). Petri dishes were incubated at 24°C in the dark for 7 days. The consistency of isolation was estimated as a percentage (%) of positive attempts.

Effect of root exudates from non-inoculated plants grown under different treatments

Collection of root exudates

One plant per each block of the treated and non-inoculated plants (n = 6) was randomly selected to collect root exudates. To this end, plants were taken out of the pots, and the substrate was carefully removed from the roots by spraying tap water until most of the substrate was removed. Finally, roots were washed by dipping them into DDW. Immediately after washing, intact plants were placed individually in cylindrical opaque PVC pots (9.5 cm in diameter × 11 cm in height; ≈ 0.5 L vol.) without drainage holes and containing 350 mL of 0.01 M $CaSO_4 \times 2\ H_2O$ (Aulakh *et al.*, 2001; López-Moral *et al.*, 2021a) with the complete root system submerged. For the fixation of the plants, polyethylene discs 10 mm in diameter were designed, with a small hole (4 mm in diameter) in the centre to fix the basal end of the stem. Then, the pot was closed with the polyethylene disc with the fixed plant, keeping the entire root system in the collection media, and the pots were incubated for 4 h at room temperature in the dark. Subsequently, the content of each pot was recovered ($CaSO_4$ + exudate solution) and double filtered, first through filter paper (43-48 µm pore size; Filter-Lab®, Filtros Anoia, Barcelona, Spain) to remove root detritus, and second through a 0.45-µm membrane filter to remove microbial cell debris. The filtrate was immediately frozen in plastic vials at -20°C until further analyses were conducted. Prior to use, vials were defrosted overnight at 4°C in the dark. The root-exudate collection method from olive plants was adapted by López-Moral *et al.* (2021a) from previous protocols described by Aulakh *et al.* (2001).

Viability of *Verticillium dahliae* microsclerotia

Soil samples from a commercial cotton (*Gossypium hirsutum* L.) field naturally infested with *V. dahliae* were collected (Villanueva de la Reina; Jaen province, Spain; Geographic coordinates 38°00'10.8"N 3°55'57.5"W). Sampling and laboratory processing were conducted as described by López-Moral *et al.* (2021b).

Sterile transparent PVC pots (100 mL vol.) with holes in the base were prepared and filled with 60 g of the natural infested soil. Subsequently, each pot was irrigated with 30 mL of the collected root exudates. Additionally, pots filled with 60 g of natural infested soil irrigated with 30 mL of $CaSO_4$ collecting solution without root exudate were included as a control. After the percolation, the pots were hermetically closed and incubated at 22 ± 2°C for 72 h in the dark. A completely randomized design was used with three replicated pots per root exudate collected from each of the three selected plants per treatment (n = 6) or control (n = 1). The experiment was conducted twice.

After incubation, the treated soil samples were removed from the pots, and they were then processed to estimate the inoculum density of *V. dahliae* as described in section 2.3.1.

Viability of *Verticillium dahliae* conidia

Conidial suspensions were obtained from 14-day-old colonies of *V. dahliae* isolate V323 growing on PDA as described before and were adjusted to 8×10^5 conidia mL^{-1} using a haematocytometer. A 5-µL drop of the conidial suspension was placed in the centre of a microscope coverslip (20 × 20 mm); subsequently, a 5-µL drop of the root-exudate solution was mixed. Coverslips were placed inside Petri dishes containing water agar, which were used as humid chambers, and incubated at 23 ± 2°C in the darkness for 24 h. A completely randomized design was used with three replicated coverslips per root exudate collected from each of the three selected plants per treatment or control (n = 7). The experiment was conducted twice.

After the incubation period, a 5-µL drop of 0.01% acid fuchsine in lactoglycerol (1:2:1 lactic acid:glycerol:water) was added to each coverslip to stop conidial germination, and the coverslips were mounted on slides. A total of 100 randomly selected conidia per coverslip were observed at ×400 magnification using a Nikon

Eclipse 80i microscope (Nikon Corp., Tokyo, Japan), and germinated and non-germinated conidia were counted. Conidia were considered germinated when the germ tube was at least one-half of the longitudinal axis of the conidia. Conidial viability was estimated as the percentage (%) of germinated conidia (CG) (n = 7; 6 treatments and control).

Data analyses

Data from the two repetitions of each experiment were combined after checking for homogeneity of the experimental error variances by the F test ($P \geq 0.05$). Subsequently, in all cases, data were tested for normality, homogeneity of variances, and residual patterns, and they were logarithmically transformed when necessary. To determine the effect of treatments on MS viability, a factorial ANOVA was previously conducted with 'MSG' as the dependent variable and 'treatments', 'exposure periods', and their interaction as independent variables. Because the interaction was significant ($P < 0.05$), independent ANOVAs were conducted to study the effect of exposure periods on MS viability for each treatment; as well as the effect of treatments on MS viability for each exposure period. For the experiment *in planta*, two one-way ANOVAs were conducted with the 'RAUDPC' or 'RDS' as the dependent variables and the 'treatment' as the independent variable. RAUDPC and RDS were compared according to Fisher's protected LSD test at $P = 0.05$ (Steel and Torrie, 1985). Data on the final DI (% of affected plants) and mortality (% of dead plants) were analysed by multiple comparisons for proportions tests at $P = 0.05$ (Zar, 2010). For the same experiment, a factorial ANOVA was conducted with the 'growth' or 'weight' of shoots as dependent variables and 'treatment', 'inoculation' and their interaction as independent variables. Regarding the experiment with root exudates, two one-way ANOVAs were conducted with 'MSG' or 'CG' as dependent variables and 'root exudates from treatments' as the independent variable. Depending on the levels of the independent variables and their interactions that were significant, the means of the treatments were compared using Fisher's protected LSD test ($n < 7$) or Tukey's HSD ($n \geq 7$) ($P = 0.05$ in all cases) (Steel and Torrie, 1985), with the exception of the effect of exposure periods on MS viability in each treatment, which was compared using orthogonal contrasts. Pearson correlation coefficients (r) between the effects of treatments on MS viability, disease development and/or root exudates were also estimated for the evaluated treatments ($n = 6$) using the

average of MSG at 14 days of exposure, disease-related parameters (DI, Mortality, RDS and RAUDPC), and CG and MSG, respectively. Data from this study were analysed using Statistix 10.0 software (Anonimous, 2013).

RESULTS

Viability of *Verticillium dahliae* microsclerotia

The effect of treatments on the viability of MS varied depending on the different combinations of treatments and exposure periods. There were significant differences between treatments, exposure periods and their interaction ($P < 0.0001$ in all cases). Therefore, the effect of the exposure periods on MS viability was analysed separately for each treatment using orthogonal contrasts (Fig. 6.1). The effect of treatments on MS viability was also analysed separately for each exposure period (Table 6.2), although the ANOVA of the 14-day period was selected as the most representative of the three evaluated periods (Fig. 6.1).

Regarding the effect of the exposure periods on MS viability for each treatment, no significant differences in MSG between the exposure periods were observed for the Water, Control N-K and ↓K treatments. However, there was a significant increase in MSG over time of exposure for the ↑NaCl treatment and a significant decrease in MSG over time of exposure for the ↑N+↑Na and ↑N+↑Na+↓K treatments (Fig. 6.1).

Concerning the effect of treatments on MSG at 14 days of exposure, the ↑NaCl treatment showed the highest MSG (157.6 ± 4.2 MS per g of CMS-200), followed by ↓K (130.6 ± 2.03 MS per g of CMS-200), whereas the ↑N+↑Na (23.7 ± 3.54 MS per g of CMS-200) and ↑N+↑Na+↓K (33.1 ± 1.1 MS per g of CMS-200) treatments showed the lowest MSG (Table 6.2).

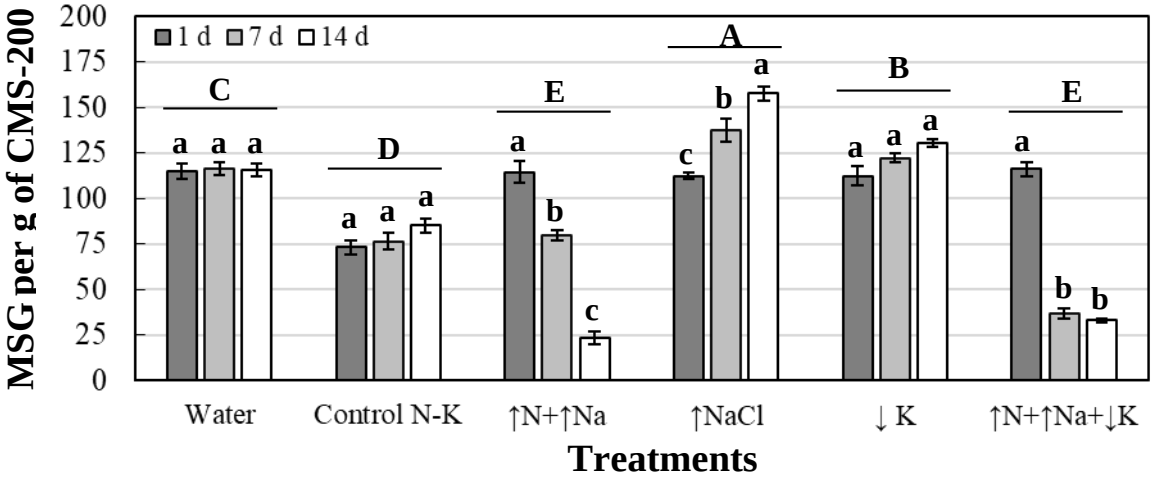

Figure 6.1. Effects of treatment and exposure period (1, 7, or 14 days) on microsclerotia germination of V. dahliae isolate V323 [MSG; number of germinated MS per g of CMS-200]. For each treatment and exposure period combination, columns represent the means of MSG values of three replicated samples of CMS-200. For each treatment, columns with a common lowercase letter do not differ significantly according to orthogonal contrasts. Treatments with a common capital letter do not differ significantly according to Fisher's protected LSD test at $P = 0.05$ for MSG at 14 days of exposure. Vertical bars represent the standard errors of the mean.

Table 6.2. Effects of treatments on microsclerotia (MS) viability of *V. dahliae* isolate V323 for each exposure period (1, 7 and 14 days) evaluated.

	MSG (MS per g of CMS-200)[a]		
Treatment	**1 day**	**7 days**	**14 days**
Water	114.8 ± 4.4 a	116.3 ± 3.6 b	115.7 ± 3.6 c
Control N-K	73.2 ± 3.9 b	76.5 ± 4.5 c	85.2 ± 3.9 d
↑N+↑Na	114.5 ± 6.2 a	79.9 ± 3.1 c	23.7 ± 3.5 e
↑NaCl	112.4 ± 1.6 a	137.7 ± 6.4 a	157.6 ± 4.2 a
↓K	112.4 ± 5.1 a	122.2 ± 2.5 b	130.6 ± 2.0 b
↑N+↑Na+↓K	116.1 ± 3.8 a	36.8 ± 2.7 d	33.1 ± 1.1 e
P-value $_{(\alpha = 0.05)}$	0.0004	≤ 0.0001	≤ 0.0001

[a]MSG: Number of germinated MS per g of CMS-200 after 1, 7 or 14 days of exposure to each treatment (MS per g of CMS-200). For each treatment and exposure period combination, values represent the means of three replicated samples of CMS-200 ± standard errors of the mean. In each column, means followed by a common letter do not differ significantly according to Fisher's protected LSD test ($P = 0.05$).

Verticillium wilt development

Symptoms of Verticillium wilt disease were only observed in inoculated plants; therefore, non-inoculated plants were excluded from this analysis. In inoculated plants, disease symptoms developed from 6 weeks to 16 weeks after inoculation, after which disease progress was stopped. All disease-related parameters analysed in the inoculated

plants showed significant differences ($P < 0.05$) between treatments (Table 3). Plants treated with ↓K showed the highest RAUDPC values (147.0 ± 11.9%), followed by those treated with ↑NaCl (119.6 ± 9.0%), with both values being significantly higher than those observed in the plants under the Control N-K treatment. However, plants treated with ↑N+↑Na or ↑N+↑Na+↓K showed significantly lower RAUDPC values than those observed in the plants under the Control N-K treatment. A similar pattern was observed for RDS since plants treated with ↑NaCl showed the highest RDS values (122.7 ± 27.4%) followed by those treated with ↓K (100.0 ± 4.6%). In this case, plants treated with ↑N+↑Na or ↑N+↑Na+↓K showed higher, but not significantly different, values of RDS than those observed in the plants under the Control N-K treatment. Conversely, plants treated with ↓K showed the highest incidence (75.0%), while in the remaining treatments, the results were similar to that observed in the plants under the Control N-K treatment. Regarding plant mortality, no significant differences between treated plants were observed, with ↑NaCl showing the lowest mortality throughout the experiment (8.3%) (Table 6.3).

Table 6.3. Disease-related parameters for olive plants treated and inoculated with *V. dahliae* isolate V323[a].

Treatment	Incidence (%)[b]	Mortality (%)[b]	RDS (%)[c,e]	RAUDPC (%)[d,e]
Control N-K	58.3 ab	16.7 ab	69.3 ± 5.3 b	108.7 ± 14.7 b
↑N+↑Na	58.3 ab	25.0 a	80.0 ± 12.2 ab	63.4 ± 7.2 c
↑NaCl	66.7 ab	8.3 b	122.7 ± 27.4 a	119.6 ± 9.0 ab
↓K	75.0 a	25.0 a	100.0 ± 4.6 ab	147.0 ± 11.9 a
↑N+↑Na+↓K	50.0 b	25.0 a	85.3 ± 14.1 ab	89.9 ± 5.9 bc
Water (+)[f]	66.7 ab	16.7 ab	100.0 ± 10.6 ab	100.0 ± 22.4 b
Water (-)[g]	0.0 c	0.0 c	0.0 ± 0.0 c	0.0 ± 0.0 d
P-value $_{(\alpha = 0.05)}$	≤ 0.0001	≤ 0.0001	0.0014	≤ 0.0001

[a]Healthy olive plants were grown in a substrate infested with a sterile cornmeal-sand mixture (CMS; sand, cornmeal and distilled water; 9:1:2, weight:weight:volume) colonized by *V. dahliae* isolate V323 and adjusted at a final theoretical inoculum density of 10^7 CFU g^{-1}. Disease-related parameters were assessed weekly for 16 weeks after inoculation with *V. dahliae*.

[b]Percentage of symptomatic plants (Incidence) or dead plants (Mortality) at 16 weeks after planting in the infested substrate with *V. dahliae* isolate V323 ($n = 24$). In each column, mean values followed by a common letter do not differ significantly according to the multiple comparisons for proportions test [28] at $\alpha = 0.05$.

[c]RDS: Relative disease severity

[d]RAUDPC: relative area under the disease progress curve.

[e]RDS, RAUDPC estimated as the relative percentage (%) to disease parameter values of the inoculated plants treated with only DDW [Water (+)] at the end of the experiment. Mean values represent the average of two experiments with 3 blocks and 4 replicated plants per block for each treatment ± standard error of the mean. In each column, mean values followed by a common letter do not differ significantly according to Fisher's protected LSD test ($P = 0.05$).

[f]Water (+): non-treated and inoculated plants.

[g]Water (-): non-treated and non-inoculated plants.

There were no significant linear correlations ($r < 0.8113$, $P > 0.05$) between the disease-related parameters and MSG at 14 days of exposure.

Regarding the effect of treatments on shoot growth, significant differences were observed only for the independent variable 'treatments' ($P = 0.0280$) and not for the independent variable 'inoculation' ($P = 0.1160$) or for the interaction 'treatments' × 'inoculation' ($P = 0.0629$). Therefore, values of 'growth' from both inoculated and non-inoculated plants were averaged for each treatment. To conduct further analysis, plants treated with water *(17.5 ± 1.9 cm)* or ↑N+↑Na+↓K *(15.7 ± 1.8 cm)* showed significantly lower increases in apical shoot length than those treated with the remaining treatments (↑N+↑Na = 20.4 ± *1.7*; ↑NaCl = 20.2 ± *1.1*; ↓K = 20.6 ± *2.2;* Control N-K = 24.6 ± 2.8 cm) (Fig. 2). Concerning the effect of treatments on the weight of the apical shoots, the factorial ANOVA did not show significant differences for either of the independent variables tested, i.e., 'treatments' ($P = 0.2493$) or 'inoculation' ($P = 0.5726$), but it did show significant differences for their interaction ($P = 0.0187$). However, all the treated means were grouped in a single group when they were compared by Tukey's HSD test according to the level of the interaction ($n = 12$). Therefore, data were represented assuming the mean values of both lots of inoculated and non-inoculated plants for each treatment. Apical shoot weight values ranged between 9.7 ± 1.6 and 6.3 ± 1.0 g for plants treated with ↑N+↑Na and ↓K, respectively (Fig. 6.2).

Finally, *V. dahliae* was consistently re-isolated from the stems of inoculated plants, with frequencies of isolation ranging from 57.1 to 85.7%. However, the pathogen was not re-isolated from non-inoculated plants. This fact confirmed that plants used for the experiment were initially healthy and indicated the success of the inoculation.

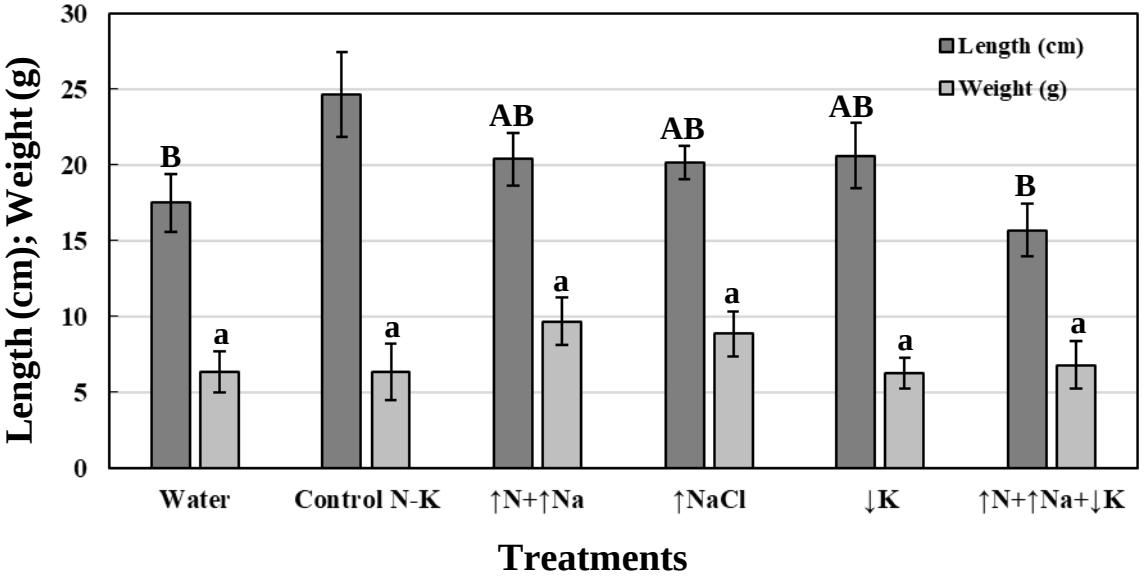

Figure 6.2. Effect of treatments on apical shoot development (length, cm; weight, g) in olive plants. For each dependent variable and treatment, columns represent the mean values of 24 plants, including both inoculated and non-inoculated plants, since no significant differences were found for the inoculation variable or its interaction with the treatments. Columns with the same uppercase or lowercase letter do not differ significantly according to Fisher's protected LSD test ($P = 0.05$) for 'length' and 'weight' variables, respectively. Vertical bars represent the standard errors of the mean.

Effects of root exudates collected from non-inoculated plants grown under different treatments

Viability of *Verticillium dahliae* microsclerotia

In the presence of root exudates from plants treated with water, the MSG was significantly increased (40.5 ± 0.5 MS per g of soil) compared to $CaSO_4$ without root exudates (36.3 ± 1.0 MS per g of soil). However, the MSG was significantly reduced under the presence of root exudates from plants subjected to the different nutrient treatments compared to $CaSO_4$ without exudates, with the exception of ↑NaCl treatment (33.2 ± 1.6 MS per g of soil) (Table 6.4).

Viability of *Verticillium dahliae* conidia

In the presence of root exudates from plants treated with Control N-K, ↑NaCl, and ↑N+↑Na+↓K, the CG was increased significantly compared to $CaSO_4$ without exudates. Conversely, significantly lower values of GC corresponding to the Water, ↑N+↑Na, and ↓K treatments were detected (Table 6.4).

Table 6.4. Effects of root exudates[a] from non-inoculated plants on conidia germination of *V. dahliae* isolate V323 and microsclerotia viability in naturally infested soil samples.

Treatments	CG (%)[b,d]	MSG (MS per g of soil[c,d])
Water	83.0 ± 1.0 c	40.5 ± 0.5 a
Control N-K	88.0 ± 0.6 b	32.5 ± 1.6 c
↑N+↑Na	85.8 ± 2.3 bc	27.9 ± 0.3 d
↑NaCl	93.5 ± 2.4 a	33.2 ± 1.6 bc
↓K	82.4 ± 0.6 c	28.4 ± 1.1 d
↑N+↑Na+↓K	88.0 ± 0.8 b	25.9 ± 0.9 d
$CaSO_4$ (-)[e]	81.2 ± 1.9 c	36.3 ± 1.0 b
P-value $_{(\alpha = 0.05)}$	0.0021	0.0003

[a]Root exudates were collected by dipping the plant roots in 0.01 M $CaSO_4$ solution for 4 h.
[b]CG: percentage (%) of germinated conidia after incubation in the root exudates at 24°C for 24 h in darkness. Values represent the means of two experiments with five replicate cover slides each ± standard errors of the mean.
[c]MSG: Number of germinated MS per g soil after 72 h of exposure to the root exudates. Values represent the means of two experiments with three replicate soil subsamples each ± standard errors of the mean.
[d]In each column, means followed by the same letter do not differ significantly according to Fisher's LSD test ($P < 0.05$).
[e]$CaSO_4$ (-): $CaSO_4$ solution (0.01 M) without root exudates was used as the negative control in this experiment.

Finally, there were no significant linear correlations between the effect of root exudates from treated plants on the CG and MSG ($r = -0.1037$, $P = 0.8450$) as well as between these two parameters and the MSG at 14 days of exposure or any of the disease-related parameters ($r < 0.5447$, $P > 0.05$).

DISCUSSION

The effect of mineral nutrition on wilt diseases has been already reported in a wide diversity of herbaceous hosts/Verticillium pathosystems (Pegg and Brady, 2002). In this regard, it is generally understood that N excess or K deficiency tends to increase or decrease, respectively, the incidence of plant diseases, including Verticillium wilts (Pegg and Brady, 2002; Prabhu *et al.*, 2007). However, other aspects such as stage of plant growth or pathogen activity, form of the elements (reduced or oxidized forms), soil conditions, and interactions with other elements can influence the plant response against diseases (Pegg and Brady, 2002; Huber and Thompson, 2007). In fact, several authors have reported that high levels of N increased *V. dahliae* susceptibility in

aubergine, cotton, potato, and tomato, whereas others showed opposite results in these same hosts, i.e., low levels of N increased the susceptibility and high levels of N reduced it (Pegg and Brady, 2002). Concerning the effect of K deficiency on plant diseases, it is well documented that plants under K starvation are more susceptible than plants under optimum K nutrition, indicating that K affects host resistance more than it directly affects the pathogen (Pegg and Brady, 2002; Prabhu *et al.*, 2007). However, the influence of mineral nutrition on VWO is still poorly studied. Despite the fact that several field observations conducted in southern Spain reveal that N excess, or more probably, imbalances of N excess and K deficiency could be related to a higher incidence and severity of the disease in the affected olive groves, this phenomenon has not yet been demonstrated. Therefore, the present work was conceived to shed light on the effects of nutritional imbalances of N and K on the infection of *V. dahliae* in olive trees.

In this study, an *in vitro* test was first conducted to determine both the effects of N-K imbalances and the influence of the exposure period to each treatment on the MSG of *V. dahliae*. Considering the 14-day exposure period, ↑NaCl and ↓K increased the MSG as the exposure period increased; whereas ↑N+↑Na and ↑N+↑Na+↓K significantly reduced the MSG compared to the Water and Control N-K treatments, the effect of N being predominant compared to that of Na in all cases.

Considering the effect of N *in planta*, plants subjected to N excess or N-K imbalances showed lower RAUDPC compared to both Control N-K and Water treatments, with only significant differences for N excess. However, non-significant differences between them were observed for RDS. The effect of N excess on VWO was not only unclear, but also opposite to our previous hypothesis. Therefore, the reduction of MSG of *V. dahliae* and the lower values of RAUDPC in olive plants treated with ↑N+↑Na or ↑N+↑Na+↓K under our experimental conditions may have been greatly influenced by the N forms used in this study. Indeed, direct effects of N ions (e.g., NO_3^-, NH_4^+) have been reported by reducing the number of the propagules of *V. dahliae* (Pegg and Brady, 2002).

The effect of K deficiency in increasing MSG was in concordance with the effect observed *in planta* since plants subjected to this treatment showed significantly higher RAUDPC and RDS compared to the Control N-K and Water treatments.

Accordingly, previous studies conducted in cotton demonstrated that susceptible cultivars to *V. dahliae* showed lower tolerance to wilt when they were grown in K-deficient soils (Pegg and Brady, 2002). Thus, the effect of K-fertilization in reducing the severity of wilt diseases has only been considered beneficial in K-deficient soils (Prabhu *et al.*, 2007). Moreover, it has been demonstrated that K controls many physiological and metabolic processes and acts as an enzyme activator, enhancing several signalling cascades similar to biotic stress responses, which could affect the host resistance against pathogens (Guardiola and García-Luis, 1990; Pegg and Brady, 2002; Prabhu *et al.*, 2007; Pettigrew, 2008; Hafsi *et al.*, 2017; Fontana *et al.*, 2020). Regarding this last aspect, plants subjected to K starvation induce H_2O_2, a reactive oxygen species, increasing the salicylic acid (SA) levels and the biosynthesis of jasmonic acid (JA), rapidly increasing the levels of this hormone (Hafsi *et al.*, 2014). Altogether, these data indicate that K deficiency negatively influences plant tolerance against diseases since the plant response against K starvation involves inducing the signal transduction pathways against environmental stresses, i.e., SA and JA pathways. However, although both SA and JA mediate the activation of plant defence pathways (Llorens *et al.*, 2017b), this host defence response is likely not enough to prevent *V. dahliae* infections in olive when induced as a consequence of K deficiency.

Concerning the N-K imbalances, notice that the effect of N prevails rather than that of K since the treatments with N excess or N-K imbalances decrease MSG or the disease progress, whereas treatments with K deficiency alone increase both parameters. Interestingly, in spite of the Na excess treatment that was included as a technical control in this study, a similar behaviour to that observed for K deficiency was shown, i.e., the MSG and RAUDPC were higher compared to the Control N-K treatment. These observations are probably due to the phytotoxic effect that NaCl excess has *per se* or because NaCl induces K deficiency, affecting the host resistance, as described previously (Guardiola and García-Luis, 1990; Hafsi *et al.*, 2017).

The non-significant effect on the plant growth development between treatments could be due to the short periods that the plants were subjected to the treatments under our experimental conditions. Although more time would be needed to evaluate the effect of mineral nutrition on plant growth, the experimental procedure in this study took into account that the experiments must concluded when the disease reached the maximum levels of severity, making it impossible to extend the evaluations. Moreover,

the non-significant differences on plant weight between inoculated and non-inoculated plants was probably due to the fact that the wilt symptoms developed late, at the end of the experiment, though the disease progressed rapidly afterwards.

Finally, the study on the effect of root exudates collected from the treated plants on the viability of conidia and MS of *V. dahliae* was conducted here because little information is currently available in the literature on this topic. Our results indicated that root exudates from treated plants reduced the germination of MS compared to those from plants treated with only Water, but they generally increased the germination of conidia. Recently, López-Moral *et al.* (2021a) demonstrated that root exudates from olive of cv. Picual significantly induced the germination of conidia and MS of *V. dahliae* compared to the control without root exudates. Thus, our results suggest that nutritional treatments can have an indirect effect on pathogen infection by reducing the ability of root exudates to stimulate the germination of MS of *V. dahliae*.

Considering all our results together, we can confirm that disease development depends on factors that may affect the plant, the pathogen or their interaction. These effects may also act at different stages of the life cycle of the pathogen, such as in the germination of MS, the infection process and colonization of the plant or enhancing the mechanisms of plant defence. In this complex system, nutrient inputs can have an effect on different processes, and it would be interesting to learn more about these. Nevertheless, little attention has been given to understanding the relationships between K and other nutrients such as N in relation to plant diseases and to understanding the mechanisms by which N and K influence diseases. Likewise, this work attempts to provide new knowledge, studying not only the effect of mineral nutrition on disease development, but also their direct effect on the propagules of *V. dahliae* and the indirect effect through possible modifications in root exudates affecting the first stages of the infection. However, we must remember to consider the conclusions of this study as a first approach, opening up new paths to be explored towards a better understanding of how mineral nutrition affects VWO development, e.g., comparing both NO_3^- and NH_4^+ forms of N, even more K-deficient treatments, the effect of NaCl, and longer exposure to treatments. Altogether, these findings will generate interesting knowledge in applied plant pathology and agronomy sciences within the framework of integrated disease management in terms of cultural practices towards the control of VWO through mineral nutrition management.

CONCLUSIONS

N excess or N-K imbalances decrease the germination of *V. dahliae* MS or the disease progression in olive plants inoculated with the pathogen, whereas K deficiency increases both parameters. Therefore, the effect of N excess on MS germination or on the disease progress predominates rather than the effect of K deficiency. Although Na excess was included as a technical control in this study, a similar behaviour to that noted for K deficiency was observed. Finally, root exudates from treated plants induced conidia germination compared to the Control N-K treatment but reduced the MS germination. This last fact could be due to an indirect effect caused by the mineral nutrition state in the plant, which should be studied more closely in the future. This study reveals that balanced nutrition must be considered as an important factor in plant resistance to diseases, opening up new paths to be explored towards a better understanding of how mineral nutrition affects VWO development, such as comparing both NO_3^- and NH_4^+ forms of N, even more K deficiency treatments, the effect of NaCl, and longer exposure to treatments.

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Chapter 7

Ave1-tolerant bacteria from tomato microbiome as biocontrol agents against *Verticillium dahliae*

ABSTRACT

The soil-borne pathogen *Verticillium dahliae* is able to manipulate the host microbiome by means of the VdAve1 effector protein towards facilitate disease establishment. Considering that microbiome plays a key role in plant defense by influencing the outcomes of plant-pathogen interactions, we hypothesize that some bacterial communities could be potential antagonistic agents against *V. dahliae*. Thus, evolving the antagonistic bacteria towards VdAve1 protein tolerance, they could be used to the biocontrol of the pathogen, providing plant protection. To this end, here we characterized the bacterial microbiome of tomato plants from both endosphere and rhizosphere tissues to screen for antagonistic bacteria against *V. dahliae* towards to obtain potential biological control agents (BCAs), which will be evolved tolerant to the VdAve1 effector protein. A total of 229 and 149 bacterial strains were collected from roots and stems of organic and nonorganic tomato plant, respectively. The bacterial communities from the rhizosphere showed highest diversity and antagonistic activity against the pathogen that those from the endosphere. In root samples, *Enterobacter* and *Pseudomonas* were the most frequent genera in organic plant; and *Microbacterium* (42.4%) and *Pseudomonas* (27.1%) in nonorganic plants. In stem samples, *Enterobacter* (17.2%) was the most frequent in organic plants, while *Herbaspirillum* sp. was the only bacteria identified in nonorganic plant. More than 60% of the bacterial strains from roots showed antagonistic effect, but only 13 resulted in reducing the *V. dahliae* growth higher than 50%, with five of them showing a slight sensitivity to VdAve1. Although further research is needed to complete this study in the near future, through this work, we will provide an important data resource to go ahead in generate targeted BCAs to *V. dahliae*.

Keywords: bacterial communities, biological control, plant-pathogen interactions, soil-borne pathogen, *Solanum lycopersicum*, Verticillium wilt

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INTRODUCTION

Plant microbiota comprises a broad diversity of microbes that together with their genomes and the environment, compound the plant microbiome (Marchesi and Ravel, 2015). The plant microbiome plays a key role in plant defense since it is able to influence the outcomes of plant-pathogen interactions (Yuan *et al.*, 2018). The protection of plant microbiome against pathogens infections involves both direct and indirect mechanisms. Therefore, beneficial microbes are able to directly suppress the pathogen by means of niche competition and antibiosis (Shalev, 2021) as well as by antagonism plant pathogens through the production of antimicrobial compounds (Helfrich *et al.*, 2018). On the other hand, the indirect mechanism of action of the beneficial microbes to pathogen inhibition involves the activation of the host immune system, which systemically enhances plant responses against biotic stresses (Mauch-Mani *et al.*, 2017; Pieterse *et al.*, 2014). Therefore, the plant microbiome can expand the plant capacity to withstand pathogen infection and colonization, with the plant associated microbial community being an additional barrier for invading pathogens.

Plant pathogens establish diseases by secreting a wide diversity of effector proteins, which are generally regarded as small, cysteine-rich, positively charged proteins (Rovenich *et al.*, 2014). Effector proteins are secreted after pathogen colonization in order to manipulate host physiology or to alter the host immune response (Stergiopolus and de Wit, 2009). Indeed, effectors have been typically regarded as proteins involved in the suppression of the host immune responses. In addition, considering the importance of the plant microbiome in the outcome of plant-pathogen interactions, it has been demonstrated that plant pathogens involve the effector proteins to evolve mechanisms to manipulate host microbiomes (Rovenich *et al.*, 2014; Snelders *et al.*, 2018; 2020a). In particular, microbiome-manipulation by effector proteins has been demonstrated for *Verticillium dahliae*, a soil-borne fungal pathogen that cause vascular wilt diseases on a broad diversity of plant species (Fradin and Thomma, 2006; Klosterman *et al.*, 2009; Snelders *et al.*, 2020a). *Verticillium dahliae* exploits life-stage dependent effector proteins to manipulate the local microbiome and suppress microbial antagonists. Likewise, recent studies revealed that the antimicrobial *V. dahliae* effector protein Ave1 (VdAve1) promotes host colonization through the manipulation of host microbiome composition. The VdAve1 is secreted during host

colonization as a virulence factor strongly antagonizing Sphingomonadales bacteria as well as supressing saprophytic growth in soil (Snelders *et al.*, 2020b).

The lifecycle of *V. dahliae* includes dormant, parasitic, and saprophytic stages. The pathogen develops microsclerotia (MS), which are dormant structures that confers its ability to survive in the soil for a long time, germinating in response to root exudates released by nearby plants (Mol and van Riessen, 1995). Then, emerging infective hyphae growth through the soil and rhizosphere and penetrate the plant roots growing towards the vascular system. Subsequently, the pathogen invades the xylem where it produces conidia that are spread throughout the vasculature by the sap stream. This systematic colonization causes the reduction of water flow, leads to water stress, and consequently, plants become wilted, showing chlorosis and necrosis of plant tissues (Ayres 1978; Pegg and Brady 2002; Fradin and Thomma, 2006).

The longevity of microsclerotia in soil that confers *V. dahliae* to survive for up to 14 years in the soil, added to the broad host range of this pathogen and the absence of effective fungicides to protect the plants once the fungus entered the xylem make its control difficult. For all these reasons, the management strategies against Verticillium wilt must to consider either complete removal of the infected plants, the usage of resistant cultivars when available for a particular crop, inoculum reduction, as well as biocontrol strategies (Yuan *et al.*, 2018; Fradin and Thomma, 2006).

Searching to novel biocontrol strategies, the modification of the plant microbiome has been described as a promising strategy to improve plant performance by increasing nutrient uptake or providing pathogen protection (del Carmen Orozco-Mosqueda *et al.*, 2020). Due to above-ground as well as below-ground plants organs are colonized by beneficial microorganisms from different taxonomic groups that are able to alleviates the pathogen impact (Mendes *et al.*, 2018), we have to consider that microbiome-mediated disease suppression is not only restricted to the rhizosphere, but also the phyllosphere play a key role in the protective action by means of its microbiome (Vogel *et al.*, 2021). Interestingly, a recent study conducted by Dong *et al.* (2019), whom sequenced different tissues of tomato plants grown in greenhouse conditions, showed that the bulk soil as well as rhizosphere soil harbor significantly richer and more diverse bacterial communities than endospheric or phyllospheric samples. In addition, as we indicated above, *V. dahliae* is able to manipulate the host

microbiome by means of the VdAve1 effector to antagonize *Sphingomonadales* bacteria towards facilitate disease establishment (Snelders *et al.*, 2020a). Therefore, we hypothesize that evolving the antagonistic bacteria towards VdAve1 protein tolerance, they could be used to the biocontrol of the pathogen, providing plant protection. Thus, the main objective of this study was to characterize the bacterial microbiome of tomato plants from both endosphere and rhizosphere tissues to screen for antagonistic bacteria against *V. dahliae* towards to obtain potential biological control agents (BCAs) by evolving them tolerant to the VdAve1 effector protein.

MATERIAL AND METHODS

Isolation and identification of bacterial microbiome from tomato plants

Bacterial isolation

The bacterial microbiome was obtained from stems and roots of organic and nonorganic tomato (*Solanum lycopersicum* L.) plants grown in a commercial nursery. Samples of each type of plant and tissue were frozen in case it was needed for further analysis, while the rest of the plant material followed the isolation process. For bacterial isolation, six 0.5 cm long segments of each sample were introduced into 2 mL tubes with 200 µL of 100 mM $MgCl_2$ and a pair of metal beads. To grind the samples, the tubes were placed in a tissue lyzer (TissueLyser II, Qiagen, Hilden Germany) for 3 × 45s at 30 Hz, switching their orientation every 45 s. Subsequently, serial dilutions (1:10, 1:100 and 1:1000) were prepared in 100 mM $MgCl_2$ and 30 µL of each dilution were plated on plates containing lysogeny broth agar (LBA; Merck, Darmstadt, Germany), tryptone soya agar (TSA; Merck, Darmstadt, Germany), or Reason´s 2A agar (R2A; Merck, Darmstadt, Germany), supplemented with 100 µg/mL of cycloheximide to prevent fungal growth, and/or supplemented with 50 µg/mL of kanamycin, 50 µg/mL of gentamycin or the combination of both antibiotics. Plates were incubated at 22ºC and regularly observed for bacterial growth for 96 hours. Bacteria colonies growing were transferred to fresh agar plates. Subsequently, single colonies were selected and grown on agar plates to obtain purified bacterial strains, which were eventually cryopreserved with 25% glycerol at -80°C.

Molecular identification of bacterial strains

The bacterial strains were characterized using gen 16S rDNA molecular identification. First, a sample of each bacterial strain grown in TSA was transferred using a sterile 5 µL plastic loop to a sterile 2 mL tube containing 1 mL of 100 mM $MgCl_2$ and shaken for 5 min. Genomic DNA was extracted from the bacterial dilution using the alkaline lysis methods described by Zhang *et al.* (2021). To this end, an aliquot of 16.6 µL of lysis buffer (25 mM NaOH and 0.2 mM Na_2-EDTA at pH 12) was added to 10 µL of each bacterial dilution in a 96-well PCR plate. Next, plates were incubated in a C1000 Touch Thermal Cycler (Bio-Rad, CA, USA) at 95ºC for 30 min. Following 15 min of incubation of plates at room temperature, 16.6 µL of neutralization buffer (40 mM Tris-HCl at pH 7.5) were added in each well and plates were stored at -20ºC. Subsequently, 16S rRNA genes were amplified by PCR with primer pair 27F CM/1492R. Reactions were prepared containing 4 µL 5× Phusion buffer GC, 0.4 µL 10 mM dNTPs, 0.2 µL Phusion HF polymerase (0.4U, Finnzymes), 0.5 µL of each primer (10 µM), 1 µL of template and 13.4 µL sterile ultrapure water. Initial denaturation was at 98ºC for 30 s, followed by 30 cycles of denaturation at 98ºC for 10 s, annealing at 56ºC for 30 s and extension at 72ºC for 45 s (Schulze-Schweifing *et al.*, 2014). Eventually, a final extension at 72ºC for 8 min was applied. The resulting amplicons were sent for Sanger sequencing to Microsynth Seqlab GmbH (Cologne, Germany). The 16S rRNA sequences were then compared against the GenBank database using the NCBI BLAST nucleotide search.

In addition, genomic DNA from plant material was obtained using PowerSoil Kit (Qiagen, Hilden, Germany) to determine the relative abundance of bacterial phyla in the root and stem microbiome of tomato plants by 16S rDNA profiling (Snelders *et al.*, 2020a).

In vitro effect of bacterial strains against *V. dahliae*

V. dahliae strains

Two *V. dahliae* strains were used in this study: a wild-type strain (JR2) and a deletion mutant strain for the VdAve1 protein gene (*VdAve1* deletion mutant) (Snelders *et al.*, 2020a). These *V. dahliae* strains are stored in 25% glycerol stock at -80ºC in the cryogenic collection of microbial strains of the Evolutionary Microbiology Group at the Institute of Plant Sciences (University of Cologne, Germany).

Dual culture assays

All bacteria strains were tested for antagonism against the *V. dahliae* strain JR2, using dual-culture assays (Weiner *et al.*, 2010). To this end, bacterial strains were grown on TSA at 28°C for 24h. Then, single colonies were selected and grown overnight in 10 mL of tryptone soy broth (TSB; Merck, Darmstadt, Germany) at 28°C with constant shaking at 200 rpm. Overnight cultures were diluted in 100 mM $MgCl_2$ (1:1, vol:vol). Subsequently, a 5-mm diameter agar plug of *V. dahliae* mycelium was harvested from 2-week-old cultures on potato dextrose agar (PDA; Merck, Darmstadt, Germany) and plated centrally upside down on PDA or TSA plates. Next, a 5 µL drop of overnight culture of 4 different bacterial strains were point-inoculated on the same plate around the plug of *V. dahliae* mycelium. As a negative control, three plates were inoculated only with the *V. dahliae* strain. Each combination of pathogen/antagonist was replicated three times in a randomized complete block design. After 14 days of incubation at 22ºC, the daily rate of mycelial growth (MGR) of *V. dahliae* was determined. In addition, the percentage of mycelial growth inhibition (MGI) was calculated using the following formula:

$$\text{MGI } \% = 100 - ((R_{Vd+B} \times 100) / R_{VdControl})$$

where, R_{Vd+B} is the radius of the *V. dahliae* colony on bacterial-inoculated plates and, $R_{VdControl}$ is the radius of the *V. dahliae* colony on control plates.

Data were tested for normality, homogeneity of variances, and residual patterns. For the dual culture assays, ANOVA was conducted with 'MGR' as the dependent variable and 'Bacterial strains' as the independent variable. For each tissue (stem or root) and type of plant (organic or nonorganic) combination, treatment means were compared by means one-sided Dunnett's Multiple Comparisons test with the positive control at $P = 0.05$. Data were analysed using Statistix 10.0 software (Analytical Software, Tallahassee, USA).

***In vitro* co-cultivation assays**

The bacterial strains showing *in vitro* antagonistic effect against mycelial growth of *V. dahliae* were used for microbial co-cultivation assays. They were grown on TSA at 28°C for 24h. Single colonies were selected and grown overnight in 10 mL of 5% TSB at 28°C with constant shaking at 200 rpm. Overnight cultures were resuspended to an optical density at 600 nm (OD_{600}) of 0.05 in 5% TSB. Subsequently, conidia of *V.*

dahliae strain JR2 and VdAve1 deletion were harvested from 2-week-old cultures on PDA and diluted to the final concentrations of 10^3, 10^4 and 10^5 conidia/mL in sterile deionized distilled water (SDDW). Aliquots of 150 µL of the conidia suspensions were added in clear 24 well flat-bottom polystyrene tissue culture plates. Next, 150 µL of overnight cultures of the bacterial strains were added to the conidial suspensions to a final OD_{600}= 0.05. In addition, 500 µL of both the conidial and bacterial suspensions were aliquoted separately in clear 24 well flat-bottom polystyrene tissue culture plates. Plates were incubated at 22°C and 120 rpm for 48 hours. Following incubation period, *V. dahliae* biomass was first observed using a microscope and eventually quantified through real-time PCR using *V. dahliae* specific primers (ITS1-Fw & St-Ve1-Rv; Snelders *et al.*, 2020a) targeting the internal transcribed spacer (ITS) region of the ribosomal DNA.

Influence of the VdAve1 effector protein on bacterial strains

Production and purification of VdAve1 protein

For heterologous protein production, *E. coli* strain BL21 (transformed strain expressing VdAve1 protein; Snelders *et al.*, 2020a) was grown in 50 mL of YT medium (4 g/L Tryptone, 2.5 g/L Yeast Extract, 1.25 g/L NaCl) supplemented with 100 µg/ml of ampicillin antibiotic (AMP) at 37ºC with constant shaking at 200 rpm overnight. Next, the 50 mL was poured in a 5L flask containing 1L of YT (with AMP) and incubated at 37ºC in constant shaking at 200 rpm for 4-5 h until reach the $OD_{600} \geq 2.0$. Protein production was induced by addition of 1 mM IPTG final concentration. Following 2 hours of protein production at 42ºC, the bacterial cells were pelleted by centrifugation for 30 min at 4600 rpm, snap-frozen in liquid nitrogen and stored at -20ºC until the purification process.

For VdAve1 protein purification, the pellet was resuspended in 40 mL of 10 mM Tris, 6 M Guanidium chloride, 10 mM β-mercaptoethanol at pH 8.0 and maintained at 5ºC overnight. Subsequently, the sample was centrifuged for 10 min at 12,000 rpm to pellet the insoluble debris, selecting only the supernatant to continue the purification process. VdAve1 protein was purified under denaturing conditions by metal affinity chromatography using a column packed with 50% His60 Ni2+ Superflow Resin (Clontech, Mountain View, CA, USA). The purified effector protein was dialysed (Spectra/Por®3 Dialysis Membrane, MWCO= 3.5 kDa) step-wise in 20 volumes of 0.25

M ammonium sulfate, 0.1 M BisTris, 10 mM reduced glutathione, 2 mM oxidized glutathione, pH 5.5 with decreasing GnHCl concentrations for refolding. Each dialysis step was allowed to proceed for at least 24 hours. Finally, proteins were dialysed against DDW. Final protein concentration was determined using the Bradford method. The bovine serum albumin protein (BSA protein) was used for the calibration curve.

Sensitivity testing of bacterial strains to VdAve1 protein

Bacterial strains with a high antagonistic effect in *in vitro* assays were selected to evaluate their sensitivity to VdAve1 protein following the protocol described by Snelders *et al*. (2020a). Bacterial strains were grown on LBA or TSA at 28°C. Single colonies were selected and grown overnight in 10 mL of lysogeny broth (LB; Merck, Darmstadt, Germany) or TSB at 28°C with constant shaking at 200 rpm. Overnight cultures were resuspended to OD_{600}= 0.05 in TSB or LB. While VdAve1 protein was diluted in SDDW to 16 µM. Then, 100 µL of the protein dilution was aliquoted in clear 96 well flat bottom polystyrene tissue culture plates. Subsequently, 100 µL of bacterial suspension was added, obtaining so a final VdAve1 concentration of 8µM, and final OD_{600} of 0.025. Plates were incubated in a CLARIOstar® plate reader at 25°C with double orbital shaking every 15 minutes (10 seconds at 300 rpm). The optical density was measured every 15 minutes at 600 nm.

Evolving bacterial strains towards VdAve1 protein tolerance

The bacterial strains effective in reducing *V. dahliae* growth and sensitive to VdAve1 were used to obtain mutants insensitive to the effector protein. For this purpose, the generation of tolerant bacterial strains were carried out through continued exposure to VdAve1 with a gradual increase in concentration in an evolution experiment as described Snelders *et al.* (2020b). The selected bacterial strains were grown on LBA at 28°C for 24h. Then, single colonies were selected and grown overnight in 10 mL of low-salt LB (8.5 mM NaCl) at 28°C with constant shaking at 160 rpm. Next, a sample of the culture was inoculated to into fresh growth medium supplemented with VdAve1. Subsequently, a subsample of the culture was transferred to fresh medium with VdAve1 every time when the stationary phase of the culture was reached ($OD_{600}\geq$ 2.0), amounting to a total of six transfers. While the initial VdAve1 concentration to which the culture was exposed was 4 µM, every second transfer the VdAve1 concentration was increased with 4 µM such that after six transfers the bacteria

grew in 12 µM. Following the last transfer into 12 µM VdAve1, the liquid culture was plated on agar medium devoid of VdAve1 and five colonies were selected and tested for increased tolerance to VdAve1 by following their growth in medium containing 6.5 µM VdAve1 as described before. The isolates did not display impaired growth in the presence of this concentration were selected for their evaluation as biocontrol agents in *in plant* assays (Snelders *et al.*, 2020b).

Evaluation of the biocontrol effect of VdAve1-tolerant bacterial strains against *V. dahliae in planta* competition assay

Bacterial strains previously evolved to be tolerant to Ave1 protein were used to elucidate their effect as biocontrol agents against Verticillium wilt in tomato plants. For this, to allow bacterial colonization of in the absence of other microbes, tomato seeds (cv. Money Maker) were incubated for five minutes in 2% sodium hypochlorite and washed three times using in SDDW. Subsequently, tomato seeds were transferred to a sterile Petri dish containing a sterile filter paper pre-moistened with bacterial suspension in SDDW (OD_{600}= 0.05) (Snelders *et al.*, 2020a). Furthermore, seeds colonized with a strain of *Sphingopyxis macrogoltabida* were included as a positive control (Snelders *et al.*, 2020a), as well as seeds treated with SDDW as a negative control. The tomato seeds were allowed to germinate *in vitro* for 5-7 days and eventually transferred to regular potting soil contained in 9 cm diameter pots. Finally, 10-day-old plants were uprooted and the roots were rinsed in water. Subsequently, the roots were dipped for 3 min in a suspension of 10^6 conidia/mL of the pathogen (JR2 and VdAve1 *V. dahliae* strains). In addition, plants were root-dipping with SDDW were included as a control (Fradin *et al.*, 2009). Finally, the plants were transferred back to the pots containing regular potting soil.

For phenotyping, stunting (%) in both inoculated and inoculated bacteria-treated plants in related to non-inoculated plants was calculated based on plant canopy area at 21 days post inoculation (dpi) as described Hanika *et al.* (2021). Next, tomato stems were collected and lyophilized prior to genomic DNA isolation with a CTAB-based extraction buffer (100 mM Tris-HCl pH 8.0, 20 mM EDTA, 2 M NaCl, 3% CTAB). *V. dahliae* biomass was quantified with real-time PCR on the genomic DNA by targeting the internal transcribed spacer (ITS) region of the ribosomal DNA. The tomato rubisco gene was used for sample calibration.

RESULTS AND DISCUSSION

A total of 229 and 149 bacterial strains were collected from organic and nonorganic tomato plant, respectively. In organic plant, 155 strains were obtained from roots and 74 from stems; while in nonorganic plant, 148 strains came from roots and only one from stems. Due to the low frequency of bacterial isolation from stem of nonorganic plant, the isolation process was repeated twice using the original frozen plant material, always obtaining the same result in any cases. This low frequency could be to chemical treatments applied to the plant just before marketing. In addition, our results are in concordance with those obtained by Dong *et al*. (2019), whom indicated that the bacterial communities from the rhizosphere of tomato plants results in highest richness and diversity, whereas the lowest bacterial diversity occurs in the phyllosphere and the lowest richness in the endosphere.

At this stage, all the bacterial strains have been evaluated by dual cultures *in vitro* against the *V. dahliae* strain JR2 to determine their antagonistic effect. Among them, 61.3% of strains from roots of organic plant significantly reduced the MG of *V. dahliae*, but only 3.2% of them caused an IMG higher than 50%. A similar pattern was observed for bacterial strains from roots of nonorganic plant, with 65.5% of strains with antagonistic effect and 5.4% of them being highly effective against the pathogen (IMG> 50%). However, bacterial strains from stems showed less antagonistic effect, not exceeding 50% IMG of *V. dahliae* in any case (Tables 7.1 and 7.2).

On the other hand, the 13 bacterial strains that caused an IMG of *V. dahliae* higher than 50% were evaluated by co-cultivation assays. Microscope observations showed that these strains were also able to reduce pathogen biomass in liquid culture (Fig. 7.1). However, it is necessary to evaluate the rest of the bacterial strains and quantify the biomass of *V. dahliae* using qPCR technique. In addition, these same strains were tested against VdAve1, with 5 out of 13 strains showing a slight sensitivity to the protein (Fig. 7.2). Nevertheless, further research is needed not only to complete the sensitivity evaluation of all bacterial strains to VdAve1, but also to evaluate their effect by suppressing *V. dahliae* infection in tomato plants.

The molecular identification of 272 out of the 378 bacterial strains collected in this study has been completed. The results obtained at this stage showed that species of the genera *Enterobacter* (30.2%) and *Pseudomonas* (36.2%) were the most frequently isolated from roots of organic plant. For the same plant tissue of nonorganic plant,

Microbacterium (42.4%) and *Pseudomonas* (27.1%) were the dominant genera. On the other hand, bacterial strains from stems of organic plant belonged to a broad diversity of genera, with *Enterobacter* (17.2%) being the most frequent, while the only bacterial strains from stems of nonorganic plant was identified as *Herbaspirillum* sp. (Table 7.3). However, we cannot give strong conclusions on the diversity and richness of bacterial communities from tomato plants until the molecular identification of all bacterial strains will be completed. This step is essential to characterise and compare the microbiome of both organic and inorganic tomato plants.

Finally, the analysis of relative abundance in organic tomato plant showed that a higher diversity of bacterial phylum was obtained using DNA from plant material compared to that obtained from cultures on agar plates (Fig. 7.3).

In summary, a diverse and rich collection of bacterial strains has been collected from tomato plants, with all they already evaluated *in vitro* for their antagonistic effect against *V. dahliae* and most of them identified at the species level by sequencing 16S rRNA. The results obtained from dual cultures as well as those from co-cultivation assays promises that some potentially antagonistic bacterial strains have been isolated. In addition, the bacterial strains with the highest antagonistic effect could be sensitive to the VdAve1 protein, and they could become potentially BCAs after evolve them VdAve1 tolerant (Snelder *et al.*, 2020a). The mutation of bacterial strains and their exact functions in tomato health against *V. dahliae* infections will be studied in the near future. Altogether will provide an important data resource to go ahead in looking for specific potentially BCAs to *V. dahliae*, which is a serious problem for farmers worldwide.

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Table 7.1. *In vitro* effect of the bacterial strains on mycelial growth of *V. dahliae* strain JR2.

Organic tomato plant / Root								
Strain ID	**MGR (mm/day)[a,b]**	**MGI (%)[a]**	**Strain ID**	**MGR (mm/day)**	**MGI (%)**	**Strain ID**	**MGR (mm/day)**	**MGI (%)**
R1	1.64 ± 0.18	6.8 ± 1.2	**R53**	1.60 ± 0.09	9.5 ± 2.9	**R105**	0.85 ± 0.03*	52.0 ±1.8
R2	1.32 ± 0.02*	25.0 ± 1.2	**R54**	1.73 ± 0.08	2.0 ± 0.4	**R106**	1.57 ± 0.08	10.8 ± 4.7
R3	1.57 ± 0. 04	10.8 ± 2.3	**R55**	1.79 ± 0.07	0.0 ± 0.2	**R107**	1.64 ± 0.04	6.8 ± 2.3
R4	1.15 ± 0.14*	34.5 ± 7.8	**R56**	1.39 ± 0.02*	21.0 ± 1.2	**R108**	1.14 ± 0.02*	35.1 ± 1.2
R5	1.68 ± 0.02	4.7 ± 1.2	**R57**	1.48 ± 0.02	16.2 ± 1.4	**R109**	1.33 ± 0.04*	31.6 ± 3.0
R6	1.32 ± 0.04*	25.0 ± 2.0	**R58**	1.62 ± 0.09	8.1 ± 2.9	**R110**	1.50 ± 0.05	19.3 ± 3.5
R7	1.36 ± 0.04*	23.0 ± 2.3	**R59**	1.42 ± 0.04*	31.8 ± 3.9	**R111**	1.45 ± 0.09	22.8 ± 6.3
R8	1.65 ± 0.03	6.1 ± 1.8	**R60**	1.61 ± 0.05	8.8 ± 1.6	**R112**	1.57 ± 0.06	14.0 ± 4.6
R9	0.92 ± 0.04*	48.0 ± 2.4	**R61**	1.45 ± 0.05	29.6 ± 4.6	**R113**	0.88 ± 0.05*	50.0 ± 2.7
R10	0.92 ± 0.01*	48.0 ± 0.7	**R62**	1.76 ± 0.02	0.90 ± 0.1	**R114**	1.48 ± 0.06	16.2 ± 3.6
R11	0.75 ± 0.05*	57.4 ± 3.1	**R63**	1.52 ± 0.05	13.5 ± 2.7	**R115**	1.69 ± 0.06	4.1 ± 1.6
R12	1.58 ± 0.07	10.1 ± 3.8	**R64**	1.52 ± 0.09	22.7 ± 8.2	**R116**	1.62 ± 0.02	8.1 ± 1.4
R13	1.47 ± 0.05	27.3 ± 4.6	**R65**	1.32 ± 0.06*	25.0 ± 3.5	**R117**	1.38 ± 0.05*	28.1 ± 3.5
R14	1.85 ± 0.03	0.0 ± 0.8	**R66**	1.52 ± 0.17	13.5 ± 4.5	**R118**	1.30 ± 0.02*	33.3 ± 1.8
R15	1.52 ± 0.02	22.7 ± 2.3	**R67**	1.38 ± 0.05*	21.6 ± 2.7	**R119**	1.23 ± 0.02*	38.6 ± 1.8
R16	1.44 ± 0.06	18.2 ± 3.4	**R68**	1.33 ± 0.05*	40.9 ± 4.6	**R120**	1.42 ± 0.06*	24.6 ± 4.6
R17	1.59 ± 0.02	15.9 ± 2.3	**R69**	1.54 ± 0.04	12.8 ± 1.7	**R121**	0.98 ± 0.05*	44.6 ± 2.7
R18	1.39 ± 0.02*	21.0 ± 1.2	**R70**	1.75 ± 0.04	0.68 ± 0.2	**R122**	0.88 ± 0.02*	50.0 ± 1.4
R19	0.80 ± 0.01*	54.7 ± 0.7	**R71**	1.27 ± 0.05*	28.0 ± 2.5	**R123**	1.05 ± 0.06*	40.5 ± 3.6
R20	1.74 ± 0.02	1.4 ± 0.4	**R72**	1.32 ± 0.04*	25.0 ± 1.7	**R124**	0.90 ± 0.02*	48.7 ± 1.4
R21	0.88 ± 0.03*	50.0 ± 1.79	**R73**	1.10 ± 0.13*	37.8 ± 7.5	**R125**	1.24 ± 0.16*	29.7 ± 8.9
R22	1.33 ± 0.02*	24.3 ± 1.35	**R74**	1.33 ± 0.06*	24.3 ± 3.6	**R126**	1.12 ± 0.02*	36.5 ± 1.4
R23	1.05 ± 0.03*	40.5 ± 1.79	**R75**	1.13 ± 0.14*	35.8 ± 8.0	**R127**	1.10 ± 0.09*	37.8 ± 4.9
R24	1.48 ± 0.06	16.2 ± 3.6	**R76**	1.35 ± 0.06*	29.8 ± 4.6	**R128**	1.00 ± 0.04*	43.2 ± 2.3
R25	0.67 ± 0.03*	62.2 ± 1.8	**R77**	1.38 ± 0.06*	21.6 ± 3.6	**R129**	1.24 ± 0.06*	29.7 ± 3.6
R26	1.07 ± 0.02*	39.2 ± 1.0	**R78**	1.21 ± 0.11*	31.1 ± 6.2	**R130**	0.90 ± 0.06*	48.7 ± 3.6

R27	1.12 ± 0.05*	36.5 ± 2.7	**R79**	1.38 ± 0.09*	21.6 ± 4.9	**R131**	1.24 ± 0.02*	29.7 ± 1.4
R28	1.14 ± 0.05*	35.1 ± 3.1	**R80**	1.13 ± 0.04*	35.8 ± 2.4	**R132**	1.14 ± 0.09*	35.1 ± 5.1
R29	1.50 ± 0.18	14.9 ± 1.2	**R81**	1.48 ± 0.20	16.2 ± 1.6	**R133**	1.31 ± 0.02*	25.7 ± 1.4
R30	1.24 ± 0.09*	29.7 ± 4.9	**R82**	1.20 ± 0.15*	31.8 ± 8.5	**R134**	1.12 ± 0.06*	36.5 ± 3.6
R31	1.57 ± 0.06	14.0 ± 6.5	**R83**	1.38 ± 0.09*	21.6 ± 4.9	**R135**	1.08 ± 0.05*	38.5 ± 3.0
R32	1.17 ± 0.09*	33.8 ± 4.7	**R84**	1.24 ± 0.05*	29.7 ± 2.7	**R136**	1.07 ± 0.04*	39.2 ± 2.3
R33	1.06 ± 0.05*	39.9 ± 3.0	**R85**	1.64 ± 0.06	8.8 ± 3.6	**R137**	1.04 ± 0.02*	41.2 ± 1.2
R34	1.08 ± 0.06*	38.5 ± 3.4	**R86**	1.48 ± 0.06	16.2 ± 3.6	**R138**	1.08 ± 0.06*	38.5 ± 3.4
R35	1.04 ± 0.04*	41.2 ± 2.3	**R87**	1.50 ± 0.04	25.0 ± 3.9	**R139**	1.02 ± 0.05*	41.9 ± 2.7
R36	1.46 ± 0.09	16.9 ± 5.4	**R88**	1.83 ± 0.04	0.0 ± 0.0	**R140**	1.02 ± 0.05*	41.9 ± 2.6
R37	1.24 ± 0.05*	29.7 ± 2.7	**R89**	1.45 ± 0.06	29.6 ± 6.0	**R141**	1.43 ± 0.08*	18.9 ± 4.7
R38	1.24 ± 0.02*	29.7 ± 1.4	**R90**	1.48 ± 0.13	16.2 ± 5.2	**R142**	1.31 ± 0.09*	25.7 ± 4.9
R39	1.19 ± 0.06*	32.4 ± 3.6	**R91**	1.52 ± 0.13	13.5 ± 4.2	**R143**	1.40 ± 0.13*	20.3 ± 7.6
R40	1.69 ± 0.02	6.8 ± 2.3	**R92**	1.13 ± 0.07*	35.8 ± 4.1	**R144**	1.52 ± 0.05	17.5 ± 3.5
R41	1.40 ± 0.06*	20.3 ± 3.6	**R93**	1.47 ± 0.07	21.1 ± 5.3	**R145**	1.55 ± 0.10	12.2 ± 5.4
R42	1.47 ± 0.02	27.2 ± 2.3	**R94**	0.89 ± 0.04*	49.3 ± 2.0	**R146**	1.33 ± 0.04*	31.6 ± 3.0
R43	1.35 ± 0.05*	23.7 ± 3.0	**R95**	1.52 ± 0.06	13.5 ± 3.6	**R147**	1.24 ± 0.06*	29.7 ± 3.6
R44	1.32 ± 0.04*	25.0 ± 2.0	**R96**	1.07 ± 0.11*	39.2 ± 3.1	**R148**	1.31 ± 0.06*	25.7 ± 3.6
R45	1.67 ± 0.10	5.4 ± 0.9	**R97**	1.28 ± 0.06*	35.1 ± 4.6	**R149**	1.35 ± 0.01*	23.7 ± 0.7
R46	1.18 ± 0.09*	33.1 ± 5.4	**R98**	1.07 ± 0.04*	39.2 ± 2.3	**R150**	1.52 ± 0.10	13.5 ± 5.9
R47	1.19 ± 0.06*	32.4 ± 3.6	**R99**	1.05 ± 0.02*	40.5 ± 1.4	**R151**	1.59 ± 0.06	15.9 ± 6.0
R48	1.38 ± 0.02*	21.6 ± 1.4	**R100**	1.74 ± 0.02	1.8 ± 0.9	**R152**	1.67 ± 0.02	5.4 ± 1.4
R49	1.56 ± 0.01	11.5 ± 0.7	**R101**	1.21 ± 0.07*	31.1 ± 4.2	**R153**	0.88 ± 0.05*	50.0 ± 2.7
R50	1.57 ± 0.04	18.1 ± 3.9	**R102**	1.50 ± 0.04	14.9 ± 2.3	**R154**	1.29 ± 0.04*	27.0 ± 2.3
R51	1.54 ± 0.06	20.5 ± 6.0	**R103**	1.12 ± 0.013*	36.5 ± 7.5	**R155**	1.10 ± 0.02*	37.8 ± 1.4
R52	1.57 ± 0.04	10.8 ± 2.3	**R104**	0.81 ± 0.005*	54.1 ± 2.7	**Control**	1.76 ± 0.06	-

Organic tomato plant / Stem

Strain ID	MGR (mm/day)[a,b]	MGI (%)[a]	Strain ID	MGR (mm/day)	MGI (%)	Strain ID	MGR (mm/day)	MGI (%)
S1	0.88 ± 0.04*	36.8 ± 3.0	**S26**	0.79 ± 0.04*	43.1 ± 3.0	**S51**	1.07 ± 0.06*	29.6 ± 6.0
S2	1.38 ± 0.17	0.0 ± 0.1	**S27**	1.07 ± 0.04*	22.4 ± 1. 8	**S52**	1.40 ± 0.13	0.0 ± 0.6

S3	1.12 ± 0.04	25.0 ± 3.9	**S28**	1.24 ± 0.06	10.3 ± 4.6	**S53**	1.31 ± 0.10	5.2 ± 0.5
S4	1.12 ± 0.05	19.3 ± 3.5	**S29**	0.93 ± 0.04*	32.8 ± 2.4	**S54**	1.17 ± 0. 09	15.5 ± 3.2
S5	1.04 ± 0.04*	31.8 ± 4.3	**S30**	1.38 ± 0.05	0.0 ± 0.1	**S55**	1.05 ± 0.06*	24.1 ± 4.5
S6	1.31 ± 0.02	6.8 ± 2.3	**S31**	1.38 ± 0.05	0.0 ± 0.1	**S56**	1.24 ± 0.06	14.3 ± 2.5
S7	1.14 ± 0.04	17.2 ± 2.1	**S32**	0.90 ± 0.06*	34.5 ± 3.7	**S57**	1.10 ± 0.09	20.7 ± 5.4
S8	1.52 ± 0.14	2.3 ± 2.3	**S33**	0.83 ± 0.09*	39.7 ± 6.2	**S58**	1.07 ± 0.07*	22.4 ± 5.2
S9	0.79 ± 0.04*	43.1 ± 2.9	**S34**	1.05 ± 0.09*	24.1 ± 5.3	**S59**	0.95 ± 0.06*	31.0 ± 4.6
S10	0.81± 0.05*	41.4 ± 3.5	**S35**	1.02 ± 0.06*	25.9 ± 3. 5	**S60**	1.24 ± 0.06	10.3 ± 2.0
S11	1.02 ± 0.06*	25.9 ± 4.6	**S36**	1.31 ± 0.06	5.2 ± 2.5	**S61**	1.35 ± 0.02	0.0 ± 0.3
S12	1.21 ± 0.04	12.1 ± 3.0	**S37**	1.40 ± 0.04	0.0 ± 0.1	**S62**	1.36 ± 0.07	0.0 ± 0.7
S13	1.33 ± 0.06	3.45 ± 0. 6	**S38**	1.19 ± 0.09	13.8 ± 3.3	**S63**	1.39 ± 0.04	0.0 ± 0.1
S14	1.21 ± 0.04	12.1 ± 3.0	**S39**	1.07 ± 0.06*	22.4 ± 3.0	**S64**	1.07 ± 0.02*	29.6 ± 1.6
S15	1.07 ± 0.05*	29.6 ± 4.6	**S40**	1.19 ± 0.06	13.8 ± 4.6	**S65**	1.14 ± 0.04	17.2 ± 2.8
S16	1.17 ± 0.06	15.5 ± 4.6	**S41**	0.83 ± 0.06*	39.7 ± 5.8	**S66**	1.12 ± 0.06	19.0 ± 4.6
S17	1.24 ± 0.06	10.3 ± 4.6	**S42**	0.79 ± 0.04*	43.1 ± 2.5	**S67**	1.02 ± 0.02*	25.9 ± 1.7
S18	0.88 ± 0.02*	36.2 ± 1.7	**S43**	1.45 ± 0.09	0.0 ± 0.1	**S68**	0.90 ± 0.02*	34.5 ± 2.7
S19	1.10 ± 0.06	20.7 ± 4.7	**S44**	1.40 ± 0.12	0.0 ± 0.0	**S69**	0.98 ± 0.05*	29.3 ± 3.5
S20	1.33 ± 0.02	3.45 ± 0.7	**S45**	1.39 ± 0.05	0.0 ± 0.1	**S70**	0.95 ± 0.02*	31.0 ± 1.8
S21	1.12 ± 0.06	19.0 ± 3.6	**S46**	1.12 ± 0.06	19.0 ± 2.5	**S71**	0.95 ± 0.02*	31.0 ± 5.4
S22	1.43 ± 0.08	0.0 ± 0.2	**S47**	1.14 ± 0.04	17.2 ± 2.4	**S72**	1.02 ± 0.06*	25.9 ± 4.6
S23	1.29 ± 0.04	6.9 ± 1.0	**S48**	0.98 ± 0.02*	29.3 ± 1.7	**S73**	1.28 ± 0.09	9.1 ± 2.2
S24	1.26 ± 0.02	8.6 ± 1.7	**S49**	1.21 ± 0.06	15.9 ± 3.0	**S74**	1.05 ± 0.06*	24.1 ± 5.4
S25	1.31 ± 0.02	5.2 ± 1.7	**S50**	0.69 ± 0.02*	50.0 ± 1.8	**Control**	1.40 ± 0.05	-

Nonorganic tomato plant / Root

Strain ID	**MGR (mm/day)[a,b]**	**MGI (%)[a]**	**Strain ID**	**MGR (mm/day)**	**MGI (%)**	**Strain ID**	**MGR (mm/day)**	**MGI (%)**
Ri1	0.90 ± 0.06*	41.5 ± 4.1	**Ri51**	0.55 ± 0.02*	64.6 ± 1.5	**Ri101**	1.17 ± 0.09	24.6 ± 5.6
Ri2	1.60 ± 0.02	0.0 ± 0.2	**Ri52**	0.93 ± 0.07*	40.0 ± 4.6	**Ri102**	1.05 ± 0.06*	32.3 ± 4.1
Ri3	1.57 ± 0.18	0.0 ± 0.5	**Ri53**	1.31 ± 0.02	17.5 ± 1.8	**Ri103**	1.00 ± 0.04*	35.4 ± 2.7
Ri4	0.90 ± 0.05*	41.5 ± 3.1	**Ri54**	0.71 ± 0.04*	53.9 ± 2.7	**Ri104**	0.86 ± 0.04*	44.6 ± 2.7
Ri5	1.45 ± 0.09	6.2 ± 1.6	**Ri55**	1.10 ± 0.05*	29.2 ± 3.1	**Ri105**	0.98 ± 0.02*	36.9 ± 1.5

Ri6	1.38 ± 0.05	10.8 ± 2.1	**Ri56**	1.05 ± 0.02*	32.3 ± 1.5	**Ri106**	1.02 ± 0.02*	33.9 ± 4.5
Ri7	0.90 ± 0.05*	41.5 ± 3.1	**Ri57**	1.12 ± 0.02*	27.7 ± 1.5	**Ri107**	1.12 ± 0.02*	27.7 ± 1.5
Ri8	0.90 ± 0.02*	41.5 ± 1.5	**Ri58**	1.19 ± 0.06	23.1 ± 4.1	**Ri108**	1.02 ± 0.06*	33.9 ± 4.1
Ri9	1.33 ± 0.04	15.8 ± 3.0	**Ri59**	1.31 ± 0.05	15.4 ± 3.1	**Ri109**	1.10 ± 0.02*	29.2 ± 3.7
Ri10	1.12 ± 0.00*	31.6 ± 0.0	**Ri60**	1.26 ± 0.06	18.5 ± 4.2	**Ri110**	1.02 ± 0.05*	33.9 ± 3.1
Ri11	1.09 ± 0.02*	33.3 ± 1.8	**Ri61**	1.02 ± 0.02*	33.9 ± 3.4	**Ri111**	1.05 ± 0.06*	32.3 ± 4.3
Ri12	0.88 ± 0.06*	49.1 ± 4.6	**Ri62**	1.33 ± 0.05	13.9 ± 2.1	**Ri112**	0.90 ± 0.02*	41.5 ± 2.9
Ri13	0.98 ± 0.06*	36.9 ± 4.1	**Ri63**	1.31 ± 0.02	15.4 ± 1.5	**Ri113**	1.05 ± 0.02*	32.3 ± 5.8
Ri14	0.90 ± 0.09*	41.5 ± 5.6	**Ri64**	1.45 ± 0.02	9.1 ± 2.3	**Ri114**	1.17 ± 0.09	24.6 ± 5.6
Ri15	0.90 ± 0.05*	41.5 ± 3.1	**Ri65**	0.52 ± 0.02*	66.2 ± 3.6	**Ri115**	1.07 ± 0.07*	30.8 ± 3.8
Ri16	1.00 ± 0.04*	35.4 ± 2.7	**Ri66**	1.40 ± 0.06	9.2 ± 2.1	**Ri116**	1.00 ± 0.04*	35.4 ± 2.7
Ri17	0.88 ± 0.02*	43.1 ± 1.5	**Ri67**	1.39 ± 0.04	10.0 ± 1.9	**Ri117**	1.29 ± 0.04	16.9 ± 2.7
Ri18	1.14 ± 0.07	26.2 ± 4.6	**Ri68**	1.24 ± 0.02	20.0 ± 1.5	**Ri118**	1.33 ± 0.05	13.9 ± 3.1
Ri19	1.43 ± 0.11	7.7 ± 1.1	**Ri69**	0.88 ± 0.06*	42.3 ± 5.7	**Ri119**	0.81 ± 0.05*	47.7 ± 3.2
Ri20	1.19 ± 0.10	23.1 ± 6.7	**Ri70**	0.83 ± 0.05*	44.6 ± 3.8	**Ri120**	0.40 ± 0.06*	73.9 ± 4.3
Ri21	0.98 ± 0.09*	36.9 ± 5.6	**Ri71**	0.79 ± 0.04*	46.9 ± 1.9	**Ri121**	0.67 ± 0.02*	56.9 ± 1.5
Ri22	0.90 ± 0.02*	41.5 ± 1.5	**Ri72**	1.07 ± 0.07*	28.5 ± 5.7	**Ri122**	1.52 ± 0.02	1.5 ± 0.7
Ri23	0.95 ± 0.06*	38.5 ± 4.1	**Ri73**	0.76 ± 0.02*	50.8 ± 4.5	**Ri123**	1.26 ± 0.05	18.5 ± 3.1
Ri24	0.95 ± 0.06*	38.5 ± 4.1	**Ri74**	0.81 ± 0.05*	47.7 ± 3.1	**Ri124**	1.45 ± 0.09	6.2 ± 1.7
Ri25	0.48 ± 0.02*	69.2 ± 1.5	**Ri75**	0.88 ± 0.02*	43.1 ± 3.7	**Ri125**	1.32 ± 0.11	14.6 ± 2.7
Ri26	1.10 ± 0.02*	29.2± 1.5	**Ri76**	0.88 ± 0.02*	43.1 ± 1.5	**Ri126**	1.07 ± 0.08*	30.8 ± 5.3
Ri27	1.26 ± 0.10	18.5 ± 6.7	**Ri77**	0.95 ± 0.02*	38.5 ± 2.5	**Ri127**	0.86 ± 0.04*	44.6 ± 2.8
Ri28	1.14 ± 0.08	26.2 ± 5.3	**Ri78**	0.83 ± 0.05*	46.2 ± 3.1	**Ri128**	1.33 ± 0.09	13.9 ± 4.3
Ri29	1.24 ± 0.02	20.0 ± 1.5	**Ri79**	0.88 ± 0.06*	43.1 ± 4.1	**Ri129**	1.00 ± 0.04*	35.4 ± 2.7
Ri30	1.21 ± 0.04	21.5 ± 2.7	**Ri80**	1.17 ± 0.05	24.6 ± 3.3	**Ri130**	0.90 ± 0.09*	41.5 ± 5.6
Ri31	1.00 ± 0.11*	35.4 ± 7.1	**Ri81**	0.98 ± 0.05*	36.9 ± 3.1	**Ri131**	0.83 ± 0.02*	46.2 ± 2.5
Ri32	1.24 ± 0.02	20.0 ± 1.5	**Ri82**	0.88 ± 0.02*	43.1 ± 1.4	**Ri132**	1.00 ± 0.07*	35.4 ± 4.6
Ri33	1.05 ± 0.06*	32.3 ± 4.1	**Ri83**	0.83 ± 0.02*	46.2 ± 5.4	**Ri133**	0.90 ± 0.02*	41.5 ± 1.5
Ri34	1.31 ± 0.06	15.4 ± 4.1	**Ri84**	0.86 ± 0.04*	44.6 ± 2.7	**Ri134**	1.29 ± 0.04	16.9 ± 2.7
Ri35	1.36 ± 0.04	12.3 ± 2.7	**Ri85**	0.98 ± 0.05*	36.9 ± 3.1	**Ri135**	1.11 ± 0.04*	28.5 ± 1.9

Ri36	1.31 ± 0.02	15.4 ± 1.5	**Ri86**	0.86 ± 0.08*	44.6 ± 5.3	**Ri136**	1.07 ± 0.04*	30.8 ± 2.7
Ri37	0.81 ± 0.02*	47.7 ± 1.5	**Ri87**	1.00 ± 0.04*	35.4 ± 2.7	**Ri137**	0.95 ± 0.06*	38.5 ± 4.1
Ri38	1.07 ± 0.04*	30.8 ± 2.7	**Ri88**	0.93 ± 0.00*	40.0 ± 0.0	**Ri138**	1.12 ± 0.09*	27.7 ± 5.6
Ri39	0.95 ± 0.05*	38.5 ± 3.1	**Ri89**	1.45 ± 0.02	6.2 ± 1.5	**Ri139**	1.10 ± 0.05*	29.2 ± 3.1
Ri40	0.52 ± 0.02*	66.2 ± 1.5	**Ri90**	1.40 ± 0.02	13.6 ± 2.3	**Ri140**	1.07 ± 0.04*	30.8 ± 2.7
Ri41	1.02 ± 0.02*	33.9 ± 1.5	**Ri91**	1.38 ± 0.02	15.9 ± 2.3	**Ri141**	1.57 ± 0.04	0.5 ± 0.6
Ri42	1.05 ± 0.09*	32.3 ± 5.6	**Ri92**	1.45 ± 0.02	9.1 ± 2.0	**Ri142**	1.48 ± 0.06	5.1 ± 0.3
Ri43	1.02 ± 0.06*	33.9 ± 4.1	**Ri93**	1.12 ± 0.07*	31.6 ± 5.3	**Ri143**	1.31 ± 0.06	15.4 ± 2.1
Ri44	1.45 ± 0.09	6.2 ± 2.6	**Ri94**	1.50 ± 0.04	3.1 ± 0.7	**Ri144**	1.54 ± 0.04	0.7 ± 0.2
Ri45	0.90 ± 0.02*	41.5 ± 1.5	**Ri95**	1.29 ± 0.07	16.9 ± 4.6	**Ri145**	1.07 ± 0.04*	30.8 ± 2.7
Ri46	0.69 ± 0.02*	55.4 ± 1.6	**Ri96**	1.31 ± 0.02	15.4 ± 1.5	**Ri146**	1.12 ± 0.05*	27.7 ± 3.1
Ri47	1.02 ± 0.02*	33.9 ± 1.4	**Ri97**	0.95 ± 0.02*	38.5 ± 2.7	**Ri147**	1.10 ± 0.06*	29.2 ± 4.1
Ri48	0.95 ± 0.02*	38.5 ± 4.5	**Ri98**	0.88 ± 0.02*	43.1 ± 1.5	**Ri148**	1.14 ± 0.04	26.2 ± 2.7
Ri49	1.38 ± 0.06	10.8 ± 2.1	**Ri99**	1.00 ± 0.00*	35.4 ± 0.8	**Control**	1.60 ± 0.02	-
Ri50	1.12 ± 0.06*	27.7 ± 4.1	**Ri100**	0.90 ± 0.02*	41.5 ± 3.9			

Nonorganic tomato plant / Stem

Strain ID	**MGR (mm/day)[a,b]**	**MGI (%)[a]**	**Strain ID**	**MGR (mm/day)**	**MGI (%)**
Si1	1.02 ± 0.02*	25.9 ± 1.7	**Control**	1.40 ± 0.05	-

[a]Mycelial Growth Rate (MGR; mm/day) and Mycelial Growth Inhibition (MGI; %) of *V. dahliae* were obtained after growing both the BCAs and *V. dahliae* in dual cultures on PDA at 22ºC for 14 days in the dark. In any cases, data represents the average of three replicate plates per BCA or control ± the standard error of the means.

[b]Means followed by an asterisk differ significantly from the "Control" according to Dunnett's multiple comparison test at $P = 0.05$.

Table 7.2. Frequency of bacterial strains according to their effectiveness inhibiting mycelial growth of *V. dahliae*.

IMG (%)	Organic tomato plant		Nonorganic tomato plant		Total
	Root	Stem	Root	Stem	
0	5/155 (3.2%)[a]	10/74 (13.5%)[a]	4/148 (2.7%)[a]	0/1 (0.0%)[a]	19/378 (5.0%)[a]
1-10	20/155 (12.9%)	16/74 (21.6 %)	14/148 (9.5%)	0/1 (0.0%)	50/378 (13.2%)
11-20	30/155 (19.3%)	16/74 (21.6 %)	24/148 (16.2%)	0/1 (0.0%)	70/378 (18.4%)
21-30	41/155 (26.4%)	16/74 (21.6 %)	27/148 (18.2%)	1/1 (100%)	85/378 (22.4%)
31-40	39/155 (25.2 %)	11/74 (14.9%)	38/148 (25.7%)	0/1 (0.0%)	88/378 (23.7%)
41-50	15/155 (9.7%)	5/74 (6.8%)	33/148 (22.3%)	0/1 (0.0%)	53/378 (14.0%)
51-60	4/155 (2.6%)	0/74 (0.0%)	3/148 (2.0%)	0/1 (0.0%)	7/378 (1.8%)
61-70	1/155 (0.7 %)	0/74 (0.0%)	4/148 (2.7%)	0/1 (0.0%)	5/378 (1.3%)
71-80	0/155 (0.0%)	0/74 (0.0%)	1/148 (0.7%)	0/1 (0.0%)	1/378 (0.2%)
81-90	0/155 (0.0%)	0/74 (0.0%)	0/148 (0.0%)	0/1 (0.0%)	0/378 (0.0%)
91-100	0/155 (0.0%)	0/74 (0.0%)	0/148 (0.0%)	0/1 (0.0%)	0/378 (0.0%)

[a]Number of bacteria within each IMG range/total bacteria isolated in each plant type and tissue combination (data expressed as percentage).
[b]Number of total bacteria within each IMG range/total bacteria isolated in this study (data expressed as percentage).

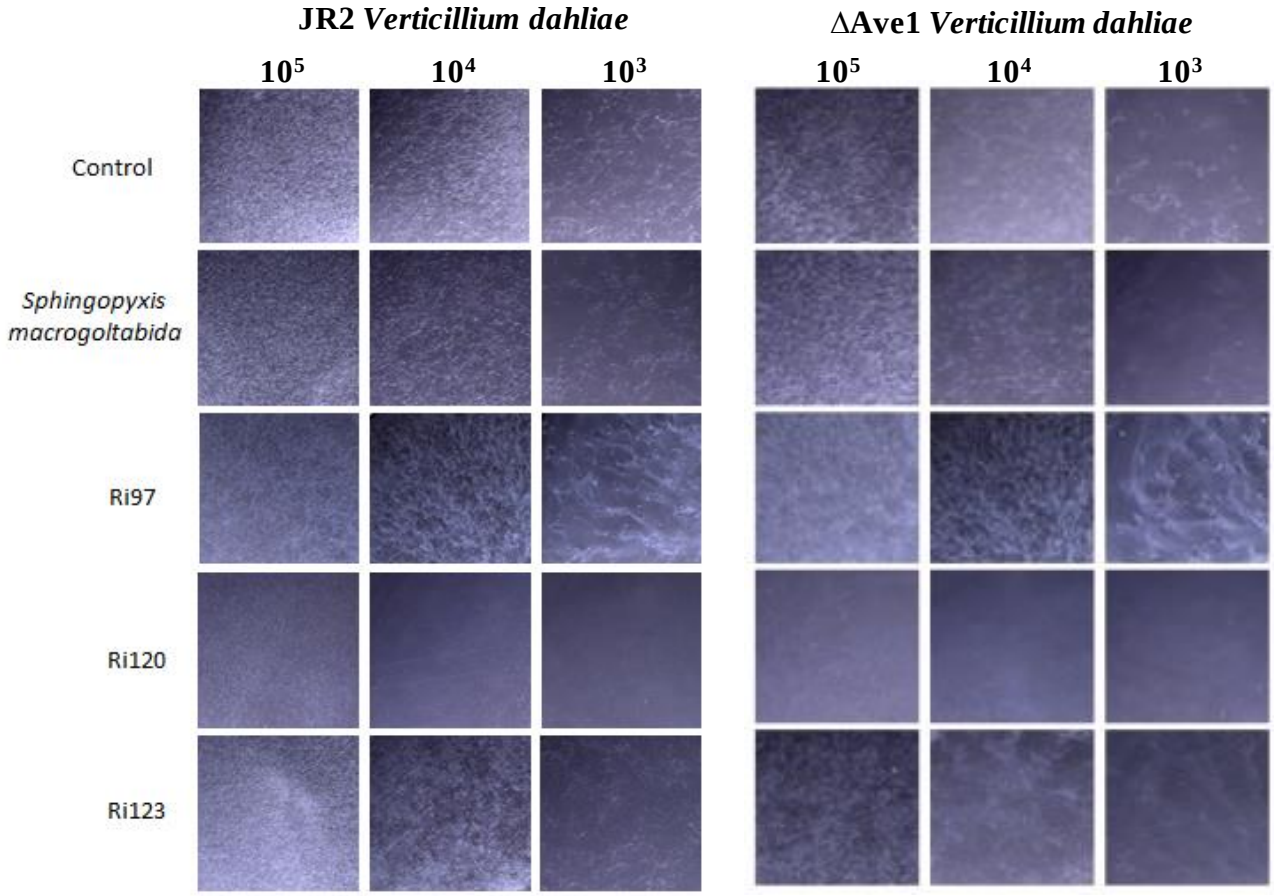

Figure 7.1. *In vitro* effect of the bacterial strains Ri97, Ri120 and Ri123 against *V. dahliae* (strains JR2 and deletion mutant) by means of co-cultivation assays

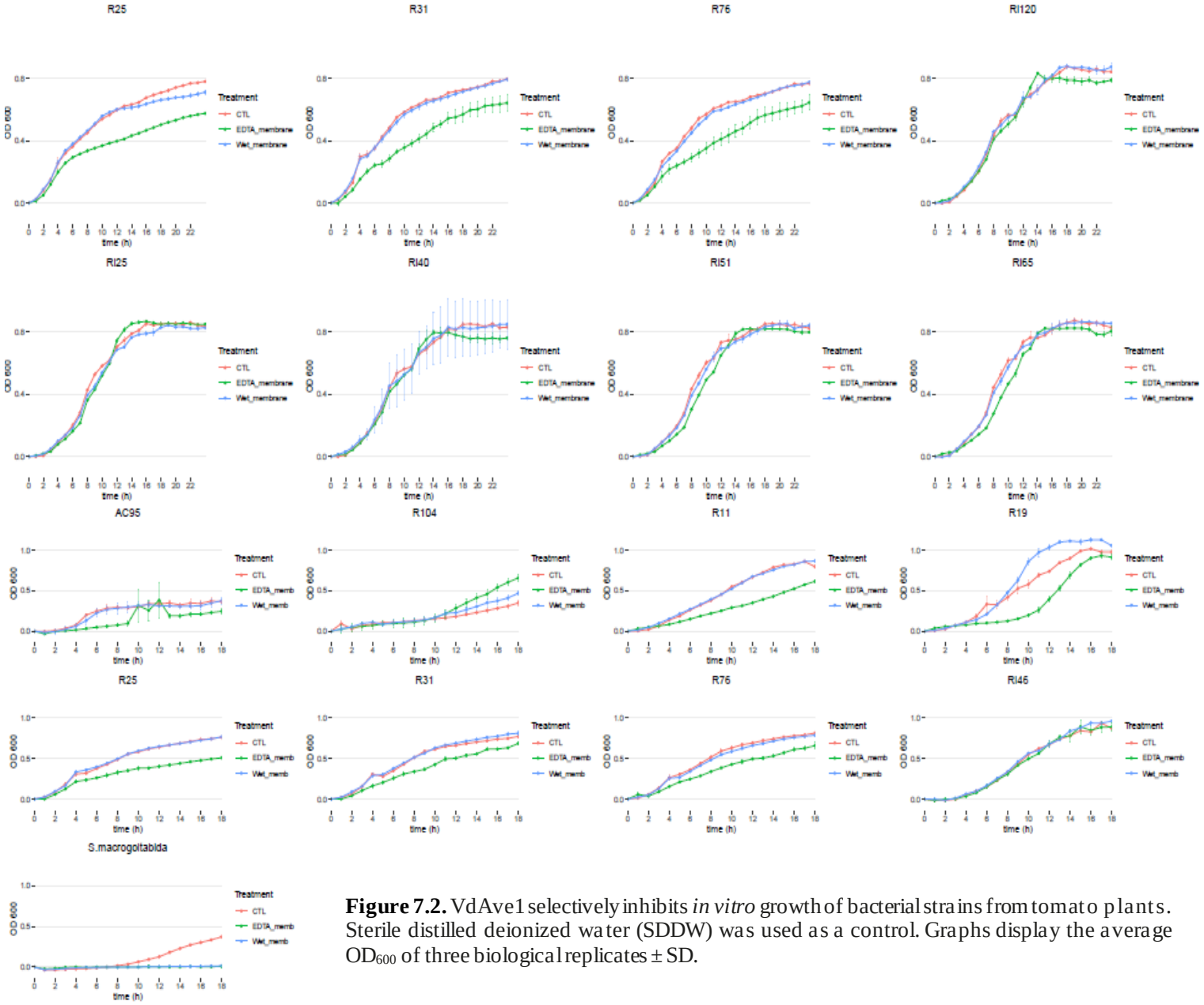

Figure 7.2. VdAve1 selectively inhibits *in vitro* growth of bacterial strains from tomato plants. Sterile distilled deionized water (SDDW) was used as a control. Graphs display the average OD_{600} of three biological replicates ± SD.

Table 7.3. Molecular identity of the bacterial strains isolated from tomato plants determined by 16S ribosomal DNA profiling.

Organic tomato plant / Root			
StrainID	**Phylum**	**Genus**	**Species**
R1	Proteobacteria	*Pseudomonas*	*P. plecoglossicida*
R2	Proteobacteria	*Enterobacter*	*E. soli*
R4	Proteobacteria	*Enterobacter*	*E. soli*
R5	Proteobacteria	*Pseudomonas*	*P. vancouverensis*
R6	Proteobacteria	*Enterobacter*	*E. soli*
R7	Proteobacteria	*Enterobacter*	*E. soli*
R8	Proteobacteria	*Pseudomonas*	*P. baetica*
R9	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R10	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R11	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R12	Proteobacteria	*Enterobacter*	*E. soli*
R13	Firmicutes	*Bacillus*	*B. oceanisediminis*
R14	Proteobacteria	*Pseudomonas*	*P. entomophila*
R16	Proteobacteria	*Enterobacter*	*E. soli*
R18	Firmicutes	*Bacillus*	*B. piscis*
R19	Proteobacteria	*Pseudomonas*	*P. corrugata*
R21	Proteobacteria	*Pseudomonas*	*P. corrugata*
R23	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R24	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R26	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R27	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R28	Proteobacteria	*Enterobacter*	*E. soli*
R29	Proteobacteria	*Pseudomonas*	*P. umsongensis*
R32	Proteobacteria	*Enterobacter*	*E. soli*
R35	Proteobacteria	*Pseudomonas*	*P. delhiensis*
R36	Proteobacteria	*Enterobacter*	*E. soli*
R37	Proteobacteria	*Enterobacter*	*E. soli*
R38	Proteobacteria	*Enterobacter*	*E. soli*
R39	Proteobacteria	*Enterobacter*	*E. soli*
R40	Proteobacteria	*Enterobacter*	*E. soli*
R42	Firmicutes	*Solibacillus*	*S. isronensis*
R43	Proteobacteria	*Enterobacter*	*E. soli*
R44	Proteobacteria	*Enterobacter*	*E. soli*
R45	Proteobacteria	*Enterobacter*	*E. soli*
R46	Proteobacteria	*Pseudomonas*	*P.citronellolis*
R47	Proteobacteria	*Pseudomonas*	*P. delhiensis*
R48	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R49	Proteobacteria	*Enterobacter*	*E. soli*
R50	Firmicutes	*Bacillus*	*B. niacini*
R51	Proteobacteria	*Enterobacter*	*E. soli*
R53	Proteobacteria	*Pseudomonas*	*P. umsongensis*
R54	Proteobacteria	*Pseudomonas*	*P. vancouverensis*
R55	Proteobacteria	*Pseudomonas*	*P. plecoglossicida*
R56	Proteobacteria	*Enterobacter*	*E. soli*
R58	Proteobacteria	*Pseudomonas*	*P. plecoglossicida*

R60	Proteobacteria	*Pseudomonas*	*P. corrugata*
R62	Proteobacteria	*Pseudomonas*	*P. oryzihabitans*
R63	Proteobacteria	*Enterobacter*	*E. soli*
R65	Proteobacteria	*Enterobacter*	*E. soli*
R66	Proteobacteria	*Pseudomonas*	*P. putida*
R67	Proteobacteria	*Enterobacter*	*E. soli*
R69	Firmicutes	*Bacillus*	*B. oceanisediminis*
R70	Proteobacteria	*Pseudomonas*	*P. plecoglossicida*
R72	Proteobacteria	*Enterobacter*	*E. soli*
R74	Actinobacteriota	*Microbacterium*	*M. esteraromaticum*
R75	Proteobacteria	*Enterobacter*	*E. soli*
R77	Proteobacteria	*Enterobacter*	*E. soli*
R80	Proteobacteria	*Pseudomonas*	*P. delhiensis*
R83	Proteobacteria	*Pseudomonas*	*P. knackmussii*
R84	Proteobacteria	*Enterobacter*	*E. soli*
R85	Proteobacteria	*Enterobacter*	*E. soli*
R87	Firmicutes	*Bacillus*	*B. cucumis*
R92	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R94	Proteobacteria	*Pseudomonas*	*P. corrugata*
R95	Proteobacteria	*Pseudomonas*	*P. umsongensis*
R96	Actinobacteriota	*Microbacterium*	*M. maritypicum*
R97	Proteobacteria	*Pseudomonas*	*P. corrugata*
R98	Proteobacteria	*Ochrobactrum*	*O. soli*
R99	Proteobacteria	*Ochrobactrum*	*O. soli*
R102	Proteobacteria	*Pseudomonas*	*P. knackmussii*
R103	Firmicutes	*Bacillus*	*B. drentensis*
R104	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R105	Proteobacteria	*Pseudomonas*	*P. knackmussii*
R106	Proteobacteria	*Pseudomonas*	*P. putida*
R107	Proteobacteria	*Pseudomonas*	*P. monteilii*
R109	Proteobacteria	*Ochrobactrum*	*O. intermedium*
R110	Proteobacteria	*Ochrobactrum*	*O. tritici*
R111	Proteobacteria	*Ochrobactrum*	*O. tritici*
R113	Proteobacteria	*Pseudomonas*	*P. corrugata*
R114	Proteobacteria	*Enterobacter*	*E. soli*
R116	Proteobacteria	*Pseudomonas*	*P. monteilii*
R120	Actinobacteriota	*Microbacterium*	*M. hominis*
R121	Bacteroidota	*Chryseobacterium*	*C. indoltheticum*
R122	Proteobacteria	*Ochrobactrum*	*O. intermedium*
R123	Proteobacteria	*Ochrobactrum*	*O. soli*
R125	Proteobacteria	*Enterobacter*	*E. soli*
R126	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R127	Proteobacteria	*Pseudomonas*	*P. citronellolis*
R128	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R129	Proteobacteria	*Enterobacter*	*E. soli*
R130	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R131	Proteobacteria	*Enterobacter*	*E. soli*
R132	Proteobacteria	*Pseudomonas*	*P. delhiensis*
R133	Proteobacteria	*Pseudomonas*	*P. delhiensis*
R134	Proteobacteria	*Enterobacter*	*E. soli*

R135	Actinobacteriota	*Paenarthrobacter*	*P. ureafaciens*
R136	Actinobacteriota	*Paenarthrobacter*	*P. ureafaciens*
R137	Actinobacteriota	*Paenarthrobacter*	*P. ureafaciens*
R138	Actinobacteriota	*Paenarthrobacter*	*Pa. ureafaciens*
R139	Actinobacteriota	*Microbacterium*	*M. foliorum*
R140	Actinobacteriota	*Microbacterium*	*M. hydrocarbonoxydans*
R142	Actinobacteriota	*Microbacterium*	*M. natoriense*
R143	Actinobacteriota	*Cellulomonas*	*Ce. soli*
R145	Actinobacteriota	*Cellulomonas*	*Ce. soli*
R147	Actinobacteriota	*Cellulomonas*	*Ce. soli*
R148	Proteobacteria	*Enterobacter*	*E. soli*
R149	Proteobacteria	*Enterobacter*	*E. soli*
R151	Firmicutes	*Paenibacillus*	*Pa. lautus*
R152	Proteobacteria	*Pseudomonas*	*P. monteilii*
R153	Actinobacteriota	*Pseudarthrobacter*	*Ps. chlorophenolicus*
R154	Proteobacteria	*Enterobacter*	*E. soli*

Organic tomato plant / Stem

StrainID	**Phylum**	**Genus**	**Species**
S1	Firmicutes	*Bacillus*	*B. pseudomycoides*
S2	Firmicutes	*Bacillus*	*B. megaterium*
S3	Firmicutes	*Paenibacillus*	*Pa. lautus*
S4	Firmicutes	*Bacillus*	*B. simplex*
S5	Proteobacteria	*Devosia*	*D. riboflavina*
S6	Firmicutes	*Paenibacillus*	*Pa. illinoisensis*
S7	Firmicutes	*Bacillus*	*B. licheniformis*
S8	Proteobacteria	*Pseudomonas*	*P. monteilii*
S10	Firmicutes	*Bacillus*	*B. zhangzhouensis*
S11	Actinobacteriota	*Glutamicibacter*	*G. halophytocola*
S12	Actinobacteriota	*Curtobacterium*	*Cu. oceanosedimentum*
S13	Proteobacteria	*Xanthomonas*	*X. campestris*
S14	Actinobacteriota	*Pseudarthrobacter*	*Ps. equi*
S15	Firmicutes	*Solibacillus*	*S. silvestris*
S16	Proteobacteria	*Enterobacter*	*E. soli*
S18	Firmicutes	*Bacillus*	*B. zanthoxyli*
S19	Actinobacteriota	*Brevibacterium*	*Br. sediminis*
S20	Actinobacteriota	*Curtobacterium*	*Cu. oceanosedimentum*
S23	Actinobacteriota	*Microbacterium*	*M. esteraromaticum*
S24	Proteobacteria	*Enterobacter*	*E. soli*
S25	Proteobacteria	*Devosia*	*D. riboflavina*
S27	Proteobacteria	*Enterobacter*	*E. soli*
S28	Proteobacteria	*Enterobacter*	*E. soli*
S29	Proteobacteria	*Aeromonas*	*A. hydrophila*
S30	Proteobacteria	*Pseudomonas*	*P. umsongensis*
S31	Proteobacteria	*Enterobacter*	*E. soli*
S32	Actinobacteriota	*Paenarthrobacter*	*Pa. ilicis*
S33	Actinobacteriota	*Pseudarthrobacter*	*Ps. equi*
S34	Proteobacteria	*Enterobacter*	*E. soli*
S35	Actinobacteriota	*Curtobacterium*	*Cu. oceanosedimentum*
S36	Actinobacteriota	*Microbacterium*	*M. oleivorans*
S37	Proteobacteria	*Pseudoxanthomonas*	*Pse. suwonensis*

S38	Proteobacteria	*Enterobacter*	*E. soli*
S40	Actinobacteriota	*Curtobacterium*	*Cu. pusillum*
S42	Proteobacteria	*Pseudomonas*	*P. knackmussii*
S43	Actinobacteriota	*Microbacterium*	*M. phyllosphaerae*
S44	Actinobacteriota	*Curtobacterium*	*Cu. pusillum*
S45	Proteobacteria	*Enterobacter*	*E. soli*
S46	Proteobacteria	*Pseudomonas*	*P. citronellolis*
S47	Actinobacteriota	*Brevibacterium*	*Br. sediminis*
S48	Actinobacteriota	*Brevibacterium*	*Br. sediminis*
S49	Actinobacteriota	*Microbacterium*	*M.schleiferi*
S51	Proteobacteria	*Devosia*	*D. riboflavina*
S52	Proteobacteria	*Kaistia*	*K. adipata*
S53	Actinobacteriota	*Curtobacterium*	*Cu. pusillum*
S54	Proteobacteria	*Enterobacter*	*E. soli*
S55	Actinobacteriota	*Brevibacterium*	*Br. anseongense*
S56	Proteobacteria	*Enterobacter*	*E. soli*
S57	Actinobacteriota	*Microbacterium*	*M. maritypicum*
S58	Actinobacteriota	*Brevibacterium*	*Br.sediminis*
S60	Actinobacteriota	*Microbacterium*	*M. phyllosphaerae*
S61	Firmicutes	*Paenibacillus*	*Pa. lautus*
S62	Proteobacteria	*Enterobacter*	*E. soli*
S63	Actinobacteriota	*Microbacterium*	*M. esteraromaticum*
S64	Firmicutes	*Exiguobacterium*	*E. artemiae*
S65	Actinobacteriota	*Cellulosimicrobium*	*Cel. cellulans*
S66	Actinobacteriota	*Microbacterium*	*M. oleivorans*
S67	Proteobacteria	*Pseudomonas*	*P. citronellolis*
S68	Proteobacteria	*Rhodanobacter*	*R. spathiphylli*
S69	Actinobacteriota	*Brevibacterium*	*Br. sediminis*
S70	Actinobacteriota	*Brevibacterium*	*Br. sediminis*
S71	Actinobacteriota	*Nocardioides*	*N. zeae*
S73	Firmicutes	*Bacillus*	*B. niacini*
S74	Actinobacteriota	*Brevibacterium*	*Br. sediminis*

Nonorganic tomato plant / Root

StrainID	**Phylum**	**Genus**	**Species**
Ri1	Proteobacteria	*Enterobacter*	*E. ludwigii*
Ri2	Proteobacteria	*Pseudomonas*	*P. migulae*
Ri3	Actinobacteriota	*Microbacterium*	*M. oleivorans*
Ri4	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri5	Actinobacteriota	*Microbacterium*	*M. phyllosphaerae*
Ri6	Proteobacteria	*Pseudomonas*	*P. mediterranea*
Ri7	Proteobacteria	*Pseudomonas*	*P. brassicacearum*
Ri8	Bacteroidota	*Chryseobacterium*	*C. wanjuense*
Ri9	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri10	Proteobacteria	*Pseudomonas*	*P. corrugata*
Ri11	Proteobacteria	*Pseudomonas*	*P. kilonensis*
Ri12	Proteobacteria	*Pseudomonas*	*P. mucidolens*
Ri13	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri14	Actinobacteriota	*Paenarthrobacter*	*Pa. nicotinovorans*
Ri15	Actinobacteriota	*Paenarthrobacter*	*Pa. nicotinovorans*
Ri17	Proteobacteria	*Rhodanobacter*	*R. spathiphylli*

Ri18	Actinobacteriota	*Microbacterium*	*M. phyllosphaerae*
Ri19	Proteobacteria	*Pseudomonas*	*P. vancouverensis*
Ri20	Proteobacteria	*Pseudomonas*	*P. corrugata*
Ri21	Proteobacteria	*Sphingobium*	*Sp. mellinum*
Ri22	Proteobacteria	*Rhodanobacter*	*R. spathiphylli*
Ri23	Actinobacteriota	*Paenarthrobacter*	*Pa. nicotinovorans*
Ri24	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri25	Proteobacteria	*Pseudomonas*	*P. kilonensis*
Ri26	Actinobacteriota	*Microbacterium*	*M. oleivorans*
Ri27	Actinobacteriota	*Microbacterium*	*M. phyllosphaerae*
Ri29	Bacteroidota	*Pedobacter*	*Pe. steynii*
Ri30	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri31	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri32	Bacteroidota	*Pedobacter*	*Pe. panaciterrae*
Ri33	Bacteroidota	*Pedobacter*	*Pe. seoulensis*
Ri34	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri35	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri36	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri38	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri39	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri40	Proteobacteria	*Pseudomonas*	*P. mucidolens*
Ri41	Actinobacteriota	*Paenarthrobacter*	*Pa. nicotinovorans*
Ri42	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri43	Proteobacteria	*Pseudomonas*	*P. corrugata*
Ri44	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri45	Actinobacteriota	*Curtobacterium*	*Cu. oceanosedimentum*
Ri46	Proteobacteria	*Pseudomonas*	*P. gessardii*
Ri47	Actinobacteriota	*Microbacterium*	*M. foliorum*
Ri48	Actinobacteriota	*Paenarthrobacter*	*Pa. nicotinovorans*
Ri49	Actinobacteriota	*Curtobacterium*	*Cu. oceanosedimentum*
Ri50	Actinobacteriota	*Microbacterium*	*M.maritypicum*
Ri51	Proteobacteria	*Pseudomonas*	*P. kilonensis*
Ri52	Firmicutes	*Bacillus*	*B. wiedmannii*
Ri54	Actinobacteriota	*Microbacterium*	*M. oleivorans*
Ri55	Bacteroidota	*Flavobacterium*	*F. hauense*
Ri57	Actinobacteriota	*Arthrobacter*	*Ar. psychrolactophilus*
Ri58	Actinobacteriota	*Microbacterium*	*M. maritypicum*
Ri59	Actinobacteriota	*Microbacterium*	*M.esteraromaticum*
Ri60	Actinobacteriota	*Microbacterium*	*M. oleivorans*
Ri61	Actinobacteriota	*Microbacterium*	*M. foliorum*
Nonorganic tomato plant / Stem			
StrainID	**Phylum**	**Genus**	**Species**
Si1	Proteobacteria	*Herbaspirillum*	*H. rhizosphaerae*

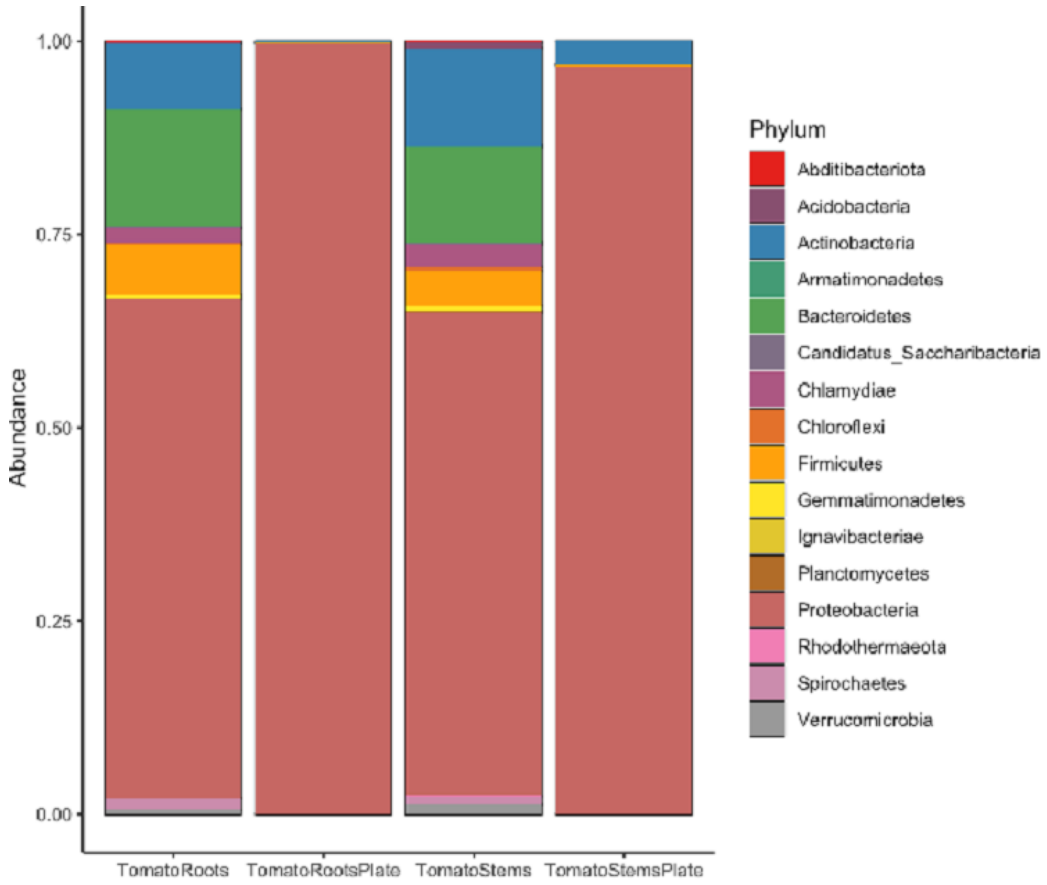

Figure 7.3. Relative abundance of bacterial phyla in the root and stem microbiome of organic tomato plants as determined by 16S ribosomal DNA profiling.

Chapter 8

GENERAL DISCUSSION

The present PhD Thesis was conceived mainly to select possible biological control treatments among beneficial microorganisms, biostimulants, copper fertilizers, nutrients and HPDI against VWO, as well as to determine the direct and indirect effect of the most effective treatments in both the biology of *V. dahliae in vitro* and the disease progress *in planta*. The aim of this thesis was to go one step further in the search for effective and eco-friendly solutions for the control of VWO. In this way, the use of biostimulants and HPDI is currently considered among the most promising biological and eco-friendly alternatives to traditional control measures, since they are able to induce innate resistance in plants, preventing pathogen attack with little or no environmental impact (Sharma *et al.*, 2014; Llorens *et al.*, 2017b; González-Hernández *et al.*, 2018 Drobek *et al.*, 2019; Barros-Rodríguez *et al.*, 2020).

Because this kind of products, which could be a novel and potentially ecological alternative control measure for VWO, has not been extensively studied against this disease (Montes-Osuna and Mercado-Blanco, 2020), the first objective of this PhD Thesis (*Chapter 2*) was to evaluate the effect of 32 products, grouped mainly under the terms BCAs, biostimulants and HPDIs, against the disease under controlled conditions. Notice that most of the products included in this first study were carefully selected for their potential activity as plant biostimulants in accordance with the most recent European regulatory framework on this subject [European Regulation (EU) 2019/1009]. The most relevant results of this first study showed that *Phoma* sp. ColPat-375 was the most effective BCA against the disease when applied by foliar way; while *B. amyloliquefaciens* PAB-024 was the most effective by root application. In addition, the fungus *A. pullulans* AP08 reduced significantly the disease by both types of applications. Regarding to chemically synthesized compounds, all of them showed higher efficacy when applied to the root, with potassium phosphite-3 and copper phosphite-3 being the most effective. Our results on the effectiveness of *Phoma* sp. by foliar application were in agreement with previous results obtained by Varo *et al.* (2016), who also showed an important effect in reducing VWO using another isolate of *Phoma* sp. In addition, the results from *Chapter 2* contribute to the selection of new potential biocontrol treatments such as *A. pullulans*, *B. amyloliquefaciens* or CuPh, which have not been previously evaluated against VWO with successful results. All they, together with other effective biocontrol products previously evaluated by several

authors including bacteria (e.g., *Pseudomonas* spp., strains of the Bacillales order etc.) and fungi (e.g., *Phoma* sp.; nonpathogenic *Fusarium oxysporum* strains; *Trichoderma* spp., etc.) (Varo *et al.*, 2016; Mulero-Aparicio *et al.*, 2019a; Montes-Osuna and Mercado-Blanco, 2020; Poveda and Baptista, 2021), as well as organic amendments, plants extracts and essential oils (Lozano-Tovar *et al.*, 2013; Varo *et al.*, 2016, 2017, 2018) will contribute to go ahead to reach the challenge of the biological control of VWO. In addition to all these previous selections of beneficial microorganisms, the positive effect of phosphites in crop protection has been already demonstrated (Guest and Grant, 1991; Cooke and Little, 2002; Gómez-Merino and Trejo-Téllez, 2015; Abbasi and Lazarovits, 2006a,b; Romero *et al.*, 2019), but their effectiveness against VWO was still uncertain before this current study.

Interestingly, among the most effective compounds *in planta* experiment, *B. amyloliquefaciens* PAB-024 and copper phosphite 3 significantly inhibited *V. dahliae* growth *in vitro*; while *A. pullulans* and potassium phosphite 3 did not show direct effect against the pathogen. These results suggested that these compounds could present different modes of action, requiring further studies to elucidate this aspect. To this end, the most effective compounds were selected to determine: *i*) the influence of the olive cultivar and biocontrol treatments on the effect of OSE on the viability of *V. dahliae* conidia (*Chapter 3*); *ii*) the influence of the olive cultivar and biocontrol treatments on the effect of root exudates of olive plants against *V. dahliae* conidia and MS (*Chapter 4*); and *iii*) their effectiveness in inducing resistance in olive plants against VWO (*Chapter 5*).

In *Chapter 3*, the influence of the olive genotype (cultivar resistance), and the interaction between cultivars and biocontrol treatments on the effect of OSE on the viability of conidia of *V. dahliae* was evaluated *in vitro*. To conduct this study, olive plants of the cvs. Frantoio (tolerant), Arbequina (moderately susceptible) and Picual (highly susceptible) were used. In addition, the most effective treatment combinations from the *Chapter 2* (Table 3.1.) and their interaction with the three olive cultivars described above were evaluated in this study. Notice that OSE from treated plants obtained by means of the Cadahía's method (Cadahía, 2008) was used to conduct this study instead of pure sap collected by Scholander chamber (Alexou and Peuke, 2013). Cadahía's method was selected here not only because it results among the easiest methods available to obtain OSE, but also for the demonstrated validity of the method

in agronomy as indicated in *Chapter 3* (Cadahía, 2008). The results of this study showed that the influence of biological treatments against *V. dahliae* depends on the genotype, since the greater the resistance of the cultivar the lower the influence of the treatments on the ability of the OSE to inhibit conidia germination. In 'Picual', the most susceptible cultivar to VWO, the BCA *B. amyloliquefaciens* PAB024, and the copper phosphite 3 were the most effective treatments in inhibiting conidia germination by the OSE. Thus, the results indicate that the highly tolerance to *V. dahliae* conferred by the 'Frantoio' genotype prevails over the treatments, even those that were more effective against the pathogen in susceptible cultivars. It could be interesting to carry out studies to determine whether the treatments applied could cause changes in the xylem microbiome of olive plants. In this way, recent studies conducted by Anguita-Maeso *et al.* (2021b) determined that the xylem microbiome of olive plants inoculated with *V. dahliae* increases the diversity of bacterial communities compared to non-inoculated plants.

Similar results were obtained from the study conducted in *Chapter 4*, which aimed to elucidate the influence of biological treatments and olive cultivar on the effect of root exudates on *V. dahliae* conidia and MS by setting up an efficient method of collecting root exudates. As a first conclusion of this study, a 0.01 M $CaSO_4$ solution and root immersion for 4 h may be considered a preferential medium and timing combination to collect root exudates from young olive plants. This combination was selected taking into consideration mainly the previous studies conducted by Aulakh *et al.* (2001) as well as those from Kirk *et al.* (1999) and Jones and Darrah (1992). Further results of this study demonstrated that root exudates from olive plants can induce germination of conidia and MS of *V. dahliae*. In addition, the genotype influenced the capacity of root exudates to induce germination of *V. dahliae* propagules, since root exudates from 'Frantoio' did not show any effect; while those from 'Arbequina' or 'Picual' induced conidia and MS germination. Finally, *A. pullulans* AP08 resulted in the most effective treatment since root exudates collected from treated plants significantly reduced the germination of conidia and MS of the pathogen.

Results from both *Chapters 3* and *4* were in concordance between them since they revealed similar patterns about the indirect effect of the biological treatments against VWO by influencing the OSE and root exudates from different olive cultivars on the viability of *V. dahliae* propagules.

At this stage, considering that both infection by vascular pathogens such as *V. dahliae* and the application of certain compounds can act by enhancing the natural defence mechanisms of the plant (Hilaire *et al.*, 2001; Rep *et al.*, 2002; Basha *et al.*, 2010; Gayoso *et al.*, 2010; Llorens *et al.*, 2017b), the following step in this PhD Thesis was to determine if the selected compounds have the ability to act as HPDI in olive plants against VWO (*Chapter 5*). Thus, the three most effective compounds (*Chapters 2-4*; *A. pullulans* AP08, *B. amyloliquefaciens* PAB-024 and the copper phosphite 3) were selected to conduct the study showed in *Chapter 5*, which aimed to evaluate their effectiveness as HRI against *V. dahliae* in olive plants of cv. Picual. This study was designed considering the evaluation of parameters involved in plant resistance which were monitored through determination and quantification of reactive oxygen species (ROS) response (H_2O_2), and evaluation of hormones (Llorens *et al.*, 2017a; González-Hernández *et al.*, 2018). In addition, the role of transcription factors previously shown to play a role in olive plant defence against *V. dahliae* (Schilirò *et al.*, 2012), such as *LOX*, *ACL*, *bHLH*, and *WRKY5* genes, was also evaluated. Our results suggests that the bacterium *B. amyloliquefaciens* PAB-024 could enhance the host resistance induction of olive plants through the SA pathway due to the treated plants showed high levels of ROS accumulation, which simultaneously accompany the up-regulation of SA-related genes (Llorens *et al.*, 2017a). On the hand, the enhancement by *A. pullulans* AP08 and Copper Phosphite 3 could be linked to the JA pathway since a strong accumulation of JA and JA-ile was observed in treated plants. The last results were in concordance with those previously shown by Gharbi *et al.* (2017), who reported that the JA, as well as the expression of the genes responsive to this hormone, could be related to the higher resistance of olive trees to *V. dahliae*. Furthermore, it is worth mentioning that the effectiveness of phosphites as HRI against different biotic and abiotic stress factors improving crop protection, yield, and harvest quality has been also demonstrated for other hosts (Navarro-Cerrillo *et al.*, 2004; Gómez-Merino and Trejo-Téllez, 2015; Barrós-Rodríguez *et al.*, 2020).

In parallel to the studies showed in the PhD Thesis, the most effective treatments against VWO mentioned above, together with others from previous PhD Thesis related to this topic (Mulero-Aparicio *et al.*, 2019b), are currently in evaluation under natural field conditions in two experimental fields located in Andújar (Jaen province) and Écija (Sevilla province). Although there are not still conclusive results because more than two

years of field evaluations are required, preliminary observations reveal that *A. pullulans* AP08 and copper phosphite 3 have an important effect reducing the DS, with this last one also stimulating the plant growth. Nevertheless, further evaluations and data analyses must be performed to draw conclusions about their effect on VWO in the field.

In addition, the influence of nutritional imbalances of N and K on olive infection by *V. dahliae* was also evaluated in this PhD Thesis (*Chapter 6*). Although several field observations carried out in southern Spain (López-Escudero *et al.*, 2011; Trapero *et al.*, 2017) reveal that N excess, or more probably imbalances of N excess and K deficiency, could be related to a higher incidence and severity of the disease in the affected olive groves, this fact was not demonstrated. Thus, this work was conceived to shed light on the effect of nutritional imbalances of N and K on the *V. dahliae* infection in olive trees. Among the results of this study, the main conclusion was that the effect of N excess on MS germination or on the disease progress predominates over the effect of K deficiency. Although the previous field observations hypothesized that N excess increase the disease incidence and severity in olive groves, our results demonstrated the inverse effect. Indeed, it is generally understood that N excess or K deficiency tends to increase or decrease (Pegg and Brady, 2007; Prabhu *et al.*, 2007), since several authors have reported that high levels of N increased *V. dahliae* susceptibility in herbaceous hosts such as aubergine, cotton, potato and tomato, whereas others showed opposite results in these same hosts (Pegg and Brady, 2007). In addition, considering the effect of N, notice that the reduction of MSG of *V. dahliae* and the lower values of RAUDPC in olive plants treated with ↑N+↑Na or ↑N+↑Na+↓K, under our experimental conditions, may greatly influenced by the N-forms used in this study. These results are in concordance with those previously obtained by several authors whose reported a direct effect of N ions (e.g., NO_3^-, NH_4^+) reducing the number of the propagules of *V. dahliae* (Pegg and Brady, 2002; Huber and Thompson, 2007). Further research is needed to better understand how mineral nutrition affects the inoculum of *V. dahliae* and the development of VWO, e.g., comparing both NO_3^- and NH_4^+ forms of N, even more K-deficiency treatments, the effect of NaCl, longer exposure to treatments, etc.

In summary, this PhD Thesis has been useful to select better candidates for future studies on biocontrol, contributing significantly to new insights into the current challenge of the biological control of VWO (*Chapters 2-5*). In addition to set up protocols to obtain OSE (*Chapter 3*) and root exudates (*Chapter 4*) from olive plants,

the indirect effect of biological treatments by influencing the effect of OSE and root exudates from treated plants on the viability of *V. dahliae* propagules has been determined. On the other hand, to date, studies on how determine the parameters related with HRI in olive plants against *V. dahliae* were poorly studied. Thus, evaluating the role of parameters such as ROS, hormones and transcription factors, among others, in olive plant defence against *V. dahliae* represents novel knowledge to better understand how biological treatments could modulate HRI in olive trees (*Chapter 5*). The study conducted in *Chapter 6* revealed that the balanced nutrition must be considered as important factor in plant resistance to diseases. This work attempts to provide new knowledge, studying not only the effect of mineral nutrition on the development of VWO, but also its direct effect on *V. dahliae* propagules and the indirect effect through possible modifications in root exudates affecting the first stages of the infection. Finally, the study conducted in *Chapter 7* is novel and original since it focuses on searching for beneficial bacterial in the tomato plant microbiome, which will be evolved insensitive to Ave1, obtaining a potential BCAs against the disease in the future. All the knowledge generated through this PhD Thesis represents a relevant step towards the biological control of VWO, and opens a variety of new paths to explore to better understand the mechanism of action of the biological treatments against *V. dahliae* in olive trees.

Conclusions

CONCLUSIONS

1. A total of 32 products were evaluated against *V. dahliae*, with root applications being more effective than foliar applications. Only three products (Aminoacids+N,P,K and S, *A. pullulans* and *Phoma* sp.) reduced the disease progress (RAUDPC) higher than 60% by foliar applications, while seven products (aluminum lignosulfonate, *A. pullulans*, *B. amyloliquefaciens*, copper gluconate 2, copper phosphite 3, potassium phosphite 3 and potassium silicate 1) did so by root application.
2. Among the most effective products *in planta* experiment, *B. amyloliquefaciens* and copper phosphite 3 significantly inhibited *V. dahliae* growth *in vitro*; while *A. pullulans* and potassium phosphite 3 did not show a direct effect against the pathogen. This suggests that these products could have different modes of action, such as antagonism or induction of host resistance.
3. The influence of biological treatments on the effect of OSE on the inhibition of the germination of *V. dahliae* conidia depends on the olive cultivar, with *B. amyloliquefaciens* and copper phosphite 3 being the most effective treatments in the susceptible cultivar Picual.
4. A reliable method to collect root exudates from young olive plants was established, which served to demonstrate that the collected root exudates can induce germination of *V. dahliae* conidia and microsclerotia.
5. The influence of biological treatments on the effect of root exudates in the inhibition of *V. dahliae* conidia and microsclerotia depends on the olive genotype, with *A. pullulans* being the most effective treatment in the susceptible cultivars.
6. The induction of resistance to *V. dahliae* in olive plants by *Bacillus amyloliquefaciens* could be regulated through the salicylic acid pathway; whereas the induction of resistance by *A. pullulans* and copper phosphite 3 could be linked to the jasmonic acid pathway.
7. N excess decreases the germination of *V. dahliae* microsclerotia and the progress of the disease in olive plants inoculated with the pathogen, while K deficiency increases both parameters. When imbalances of the two nutrients are applied simultaneously, the effect of N excess predominates over that of K deficiency.
8. This study has allowed us to select potential candidates for the biocontrol of *V. dahliae* due to their effectiveness as host resistance inducers, showing hopeful

results for the control of VWO. These results, together with future studies on antagonistic microorganisms derived from the host plant microbiome, will provide a relevant data resource to advance the generation of BCAs against *V. dahliae*.

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Appendix: Scientific production during the PhD

Scientific production during the PhD

PEER-REVIEWED JOURNAL PUBLICATIONS

1. **López-Moral, A.,** Sánchez-Rodríguez, A.R., Trapero, A., Agustí-Brisach, C. 2022. Elucidating the role of root exudates of olive plants against *Verticillium dahliae*: effect of the cultivar and biocontrol treatments. *Plant and Soil* (*under review*).

2. **López-Moral, A.,** Agustí-Brisach, C., Ruiz-Blancas, C., Antón-Domínguez, B., Alcántara, E., Trapero, A., 2022. Elucidating the effect of nutritional imbalances of N and K on the infection of *Verticillium dahliae* in olive. *Journal of Fungi* 8:139. (IF: 5.816; Q1).

3. **López-Moral, A.,** Lovera, M., Antón-Domínguez, B.I., Gámiz, A.M., Michailides, T.J., Arquero, O., Trapero, A., Agustí, Brisach, C. 2022. Effects of cultivar susceptibility, branch age, and temperature on the infection by Botryosphaeriaceae and *Diaporthe* fungi in English walnut (*Juglans regia* L.). *Plant Disease* (*accepted*). (IF:4.438; Q1).

4. **López-Moral, A.,** Agustí-Brisach, C., Leiva-Egea, F.M., Trapero, A. 2022. Influence of the cultivar and biocontrol treatments on the effect of olive stem extracts on the viability of *Verticillium dahliae* conidia. *Plants* (*accepted*). (IF:2.762; Q1).

5. **López-Moral, A.,** Llorens, E., Scalschi, L., García-Agustín, P., Trapero, A., Agustí, Brisach, C. 2022. Resistance induction in olive tree (*Olea europaea*) against Verticillium Wilt by two beneficial microorganisms and a copper phosphite fertilizer. *Frontiers in Plant Science* 13:831794 (IF:5.753; Q1).

6. **López-Moral, A.,** Agustí-Brisach, C., Raya, M.C., Lovera, M., Trapero, C., Arquero, A., Trapero, A. 2022. Etiology of Septoria Leaf Spot of Pistachio in Southern Spain. *Plant Disease* https://doi.org/10.1094/PDIS-02-21-0331-RE (IF:4.438; Q1).

7. Moral, J., Agustí-Brisach, C., Raya, M.C., Jurado-Bello, J., **López-Moral, A.**, Roca, L.F., Chattaoui, M., Rhouma, A., Nigro, F., Sergeeva, V., Trapero, A., 2021. Diversity of *Colletotrichum* species associated with olive anthracnose worldwide. *Journal of Fungi* 7:741 (IF: 5.816; Q1).

8. **López-Moral, A.**, Agustí-Brisach, C., Trapero, A. 2021. Plant biostimulants: new Insights into the biological control of Verticillium Wilt of Olive. *Frontiers in Plant Science* 12:662178. (IF:5.753; Q1).

9. Agustí-Brisach, C., Jiménez-Urbano, J.P., Raya, M.C., **López-Moral, A.**, Trapero, A. 2020. Vascular Fungi Associated with branch dieback of olive in super-high-density systems in southern Spain. *Plant Disease* 105:797-818 (IF:4.438; Q1).

10. **López-Moral, A.** Agustí-Brisach, C., Lovera, M., Arquero, O., Trapero, A. 2020. Almond anthracnose: current knowledge and future perspectives. *Plants* 9:945 (IF:2.762; Q1).

11. **López-Moral, A.**, Raya, M.C., Ruiz-Blancas, C., Medialdea, I., Lovera, M., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Aetiology of branch dieback, panicle and shoot blight of pistachio associated with fungal trunk pathogens in southern Spain. *Plant Pathology* 69:1237-1269 (IF:2.169; Q1).

12. **López-Moral, A.**, Lovera, M., Raya, M.C., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Etiology of branch dieback and shoot

blight of English walnut caused by Botryosphaeriaceae and *Diaporthe* fungi in southern Spain. *Plant Disease* 104:533-550 (IF:3.809; Q1).

13. López Moral, A., Agustí-Brisach, C., Lovera, M., Luque, Roca, L.F., Arquero, O., Trapero, A. 2019. Effects of cultivar susceptibility, fruit maturity, leaf age, fungal isolate and temperature on infection of almond by *Colletotrichum* spp. *Plant Disease* 103:2425-2432 (IF:3.809; Q1).

14. Agustí-Brisach, C., **López-Moral, A.**, Raya-Ortega, M.C., Franco, R., Roca, L.F., Trapero, A. 2018. Occurrence of grapevine trunk diseases affecting the native cultivar Pedro Ximénez in southern Spain. *European Journal of Plant Pathology* 153:599-625 (IF:1.582; Q2).

15. López Moral, A., Agustí-Brisach, C., Raya-Ortega, M.C., Roca, L.F., Lovera, M., Arquero, O., Trapero, A. 2018. First report of *Alternaria alternata* causing leaf blight in *Pistacia terebinthus* in Spain. *Plant Disease* 102:11 (IF:3.809; Q1).

16. Agustí-Brisach, C., Raya-Ortega, M.C., Trapero, C., Roca, L.F., Luque, F., **López-Moral, A.**, Fuentes, M., Trapero, A. 2018. First report of *Fusarium pseudograminearum* causing crown rot of wheat in Europe. *Plant Disease* 102:8 (IF:3.809; Q1).

17. López Moral, A., Raya-Ortega, M.C., Agustí-Brisach, C., Roca, L.F., Lovera, M., Luque, F., Arquero, O., Trapero, A. 2017. Morphological, pathogenic, and molecular characterization of *Colletotrichum acutatum* isolates causing almond anthracnose in Spain. *Plant Disease* 101:2034-2045 (IF:3.809; Q1).

SCIENTIFIC AND EXTENSION JOURNAL PUBLICATIONS

1. Trapero, A., Varo, A., Sánchez, M.E., Roca, L.F., **López-Moral, A.,** Agustí-Brisach, C. 2021. Enfermedades del algarrobo (*Ceratonia siliqua* L.). *Fruticultura* (*in press*).

2. Trapero, A., Roca, L.F., Segura, R., Luque, F., Romero, J., Raya, M.C., **López-Moral, A.,** Agustí-Brisach, C. 2021. Hacia el control biológico de las enfermedades aéreas del olivar. *Vida Rural* 504:50-56.

3. Agustí-Brisach, C., José Pablo Jiménez-Urbano, Raya, M.C., **López-Moral, A.**, Trapero, A. 2021. Hongos vasculares asociados a la 'seca' de ramillas en olivar superintensivo en Andalucía. *Phytoma* 326:2-6.

4. Lovera, M., **López-Moral, A.,** Raya, M.C., Ruíz-Blancas, C., Medialdea, I., Arquero, O., Trapero, A., Agustí-Brisach, C. 2020. Etiología de la seca de ramas y de la marchitez de brotes y panículas del pistachero en el sur de España. *Fruticultura* 78:12-27.

5. Mulero-Aparicio, A., Romero, J., Varo, A**., López-Moral, A.,** Agustí-Brisach, C., Roca, L.F., Raya, M.C., Santos-Rufo, A., López-Escudero, F.J., Narrillos, C., Salido-Navarro, L., Trapero, A. 2020. Diseño y evaluación de formulados precomerciales para el control biológico de la Verticilosis del olivo. *Phytoma* 321:30-36.

6. Agustí-Brisach, C., Roca, L.F., Antón-Domínguez, B.I., **López-Moral, A.,** Raya, M.C., Lovera, M., Arquero, O., Trapero, A. 2020. Decaimiento del almendro en plantaciones jóvenes en Andalucía. *Vida Rural* 476:36-43.

7. Mulero-Aparicio, A., **López-Moral, A.,** Agustí-Brisach, C., Varo, A., Roca, L.F., Raya, M.C., Romero, J., López-Escudero, F.J., Trapero, A. 2019. Avances en el control biológico de la verticilosis del olivo. *Vida Rural* 474:38-45.

8. Lovera, M., **López-Moral, A.,** Raya, M.C., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C. 2019. Etiología de la seca de ramas y marchitez de brotes del nogal en el sur de España. *Fruticultura* 72:6-19.

9. López-Moral, A., Agustí-Brisach, C., Lovera, M., Trapero, A, Raya, M.C., Roca, L.F., Arquero, O., Trapero, A. 2018. La septoriosis del pistachero. *Fruticultura* 64:134-143.

10. Agustí Brisach, C., **López-Moral, A.**, Raya, M.C., Roca, L.F., Beltrán, J.A., Carranza, R., Sánchez, M., Fuentes, M., Trapero, A. 2018. El pie negro de la colza. Una amenaza para un cultivo emergente. *Vida Rural* 450:38-45.

11. López-Moral, A., Agustí-Brisach, C., Lovera, M., Trapero, C., Roca, L.F., Arquero, O., Trapero, A. 2018. Enfermedades del pistachero en España (II). *Vida Rural* 441:12-16.

12. López-Moral, A., Agustí-Brisach, C., Lovera, M., Trapero, C., Roca, L.F., Arquero, O., Trapero, A. 2017. Enfermedades del pistachero en España (I). *Vida Rural* 439:52-62.

13. Agustí-Brisach, C., Franco, R., **López-Moral, A.,** Raya-Ortega, M.C., Roca, L.F., Trapero, A. 2017. Enfermedades de la madera de la vid. Resultados de prospecciones en Montilla-Moriles afectando a la variedad autóctona Pedro Ximénez. *Vida Rural* 432:30-38.

14. López-Moral, A., Agustí-Brisach, C., Raya-Ortega, M.C., Lovera, M., Roca, L.F., Luque, F., Arquero, O., Trapero, A. 2016. La antracnosis del almendro, susceptibilidad varietal en Andalucía. *Vida Rural* 423:56-62.

INTERNATIONAL CONFERENCES PAPERS

1. López-Moral, A., Agustí-Brisach, C., Mulero-Aparicio, A., Varo, A., Roca, L.F., Raya, M.C., Romero, J., López-Escudero, F.J., Trapero, A. Massive screening of natural and biological compounds to select the best candidates for biocontrol of Verticillium Wilt of Olive in the Mediterranean basin. 8th IOBC Meeting on integrated protection of olive crops, Lisboa, Portugal, 26-29 October 2021. Oral presentation.

2. López-Moral, A., Llorens, E., Scalschi, L., García-Agustín, P., Trapero, A., Agustí-Brisach, C. Biological treatments enhancing the plant immune system of olive (*Olea europaea*) against *Verticillium dahliae*. 8th IOBC Meeting on integrated protection of olive crops, Lisboa, Portugal, 26-29 October 2021. Poster.

3. Agustí-Brisach, C., **López-Moral, A.,** Raya, M.C., Franco, R., Roca, L.F., Trapero, A., Current situation of the grapevine trunk diseases affecting the native cultivar Pedro Ximénez in the Montilla-Moriles region, southern Spain. 'I Congreso Internacional sobre los Vinos Tradicionales de Andalucía', Madrid, Spain, 1-4 April 2019. Poster.

4. López-Moral, A., Xaviér, C., Escalona, A., Vioque, A., Agustí-Brisach, C., Moral, J., Trapero, A. Differences in virulence and sensitivity to fungicides within

Colletotrichum isolates causing olive anthracnose. 6th International Conference on Olive Tree and Olive Products-Olive Bioteq, Sevilla, Spain, 15-16 October 2018. Poster

5. Agustí-Brisach, C., Jiménez-Urbano, J.P., Raya, M.C., **López-Moral, A.,** Trapero, A. Fungal vascular diseases associated with branch dieback of olive in Andalusia region (southern Spain). 6th International Conference on Olive Tree and Olive Products-Olive Bioteq, Sevilla, Spain, 15-16 October 2018. Poster

6. López-Moral, A., Raya-Ortega, M.C., Agustí-Brisach, C., Roca, L.F., Lovera, M., Luque, F., Arquero, O., Trapero, A. Etiology of almond anthracnose in southern Spain. VII International Symposium on Almonds and Pistachios, Adelaida, Australia, 05-11 November 2017. Poster

7. López Moral, A., Raya-Ortega, M.C., Agustí-Brisach, C., Roca, L.F., Lovera, M., Luque, F., Arquero, O., Trapero, A. Characterization of *Colletotrichum acutatum* isolates causing almond anthracnose in Spain. 15th Mediterranean Phytopathological Union Congress, Córdoba, Spain, 20-23 June 2017. Poster

NATIONAL CONFERENCES PAPERS

1. Agustí-Brisach, C., **López-Moral, A.,** Lovera, M., Roca, L.F., Raya, M.C., Luque, F., Arquero, O., Trapero, A. Enfermedades emergentes del almendro en Andalucía: causas de su emergencia. Encuentro Internacional 2020-Año Internacional de la Sanidad Vegetal. Phytoma. Córdoba, Spain, 1-2 December 2021 (Phytoma, 334: 133). Poster.

2. Mulero-Aparicio, A., **López-Moral, A.,** Agustí-Brisach, C.**,** Varo, A., Roca, L.F., Raya, M.C., Romero, J., López-Escudero, F.J., Trapero, A. Desarrollo de una estrategia para el control biológico de la Verticilosis del olivo. Encuentro Internacional 2020-Año Internacional de la Sanidad Vegetal. Phytoma. Córdoba, Spain, 1-2 December 2021 (Phytoma, 334: 132). Poster.

3. Mulero-Aparicio, A., **López-Moral, A.,** Agustí-Brisach, C., Varo, A., Roca, L.F., Raya, M.C., Romero, J., López-Escudero, F.J., Trapero, A. Desarrollo de una estrategia para el control biológico de la Verticilosis del olivo. Encuentro Internacional Año Internacional de la Sanidad Vegetal. Phytoma. Córdoba, Spain, 1-2 December 2021. Poster.

4. López-Moral, A., Agustí-Brisach, C., Llorens, E., Scalschi, L., Sánchez-Rodríguez, A.R., García-Agustín, P., Trapero, A. Bioestimulantes e inductores de resistencia como alternativas para el control biológico de la Verticilosis del olivo. 'XVI Congreso nacional de ciencias hortícolas (SECH)', Córdoba, Spain, 17-22 October 2021. Oral presentation.

5. López-Moral, A., Agustí-Brisach, C., Lovera, M., Raya, M.C., Luque, F., Roca, L.F., Arquero, O., Trapero, A. Gestión integrada de la Antracnosis del almendro en Andalucía. 'XVI Congreso nacional de ciencias hortícolas (SECH)', Córdoba, Spain, 17-22 October 2021. Oral presentation.

6. Lovera, M., **López-Moral, A.,** Luque, F., Ollero-Lara, A., Mateos-Cobacho, N.; Ruiz-Prados, M.D., Romero, J., Agustí-Brisach, C., Arquero, O., Trapero, A. Bases para la gestión integrada de la mancha ocre del almendro en Andalucía.

'XVI Congreso nacional de ciencias hortícolas (SECH)', Córdoba, Spain, 17-22 October 2021. Poster.

7. López-Moral, A., Lovera, M., Antón-Domínguez, B.I., Gámiz, A.M., Arquero, O., Trapero, A., Agustí-Brisach, C. Caracterización biológica de hongos asociados a la seca de ramas y brotes del nogal. 'XVI Congreso nacional de ciencias hortícolas (SECH)', Córdoba, Spain, 17-22 October 2021. Poster.

8. López-Moral, A., Agustí-Brisach, C., Trapero, A. Bioestimulantes: nuevas alternativas para el control biológico de la Verticilosis del olivo. 'Congreso en red de Olivicultura, Citricultura y Fruticultura de la Sociedad Española de Ciencias Hortícolas (SECH)', Online, 23-25 March 2020. Oral presentation.

9. Agustí-Brisach, C., **López-Moral, A.,** Lovera, M., Raya, M.C., Roca, L.F., Antón-Domínguez, B.I., Arquero, O., Trapero, A. Etiología de la seca de ramas y decaimiento de árboles en los cultivos de frutos secos en Andalucía. 'Congreso en red de Olivicultura, Citricultura y Fruticultura de la Sociedad Española de Ciencias Hortícolas (SECH)', Online, 23-25 March 2020. Oral presentation.

10. Trapero, A., **López-Moral, A.,** Lovera, M., Roca, L.F., Raya, M.C., Luque, F., Arquero, O., Agustí-Brisach, C. Enfermedades emergentes del almendro en Andalucía. 'XI Jornada Fruticultura Sociedad Española de Ciencias Hortícolas (SECH)', Sevilla, Spain, 18-19 September 2019. Oral presentation.

11. Lovera, M., Arquero, O., Roca, L.F., **López-Moral, A.,** Raya, M.C., Agustí-Brisach, C., Trapero, A. Evaluación de tratamientos fungicidas para el control de las enfermedades de la parte aérea del almendro. 'XI Jornada Fruticultura Sociedad Española de Ciencias Hortícolas (SECH)', Sevilla, Spain, 18-19 September 2019. Oral presentation.

12. López-Moral, A., Agustí-Brisach, C., Lovera, M., Raya, M.C., Luque, F., Roca, L.F., Arquero, O., Trapero, A. La Antracnosis del almendro en Andalucía: etiología, epidemiología y control. 'XI Jornada Fruticultura Sociedad Española de Ciencias Hortícolas (SECH)', Sevilla, Spain, 18-19 September 2019. Oral presentation.

13. López-Moral, A., Agustí-Brisach, C., Raya, M.C., Lovera, M., Trapero, C., Roca, L.F., Arquero, O., Trapero, A. Caracterización fenotípica, molecular y patogénica de *Septoria pistaciarum*, agente causal de la septoriosis del pistachero en España. 'XI Jornada Fruticultura Sociedad Española de Ciencias Hortícolas (SECH)', Sevilla, Spain, 18-19 September 2019. Poster.

14. López-Moral, A., Lovera, M., Raya, M.C., Cortés-Cosano, N., Arquero, O., Trapero, A., Agustí-Brisach, C**.** Etiología de la marchitez de brotes y seca de ramas de nogal causada por Botryosphaeriaceae y Diaporthaceae en el sur de España. 'XI Jornada Fruticultura Sociedad Española de Ciencias Hortícolas (SECH)', Sevilla, Spain, 18-19 September 2019. Poster.

15. Agustí-Brisach, C., Roca, L.F., Antón-Domínguez, B.I., **López-Moral, A.,** Raya, M.C., Lovera, M., Arquero, O., Trapero, A., Etiología del decaimiento del almendro en plantaciones jóvenes. Una nueva enfermedad en Andalucía. 'XIX Congreso de la Sociedad Española de Fitopatología', Toledo, Spain, 08-10 October 2018. Oral presentation.

16. **López Moral, A.,** Agustí-Brisach, C., Lovera, M., Trapero, C., Raya, M.C., Roca, L.F., Arquero, O., Trapero, A. Etiología de la Septoriosis del pistachero en

Andalucía. 'XIX Congreso de la Sociedad Española de Fitopatología', Toledo, Spain, 08-10 October 2018. Poster.

17. Agustí-Brisach, C., **López-Moral, A.,** Raya, M.C., Franco, R., Roca, L.F., Trapero, A. Situación actual de las enfermedades de la madera de la vid en la DOP Montilla-Moriles afectando a la variedad Pedro Ximénez. 'XIX Congreso de la Sociedad Española de Fitopatología', Toledo, Spain, 08-10 October 2018. Poster.

18. López-Moral, A., Agustí-Brisach, C., Lovera, M., Luque, F., Arquero, A., Trapero, A. Susceptibilidad de variedades del almendro a la antracnosis causada por *Colletotrichum* spp. 'XIX Congreso de la Sociedad Española de Fitopatología', Toledo, Spain, 08-10 October 2018. Poster.

19. López-Moral, A., Xaviér, C., Escalona, A., Vioque, A., Agustí-Brisach, C., Moral, J., Trapero, A. Variabilidad de especies de *Colletotrichum* asociadas con la antracnosis del olivo y del almendro. 'XIX Congreso de la Sociedad Española de Fitopatología', Toledo, Spain, 08-10 October 2018. Poster.

20. López- Moral, A., Raya, M.C., Luque, F., Arquero, O., Trapero, A. Caracterización morfológica, patogénica y molecular de *Colletotrichum acutatum*, agente causal de la antracnosis del almendro. 'XVIII Congreso de la Sociedad Española de Fitopatología', Palencia, Spain, 20-23 September 2016. Oral presentation.

PARTICIPATION IN RESEARCH PROJECTS

1. AGL2016-76240-R. Efficacy of new genotypes and biological treatments for the control of Verticillium Wilt of Olive in the field.(VERTOLEA). PI. PI: Antonio Trapero Casas; F. Javier López-Escudero. Participation as researcher. 01/12/2021-30/11/2023. 115,000,00 €.

2. PPTRATRA-2016.00.6. `Transforma de Fruticultura Mediterránea´. IFAPA, Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía. PI: Octavio Arquero Quiles (IFAPA). Participation as work-team member. 10/2014-10/2017. 230.000,00 €

3. PP.TRA.TRA2019.002-'Transferencia en Fruticultura Mediterránea'. IFAPA, Consejería de Agricultura, Pesca y Desarrollo Rural, Junta de Andalucía. PI: Octavio Arquero Quiles (IFAPA). Participation as work-team member. 02/2018-Present. 351.613,00 €

4. AGL2016-76240-R. 'Gestión Integrada de la Verticilosis del Olivo mediante resistencia genética, prácticas agronómicas y control biológico'. MICINN. PIs: Antonio Trapero Casas; Francisco Javier López Escudero (University of Cordoba). Participation as predoctoral researcher. 01/2017-12/2020. 200.000,00 €

PARTICIPATION IN CONTRACTS WITH PRIVATE COMPANIES (Article 83)

1. Contract for the Provision of Services between Fertinyect S.L. and the University of Cordoba. 'Evaluation of endotherapy treatments against grapevine trunk diseases'. PI: Antonio Trapero Casas, Carlos Agustí Brisach (UCO). 01/04/2021-31/03/2023. 7,475.00 €.

2. Contract for the Provision of Services between Bodegas Fundador S.L.U. and the University of Cordoba. 'Research project and training for integrated management against trunk diseases in new vineyards' PI: Antonio Trapero Casas, Carlos Agustí Brisach (UCO). 01/09/2021-31/08/2022. 16,064.54 €.

3. Contract for the Provision of Services between the Institute for Agricultural and Fisheries Research and Training (IFAPA) and the University of Cordoba. 'Technical assistance about diagnosis and phytopathological analyses of gravenine trunk diseases: field and nursery surveys in Jerez area. PI: Antonio Trapero Casas, Carlos Agustí Brisach (UCO). 01/04/2021-01/01/2022. 28,862.00 €.

4. Contract for the Provision of Services between the Institute for Agricultural and Fisheries Research and Training (IFAPA) and the University of Cordoba. 'Diagnosis of grapevine trunk diseases in the protected designation of origin Jerez-Xérès-Sherry'. PIs: Antonio Trapero Casas, Carlos Agustí Brisach (University of Cordoba). Participation as work-team member. 01/10/2020-31/12/2020. 7,900.00 €.

5. Contract for the Provision of Services between Arysta Lifescience Company and the University of Cordoba. `Evaluación de un fungicida frente a enfermedades del olivo (repilo, antracnosis y tuberculosis) y del almendro (antracnosis)´. PIs: Antonio Trapero Casas (University of Cordoba). Participation as contrated researcher. 12/2015-12/2017. 31,900.00 €

6. Contract for the Provision of Services between the Spanish Olive Oil Interprofessional and the University of Cordoba. `Control biológico de la Verticilosis del olivo´. PI: Antonio Trapero Casas (University of Cordoba). Participation as contrated researcher. 05/2011-12/2016. 123,600.00 €

SUPERVISION OF BSc DEGREE THESIS

1. Esther Aguilera Cuenca. Efecto de tratamientos fungicidas, bioestimulantes y microorganismos sobre suelo naturalmente infestado con *Verticillium dahliae*. GIAMR, ETSIAM, UCO. (Evaluation 09/2020).

2. Francisco Manuel Leiva Egea. 'Efecto de tratamientos fungicidas, bioestimulantes y biológicos sobre *Verticillium dahliae* en condiciones controladas´. GIAMR, ETSIAM, UCO. (Evaluation 07/2020).

3. Azahara Escalona García-Escribano. 'Sensibilidad in vitro a fungicidas de especies de *Colletotrichum* causantes de la antracnosis del olivo y del almendro´. GIAMR, ETSIAM, UCO. (Evaluation 09/2018).

SUPERVISION OF MSc DEGREE THESIS

1. Cristina Ruíz Blancas. Efecto de desequilibrios nutricionales de N y K sobre *Verticillium dahliae* mediante ensayos *in vitro* e *in planta* en olivo. Master in Agricultural Engineering, ETSIAM, UCO. (Evaluation 09/2021).

2. Francisco Manuel Leiva Egea. 'Efecto sobre el crecimiento y propiedades de la savia de plantones de olivo tratados con diversos compuestos e inoculados con

Verticilium dahliae.' Master in Agricultural Engineering, ETSIAM, UCO. (Evaluation 09/2021).

3. **Azahara Escalona García-Escribano.** 'Efecto *in vitro* de fungicidas sobre *Septoria pistaciarum*, agente causal de la septoriosis del pistachero en Andalucía´. Master in Agricultural Engineering, ETSIAM, UCO. (Evaluation 10/2020).
4. **Andrés Venancio Vioque Maynez.** 'Efecto de fungicidas sobre *Colletotrichum acutatum*, agente causal de la antracnosis del olivo y del almendro, mediante bioensayos en manzana' Master in Agricultural Engineering, ETSIAM, UCO. (Evaluation 12/2019).
5. **Marina Rosa Fuentes Morales.** 'Efecto de extractos de savia de olivo sobre la germinación de conidios de *Verticillium dahliae*'. Master in Agricultural Engineering, ETSIAM, UCO. (Evaluation 12/2018).

AWARDS

1. **Extraordinary Master Award.** Master in Plant Production, Protection and Breeding, Course 2014/15, University of Cordoba (10/2016).
2. **Poster Award of the section 'T04-Plant Protection'**- '*Fungal vascular diseases associated with branch dieback of olive in Andalusia region (southern Spain)*'; '6th International Conference on Olive Tree and Olive Products-Olive Bioteq', Sevilla, Spain (10/2018).
3. **Mention in the 'XXI Edición Premios Nacionales a la Investigación y Defensa en Producción Ecológica "Andrés Núñez de Prado", Reseacrh modality**- '*Posibilidades del uso de sustancias de origen natural para el control biológico de la Verticilosis del olivo en Andalucía*' en su modalidad de investigación'; Ecovalia, Sevilla, Spain (11/2019).