A split-root system to assess biocontrol effectiveness and defense-related genetic responses in above-ground tissues during the tripartite interaction *Verticillium dahliae*-olive-*Pseudomonas fluorescens* PICF7 in roots

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Abstract

Background and aims The olive root endophyte *Pseudomonas fluorescens* PICF7 is an effective biocontrol agent of *Verticillium* wilt of olive (VWO). Colonization of olive roots either by strain PICF7 or by *Verticillium dahliae* triggers differential systemic transcriptomic responses, many of them related with defense-related genes. The aims were to develop an olive split-root system for assessing VWO development and biocontrol effectiveness of strain PICF7 in plants with a divided root architecture, and

for evaluating systemic defense responses during this tripartite interaction when strain PICF7 and *V. dahliae* are spatially separated.

Methods An olive split-root system was generated and disease development, biocontrol effectiveness and systemic genetic responses in these plants upon strain PICF7 and *V. dahliae* colonization were compared to those reported and observed in olive plants grown under standard conditions (single pots). Specific defense-related genes, previously identified during PICF7- and/or *V. dahliae*-olive root interactions were selected and their expression patterns assessed in above-ground tissues by real-time qPCR analyses.

Results Symptoms of VWO developed similarly both in split-root and single-root plants. However, even though PICF7 triggered systemic defense responses in aerial tissues prior to the infection by *V. dahliae*, effective biocontrol was not observed under these experimental conditions. While most of studied genes showed similar expression patterns along time in both systems (i.e. split root and single pot), some of them (e.g. the *caffeoyl-O-methyltransferase* coding gene) varied depending on whether strain PICF7 and *V. dahliae* were spatially separated or shared the same compartment.

Conclusions A successful split-root system was generated to investigate genetic events taking place during the tripartite interaction olive-*V. dahliae*-*P. fluorescens* PICF7. VWO biocontrol by strain PICF7 must rely on mechanisms other than induction of systemic resistance responses. The expression pattern of specific defense-related olive genes depended on whether or not the biocontrol agent and the pathogen share the same root/soil region.

Keywords Biological control, Host defense response, *Olea europaea* L., Plant-microbe interaction, Verticillium wilt

Introduction

Plant-microbe interactions occurring at the root/rhizosphere level are increasingly studied since the events (molecular, genetic, etc.) taking place in this extraordinary ecological niche are crucial for above-ground tissues, influencing plant's health and productivity (Lakshmanan et al. 2014). The positive and negative effects observed in aerial tissues can be exerted by beneficial or pathogenic components present in the soil/root-associated microbiomes, or through their activities leading to litter decomposition, nutrients solubilization and cycling (Glick 1995), secretion of plant growth hormones (Ali et al. 2009; Mishra et al. 2009), antagonism of pathogens (Kloepper et al. 2004), and induction of the plant immune system (Rudrappa et al. 2008, 2010). In nature, interactions between a single plant and individualized soil-borne microorganisms do not occur. On the contrary, complex multipartite relationships are dynamically established. These multitrophic scenarios are now increasingly investigated from holistic perspectives, although predominantly focusing on herbaceous plants (Narisawa et al. 2002; Li et al. 2011; Cwalina-Ambroziak et al. 2012; Gafni et al. 2015; Palazzini et al. 2016). However, studies on plant gene expression during the interaction of the host with both biological control agents (BCA) and pathogens are still limited (Gallou et al. 2009; Mejía et al. 2014; Mayo et al. 2016; Mgbeahurike et al. 2017). Nowadays, it is becoming an increasingly widespread practice to add beneficial microorganisms (i.e. BCA and/or plant growth promoting rhizobacteria) to the soil to counteract the negative effects of some pathogens and to stimulate the plant growth (Bashan et al. 2014; Ciancio et al. 2016). However, when this strategy is implemented uneven distribution leading to reduced colonization of the soil and/or the plant roots by

the BCA may happen, especially in the case of woody plants with usually large radical systems. This may affect the effectiveness of the BCA, either because direct contact with the target pathogen is hampered or does not take place at all (i.e. failure of direct antibiosis or competition) or the effectiveness of defense systemic responses triggered by the BCA is attenuated.

In the present work we focus our attention on a tripartite interaction involving olive (*Olea europaea* L.), the beneficial root endophytic bacterium *Pseudomonas fluorescens* PICF7, and the soil-borne fungal pathogen *Verticillium dahliae* Kleb., the etiological agent of Verticillium wilt of olive (VWO). This is one of the most important biotic constraints affecting this woody crop. During the last decades VWO has alarmingly spread within the Mediterranean Basin, the region where olive is mainly cultivated. Moreover, the disease is very difficult to manage because of a number of contributing factors (Tsrör 2011), and no effective control measure is currently available when applied individually. Thus, the implementation of an integrated disease management strategy for the effective control of VWO is strongly recommended (López-Escudero and Mercado-Blanco 2011). A promising control tool within this holistic framework is the use of microorganisms showing effective biocontrol activity against *V. dahliae*. This holds particularly interesting when they are used as a preventive measure during the plant propagation stage at nurseries (Mercado-Blanco et al. 2004).

Pseudomonas fluorescens PICF7 is a natural colonizer of olive roots (Martínez-García et al. 2015), an effective *in vitro* antagonist against *V. dahliae* and an efficient BCA of VWO in nursery-produced olive plants (Mercado-Blanco et al. 2004; Prieto et al. 2009; Maldonado-González et al. 2015b). It has been demonstrated that strain PICF7 is able to endophytically colonize olive root tissues under different experimental conditions (Prieto and Mercado-Blanco 2008; Prieto et al. 2011; Maldonado-González

et al. 2015b). Moreover, effective suppression of the disease requires the presence of the BCA at both the surface and the interior of olive roots prior to colonization by *V. dahliae* (Prieto et al. 2009). While our knowledge on PICF7 traits involved in endophytic lifestyle and biocontrol activity is still limited (Maldonado-González et al. 2015a; 2015b), broad changes at the transcriptomic level have been earlier identified in olive tissues (roots and stems) upon root colonization by PICF7. Indeed, the presence of this bacterium in olive root tissues induced, among others, a range of defense responses at the local level (i.e. roots), including genes related to salicylic acid (SA) (e.g. acetone cyanohydrin lyase, [ACL]) and jasmonic acid (JA) (e.g. lipoxygenase [LOX] and transcription factor *bHLH*) signaling pathways, and phenylpropanoids biosynthesis (phenylalanine ammonia lyase [PAL]) (Schilirò et al. 2012). Systemic responses are also up-regulated in aerial tissues, many of them involved in plant defense responses to different stresses. For instance, genes involved in phenylpropanoids biosynthesis, JA, SA and ethylene (ET) signaling pathways (e.g. *PAL*, *LOX-2*, *ACL*, 1-aminocyclopropane-1-carboxylate oxidase [ACO], etc.), oxidative stress (catalase [CAT]) and Ca²⁺ metabolism implicated in systemic defensive responses were induced in above-ground organs. Besides, expression of several transcription factors (TF) related to plant defense were also up-regulated (e.g. *JERF*, *bHLH* and *WRKY*) (Gómez-Lama Cabanás et al. 2014).

Broad transcriptomic changes in olive aerial tissues upon root inoculation with the highly-virulent, defoliating (D) pathotype of the pathogen have also been reported. Many of the olive genes induced (e.g. calmodulin-binding family protein, *ACL*, or the TF *GRAS1*) or repressed (e.g. defensin or chloroplastic 6-phosphogluconolactonase) were involved in defense responses against diverse (a)biotic stresses, similarly to the situation observed during the interaction with strain PICF7. While most of the genetic

responses observed in olive tissues were distinctive for each olive-microorganism interaction, some of them were found to occur in both interactions (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014, 2015). These studies have provided crucial information on genetic responses upon inoculation of olive roots with soil-borne microorganisms (either beneficial or deleterious), but so far only during dual interactions. However, there is a complete lack of knowledge on olive genetic responses when these two soil-borne microorganisms interact with their host at below ground level.

Defense-related genetic responses observed in aerial tissues upon olive root inoculation with strain PICF7 suggested that this endophytic BCA could trigger an induced systemic resistance response potentially effective against other phytopathogens. Moreover, results also showed that PICF7 was able to control infection by *Botrytis cinerea* De Bary. in leaves of *Arabidopsis thaliana* L. when the BCA was applied to the roots (Maldonado-González et al. 2015a). In contrast, the presence of strain PICF7 in olive roots does not prevent olive knot disease caused by *Pseudomonas savastanoi* pv. *savastanoi* (Maldonado-González et al. 2013). In view of these contradicting results the question arises whether defense responses triggered in above-ground olive tissues after root colonization by PICF7 are effective when this BCA and a given pathogen are spatially separated. Moreover, whether an induced resistance-based biocontrol mechanism, operating beyond the initially-colonized tissue, is responsible of the effective VWO control exerted by this BCA remains to be demonstrated. In this tripartite interaction, however, both microorganisms share the same ecological niche: the olive root/rhizosphere. Therefore, to demonstrate the existence of an effective defense response against *V. dahliae* occurring at long distances in the radical system, direct interaction between the two microorganisms must be ruled out. This can be achieved by ensuring spatial separation of the BCA and the pathogen, as demonstrated

for other pathosystems (Pieterse et al. 2014).

Split-root systems have been designed and used to confirm the involvement of induced resistance mechanisms triggered by a range of BCAs effective against different phytopathogens, although most of the available examples are for herbaceous plants (He et al. 2002; Pozo et al. 2002; Siddiqui et al. 2002; Li et al. 2010; Aimé et al. 2013; Martínez-Medina et al. 2016). The implementation of the split-root approach in woody plants is more complicated because of inherent characteristics of these plants (Cazorla and Mercado-Blanco 2016). However, a few examples are available although those in which a split-root system has been particularly designed to study the interaction with soil-borne microorganisms in woody plants are scarce (Boukcim et al. 2006; Dry and Loveys 1999; Qiang-Sheng et al. 2016; Wang et al. 2016). In olive, a split-root system was used to evaluate different water regimes in cultivars Picholine Marocaine and Picholine Languedoc (Aganchich et al. 2007), Chetoui (Dbara et al. 2016) and Picual (García-Tejera et al. 2017).

Considering the previous information and premises, the objectives of this study were: (i) to develop an olive split-root system to assess effectiveness of above-ground defense responses in olive upon PICF7 root colonization when this BCA and *V. dahliae* are spatially separated, (ii) to evaluate whether VWO symptoms develop in this system and whether PICF7 is able to control *V. dahliae* when both microorganisms are spatially separated and sequentially applied, (iii) to assess whether systemic responses triggered are similar regardless of whether or not both microorganisms are separated in the olive rhizosphere, and (iv) to check whether *V. dahliae* is able to move from inoculated to non-inoculated root compartments, firstly acropetally and then basipetally. We aimed to test the hypothesis that an induced resistance response is involved in the effective VWO biocontrol exerted by strain PICF7.

Materials and Methods

Production of olive cuttings to set up a split-root study system

Olive plant material was collected from healthy trees in a forty-year-old olive orchard located at Aguilar de la Frontera (Córdoba province, Southern Spain) and planted with cultivar Picual (highly susceptible to *V. dahliae*) (López-Escudero and Mercado-Blanco 2011). Four *V. dahliae*-free olive trees were eventually selected in the orchard to collect plant material for further propagation. In these trees, five stems were collected at random from the canopy for assessing the absence of natural infections of the pathogen by PCR analysis (Mercado-Blanco et al. 2003). Additionally, *V. dahliae* was not detected in samples from the soil of the orchard that were examined by the wet sieving method (Huisman and Ashworth 1974) and culturing on a semi-selective modified sodium pectate agar (Butterfield and De Vay 1977). For this, an area of 400 m² from where selected trees originated was analyzed. One 200 g soil sample was taken from the upper 20 cm along the tree rows at 30 cm from the trunk of trees with a cylindrical (3.5 cm x 22 cm) auger. Thereafter, collected sub-samples (a total of 25) were bulked, crumbled, and mixed. Young sprouts (60-cm long, approximately 1-cm wide), growing from the base of the main trunk of the selected olive trees were collected and preserved at 4°C. Then, cuttings (15-cm long) were obtained by producing a longitudinal incision (approximately 5-cm deep, 2.5-mm wide) with a professional saw (Metabo BAS 260 Swift Band, Herramientas Metabo, S.A., Boadilla del Monte, Madrid, Spain). Since no information on the rooting success rate was available for this material, a fair number of cuttings (>200) were initially prepared for propagation. Cuttings were rooted according to the technique of soft-wood under mist conditions (Caballero and Del Río 2010). First, cuttings were dipped by the incision end in a 40% (v/v) ethanol solution containing

indol-3-butyric acid (4 g/l) during 5 s. Each part of the cleft cutting was then inserted (up to 4-cm deep) into two adjacent plastic pots (Figure 1A) containing 40% peat, 60% silt, and a slow-release fertilizer (Nutricote Total T270 [15-9-10]), Arista Life Science, Dusseldorf, Germany). These paired pots will eventually harbor two independent radical systems developed separately from each part of the cleft cutting. Double pots were maintained at 20-25°C in a controlled chamber, with 90% of relative humidity provided by intermittent mist (Figure 1B). After two months, rooted double cuttings were transferred to benches in a greenhouse, and grown for 45 days under natural lighting until shoots produced around 50 pairs of leaves. Prior to PICF7 treatment, plants were acclimated for 3 weeks in the greenhouse where the experiment was performed. By this time, plants were ready for inoculation (root dipping) (Figure 1C). For each treatment (see below) five healthy plants with well-developed foliage were selected for the time-course of gene expression experiment, and 15 additional plants to assess VWO progress.

Inoculation strategy of *Pseudomonas fluorescens* PICF7 and *Verticillium dahliae* D pathotype in the split-root system

Five treatments were included in the split-root study system (Figure 2): T1) true control treatment; that is, plants non-manipulated at all; T2) plant manipulation control treatment; that is, radical systems developed in each compartment were dipped in 10mM SO₄Mg·7 H₂O (15 min) or sterile distilled water (30 min) at the time each microbial inoculation (the BCA and/or the pathogen) was performed (see below); T3) BCA control treatment (plants inoculated only with strain PICF7); that is, roots of one compartment were dipped in a suspension of PICF7 cells and the roots of the other compartment in sterile distilled water; T4) Disease control treatment (plants inoculated

only with *V. dahliae*); that is, roots of one compartment were dipped in 10 mM $\text{SO}_4\text{Mg}\cdot 7\text{H}_2\text{O}$ and roots of the other compartment in a conidia suspension of *V. dahliae*; and T5) Plants inoculated with the BCA and the pathogen; that is, the radical system of one compartment was first dipped in a suspension of strain PICF7 cells, and seven days later the roots of the other compartment in a conidia suspension of *V. dahliae*.

Plants used in the split-root system were inoculated with *P. fluorescens* PICF7 and/or *V. dahliae* as previously described (Prieto and Mercado-Blanco 2008; Schilirò et al. 2012). The inoculation strategy of the BCA and the pathogen was as follows. Firstly, roots developed in one of the compartments (treatments T3 and T5) were carefully uprooted from the original substrate, thoroughly washed in tap water without intentional wounding (Figure 1D), and dipped in a suspension of PICF7 cells (10mM SO_4Mg ; $1\cdot 10^8$ cells ml^{-1}) during 15 min. At the same time, radical systems for one of the compartments of treatments T2 and T4 were manipulated in the same way but dipped just in 10mM $\text{SO}_4\text{Mg}\cdot 7\text{H}_2\text{O}$. Then, roots of these compartments were transplanted into polypropylene pots containing a sterile sandy substrate (Prieto and Mercado-Blanco 2008). Plants were maintained in a controlled growth chamber ($23 \pm 1^\circ\text{C}$, 60–90% relative humidity, 14- h photoperiod of fluorescent light at $360\mu\text{E m}^{-2}$) until pathogen inoculation. One week later, inoculation of the other compartments with *V. dahliae* isolate V937I (representative of the D, highly virulent pathotype) (Maldonado-González et al. 2015a; 2015b) was carried out for treatments T4 and T5 by immersion of the roots in a conidia suspension (30 min, $1\cdot 10^7$ conidia ml^{-1}), as described by Mercado-Blanco et al. (2002). Radical systems developed in the corresponding compartments of treatments T2 and T3 were dipped in water. Non-manipulated plants (treatment T1) were used as genuine controls (no manipulation, no inoculation). Plants were cultured under conditions indicated above during 80 days.

Olive-*Pseudomonas fluorescens* PICF7-*Verticillium dahliae* bioassay using single pots

To evaluate whether some of the early- and medium-term systemic responses triggered by strain PICF7 and PICF7/*V. dahliae*, respectively, followed the same expression pattern when the BCA and the pathogen share the same ecological niche (i.e. the olive rhizosphere), a complementary experiment in which *P. fluorescens* PICF7 and *V. dahliae* were inoculated in the same pot was carried out. The microorganisms were applied in the same temporal sequence (i.e. first the BCA and 7 days later the pathogen). The standard bioassay routinely performed in our group was conducted (i.e. Prieto et al. 2009; Maldonado-González et al. 2015b) but with minor modifications. Briefly, five-month-old, nursery-produced potted ‘Picual’ plants were used and three treatments were included: i) Control plants (non-manipulated, non-inoculated), ii) strain PICF7-inoculated plants, and iii) PICF7- and *V. dahliae*-inoculated plants. PICF7 suspension cells and *V. dahliae* conidia inoculum were prepared as described above. In this case, however, inoculation was performed by adding 150 ml per pot, first with a PICF7 cells suspension and seven days later with a *V. dahliae* conidia suspension. Control plants were watered with the same volume of 10mM SO₄Mg·7H₂O and sterile distilled water.

Assessment of *Verticillium* wilt of olive progress

To evaluate VWO progress in inoculated plants, disease was assessed twice a week over a period of 73 days severity using a 0 to 16 rating scale. This scale estimated percentage of affected tissue using four main categories or quarters (≤ 25 , 26-50, 51-75, and 76-100%) with four values per each category. Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) were linearly related to the

percentage of affected tissue (Y) by the equation: $Y = 6.25X - 3.125$ (Varo et al. 2017). The relative area under the disease progress curve (RAUDPC) was calculated from the disease severity values by the trapezoidal integration method (Campbell and Madden 1990) after 80 days of PICF7 inoculation (73 days after *V. dahliae* inoculation) for SO₄Mg/*V. dahliae* and PICF7/*V. dahliae* inoculated plants (treatments T4 and T5, respectively). RAUDPC data of treatments were analyzed using ANOVA and significant differences among means were compared using the Fisher's Least Significant Difference (LSD) test ($P < 0.05$). ANOVA were performed with Statistix 9.0 (Statistix for Windows Manual. 1985-2008. Analytical Software. Version 9.0, Tallahassee FL, 2008). In addition, the disease incidence or percentage of symptomatic plants and percentage of dead plants were recorded to assess the intensity of the reactions (López-Escudero et al. 2004).

In planta* molecular detection of *Verticillium dahliae

In order to check effective infection by *V. dahliae* in the root compartments where the pathogen was inoculated, as well as to assess either accidental (i.e. cross contamination) or active (i.e. effective movement of *V. dahliae* throughout the vascular system of the plant) transfer of the fungus to the adjacent compartment in the split-root system, root samples were examined for the presence of *V. dahliae* DNA by qualitative real time PCR assays. Roots sampled from both compartments of six *ad hoc* selected plants (three of them showing severe VWO symptoms and three only displaying moderate/null disease symptoms) were collected at the end of the experiment, 73 days after inoculation (DAI) with the pathogen. Plants were uprooted from the pots, their roots washed under tap water to remove soil particles, lyophilized and ground to a fine powder in 2-ml tubes with glass beads using a MM 301 mixer mill (Retsch GmbH,

Haan, Germany). DNA was extracted from ground roots using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. DNA purity and concentration were determined by using a ND-1000 Spectrophotometer (NanoDrop Thermo Fisher Scientific Technologies, Wilmington, DE, USA) and Qubit® 2.0 Fluorometer (Invitrogen, UK) and DNA solutions were stored at -20°C until used. PCR experiments and analyses were done in a thermal cycler iQ5 Real-Time PCR System (BioRad, Hercules, CA, USA) and PCR reactions were performed containing 25 ng of DNA, 0.1µM of *V. dahliae* specific primers DB19 (Carder et al. 1994) and espDEF01 (Mercado-Blanco et al. 2003), 2x iQ™ SYBR Green Supermix (BioRad) and H₂O up to a total volume of 20 µL. PCR protocol was: a denaturation step for 4 min at 95°C, followed by 50 cycles of 1 min at 94°C, 45 s at 59°C and 45s at 72°C and a final extension step of 10 min at 72°C. Melting curves of products were assessed from 59°C to 100°C with a 0.5°C transition rate every 10s with an initial denaturation step of 5 min at 95°C to confirm the presence of single PCR amplicons. For each root compartment, presence of *V. dahliae* DNA was verified twice in independent experiments, and three replicates per plate were included.

mRNA purification and time-course expression of selected olive genes by real-time qPCR

The olive tissue sampling strategy was as follows. For plants used in the split-root experiment, above-ground tissues (leaves) were sampled at different DAI either with strain PICF7 or *V. dahliae*. Thus, aerial tissues from treatment T2 (manipulated control plants) and T3 (PICF7 control plants) were sampled at 1, 7, 8 and 14 DAI. T4 (*V. dahliae* control plants) and T5 (double-inoculated plants) were collected at 7, 8 and 14 DAI. Each sample (treatment/time-point) consisted of a pool of leaves collected from

five different plants for each treatment. Moreover, two different pools were collected for each treatment/time-point (i.e. two biological replicates).

For plants used in the single-pot experiment, leaves were collected as follows: i) Non-manipulated control plants were sampled at the beginning of the assays (day 0), ii) samples from PICF7 control plants were collected at 1 DAI, and iii) leaves from PICF7/*V. dahliae* inoculated plants were collected at 7 DAI with the pathogen (i.e. 14 DAI with strain PICF7). Two biological replicates were also collected, consisting of pool of leaves from two different plants for each treatment/time-points.

Isolation of total RNA from each sample was performed using easyBLUE™ (iNtRON Biotechnology Inc, Sangdaewon-Dong, South Korea), following the manufacturer's protocol for plant tissues. Genomic DNA in samples was removed by DNaseI treatment (Roche Applied Science, Mannheim, Germany) according to the manufacturer's recommendations. Purity and quality of RNA samples were checked by agarose gel electrophoresis, spectrophotometry using a ND-1000 Spectrophotometer (NanoDrop Thermo Fisher Scientific Technologies, Wilmington, DE, USA) and Qubit® 2.0 Fluorometer (Invitrogen, UK).

Selection of olive genes for time-course expression analysis was done based on our previous results from the PICF7-olive (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014) and *V. dahliae*-olive (Gómez-Lama Cabanás et al. 2015) interactions. Seven genes related to defense responses (Table 1) were thus selected to examine their expression pattern within a 14-day interval in plants used in the split-root system: *PAL*, *14-3-3* and brassinosteroid up-regulated gene (*BRUI*) (up-regulated in olive roots and stems [*PAL*], stems [*14-3-3*] and roots [*BRUI*] during the interaction with strain PICF7), *CAT* (up-regulated in olive roots and stems during the interaction with PICF7 but down-regulated in olive stems upon *V. dahliae* inoculation), caffeoyl-O-methyltransferase

(*CO-MT*, up-regulated in olive roots inoculated with PICF7 and in olive stems upon *V. dahliae* olive roots inoculation), *WRKY2* transcription factor (*WRKY2*, down-regulated in olive stems during the *V. dahliae*-olive interaction) and *ACO* (down-regulated in stems during the *V. dahliae*-olive interaction but up-regulated during PICF7-olive interaction) (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014, 2015). Four of these genes (*14-3-3*, *ACO*, *WRKY* and *CO-MT*) were chosen for their evaluation in plants used in the single-pot experiment at one DAI with strain PICF7, for assessing early-term responses against BCA colonization, and at seven DAI with *V. dahliae* (i.e. 14 DAI with PICF7), for comparing medium-term responses.

The “CLC Main Workbench 6.8.1” (CLC bio, Aarhus, Denmark) software was used to design specific primer pairs for *WRKY2*, *14-3-3* and *BRU1* olive gene sequences (accession numbers JZ844803, AAY67798 and ACJ85040, respectively) deposited in Genbank database from EST libraries previously identified (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014) (Table 1). Performance of these primer pairs was empirically tested by conventional PCR (temperature range 53°–63°C). Primer pair sequences for *CO-MT*, *PAL*, *CAT* and *ACO* olive genes have been previously reported (Gómez-Lama Cabanás et al. 2014) (Table 1). To determine the range of concentrations at which target cDNA and cycle thresholds (Ct) values were linearly correlated and to estimate PCR efficiencies, specific real-time qPCR assays were conducted using cDNAs synthesized from 10-fold serially diluted (1 µg, 100 ng, 10 ng, 1 ng, 100 pg) RNA samples. Standard curves were generated for each selected transcript using reverse transcribed cDNA from serial dilutions (20 ng, 2 ng, 200 pg and 20 pg). Synthesis of cDNA was performed from 100 ng of total RNA of PrimeScript™ RT Master Mix (Takara, Kyoto, Japan) following the manufacturer’s instructions. Real-time qPCR experiments and analyses were performed in a thermal cycler iQ5 Real-Time PCR

System (BioRad) equipped with a 96-well sample block. Relative expression for each studied gene was repeated at least once for each biological replicate (i.e. different olive above-ground tissue pools) in independent Real-time qPCR assays (plates), and three technical replicates per sample and per plate were routinely included. All real-time qPCR experiments were carried out containing 2 μ L of cDNA, 0.5 mM of each primer, and 10 μ L of SYBR[®] Green Supermix (BioRad) and dH₂O up to a total volume of 20 μ L. The following thermal cycling profile was used for all PCR reactions: 94°C for 5 min, 50 cycles of 94°C for 30 s, 55°C for 30 s (except for gene *BRU1*, 58°C) and 72°C for 40 s. Linear equations, correlation coefficients (R^2) and PCR efficiencies were calculated in all cases (Table 1). Melting curves of real-time qPCR products were assessed from 55°C to 95°C to verify specificity of the reactions, under the following conditions: initial denaturation for 5 min at 95°C, cooling to 55°C and melting from 55°C to 95°C with a 0.5°C transition rate every 10 sec. Data resulting from real-time qPCR were normalized to the *O. europaea* 60S-RBP L18-3 gene (coding for 60S ribosomal protein L18-3) (Ray and Johnson 2014). Ct values and the logarithm of cDNA concentrations were linearly correlated for each of the examined genes and PCR efficiencies were calculated by iQ5 optical system software v.2.1 (BioRad, Hercules, CA). Relative expression (RE) level values (log₂-fold-change values) were calculated according to the $2^{-\Delta\Delta C_t}$ method Livak and Schmittgen (2001). The average of each expression gene fold change was categorized as follows: “low” ≥ -1.0 to ≤ 1.0 ; “medium” ≥ -2.0 to < -1.0 or > 1.0 to ≤ 2.0 ; and “high,” < -2.0 or > 2.0 (Kim et al. 2008). All RE data within a 14-day interval for each gene were graphically represented as means $\pm$ STD of at least two separate experiments, each performed with triplicate samples. Paired sample T-test was performed to determine whether there were significant differences between average values of each relative gene expression in independent experiments (plates), and

between treatments at each time-point studied. Paired T-test analysis was performed using the Statistix software (Version 10 for Windows).

Results

Production of olive cuttings for the split-root study system

The process here developed for producing olive plants with split-root systems was complex but successful. A high-moderate percentage in rooting rate (62%) at both sides of the cleft cutting was achieved. Throughout the whole process of plant production, 17% of cuttings eventually did not root at all. In some of these plants, production of small shoots, likely arising from the reserves present in the stem cutting, was observed. However, they became wilted and necrotic in a few weeks. Finally, a single-root system formed only at one of the two sides of the cleft was observed in 21% of the plants. In some of these cases, rooting failure was likely due to clefts performed in an off-center manner. The lack of the radical system in one of the compartments of the cutting was confirmed by removing the substrate of the pot. Plants in which radical system was formed at both sides showed a normal vegetative growth and produced healthy shoots. Prior to the inoculation procedure, direct observation in a sub-sample of plants, by carefully removing the substrate of the two pots, proved that roots were indeed properly developed at both sides of the cutting (Figure 1D). In addition, the plants were robust and mechanically stable, what facilitated their handling during inoculation. None of the plants showed damage symptoms as the result of the two-step inoculation process.

Verticillium wilt of olive development in the split-root study system

As expected, no VWO symptoms were observed in non-manipulated control plants (T1),

manipulated control plants (T2) and plants inoculated only with strain PICF7 (T3). The disease, however, developed in *V. dahliae*-inoculated plants although average values of final symptom severity were not very high, neither for T4 (average disease severity 3.2) nor for T5 (3.68) treatments. No plant died at the end of the experiment, and disease severity was always lower than 12 (0-16 symptoms severity scale; data not shown). Analysis of variance (ANOVA) showed no significant differences ($P > 0.05$) in RAUDPC between $\text{SO}_4\text{Mg}/V. dahliae$ (treatment T4) and PICF7/*V. dahliae* (treatment T5) (mean values 9.403 and 11.104, respectively) plants. Therefore, no effective biocontrol of VWO was observed under experimental conditions used here, although final disease incidence was slightly higher in T4 (86.7%) than in T5 (73.3%) plants. Remarkably, a frequently-observed phenomenon in VWO affected plants was that defoliation mostly occurred on stems located close to the compartment where the pathogen was inoculated (Figure 3).

***Verticillium dahliae* does not translocate between root compartments**

Qualitative real-time PCR assays demonstrated the presence of pathogen DNA in all root samples from compartments inoculated with *V. dahliae* (73 DAI with the pathogen), regardless of whether plants *ad hoc* selected for molecular detection analysis from treatments T4 and T5 showed severe (severity values 11, 10 and 6) or moderate (1, 2 and 3) VWO symptoms (data not shown). On the contrary, roots sampled from compartments non-inoculated with the pathogen always yielded negative results (data not shown). These results confirmed the absence of accidental cross contamination events between adjacent compartments in the split-root system. Furthermore, active movement of *V. dahliae* throughout the plant vascular system from the pathogen-inoculated compartment to the non-inoculated, adjacent one can be ruled out.

448

449 **Gene expression patterns of *ACO*, *CO-MT*, *WRKY2*, *14-3-3*, *BRUI*, *PAL* and *CAT***
450 **genes in ‘Picual’ plants grown in a split-root system**

451 Relative expression patterns of selected genes involved in defense responses during the
452 tripartite interaction were assessed at 1, 7, 8 and 14 DAI with PICF7 under three
453 different situations: PICF7/H₂O (treatment T3), SO₄Mg/*V. dahliae* (T4) and PICF7/*V.*
454 *dahliae* (T5). The parameters of these real-time qPCR experiments are shown in Table 1.
455 Results from RE for each gene/situation are shown in Figure 4.. Data collected from
456 these experiments allowed us to confirm that systemic defense responses are triggered
457 in aerial olive tissues upon root colonization by strain PICF7 and *V. dahliae* in plants
458 grown in a split-root system, as previously reported for olive plants grown under
459 standard conditions (i.e. single pot) during dual interactions (i.e. olive/PICF7 and
460 olive/*V. dahliae*). Overall, results from time-course gene expression analysis showed a
461 consistent trend for each gene, treatment and time-point evaluated (Figure 4). However,
462 results from each biological replicate tested (i.e. two different pools of leaves sampled
463 from five plants) occasionally showed variable RE levels resulting in some cases in
464 large STD values (Figure 4). No significant difference ($P>0.05$) between experiments
465 was found except for the *PAL* gene ($P=0.0319$). The vast majority of RE fold changes
466 were assigned to medium ($> +1.0$ to $\leq + 2.0$ or $< - 1.0$ to $\geq - 2.0$) or low (≥ -1.0 to
467 $\leq +1.0$ or ≤ 1.0 to ≥ -1.0) categories of differential expression. Differential responses
468 were found depending on the tested gene. Thus, the expression pattern of some genes
469 was not affected independently of the microbial input (e.g. *14-3-3* gene). Some showed
470 opposite expression patterns related to the time PICF7 or *V. dahliae* were inoculated (e.g.
471 the TF *WRKY2*). Finally, others only responded upon the presence of the pathogen (e.g.
472 *BRUI* and *CO-MT* genes). Complete results concerning RE of all tested genes are

473 shown in Figure 4 (and in Table 3 and in Supplementary Table 1). Some of the most
 474 interesting observations are now summarized. For instance, when PICF7 is the only
 475 microorganism introduced in the split-root system, no significant variation was
 476 observed in the RE of *BRUI* gene, its expression remaining virtually unaltered along the
 477 time interval analyzed (Figure 4; PICF7/H₂O treatment). However, a strong up-
 478 regulation was observed at 1 DAI with *V. dahliae*, independently of whether the
 479 pathogen was the only microorganism introduced in the system (Figure 4; SO₄Mg/*V.d*
 480 treatment) or the BCA was already present in the adjacent compartment (Figure 4;
 481 PICF7/*V.d* treatment). Nevertheless, the up-regulation of *BRUI* was transient, returning
 482 to normal RE levels 14 DAI with PICF7 (i.e. 7 DAI with *V. dahliae*) (Figure 4).
 483 Changes were also observed in the RE of *CO-MT* gene (Figure 4; PICF7/H₂O
 484 treatment); that is, a shift from moderate up-regulation at 1 and 7 DAI to down-
 485 regulation from 8 to 14 DAI with PICF7. In this case, introduction of the pathogen in
 486 the system caused a constant medium-level up-regulation regardless of whether or not
 487 the BCA was present (Figure 4; PICF7/*V.d* and SO₄Mg/*V.d* treatments). PICF7
 488 inoculation led to a down-regulation in the RE of *CAT* gene from 7 to 14 DAI (Figure 4;
 489 PICF7/H₂O treatment). Conversely, the presence of *V. dahliae* transiently induced the
 490 RE of *CAT* gene at medium-level (Figure 4; SO₄Mg/*V.d* treatment), an up-regulation
 491 that was attenuated by the early presence of PICF7 (Figure 4; PICF7/*V.d* treatment).
 492 Increasing down-regulation of the *ACO* gene was observed from 7 to 14 DAI with
 493 PICF7 (Figure 4; PICF7/H₂O treatment) whereas no significant variation was observed
 494 when *V. dahliae* was inoculated in control plants (Figure 4; SO₄Mg/*V.d* treatment).
 495 Interestingly, one DAI inoculation with *V. dahliae* in plants where PICF7 was already
 496 present, a medium-level up-regulation was scored (Figure 4; PICF7/*V.d* treatment).
 497 Finally, the RE pattern of *WRKY2* gene varied depending on the time PICF7 or *V.*

dahliae were inoculated. Indeed, a slight up-regulation was observed upon immediate *V. dahliae* inoculation (Figure 4; SO₄Mg/*V.d* treatment). However, when both microorganisms are introduced in the system (Figure 4; PICF7/*V.d* treatment) a pronounced down-regulation when PICF7 was already present was evidenced (Figure 4; PICF7/H₂O treatment).

Gene expression patterns of selected defense response genes in olive cv. Picual grown under standard conditions

In order to assess whether the expression pattern of defense-related genes in above-ground tissues differs between olive plants grown under split-root (i.e. no direct contact between the BCA and the pathogen) and standard conditions (i.e. both microorganisms coexisting in the root/rhizosphere), a real-time qPCR time-course study was carried out. In this case, four genes representative of the different expression patterns observed in the split-root experiment (namely, *ACO*, *CO-MT*, *WRKY2* and *14-3-3*) were selected for evaluation in ‘Picual’ plants growing under standard conditions (single pot). The parameters of these PCR experiments are shown in Table 2. The RE averages for each gene at different time-point/treatment situations evaluated are indicated in Table 3. The most relevant observations are now summarized. Unlike in the split-root system, the RE of *ACO* gene showed up-regulation 1 DAI with PICF7 (Table 3; PICF7 treatment). After seven days of PICF7 and *V. dahliae* coexistence (Table 3; PICF7/*V.d* treatment), a slight down-regulation of the *ACO* gene was observed. The RE of the *14-3-3* gene did not show major differences for the two growing systems. Up-regulation of the *WRKY2* gene was observed in both treatments and time points (Table 3; PICF7 and PICF7/*V.d*), although to a lower extent than that observed in the split-root system. Finally, the most relevant difference found between both systems was that observed for the *CO-MT* gene.

While this gene showed as slightly up-regulated at 1 DAI with the BCA in both situations (Table 3; PICF7 treatment), opposite RE patterns depending on the growing system were observed upon prolonged presence of *V. dahliae* (i.e. 7 DAI with the pathogen; Table 3; PICF7/*V.d* treatment). Indeed, this gene was repressed in the single-pot system but clearly induced when the BCA and the pathogen were spatially separated.

Discussion

Plants do not live as hermetic compartments but as systems in which complex interactions are dynamically established with their associated microbiomes which include both pathogenic and beneficial organisms. The use of beneficial microorganisms is gaining attention as an alternative and sustainable practice to control phytopathogens. However, our current information on how BCAs exert their beneficial effects under natural conditions (i.e. mechanisms involved) against deleterious agents, and what responses are triggered in the target host as consequence of the multipartite interactions that can be established are still limited. This situation is even more complex in an ecological niche such as the rhizosphere.

One of the possible mechanisms deployed by BCA is the induction of resistance and/or defense responses in the host which can be effective against a range of pathogens at long distances (e.g. ISR and SAR responses). Alternatively, mechanisms such as antibiosis and/or competition for nutrients and niches are operative as well. These mechanisms are not mutually exclusive, and a BCA can exert their beneficial effects by a combination of them. Moreover, these mechanisms can be influenced by different (a)biotic factors present in the ecological niche where they are deployed, modifying their performance and effectiveness along time and space.

Our previous works have shown that the olive root endophyte *P. fluorescens* PICF7 is an effective BCA against VWO (Prieto et al. 2009; Maldonado-González et al. 2015b). Colonization of olive roots by PICF7 triggers a set of defensive responses in olive at both local (roots) and systemic (aerial tissues) level upon root colonization (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014). Additionally, when this BCA is applied to *A. thaliana* roots it is also able to control the leaf pathogen *Botrytis cinerea*. This result supports the idea that strain PICF7 triggers effective systemic resistance against this foliar pathogen (Maldonado-González et al. 2015b). Moreover, this may suggest that PICF7 could be an effective BCA against other olive pathogens by means of inducing resistance in the host, although additional biocontrol mechanisms (i.e. antibiosis, nutrient competition) cannot be discarded. Indeed, strain PICF7 effectively antagonized another relevant olive pathogen, *Pseudomonas savastanoi* NCPPB 3335 (*Psv*). Moreover, when both microorganisms were co-inoculated in stems, developed tumors showed an altered anatomy and pathogen cells tended to be confined in the interior of the hyperplasic tissue. However, *in planta* bioassays in which the pathogen and the BCA were spatially separated (PICF7 inoculated in roots and *Psv* in stems) showed that PICF7 was unable to hamper knots development (Maldonado-González et al. 2013). This suggests that systemic defense responses induced by PICF7 are not fully effective; at least against *Psv*.

Regarding the olive-*V. dahliae* interaction, defense-related responses were also found in host aerial tissues upon root colonization by the pathogen, some of them being common to those observed during the interaction with PICF7 (Gómez-Lama Cabanás et al. 2015). Most of the studies focused on dual interactions, either involving the host plant with the BCAs (e.g. Conn et al. 2008; Bordiec et al. 2011) or with the pathogen (e.g. Zheng et al. 2006). In this present work, we developed a successful strategy to

study a tripartite interaction involving a relevant woody species, a beneficial endophytic rhizobacterium, and a soil-borne fungal pathogen. We have particularly focused on the expression of specific genes involved in plant defense response triggered in above-ground tissues upon preventive application of the BCA. Since both microorganisms naturally share the same ecological niche (i.e. the olive rhizosphere), and in order to assess whether systemic resistance responses are involved in the biocontrol exerted by strain PICF7 against VWO, spatial separation of the BCA and the pathogen must be guaranteed. To achieve that split-root systems are extremely useful. However, the development of these study systems for woody plants poses difficulties because of their inherent characteristics. For olive, only a few examples are available in the literature but none of them devoted to the study of plant-microbe interactions. The process leading to obtain a fair number of plants with suitable split-root systems was time consuming and complex. On the one hand, some stem cuttings to which incisions were made failed to produce well-developed and similar radical systems in both adjacent compartments (pots), or did not produce roots at all in one of the compartments. On the other hand, not all plants developed suitable and enough foliage either for the scheduled leaf sampling process or for the VWO symptoms development assessment. Percentage of cuttings producing satisfactory root systems (around 62%) was lower than the rooting ability reported in soft-wood cuttings for cv. Picual grown under standard conditions (79.2%) (Caballero and Del Río 2010). This fact could be due to the thickness (1 cm in diameter) of the cuttings used in this work, considerably wider than those used in standard production systems (2-3 mm; 15 cm long). In favor of using thicker cuttings, greater amount of reserves may help in further development of new tissues as well as better counteract damages provoked by incisions. It is also worth mentioning that this plant architecture likely helped to overcome damages caused by transplanting during the two-

step inoculation process since no visible symptoms were observed in any of the plants. Damages during transplanting and manipulating are commonly observed when cuttings with a standard root system are used. This usually implies that young olive plants used for this type of experiments need to be protected from direct light exposure to avoid excessive stress during the first days after inoculation (Gómez-Lama Cabanás et al. 2014), a strategy that was not necessary to implement in our study. In fact, during inoculations of olive trees in evaluation programs of resistance to *Verticillium* wilt of olive, transplanting damages usually occur in percentages between 10-20% of plants, depending on the cultivar (López-Escudero et al. 2004; Martos-Moreno et al. 2006). A possible explanation for this outcome is that split-root plants can much better overcome stress during manipulation since during the double inoculation procedure one of the two root compartments remained protected by the substrate.

The use of the olive split-root system allowed several important observations. Firstly, the VWO epidemics in plants grown in this system developed similarly to the pattern observed when standard growing conditions are used (i.e. Mercado-Blanco et al. 2004; Prieto et al. 2009; Maldonado-González et al. 2015b). However, severity of symptoms was moderate on average and usually affected only one half of the plant. Indeed, defoliation of green leaves (the representative symptom of infection by *D* isolates) was mostly observed in stems which were closer to the root compartment where *V. dahliae* had been inoculated (Figure 3). This is explained by the fact that olive plants/trees are highly sectorized, showing direct vascular connection of specific roots and shoots. This explains that infection of a major root by the pathogen usually leads to severe symptoms in one part of the tree canopy while other sectors remain symptomless (Lavee 1996; Levin et al. 2003; López-Escudero and Mercado-Blanco 2011). This phenomenon is frequently observed on trees infected under natural conditions. The use

of the split-root system developed here can easily visualize and demonstrate this fact.

Secondly, *V. dahliae* was only detected in those compartments inoculated with the pathogen. This demonstrated that no propagule of the fungus was able to translocate to roots located in the adjacent compartment from vascular vessels of above-ground tissues, even though some of the plants analyzed showed severe VWO symptoms. Thus, there was no evidence of basipetal movement of *V. dahliae* propagules (i.e. conidia and hyphae) from infected aerial vascular tissues toward non-infected roots, confirming that spread of *V. dahliae* in olive plants always proceeded *via* acropetal movement from initial infection sites in the roots (Hiemstra and Harris 1998; Pegg and Brady 2002). In addition, molecular tests also confirmed that there was no cross contamination during the bioassay conferring great solvency and robustness to the olive split-root system here developed.

A third conclusion was that no biocontrol of VWO was observed under experimental conditions used in our split-root system. That is, strain PICF7 was not effective when inoculated in sectors of the olive roots where the pathogen was not present, even though the BCA was introduced in the system seven days earlier than the pathogen, thereby providing enough advantage to PICF7 in terms of root colonization (Prieto et al. 2009) and triggering of defense-related responses (Schilirò et al. 2012; Gómez-Lama Cabanás et al. 2014). These results suggested that induction of systemic defense responses in distant olive root tissues does not take place or is not involved (at least in a decisive way) in the effective biocontrol of VWO by PICF7, which is, consistently demonstrated in single-pot bioassays under a range of experimental conditions where both microorganisms share the same niche (Mercado-Blanco et al. 2004; Prieto et al. 2009; Maldonado-González et al. 2015b). Thus, while a range of defense responses are triggered at the local and systemic level, the hypothesis that an

induced resistance response is involved in the effective VWO biocontrol by strain PICF7 is not supported by our results. Alternatively, defense responses triggered by this BCA are not effective enough to control *V. dahliae* under experimental conditions here used. Effective VWO biocontrol by PICF7 must thus rely on other modes of action (e.g. competition for space and/or nutrients, antibiosis, etc.) and/or triggering of host-mediated defense mechanisms are only effective under specific spatial or temporal circumstances (see below).

Fourth, although PICF7 did not show effective control of VWO in plants grown in the split-root system, time-course expression of selected olive genes related to defense responses confirmed our previous results on the ability of *V. dahliae* and PICF7 to trigger systemic responses in above-ground olive tissues (Gómez-Lama Cabanás et al. 2014, 2015), that were further supported by results here obtained in the single-pot experiment (Table 3). In order to compensate for possible differences in gene expression levels among plants in the split-root experiment, pools of leaves from five different plants were collected at each time-point. This sampling strategy aimed to ‘buffer’ or avoid the effects that differences on root colonization ability of the BCA and/or the pathogen, or varying amounts of the pathogen within plant tissues due to the usual non-homogenous distribution of *V. dahliae* biomass in the plant, may cause on the gene expression patterns among sampled plants. Indeed, differences in RE patterns have occasionally been found in biological replicates (i.e. individual olive plants) in previous studies (Gómez-Lama Cabanás et al. 2014), regardless of whether plants originated from the same propagation batch at the nursery and inoculation procedures were performed with all cautions. This sampling strategy has been also used to increase reliability of *in planta V. dahliae* molecular detection procedures (Karajeh and Masoud 2006; Keykhasaber et al. 2016). Even though some of the RE patterns here obtained

would require a deeper knowledge of defense responses in olive for proper explanation, the development of the olive split-root system offered for the first time the possibility to study systemic responses triggered during a tripartite interaction taking place at the belowground level. In addition, it enables to investigate the potential mode(s) of action of PICF7-mediated VWO biocontrol. Results of RE patterns showed differential responses depending on the studied gene. Thus, in the split-root system, some genes only significantly responded upon the presence of the pathogen. For instance, the olive *BRUI* gene (coding for an endotransglycosylase/xyloglucan hydrolase and regulated by brassinosteroids; Bourquin et al. 2002; Sharmin et al. 2012) could be considered as an early-term responsive gene against *V. dahliae* attack. Another example is the *CO-MT* gene, involved in the phenylpropanoids biosynthesis, with an early/medium-term response against *V. dahliae*. Other genes were not affected regardless of the microbial input, e.g. the *14-3-3* gene (Robert et al. 2002; Manosalva et al. 2011). Finally, other genes showed opposite expression patterns depending on the moment the BCA or the pathogen were inoculated. This was the case of the *WRKY2* gene that belongs to a superfamily of TF playing important roles in plant growth, development and responses to (a)biotic stresses (Zheng et al. 2006; Kjellin 2012; Chi et al. 2013) and plant defense signaling (Pandey and Somssich 2009).

Remarkably, some genes varied in their RE pattern depending on whether the two microorganisms were spatially separated (split-root system) or shared the same compartment (single-pot system). In this regard it is worth discussing the RE patterns of the olive *CO-MT* and *ACO* genes since RE patterns of the *14-3-3* and *WRKY2* genes did not show remarkable differences. Indeed, one of the most relevant results concerned the *CO-MT* gene that was consistently up-regulated along time in response to pathogen inoculation under split-root conditions, regardless of whether or not the BCA was

present in the adjacent compartment. However, when both microorganisms were in the same compartment (single pot) the expression of this gene was repressed (Table 3). This observation deserves attention since the expression of some defense-related genes may differ depending on whether soil/root-borne microorganisms share or not the same niche. The RE of the olive *ACO* gene also showed a variable pattern depending on whether or not the root was divided (Table 3). This gene codes for an enzyme responsible of ET synthesis, a plant hormone involved, among other processes, in the complex cross talk established among signaling pathways regulating plant defense responses to microbial infection. For instance, reduced ET production caused by virus-induced silencing of the *ACO* gene in *Nicotiana benthamiana* Domin affected the susceptible plant response to infection by *Colletotrichum orbiculare* Berk. & Mont. (Shan and Goodwin 2006). It is tempting to speculate that negligible level of *ACO* gene expression scored for above-ground tissues in plants grown in the split-root system compared to that observed for single-pot plants (1 DAI with PICF7; Table 3) could be due to the fact that only half of the whole root system was in contact with PICF7, thereby causing lower stress to the plant. On the contrary, in plants grown in single pots, the entire root system was susceptible of being endophytically colonized by strain PICF7, a scenario potentially causing higher stress to the plant (Mercado-Blanco and Lugtenberg 2014) leading to increased RE of the *ACO* gene. The low systemic RE of the *ACO* gene might explain, at least partially, the lack of VWO biocontrol observed in the split root system. However, this hypothesis will need further experimental evidence.

Conclusions

We succeeded in generating olive plants with suitable root architecture to investigate the olive-*V. dahliae* pathosystem. This poses novelty degree since split-root

723 systems are not frequently developed for woody plant research. We have been able to
724 demonstrate a normal development of VWO epidemics when the plant is attacked by
725 the pathogen only in one section of the radical system, a scenario closer to field
726 conditions (i.e. large root systems and uneven distribution of pathogen's propagules in
727 the soil). Moreover, strong compartmentalization of symptoms was found, explained by
728 the vascular connection existing between the roots inoculated with the pathogen and the
729 stems showing symptoms located above that compartment, a situation frequently
730 observed in naturally-infected olive trees. The split-root system here developed was also
731 helpful for assessing above-ground genetic responses triggered by *V. dahliae* and an
732 endophytic biocontrol rhizobacterium when both colonized the olive root system but are
733 spatially separated and sequentially applied. This enabled us to study for the first time
734 this tripartite interaction with the aim to demonstrate whether the induction of resistance
735 responses triggered by strain PICF7 mediate biocontrol against *V. dahliae* when the
736 pathogen attacks distant roots. Results obtained may also have relevant practical
737 implications related to the way this BCA should be applied under field conditions,
738 considering the huge radical systems developed by woody hosts such as olive. Since
739 strain PICF7 was not able to control VWO under split-root conditions here used, its
740 application in olive orchards (e.g. by dripping systems) must ensure coverage of most of
741 the tree's radical system. The olive split-root system allowed us to conclude that while
742 strain PICF7 is able to induce a range of systemic responses, many of them related to
743 defense to different stresses, they were not effective or powerful enough to control
744 subsequent attacks by *V. dahliae* occurring at other sections of the olive radical system.
745 Consequently, VWO control by strain PICF7 must rely at least partially on mechanisms
746 other than induction of systemic resistance responses, at least under the experimental
747 conditions here used. Differences found in RE patterns of some olive genes depending

on whether the BCA and the pathogen were spatially separated (split-root system) or not (single-root system) was also an interesting outcome. While most of the olive genes here assessed showed similar RE patterns regardless of whether strain PICF7 or *V. dahliae* coexist in the same portion of the olive rhizosphere, a few of them (e.g. *CO-MT*) showed differential RE patterns. This highlights the importance that contact between the BCA and the pathogen might have in triggering specific defense responses in the host. The significance of being spatially separated or not during this tripartite interaction to explain effective VWO biocontrol by PICF7 warrants more in-depth studies in the future.

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References

- Aganchich B, Tahi H, Wahbi S, Elmodaffar C, Serraj R (2007) Growth, water relations and antioxidant defence mechanisms of olive (*Olea europaea* L.) subjected to Partial Root Drying (PRD) and Regulated Deficit Irrigation (RDI). *Plant Biosyst* 141: 252–264
- Aimé S, Alabouvette C, Steinberg C, Olivain C (2013) The endophytic strain *Fusarium oxysporum* Fo47: a good candidate for priming the defense responses in tomato roots. *Mol Plant Microbe Interact* 26: 918–926

773

774 Ali A, Ahmad T, Abbasi NA, Hafiz I.A (2009) Effect of different concentrations of
 775 auxins on in vitro rooting of olive cultivar ‘Moraiolo’. Pak J Bot 41: 1223–1231

776

777 Bordiec S, Paquis S, Lacroix H, Dhondt S, Barka EA, Kauffmann S, Jeandet P,
 778 Mazeyrat-Gourbeyre F, Clément C, Baillieul F, Dorey S (2011) Comparative analysis of
 779 defence responses induced by the endophytic plant growth-promoting rhizobacterium
 780 *Burkholderia phytofirmans* strain PsJN and the non-host bacterium *Pseudomonas*
 781 *syringae* pv. pisi in grapevine cell suspensions. J Exp Bot 62: 595–603

782

783 Boukcim H, Pagès L, Mousain D (2006) Local NO_3^- or NH_4^+ supply modifies the root
 784 system architecture of *Cedrus atlantica* seedlings grown in a split-root device. J Plant
 785 Physiol 163: 1293–1304

786

787 Bourquin V, Nishikubo N, Abe H, Brumer H, Denman S, Eklund M, Christiernin M,
 788 Teeri TT, Sundberg B, Mellerowicz EJ (2002) Xyloglucan endotransglycosylases have a
 789 function during the formation of secondary cell walls of vascular tissues. Plant Cell 14:
 790 3073–3088

791

792 Butterfield EJ, Devay JE (1977) Reassessment of soil assays for *Verticillium dahliae*.
 793 Phytopathology 67: 1073-1078

794

795 Caballero JM, Del Río C (2010) Propagation methods. Pages 83-112 in: Olive growing.
 796 D. Barranco, R. Fernández-Escobar, and L. Rallo, eds. Junta de Andalucía/Mundi
 797 Prensa/RIRDC/AOA, Pendle Hill, Australia

798

799 Campbell CL, Madden LV (1990) Introduction to Plant Disease Epidemiology. John
800 Wiley & Sons, New York, NY, USA

801

802 Carder JH, Morton A, Tabrett AM, Barbara DJ (1994) Detection and differentiation by
803 PCR of subspecific groups within two *Verticillium* species causing vascular wilts in
804 herbaceous hosts. In: SchotsA, DeweyFM, OliverR, eds. Modern Assays for Plant
805 Pathogenic Fungi. Oxford, UK: CAB International, 91–7

806

807 Cazorla FM and Mercado-Blanco J (2016) Biological control of tree and woody plant
808 diseases: an impossible task? BioControl 61: 233–242

809

810 Ciancio A, Pieterse CMJ, Mercado-Blanco J (2016) Editorial: Harnessing Useful
811 Rhizosphere Microorganisms for Pathogen and Pest Biocontrol. Front Microbiol 7:1620

812

813 Chi Y, Yang Y, Zhou Y, Zhou J, Fan B, Yu JQ, Chen Z (2013) Protein-protein
814 interactions in the regulation of WRKY transcription factors. Mol Plant 6: 287–300

815

816 Conn VM, Walker AR, Franco C.M (2008) Endophytic actinobacteria induces defense
817 pathways in *Arabidopsis thaliana*. Mol Plant Microbe Interact 21: 208–218

818

819 Cwalina-Ambroziak B, Nowak M (2012) The effects of biological and chemical
820 controls on fungal communities colonising tomato (*Lycopersicon esculentum* Mill.)
821 plants and soil. Folia Hort 24: 13–20

822

823 Dbara S, Haworth M, Emiliani G, Ben Mimoun M, Gómez-Cadenas A, Centritto M
824 (2016) Partial root-zone drying of olive (*Olea europaea* var. 'Chetoui') induces reduced
825 yield under field conditions. PLoS ONE 11: e0157089
826

827 Dry PR, Loveys BR (1999) Grapevine shoot growth and stomatal conductance are
828 reduced when part of the root system is dried. Vitis 38: 151–156
829

830 Gafni A, Calderon CE, Harris R, Buxdorf K, Dafa-Berger A, Zeilinger-Reichert E, Levy
831 M (2015) Biological control of the cucurbit powdery mildew pathogen *Podosphaera*
832 *xanthii* by means of the epiphytic fungus *Pseudozyma aphidis* and parasitism as a mode
833 of action. Front Plant Sci 6: 132
834

835 Gallou A, Cranenbrouck S, Declerck S (2009) *Trichoderma harzianum* elicits defence
836 response genes in roots of potato plantlets challenged by *Rhizoctonia solani*. Eur Plant
837 Pathol 124: 219–230
838

839 García-Tejera O, López-Bernal A, Testi L, Villalobos FJ (2017) A soil-plant-atmosphere
840 continuum (SPAC) model for simulating tree transpiration with a soil multi-
841 compartment solution. Plant Soil 412: 215–233
842

843 Glick BR, Karaturović DM, Newell PC (1995) A novel procedure for rapid isolation of
844 plant growth promoting pseudomonads. Can J Microbiol 41: 533–536
845

846 Gómez-Lama Cabanás C, Schilirò E, Valverde-Corredor A, Mercado-Blanco J (2014)
847 The biocontrol endophytic bacterium *Pseudomonas fluorescens* PICF7 induces systemic

848 defense responses in aerial tissues upon colonization of olive roots. *Front Microbiol* 5:
 849 427
 850
 851 Gómez-Lama Cabanás C, Schilirò E, Valverde-Corredor A, Mercado-Blanco J (2015)
 852 Systemic responses in a tolerant olive (*Olea europaea* L.) cultivar upon root
 853 colonization by the vascular pathogen *Verticillium dahliae*. *Front Microbiol* 6: 928
 854
 855 He CY, Hsiang T, Wolyn DJ (2002) Induction of systemic disease resistance and
 856 pathogen defence responses in *Asparagus officinalis* inoculated with nonpathogenic
 857 strains of *Fusarium oxysporum*, *Plant Pathol* 51: 225–230
 858
 859 Hiemstra JA, Harris DC (eds) (1998) A compendium of *Verticillium* wilts in tree species.
 860 Ponsen & Looijen, Wageningen
 861
 862 Huisman OC, Ashworth LJ (1974) Quantitative assessment of *Verticillium albo-atrum*
 863 in field soils: Procedural and substrate improvements. *Phytopathology* 64: 1043–1044
 864
 865 Karajeh MR, Masoud SA (2006) Molecular detection of *Verticillium dahliae* Kleb. in
 866 asymptomatic olive trees. *J Phtopathol* 154: 496-499
 867
 868 Keykhasaber M, Pham KTK, Thomma BPHJ Hiemstra JA (2016) Reliable detection of
 869 unevenly distributed *Verticillium dahliae* in diseased olive trees. *Plant Pathol*
 870 doi:10.1111/ppa.12647
 871
 872 Kim JH, Yu J, Mahoney N, Chan KL, Molyneux RJ, Varga J, Bhatnagar D, Cleveland

873 TE, Nierman WC, Campbell BC (2008) Elucidation of the functional genomics of
 874 antioxidant-based inhibition of aflatoxin biosynthesis. *Int J Food Microbiol* 122: 49–60
 875

876 Kjellin J (2012) Role of WRKY20 Transcription factor and raffinose in plant defense
 877 responses upon *Bacillus amyloliquefaciens* strain 5113-mediated priming in *Arabidopsis*
 878 *thaliana*. Bachelor's Thesis, Dept. of Plant Biology and Forest Genetics University of
 879 Uppsala. Kjellin
 880

881 Kloepper JW, Ryu C-M, Zhang S (2004) Induced systemic resistance and promotion of
 882 plant growth by *Bacillus* spp. *Phytopathology* 94: 1259–1266
 883

884 Lakshmanan V, Selvaraj G, Bais HP (2014) Functional soil microbiome: belowground
 885 solutions to an aboveground problem. *Plant Physiol* 166: 689–700
 886

887 Lavee S (1996) Biology and physiology of the olive. In: *World Olive Encyclopedia*.
 888 International Olive Oil Council, Madrid, 59-110
 889

890 Levin AG, Lavee S, Tsrur L (2003) Epidemiology of *Verticillium dahliae* on olive
 891 (Picual) and its effect on yield under saline conditions. *Plant pathology* 52: 212–218
 892

893 Li Y, Yanagi A, Miyawaki Y, Okada T, Matsubara Y-I (2010) Disease tolerance and
 894 changes in antioxidative abilities in mycorrhizal strawberry plants. *J Jpn Soc Hortic Sci*
 895 79: 174–178
 896

897 Li H, Li H, Bai Y, Wang J, Nie M, Li B, Xiao M (2011) The use of *Pseudomonas*

898 *fluorescens* P13 to control sclerotinia stem rot (*Sclerotinia sclerotiorum*) of oilseed rape.
 899 J Microbiol 49: 884–889
 900

901 Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-
 902 time quantitative PCR and the $2^{-\Delta\Delta C_t}$. Methods 25: 402–408
 903

904 López-Escudero FJ, del Río C, Caballero JM, Blanco-López MA (2004) Evaluation of
 905 olive cultivars for resistance to *Verticillium dahliae*. Eur J Plant Pathol 110: 79–85
 906

907 López-Escudero FJ, Mercado-Blanco J (2011) Verticillium wilt of olive: a case study to
 908 implement an integrated strategy to control a soil-borne pathogen. Plant Soil 344: 1–50
 909

910 Maldonado-González MM, Bakker PAHM, Prieto P, Mercado-Blanco J (2015a)
 911 *Arabidopsis thaliana* as a tool to identify traits involved in *Verticillium dahliae*
 912 biocontrol by the olive root endophyte *Pseudomonas fluorescens* PICF7. Front
 913 Microbiol 6: 266
 914

915 Maldonado-González MM, Prieto P, Ramos C, Mercado-Blanco J (2013) From the root
 916 to the stem: interaction between the biocontrol root endophyte *Pseudomonas*
 917 *fluorescens* PICF7 and the pathogen *Pseudomonas savastanoi* NCPPB3335 in olive
 918 knots. Microb Biotechnol 6: 275–287
 919

920 Maldonado-González MM, Schilirò E, Prieto P, Mercado-Blanco J (2015b) Endophytic
 921 colonization and biocontrol performance of *Pseudomonas fluorescens* PICF7 in olive
 922 (*Olea europaea* L.) are determined neither by pyoverdine production nor swimming

923 motility. Environ Microbiol 17: 3139–3153

924

925 Manosalva PM, Bruce M, Leach JE (2011) Rice 14-3-3 protein (GF14e) negatively

926 affects cell death and disease resistance. Plant J 68: 777–787

927

928 Martínez-García PM, Ruano-Rosa D, Schilirò E, Prieto P, Ramos C, Rodríguez-

929 Palenzuela P, Mercado-Blanco J (2015) Complete genome sequence of *Pseudomonas*

930 *fluorescens* strain PICF7, an indigenous root endophyte from olive (*Olea europaea* L.)

931 and effective biocontrol agent against *Verticillium dahliae*. Stand Genomic Sci 10: 10

932

933 Martínez-Medina A, Fernandez I, Lok GB, Pozo MJ, Pieterse CM, Van Wees S (2016)

934 Shifting from priming of salicylic acid to jasmonic acid regulated defences by

935 *Trichoderma* protects tomato against the root knot nematode *Meloidogyne incognita*.

936 New Phytol 213: 1363–1377

937

938 Martos-Moreno C, López-Escudero FJ, Blanco-López MA (2006) Resistance of olive

939 cultivars to the defoliating isolate of *Verticillium dahliae*. Hortscience 41: 1–4

940

941 Mayo S, Cominelli E, Sparvoli F, González-López O, Rodríguez-González A, Gutiérrez

942 S, Casquero PA (2016) Development of a qPCR strategy to select bean genes involved

943 in plant defense response and regulated by the *Trichoderma velutinum*–*Rhizoctonia*

944 *solani* interaction. Front Plant Sci 7:1109

945

946 Mejía LC, Herre EA, Sparks JP, Winter K, García MN, Van Bael SA, Stitt J, Shi

947 Z, Zhang Y, Gultinan MJ, Maximova SN (2014) Pervasive effects of a dominant foliar

948 endophytic fungus on host genetic and phenotypic expression in a tropical tree. *Front*
949 *Microbiol* 5: 479

950

951 Mercado-Blanco J, Lugtenberg BJJ (2014) Biotechnological applications of bacterial
952 endophytes. *Curr Biotechnol* 3:60–75

953

954 Mercado-Blanco J, Rodríguez-Jurado D, Pérez-Artés E, Jiménez-Díaz RM (2002)
955 Detection of the defoliating pathotype of *Verticillium dahliae* pathotype in infected olive
956 plants by nested PCR. *Eur J Plant Pathol* 108: 1–13

957

958 Mercado-Blanco J, Rodríguez-Jurado D, Parrilla-Araujo S, Jiménez-Díaz RM (2003)
959 Simultaneous detection of the defoliating and nondefoliating *Verticillium dahliae*
960 pathotypes in infected olive plants by duplex, nested polymerase chain reaction. *Plant*
961 *Dis* 87: 1487–94

962

963 Mercado-Blanco J, Rodríguez-Jurado D, Hervás A, Jiménez-Díaz RM (2004)
964 Suppression of *Verticillium* wilt in olive planting stocks by root-associated fluorescent
965 *Pseudomonas* spp. *Biol Control* 30: 474–486

966

967 Mgbeahuruike AC, Kovalchuk A, Ubhayasekera W, Nelson DR, Yadav JS (2017)
968 CYPome of the conifer pathogen *Heterobasidion irregulare*: Inventory, phylogeny, and
969 transcriptional analysis of the response to biocontrol. *Fungal Biology* 121(2): 158-171

970

971 Mishra PK, Mishra S, Selvakumar G, Bisht JK, Kundu S, Gupta HS (2009) Co-
972 inoculation of *Bacillus thuringiensis*-KR1 with *Rhizobium leguminosarum* enhances

973 plant growth and nodulation of pea (*Pisum sativum* L.) and lentil (*Lens culinaris* L.)
 974 World J Microbiol Biotechnol 25: 753–761
 975
 976 Narisawa K, Kawamata H, Currah RS, Hashiba T (2002) Suppression of Verticillium
 977 wilt in eggplant by some fungal root endophytes. Eur J Plant Pathol 108: 103–109
 978
 979 Palazzini JM, Dunlap CA, Bowman MJ, Chulze SN (2016) *Bacillus velezensis* RC 218
 980 as a biocontrol agent to reduce Fusarium head blight and deoxynivalenol accumulation:
 981 Genome sequencing and secondary metabolite cluster profiles Microbiol Res 192: 30–
 982 36
 983
 984 Pandey SP, Somssich IE (2009) The role of WRKY transcription factors in plant
 985 immunity. Plant Physiol 150: 1648–1655
 986
 987 Pegg GF, Brady BL (2002) Verticillium wilts. CABI Publishing International,
 988 Wallingford, UK
 989
 990 Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SC, Bakker PA
 991 (2014) Induced systemic resistance by beneficial microbes. Ann Rev Phytopathol 52:
 992 347–375
 993
 994 Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM Azcón-Aguilar C (2002)
 995 Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses
 996 to Phytophthora infection in tomato plants. J Exp Bot 53: 525–534
 997

998 Prieto P, Mercado-Blanco J (2008) Endophytic colonization of olive roots by the
 999 biocontrol strain *Pseudomonas fluorescens* PICF7. FEMS Microb Ecol 64: 297–306
 1000
 1001 Prieto P, Navarro-Raya C, Valverde-Corredor A, Amyotte SG, Dobinson KF, Mercado-
 1002 Blanco J (2009) Colonization process of olive tissues by *Verticillium dahliae* and its *in*
 1003 *planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7.
 1004 Microb Biotechnol 2: 499–511
 1005
 1006 Prieto P, Schilirò E, Maldonado-González, MM, Valderrama R, Barroso- Albarracín JB,
 1007 aMercado-Blanco J (2011) Root hairs play a key role in the endophytic colonization of
 1008 olive roots by *Pseudomonas* spp. with biocontrol activity. Microb Ecol 62: 435–445
 1009
 1010 Qiang-Sheng W, Ming-Qin C, Ying-Ning Z, Chu W, Xin-Hua H (2016) Mycorrhizal
 1011 colonization represents functional equilibrium on root morphology and carbon
 1012 distribution of trifoliolate orange grown in a split-root system. Sci Hort 199: 95–102
 1013
 1014 Ray DL, Johnson JC (2014) Validation of reference genes for gene expression analysis
 1015 in olive (*Olea europaea*) mesocarp tissue by quantitative real-time RT-PCR. BMC Res
 1016 Notes 7: 304
 1017
 1018 Roberts MR, Salinas J, Collinge DB (2002) 14-3-3 proteins and the response to abiotic
 1019 and biotic stress. Plant Mol Biol 50: 1031–1039
 1020
 1021 Rudrappa T, Czymmek KJ, Paré PW, Bais HP (2008) Root-secreted malic acid recruits
 1022 beneficial soil bacteria. Plant Physiol 148: 1547–1556

1023

1024 Rudrappa T, Biedrzycki ML, Kunjeti SG, Donofrio NM, Czymmek KJ, Paré P W, Bais

1025 HP (2010) The rhizobacterial elicitor acetoin induces systemic resistance in *Arabidopsis*

1026 *thaliana*. Commun Integr Biol 3: 130–138

1027

1028 Schilirò E, Ferrara M, Nigro F, Mercado-Blanco J (2012) Genetic responses induced in

1029 olive roots upon colonization by the biocontrol endophytic bacterium *Pseudomonas*

1030 *fluorescens* PICF7. PLoS ONE . doi:10.1371/journal.pone.0048646

1031

1032 Shan XC, Goodwin PH (2006) Silencing an ACC oxidase gene affects the susceptible

1033 host response of *Nicotiana benthamiana* to infection by *Colletotrichum orbiculare*.

1034 Plant Cell Rep 25: 241–247

1035

1036 Sharmin S, Azam MS, Islam MS, Sajib AA, Mahmood N, Hasan AM, Ahmed R,

1037 Sultana K, Khan H (2012) Xyloglucan endotransglycosylase/hydrolase genes from a

1038 susceptible and resistant jute species show opposite expression pattern following

1039 *Macrophomina phaseolina* infection. Commun Integr Biol 5: 598–606

1040

1041 Siddiqui IA, Shaukat SS (2002) Rhizobacteria-mediated induction of systemic

1042 resistance (ISR) in tomato against *Meloidogyne javanica*. J Phytopathol 150: 469–473

1043

1044 Tsrer L (2011) Epidemiology and control of Verticillium wilt on olive. Isr J Plant Sci 59:

1045 59–69

1046

1047 Varo A, Mulero-Aparicio A, Adem M, Roca LF, Raya-Ortega MC, López-Escudero FJ,

1048 Trapero A (2017) Screening water extracts and essential oils from Mediterranean plants
1049 against *Verticillium dahliae* in olive. Crop Protection 92: 168-175
1050
1051 Wang L, Shi H, Wu J, Cao F (2016) Alternative partial root-zone irrigation enhances
1052 leaf flavonoid accumulation and water use efficiency of *Ginkgo biloba*. New Forest 47:
1053 377–391
1054
1055 Zheng Z, Qamar SA, Chen Z, Mengiste T (2006) *Arabidopsis* WRKY33 transcription
1056 factor is required for resistance to necrotrophic fungal pathogens. Plant J 48: 592–605
1057
1058

1059 **Table 1.** List of genes whose relative expression (RE) pattern over time was studied by real-time qPCR experiments in aerial tissues of olive
1060 plants (cv. Picual) grown under split-root conditions and upon *Pseudomonas fluorescens* PICF7 and/or *Verticillium dahliae* V937I inoculation.

Olive gene	Process	Primer pair	Real-time qPCR parameters		
			Linear equation	R ²	PCR efficiency
1-Aminocyclopropane-1-carboxylate oxidase (<i>ACO</i>)	Ethylene biosynthesis	Fw: CTCAAGTTGATCCCCAAT Rv: GCATTCCATGGCTCTAAA	y=-3.448x+20.179	1.00	95.0 %
Caffeoyl-o- methyltransferase (<i>CO-MT</i>)	Phenylpropanoids biosynthesis	Fw: ACCAGAGGCCATGAAAGAAC Rv: ATTGCCAAAATCTTCCCATC	y=-3.522x+20.687	0.99	92.3 %
Transcription factor WRKY2 (<i>WRKY2</i>)	Signal transduction defense response	Fw: CAAGCCCCTCATACACTA Rv: GCAACGACTCCAAGAACA	y=-3.436x+20.771	0.99	95.0 %
14-3-3 protein (<i>14-3-3</i>)	Regulator of metabolism and defense response	Fw: CACTTCAAAAACCCGAATCT Rv: ACGCCTTATAACTGACTCT	y=-3.475x+22.954	0.99	94.0 %
Phenylalanine ammonia-lyase (<i>PAL</i>)	Phenylpropanoids biosynthesis	Fw: ACATAGGAGGACCAAACAA Rv: GTAGGATAAAGGGACAAGA	y=-3.245x+19.056	0.99	103.3 %
Brassinosteroid up-regulated (<i>BRU1</i>)	Cell Wall process	Fw: CCCAGGATCTCTACGATT Rv: TCTGGAGCATCTTGTCAT	y=-3.443x+17.806	1.00	95.2 %
Catalase (<i>CAT</i>)	Oxidation-reduction	Fw: CCCAGGATCTCTACGATT Rv: TCTGGAGCATCTTGTCAT	y=-3.440x+20.714	0.99	95.3 %
<i>Olea europea</i> 60S ribosomal protein L18-3 (<i>RBP</i>)	Ribosomal protein	Fw: ACGGCATACTACTTATCTTC Rv: CCCTTGCCTTGAGTAAACA	y=-3.343x+22.046	0.99	99.1 %

1061 For all transcripts, RE analysis was repeated at least two times in independent real-time qPCR experiments (see text and Figure 4). Gene names,
1062 processes, linear equations, correlation coefficients (R²) and PCR efficiencies are indicated. Fw: forward; Rv: reverse.
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Table 2. List of genes whose relative expression (RE) was analyzed in aerial tissues of olive plants (cv. Picual) grown under standard conditions (single pot) and submitted to dual and sequential root inoculation, first with *Pseudomonas fluorescens* PICF7 and seven days later with *Verticillium dahliae*.

Olive gene	Real-time qPCR parameters		
	Linear equation	R ²	PCR efficiency
1-Aminocyclopropane-1-carboxylate oxidase (<i>ACO</i>)	y=-3.450x+16.015	0.99	94.9 %
Caffeoyl-o-methyltransferase (<i>CO-MT</i>)	y=-3.328x+19.623	0.99	99.7 %
Transcription factor WRKY2 (<i>WRKY2</i>)	y=-3.301x+19.960	0.99	100.9 %
14-3-3 protein (<i>14-3-3</i>)	y=-3.542x+20.734	0.99	91.6 %
<i>Olea europea</i> 60S ribosomal protein L18-3 (<i>RBP</i>)	y=-3.441X+18.519	0.99	95.3 %

For all transcripts, RE analysis was repeated at least two times in independent real-time qPCR experiments. Gene names, linear equations, correlation coefficients (R²) and PCR efficiencies are indicated.

Table 3. Relative expression (RE) averages for *ACO*, *14-3-3*, *WRKY2* and *CO-MT* genes in above-ground tissues of olive plants (cv. Picual) grown under split-root or standard conditions (single pot).

Treatment and sampling time (T) ¹	Gene relative expression (RE) ²	
	Split-root system	Single-pot system
	<i>ACO</i>	
PICF7 [T1]	0.09 ±0.08	1.17± 0.03
PICF7/ <i>V. dahliae</i> [T14/T7]	0.20± 0.22	-0.65±0.50
	<i>14-3-3</i>	
PICF7 [T1]	1.02± 0.25	1.08±0.17
PICF7/ <i>V. dahliae</i> [T14/T7]	0.57± 0.29	-0.51±0.67
	<i>WRKY2</i>	
PICF7 [T1]	0.98± 0.12	0.14±0.003
PICF7/ <i>V. dahliae</i> [T14/T7]	0.49 ±0.12	0.19±0.07
	<i>CO-MT</i>	
PICF7 [T1]	0.43 ±0.08	0.90±0.12
PICF7/ <i>V. dahliae</i> [T14/T7]	1.47 ±0.28	-1.06±0.36

¹ T1: One day after inoculation (DAI) with *Pseudomonas fluorescens* PICF7; T14/T7: 14 DAI with *P. fluorescens* PICF7 and 7 DAI with *Verticillium dahliae* V937I. ² RE averages ± standard deviations of two biological replicates for each treatment and sampling time combination (see text for details). Major differences in gene relative expression according to the scale by Kim et al. (2008) between the two radical systems studied are indicated in bold type.

1088 **Fig 1.** Olive cuttings (cv. Picual) manipulated to develop a split-root study system (see Materials
1089 and Methods for details). (a) Close-up of an olive cutting showing the longitudinal incision and
1090 inserted between two adjacent pots; (b) General appearance of the cuttings after 45 days, (c)
1091 Appearance of a representative ‘Picual’ plant grown in the split-root system at the time first
1092 inoculation was performed; (d) Representative radical system developed in one of the compartments
1093 in the split-root system after washing and prior to inoculation. Plants must show similar radical
1094 systems in each compartment (i.e. good development, enough biomass) before each inoculation,
1095 otherwise they were discarded.

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1097 **Fig 2.** Scheme of the split-root study system showing treatments and time (days) each compartment
1098 was manipulated/inoculated. (T1) Non-manipulated control plants, (T2) manipulated control plants,
1099 (T3) *Pseudomonas fluorescens* PICF7-inoculated control plants, (T4) *Verticillium dahliae* 937I-
1100 inoculated (*V.d*) control plants, and (T5) PICF7/*V. dahliae*-double inoculated plants.

1101

1102 **Fig 3.** An olive cv. Picual plant (from treatment T4; see Materials and Methods for details) grown in
1103 the split-root system and showing the typical severe symptom (defoliation of green leaves) of
1104 *Verticillium* wilt. Defoliation was mainly observed in stems located above the compartment where
1105 *Verticillium dahliae* V937I was inoculated 35 days earlier. The white label indicates the
1106 compartment to which SO_4Mg was added, and the pink label corresponds to the *V. dahliae*-
1107 inoculated compartment.

1108

1109 **Fig 4.** Relative expression (RE) average of seven *ad hoc* chosen defense response-related genes in
1110 above-ground tissues of olive (cv. Picual) plants grown in a split-root system at different time points
1111 after *Pseudomonas fluorescens* PICF7 and/or *Verticillium dahliae* V937I (*V.d*) inoculation in roots
1112 (see main text and Figures 1 and 2 for details on the split-root system setup). *BRU1*,
1113 endotransglycosylase/xyloglucan hydrolase enzyme, *CO-MT*, caffeoyl-o-methyltransferase; *14-3-3*,

1114 14-3-3 protein; *WRKY2*, WRKY2 transcription factor; *PAL*, phenylalanine ammonia lyase; *ACO*, 1-
1115 aminocyclopropane-1-carboxylate oxidase; and *CAT*, catalase. Error bars represent the SD from at
1116 least two independent qRT-PCR experiments. RE values (log2-fold-change values) were calculated
1117 according to the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). Day 1: one day after PICF7
1118 inoculation; Day 7: seven days after PICF7 inoculation; Day 8: eight days after PICF7 inoculation
1119 and one day after *V. dahliae* inoculation; Day 14: fourteen days after PICF7 inoculation and seven
1120 days after *V. dahliae* inoculation.

Table S1. P-values for the comparison of treatments at each time-point (Paired sample T-test).

TIME (days)	Compared treatments	GENES					
		<i>BRU</i>	<i>CO-MT</i>	<i>14-3-3</i>	<i>WRKY2</i>	<i>ACO</i>	<i>CAT</i>
7	PICF7 vs <i>V. dahliae</i>	NS	0.0287	NS	0.0394	NS	0.0214
	<i>V. dahliae</i> vs PICF7/ <i>V. dahliae</i>	NS	NS	NS	0.0292	NS	NS
	PICF7 vs PICF7/ <i>V. dahliae</i>	0.0253	0.0303	NS	0.0177	NS	NS
8	PICF7 vs <i>V. dahliae</i>	0.0086	0.002	NS	NS	NS	0.0021
	<i>V. dahliae</i> vs PICF7/ <i>V. dahliae</i>	NS	NS	NS	NS	NS	NS
	PICF7 vs PICF7/ <i>V. dahliae</i>	0.0053	0.0132	NS	NS	0.0260	NS
14	PICF7 vs <i>V. dahliae</i>	NS	0.0211	NS	NS	0.0212	NS
	<i>V. dahliae</i> vs PICF7/ <i>V. dahliae</i>	0.0468	NS	NS	NS	NS	NS
	PICF7 vs PICF7/ <i>V. dahliae</i>	NS	0.0366	NS	NS	0.0237	0.0387

NS, non significant differences. Only significant differences (P<0.05) are shown. PICF7, *Pseudomonas fluorescens* PICF7-inoculated control plants. *V. dahliae*, *Verticillium dahliae* 937I- inoculated control plants. PICF7/*V. dahliae*, double inoculated plants (see text for details).