Colonization process of olive tissues by *Verticillium dahliae* and its *in planta* interaction with the biocontrol root endophyte *Pseudomonas fluorescens* PICF7

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Summary

The colonization process of *Olea europaea* by the defoliating pathotype of *Verticillium dahliae*, and the *in planta* interaction with the endophytic, biocontrol strain *Pseudomonas fluorescens* PICF7 were determined. Differential fluorescent protein tagging was used for the simultaneous visualization of *P. fluorescens* PICF7 and *V. dahliae* in olive tissues. Olive plants were bacterized with PICF7 and then transferred to *V. dahliae*-infested soil. Monitoring olive colonization events by *V. dahliae* and its interaction with PICF7 was conducted using a non-gnotobiotic system, confocal laser scanner microscopy and tissue vibratoming sections. A yellow fluorescently tagged *V. dahliae* derivative (VDAT-36I) was obtained by *Agrobacterium tumefaciens*-mediated transformation. Isolate VDAT-36I quickly colonized olive root surface, successfully invaded root cortex and vascular tissues via macro- and micro-breakages, and progressed to the aerial parts of the plant through xylem vessel cells. Strain PICF7 used root hairs as preferred penetration site, and once established on/in root tissues, hindered pathogen colonization. For the first time using this approach, the entire colonization process of a woody plant by *V. dahliae* is reported. Early and localized root surface and root endophytic colonization by *P. fluorescens* PICF7 is needed to impair full progress of verticillium wilt epidemics in olive.

Introduction

Verticillium wilts, caused by the soil-borne fungal phytopathogen *Verticillium dahliae* Kleb., are diseases causing important losses on many economically relevant herbaceous and woody crops (Pegg and Brady, 2002, and references therein). Olive (*Olea europaea* L.) is one of the most traditional and important tree crops in the Mediterranean region. Among the biotic constraints affecting olive cultivation, verticillium wilt of olive (VWO) poses the most serious threat for this commodity worldwide (Jiménez-Díaz *et al.*, 1998), causing harsh yield losses and tree mortality (Levin *et al.*, 2003).

In *Verticillium*-susceptible hosts several phases can be distinguished in the parasitic phase of *V. dahliae* life cycle: (i) infection of the root, (ii) colonization of the vascular system, and (iii) symptom development (Hiemstra, 1998). In addition, *V. dahliae* can survive in soils for prolonged period of time by means of the production of latent, resistant structures [microsclerotia (MS)] which upon exposure to appropriate stimuli may germinate (Schreiber and Green, 1963). Germinating hyphae can then penetrate into root tissues initiating the infection process. Damaged root hairs or dead epidermal cells are preferred microsites for *Verticillium* spp. penetration. In addition, in nature extensive root damage by human or animal activities can provide pathogen penetration sites (Pegg, 1985; Pegg and Brady, 2002). Following penetration into the root vascular system, pathogen colonization of above-ground vascular tissues of trees can be very rapid, finally reaching leaf petioles (Tjamos and Botseas, 1987; Rijkers *et al.*, 1992).

So far, no efficient control measure of VWO is available. Therefore, management of VWO should be primarily by means of an integrated disease management strategy, with emphasis on the implementation of before-planting measures (Tjamos, 1993). A promising element of such a preventive strategy is the protection of pathogen-free planting material from early *V. dahliae* infection during plant propagation and/or at transplanting. This could be accomplished by the use of biocontrol agents (BCAs), an
approach which would satisfy modern, sustainable agriculture requirements for environmentally friendly diseases control.

\textit{Pseudomonas} spp. strains are known to be beneficial to plants because of their ability to promote plant-growth and/or act as BCAs against a number of plant diseases and pests (Mercado-Blanco and Bakker, 2007), and some beneficial \textit{Pseudomonas} spp. can also be endophytically established (Rosenblueth and Martínez-Romero, 2006; Mercado-Blanco and Bakker, 2007). Our previous studies have revealed that \textit{Pseudomonas} spp. strains native to olive roots can antagonize \textit{V. dahliae in vitro}, and effectively suppress VWO caused by the most aggressive [defoliating (D)] pathotype (Mercado-Blanco et al., 2004). Furthermore, we have recently shown that \textit{Pseudomonas fluorescens} strain PICF7 can endophytically colonize the intercellular spaces of the root cortex (Prieto and Mercado-Blanco, 2008).

Valuable information about the \textit{in situ} localization of microorganisms in the rhizosphere has been obtained by light, transmission and scanning electron microscopy (Foster and Rovira, 1976; Campbell and Porter, 1982). Nevertheless, it is often difficult to obtain high-quality images due to the complex environment, the small size of the bacteria, and the harsh treatment of the specimens during preparation (Foster and Rovira, 1976; Bennett and Lynch, 1981; Campbell and Porter, 1982). The development of confocal laser scanning microscopy (CLSM) has significantly reduced most of these problems and the resolution is generally higher than that of conventional fluorescence microscopy. It is also possible to make three-dimensional reconstructions of the images, and problems with autofluorescence are much reduced (Bloemberg et al., 2000; Bolwerk et al., 2003; Eynck et al., 2007). Moreover, the possibility of using fluorescent-tagged microorganisms has significantly facilitated \textit{in vivo} studies and allowed the assessment of the endophytic behaviour of diverse beneficial bacteria in different plant hosts (Elbeltagy et al., 2001; Compat et al., 2005; Germaine et al., 2006; Prieto and Mercado-Blanco, 2008).

Numerous microscopic studies have examined the interaction of various hosts with \textit{V. dahliae}, and have largely focused on anatomic aspects of colonization (Garber and Houston, 1966; Perry and Evert, 1983; Gerik and Huisman, 1988; Rodriguez-Jurado et al., 1993; Zhou et al., 2006; Eynck et al., 2007). To date, only one study has followed the colonization process through the entire disease cycle (Vallad and Subbarao, 2008) and none has examined \textit{V. dahliae}–biocontrol bacteria interaction \textit{in planta}. Thus the objectives of this study were to: (i) construct an enhanced yellow fluorescent protein (EYFP)-tagged transformant of a \textit{V. dahliae} D pathotype isolate; (ii) monitor by CLSM the infection and colonization of the entire olive plant by the \textit{V. dahliae} transformant; (iii) assess the biocontrol activity of an enhanced green fluorescent protein (EGFP)-tagged \textit{P. fluorescens} PICF7 derivative in young olive plants (cv. Arbequina) against VWO caused by the D pathotype; and (iv) analyse by CLSM the interaction between the EYFP-tagged \textit{V. dahliae} isolate and the \textit{P. fluorescens} EGFP-tagged PICF7 strain on/in olive roots.

Results

Characterization of the \textit{V. dahliae} EYFP-tagged transformant strain VDAT-36I

Transformation of \textit{V. dahliae} V937I conidia with the \textit{Agrobacterium tumefaciens} strain which carried the binary vector encoding the neomycin (geneticin)-resistance gene and \textit{eyfp} gene, as described in Experimental procedures, led to the appearance of geneticin-resistant colonies within approximately 5–7 days after transfer to complete medium (CM) containing geneticin. Of the several different stable transformants generated and analysed, VDAT-36I was primarily selected because it showed consistent, strong and uniform yellow fluorescence in all fungal structures: hyphae, conidia and developing MS (Fig. 1A and B). Presence of the \textit{eyfp} gene was confirmed by DNA–DNA blot hybridization (data not shown). Transformant VDAT-36I was further characterized to check for possible phenotypic alterations compared with its parental strain V937I. Indeed, when incubated for 25 days at various temperatures (22°C, 25°C and 28°C) on diverse culture media [CM, potato dextrose agar (PDA), water agar (WA) and agar sodium polypectate medium (ASPM)], no differences in fungal colony morphology, growth rate, and morphology and number of MS produced were found between the wild-type isolate V937I and its derivative VDAT-36I (data not shown). Lastly, the results of the pathogenicity test performed in cv. Arbequina [disease assessment bioassay (DAB)] showed that both the mean final disease intensity index (DII) and the standardized area under the disease progress curve (SAUDPC) of DII [at 60 days after inoculation (DAI)] caused by VDAT-36I did not differ significantly ($P_{\text{DII}} = 0.8281$, $P_{\text{SAUDPC}} = 0.6788$) from those of its parental V937I. Moreover, the severity of the symptoms observed (including heavy defoliation of green leaves) in the DAB experiment was characteristic of the highly virulent D pathotype. Therefore, EYFP-tagged isolate VDAT-36I was considered suitable for further studies.

\textbf{Biocontrol of VWO by \textit{P. fluorescens} PICF7(pMP4655)}

Results from the biocontrol and colonization bioassay (BCB) showed that strain PICF7(pMP4655) was effective
in suppressing to some degree VWO caused by isolate VDAT-36I in ‘Arbequina’ plants. Although first symptoms were scored at the same time (21 days after transplanting to infested soil) for both PICF7-treated and non-treated plants, the final DII and SAUDPC parameters were significantly different ($P < 0.05$) between the two treatments at the end of the bioassay (60 days after VDAT-36I inoculation). Indeed, the mean SAUDPC for PICF7(pMP4655)-treated plants was 0.27 compared with 0.46 for non-bacterized plants. Similarly, the final DII for PICF7(pMP4655)-treated plants was 0.50 compared with that of non-bacterized plants (0.76). Finally, 53% of the non-bacterized plants (eight out of 15) were dead by the end of the bioassay, whereas none of the PICF7(pMP4655)-treated plants had died.

The infection and colonization process of young olive plants by V. dahliae D pathotype strain VDAT-36I

Detailed microscopic observations of ‘Arbequina’ olive tissues have been carried out. Weak autofluorescence from cell walls was always observed, but did not interfere with detection of the tagged pathogen (and bacteria, see below), even helping to image plant tissues and cells morphology and avoiding extra tissue staining. Likewise, no autofluorescent native microorganisms were detected on/in olive tissues at any time. VDAT-36I conidia were readily detected on the root surface just 1 DAI, and at this time it was consistently observed that on average half of the conidia present in the microscopy samples were germinating (Fig. 2A). Nonetheless, a mixed population of non-germinated conidia, germinating conidia, and hyphae of variable lengths was observed during the first 2 days of root sampling. Profuse VDAT-36I colonization of the entire root surface was found at these sampling times, without differences among the meristematic, elongation and differentiation zones (Fig. 2B). However, colonization differences were observed among these zones by 3 DAI. Indeed, at this time, the differentiation and the elongation zones were more abundantly colonized by VDAT-36I than was the meristematic zone. In the former zones, conidia had germinated profusely, producing elongating hyphae interspersed among root hairs (Fig. 2C). In fact, most of the conidia were germinated at 3 DAI, and a hank of hyphae was then observed wrapping the root surface. Although many hyphae grew in parallel to the longitudinal axis of the roots within the junctions of epidermal cells, others did not exhibit such a clear thigmotropic growth. Indeed, most of the samples from this time-point showed a non-specific growth pattern, resulting in a complicated network of hyphae covering the root epidermis (Fig. 2D).

Fungal biomass increased over time, and was associated with hyphae proliferating around the root epidermis and the development of MS on both the elongation and differentiation zones as early as 6 DAI (Fig. 2E). At this stage, MS still showed as fluorescent structures, in contrast to mature MS detected at later stages on the root surface, which did not fluoresce but could be recognized as dark pigmented structures by light microscopy (data not shown).
Transverse and longitudinal root vibratome sections were prepared routinely to assess the possibility of internal root colonization by VDAT-36I. In the absence of root breakages (see below), hyphae or MS were detected over the root surface in the vast majority of images (Fig. 2F), although we have occasionally captured images of intracellular colonization of epidermal cells at 7 DAI (Fig. 2G). Presence of cell wall breaks in these cases, which could not be captured by CLSM imagery, cannot be completely ruled out.

The sampling time-course strategy as well as the exhaustive CLSM observation of root samples confirmed that *V. dahliae* used macro- (several cell layers) and micro- (a single cell) wounds as preferred penetration sites within olive root tissues (data not shown). Conidia or hyphal penetration, via either the intercellular space between two epidermal cells or directly through an epidermal cell, was never observed.

Upon *V. dahliae* penetration of the root, a massive internal proliferation of pathogen hyphae was observed within the root cortex 9 DAI, both inter- and intracellularly (Fig. 2H). Hyphal ramification and appressoria were consistently observed therein, with elongating hyphae proliferating along cortical cells, and invading large areas of the cortex. Appressoria were situated between cortical cell junctions just at the site of penetration of the elongating hyphae to an adjacent cell (Fig. 2I). Hyphal swellings were also visible intracellularly, between adjacent cortical cells (Fig. 2I). Branching of VDAT-36I hyphae was observed within cortical cells as well (Fig. 2I). The first observation of xylem vessel colonization was also made at this time, and proliferation of pathogen hyphae within the xylem vessel cells took place both longitudinally and transversally (Fig. 2H and J). From this time-point on, no substantial changes in the pathogen colonization process within root tissues were detected over the next 2–3 days.

A progressive reduction in detectable VDAT-36I biomass within the root tissue was observed at 15 DAI (data not shown), and the pathogen was nearly undetectable at 20 DAI. From this time-point on, fluorescent hyphae were detected only within above-ground vascular tissues. In fact, at 25 DAI, VDAT-36I was found exclusively within the secondary xylem vessel cells along the main stem and lateral branches (Fig. 2K and L). Petioles of both fallen (because of the disease) and still-attached leaves were analysed by CLSM from 30 DAI. At this time, VDAT-36I was only detected in the secondary xylem vessel cells of fallen leaves petioles (Fig. 2M). In contrast, by 40 DAI all sampled leaves from diseased plants showed VDAT-36I hyphae within secondary xylem vessels of their petioles, regardless of their being fallen or still attached (data not shown). Tissue sampling was maintained until 60 DAI but results scored were similar to those described for 40 DAI. Biomass of VDAT-36I was always confined to the vascular tissue in above-ground organs.

**Interaction between *V. dahliae* and *P. fluorescens* PICF7 in olive roots**

Consistent with previous data (Prieto and Mercado-Blanco, 2008), the olive root rhizoplane was rapidly colonized by PICF7. Remarkably, intracellular colonization of root hairs by tagged-bacteria has been repeatedly detected (Fig. 3A, E and F). On the contrary, root penetration by strain PICF7 via the intercellular spaces of epidermal cells was not scored at any time.
Pseudomonas fluorescens PICF7 and *V. dahliae* VDAT-36I were simultaneously detected on olive root surface at one to 2 DAI, both organisms sharing the same ecological niche (Fig. 3B and C). The presence of PICF7 did not completely impair VDAT-36I conidia germination on the examined tissue, although less geminating conidia or elongating hyphae were detected when the BCA was present (Fig. 3B and C). This was in contrast to root samples from non-bacterized plants examined at the same sampling time-point (Fig. 2B). Moreover, direct contact between PICF7 cells and VDAT-36I hyphae was detected at early stages (1–5 DAI) and colonization of pathogen hyphae by bacterial cells was evident (Fig. 3B–D). The same phenomena were reliably observed at later stages (up to 8 DAI). Furthermore, pathogen biomass was consistently reduced in those areas where *P. fluorescens* PICF7 was established. Indeed, profusely bacterially colonized root hairs in the differentiation zone showed almost no germinating conidia or hyphae which, if present, were very difficult to detect by CLSM because they showed low or no fluorescence (Fig. 3E).

Although the above observations indicated that *P. fluorescens* PICF7 was hampering VDAT-36I conidia germination and colonization of ‘Arbequina’ roots, pathogen internal colonization of olive root cortex did occur, as shown in Fig. 3F (10 DAI). Interestingly enough, no pathogen biomass was visible on the root surface near the area colonized by PICF7 (Fig. 3F).

A decrease (in same cases a complete absence) of detectable VDAT-36I biomass within root regions where *P. fluorescens* PICF7 had already endophytically established was consistently monitored from 10 DAI onward. Figure 4 shows a comparison between a transverse cross-section of a non-bacterized ‘Arbequina’ plant,

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**Fig. 3.** Confocal laser scanning microscopy showing simultaneous colonization of olive cv. Arbequina tissues by *Pseudomonas fluorescens* PICF7 and *Verticillium dahliae* VDAT-36I.

A. Detection of EGFP-tagged PICF7 cells inside a root hair cell, 3 DAI. Three non-colonized root hairs are also shown. Expression of *eyfp* and *egfp* genes in VDAT-36I and PICF7 strains, respectively, on the differentiation zone of the olive root surface at (B) 1, (C) 2, (D) 5 and (E) 8 DAI. Confocal analysis was performed on whole representative roots (B–E) or on vibratome olive tissue sections (F) to show the VDAT-36I/ PICF7 interaction on the olive root surface and internally respectively. Images were single confocal optical sections (B, C), projections of 10 (D), 20 (F) and 30 adjacent confocal optical sections (A and E). The focal step size between confocal optical sections was 1 μm (A, D–F). VDAT-36I hyphae colonized by strain PICF7 cells are indicated by arrows in B–D. Arrows in E show two non-fluorescent hyphae. F. Vibratome longitudinal section of a representative olive root from a plant sampled 10 DAI showing PICF7 inside the root hairs and VDAT-36I hyphae localized intracellularly within the cortex region. Asterisks in E and F indicate root hairs colonized by PICF7. Bars, 20 μm in all the panels except in D where it represents 15 μm. rh, root hair; co, cortex; hy, hypha.
where yellow dots denoted inter- and intra-cellular presence of VDAT-36I hyphae in the root cortex and vascular tissue (Fig. 4A). In contrast, PICF7-treated plants sampled at the same time (10 DAI) showed the presence of bacteria microcolonies among the intercellular spaces of the root cortex, proximal to the vascular tissue, without detectable trace of any VDAT-36I biomass (Fig. 4B). From this stage until 60 DAI, no significant change with regards to PICF7 colonization was observed. Finally, VDAT-36I could be detected within the secondary xylem vessel cells of stems and petioles of plants where pathogen colonization was not fully impaired by PICF7 (data not shown).

Discussion

Diverse Pseudomonas spp. strains have been described as beneficial for plants, but bacterial traits that can be exploited to improve plant growth and/or health are worthless without the successful establishment and persistence of bacteria either on the root surface or as endophytes (Mercado-Blanco and Bakker, 2007).

Beneficial endophytic bacteria most likely gain entrance into plant roots through tissue breakages or zones of lateral root emergence or root elongation and differentiation (Rosenblueth and Martinez-Romero, 2006). In the current study we have demonstrated that olive root hairs are a preferred site for penetration into olive roots by P. fluorescens PICF7. Colonization by diverse biocontrol Pseudomonas strains of root epidermal cells has been observed by CLSM (Bloemberg et al., 2000; Bianciotto et al., 2001; Bolwer et al., 2003). However, all of these reports dealt exclusively with surface colonization of the root epidermis, always in herbaceous hosts and using gnotobiotic or axenic study systems. To the best of our knowledge, this work is the first to demonstrate, by CLSM imagery, internal root hairs colonization of a woody host by a beneficial Pseudomonas strain using a non-gnotobiotic system.

The mechanism by which strain PICF7 attaches and penetrates olive root hairs is unclear. The necessity of chemotactic sensing and active migration in Pseudomonas spp. and other bacteria for successful root colonization has been a subject of debate (Scher et al., 1988; Misaghi et al., 1992; Vande Broek and Vanderleyden, 1996). Our observations indicate that if a chemotactic driving force for strain PICF7 attachment to olive root hair cells is taking place, it is occurring in a very selective way. Indeed, the fact that only a minority of root hairs were colonized by EGFP-tagged bacteria suggests that few root hairs are suitable for PICF7 attachment and entrance. Although we did not detect at any time root penetration of PICF7 cells through sites other than root hair cells, passive entrance through root breakages could not be discarded. Nevertheless, wounds from plant han-
...would not explain the precise root hair colonization events that we observed. It also remains to be explained how PICF7 cells progress from root hairs towards the intercellular spaces of the cortex (Prieto and Mercado-Blanco, 2008; this study).

The use of fluorescent tagging in conjunction with three-dimensional tissue sectioning and CLSM detection made it possible to differentially detect both *V. dahliae* and the *V. longisporum* in the *V. dahliae* D pathotype was successfully transformed and tagged with the *eyfp* reporter gene. Green fluorescent protein-tagged *V. dahliae* and *V. longisporum* transformants have been previously used to assess colonization patterns in lettuce (*Lactuca sativa* L.) (Vallad and Subbarao, 2008) and oilseed rape (*Brassica napus* L.) (Eynck et al., 2007). To our knowledge, the present study is also the first to report the use of the EYFP marker to monitor *V. dahliae* colonization in a woody host such as olive using olive plantlets with fully developed root systems that harbour naturally resident fungal and bacteria cohorts (Mercado-Blanco et al., 2001).

Vallad and Subbarao (2008) have reported *V. dahliae* conidia germ tubes that extended along lettuce root epidermal cells longitudinally, and the presence of pathogen appressoria formed within the cell junctions at apparent penetration sites. Studies conducted on *V. longisporum* colonization of oilseed rape showed slight hyphal swellings before direct penetration of epidermal cells as well (Eynck et al., 2007). In contrast, under our experimental conditions, events showing VDAT-36I entry into root epidermal cells by an active mechanism were absent or, if any, must be very rare (see Fig. 2G). Therefore, we conclude that inner colonization of olive root tissues by *V. dahliae* overwhelmingly takes place by means of a passive entrance mechanism through either micro- or macro-breakages. This situation is likely operating in plant root systems growing under natural soil conditions (Pegg, 1985; Pegg and Brady, 2002).

We detected VDAT-36I MS development on the olive root surface as early as 6 DAI. Microsclerotia appeared as this stage as irregular fluorescent bodies, within a compact mass of interwoven hyphae. Production of MS on the olive root surface took place much earlier than that described for *V. dahliae* and *V. longisporum* in oilseed rape (Eynck et al., 2007). These authors suggested that massive production by *V. dahliae* of conidia and MS outside oilseed rape root is because this plant is not a suitable host for *V. dahliae* (in contrast to *V. longisporum*, which did produce MS in the root cells). This explanation would not apply for the pathosystem under study here, because olive cv. Arbequina is a susceptible host for *V. dahliae*. Therefore, as production of these latent structures is a likely response to adverse conditions, the early formation of VDAT-36I MS on olive root tissue pointed to pathogen preparation for survival, either due to nutrient shortage following inoculation, and/or the failure to penetrate root tissues.

At 6–9 DAI, CLSM images showed an abundance of VDAT-36I biomass on/in root tissues. These observations agreed with the previously detected boost of *V. dahliae* DNA in roots of different olive genotypes at 7 DAI with the fungus (Mercado-Blanco et al., 2003). From that time-point a reduction of pathogen DNA was then observed, which correlated with our present microscopy observations showing lower amount of fluorescent hyphae in roots over time, and the translocation of the pathogen to above-ground vascular tissues.

After successful penetration into the roots, *V. dahliae* VDAT-36I quickly invaded (both inter- and intracellularly) the root cortex, generating an extensive and irregular hyphal network that reached the vascular tissue within 9 DAI. Our observations also showed that *V. dahliae* VDAT-36I densely colonized the olive root cortex with little impediment to final invasion of the root vascular tissue as described in lettuce (Vallad and Subbarao, 2008). Finally, the manner in which VDAT-36I colonized the root cortex was similar to that described for *V. longisporum* in oilseed rape, and clearly in contrast to *V. dahliae* in this host, where intercellular rather than intracellular colonization of *V. dahliae* was found (Eynck et al., 2007).

Once *V. dahliae* VDAT-36I has invaded the root vascular tissue, it quickly progressed to the above-ground tissues. There, the pathogen was detected by CLSM exclusively within the xylem vessels cell of both stems and leaves petioles, but not in the surrounding mesophyll cells at any time. However *V. dahliae* VDAT-36 never managed to colonize the entire vascular tissue of the stem, as has been described for *V. longisporum* in *B. napus* (Eynck et al., 2007). We could not rule out the complete obstruction of the vessel cells by the accumulation of degradation polysaccharide-type materials not detectable by CLSM, as otherwise observed by electron scanning microscopy in cv. Picual (Baídez et al., 2007).

*Pseudomonas fluorescens* PICF7 harbouring plasmid pMP4655 effectively controlled VWO in ‘Arbequina’ plants, confirming the biocontrol activity of wild-type strain PICF7 in ‘Picual’ plants (Mercado-Blanco et al., 2004). Although fluorescence was generally uniform in conidia and hyphae of isolate VDAT-36I, hyphal dimorphism (vacuolated and non-vacuolated hyphae) could also be observed, as reported by Eynck and colleagues (2007). However, in this present study, vacuolation could not be correlated to the presence or absence of nearby PICF7 cells, in contrast to studies carried out in a *Fusarium*–tomato pathosystem (Bolwerk et al., 2003). Without excluding a negative influence of PICF7 on the metabolic activity of VDAT-36I hyphae, we rather related the absence/presence of hyphae vacuoles to different...
metabolic stages of the pathogen or to hyphal aging (Moore-Landecker, 1996), regardless the presence or not of the BCA.

An important conclusion from our study was that upon PICF7 establishment on a defined olive root area, V. dahliae colonization of the root surface seemed to be impaired. Indeed, we qualitatively detected less VDAT-36I germinated conidia and elongating hyphae in the presence than in the absence of strain PICF7. The diminished or null hyphal fluorescence observed in these locations could be associated with a reduced metabolic activity (or even dying) because of in situ antagonistic activity of the BCA. In addition, our results suggested that once P. fluorescens PICF7 had endophytically colonized specific areas within the cortex, V. dahliae inner root colonization was impaired to some degree. Therefore, effective root colonization by PICF7, including endophytic establishment, seemed to be necessary for controlling VWO under our experimental conditions. However, PICF7 did not completely prevent VDAT-36I colonization and further disease development in some plants. This failure could likely be due to the presence of major wounds in roots, which facilitated the entrance of excessive amounts of the pathogen. In this situation, neither external nor internal efficient root colonization by the BCA was enough to prevent the pathogen penetration and its subsequent quick progression to the aerial vascular tissues.

In conclusion, we have provided herein evidence that effective control of VWO requires that intact roots be persistently colonized with P. fluorescens PICF7, at both superficial and endophytic levels, prior to exposure to V. dahliae. Molecular work is currently in progress to elucidate the mechanism(s) operating in the PICF7-mediated biocontrol of this disease.

Experimental procedures

Pseudomonas fluorescens culture conditions and inoculum production

Pseudomonas fluorescens PICF7 (Mercado-Blanco et al., 2004) was used in this study, and harbours plasmid pMP4655, which carries the EGFP marker (Bloemberg et al., 2000). Culture conditions and inoculum preparation of P. fluorescens PICF7 (pMP4655) to be used in VWO assessment and biocontrol bioassays (see below) have been previously described in detail (Prieto and Mercado-Blanco, 2008). The bacterial cell density in the final inoculum used for root-dip inoculation of olive plants was prepared in 10 mM MgSO₄·7H₂O, was 1 × 10⁶ cfu ml⁻¹.

Verticillium dahliae culture and inoculum production conditions

Single-spore V. dahliae isolate V937I (representative of the olive D pathotype), and an EYFP-tagged derivative of V937I (transformant VDAT-36I; see below) were used in this study. Isolate V937I originates from a verticillium wilt-diseased olive tree (cv. Arbequina) at an orchard located in Córdoba province (southern Spain). The isolate has been previously characterized at the molecular (amplified fragment length polymorphism and specific-PCR fingerprinting), genetic [vegetative compatibility group (VCG)] (Collado-Romero et al., 2006), and pathogenic (artificial inoculation of susceptible olive cultivars) (J. Mercado-Blanco and D. Rodríguez-Jurado, unpubl. results) levels. This isolate is deposited in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible (CSIC), Córdoba, Spain. For long-term storage of V937I and VDAT-36I, cultures were grown on plum-lactose-yeast extract agar (PLYA) (Talboys, 1960), overlaid with liquid paraffin (Bejarano-Alcázar et al., 1996), and stored at 4°C in the dark. Cultures of V937I and VDAT-36I were reactivated on 2% (w/v) WA amended with chlorotetracycline (0.3 g l⁻¹) (Sigma-Aldrich, Madrid, Spain), and further subcultured on PDA (Difco Laboratories, Detroit, MI), or alternatively, PLYA. Verticillium dahliae spores used for genomic fungal DNA extraction were obtained from cultures grown at 25°C for 5 days on CM (Bennett and Lasure, 1991), with shaking at 200 r.p.m. For comparative studies of growth rate and colony morphology of V. dahliae V937I and its fluorescently tagged derivative VDAT-36I, fungal cultures were grown on CM, PDA, WA or ASPM (Huisman and Ashworth, 1974; Butterfield and DeVay, 1977), and were cultured for 25 days in the dark, at 22°C, 25°C or 28°C. Growth experiments were carried out three times, and each repetition contained two replicate cultures from each culture medium.

For disease assessment and biocontrol bioassays, V937I and VDAT-36I inocula were prepared from conidial suspensions of cultures grown in potato-dextrose broth. Cultures were incubated on an orbital shaker (125 r.p.m.) at 25°C in the dark for 7 days. Conidia produced from these cultures were obtained by filtering through layers of sterile cheese-cloth, counted with a haemocytometer, and the inoculum concentrations adjusted as necessary. Final inoculum concentration in infested soil was 5 × 10⁶ conidia g⁻¹ soil.

Construction of V. dahliae D pathotype transformants tagged with the yellow fluorescent protein

Tagging of V. dahliae V937I with the eyfp reporter gene was achieved by Agrobacterium-mediated transformation (Mullins et al., 2001), as modified by Dobinson and colleagues (2004) with vector pSK1035 (Klimes et al., 2008). Neomycin (geneticin)-resistant transformants were single-spore purified as previously described (Dobinson, 1995), and characterized by DNA–DNA hybridization (see below), and analysis of the acquired fluorescence phenotype. For the latter analysis, transformants were grown for 2–3 days on basal medium (Neumann and Dobinson, 2003) in depression well slides, and visualized by confocal microscopy with a Leica TCS SP2 confocal laser scanning microscope as described in Klimes and colleagues (2008).

The transformant selected for use in this study (VDAT-36I) was further characterized at the morphological (colony and MS morphology), physiological (growth at different temperatures in different culture media; see above) and pathological
(virulence on olive nursery-produced plants) levels, and its phenotypic traits compared with those of the parental strain V937I. Morphological comparison of MS produced by V937I and VDAT-36I was carried out as previously described (López-Escudero and Blanco-López, 2005).

Nucleic acid manipulations

Verticillium dahliae genomic DNA used for DNA blot hybridizations was purified from spores grown in liquid CM, using a glass bead breakage method (Dobinson, 1995). Plasmids were purified from bacterial cultures by an alkaline lysis method using the QiAprep miniprep kit (Qiagen GmbH, Hilden, Germany). DNA purity and concentration were determined spectrophotometrically (NanoDrop Technologies, Wilmington, DE). For DNA blot hybridizations, 600 ng aliquots of genomic DNA from V937I and VDAT-36I isolates were digested with either EcoRI, HindIII, BamHI or XhoI restriction enzymes (New England Biolabs, Ipswich, MA), fractionated through 0.8% agarose gels, and transferred by capillary blotting to Nylon membranes, positively charged (Roche Diagnostics SL, Applied Science, Barcelona, Spain). Digoxigenin (DIG)-labelled hybridization probes were prepared using the DIG DNA labelling and detection kit (Roche Diagnostics SL), and labelled hybridization probes were prepared using a QIAquick PCR purification kit (Qiagen), and used as a DIG-labelled probe in DNA blot hybridizations. PCR primers used were Neo-F: 5′-GGGCGCGGTTTCTTTTTT G-3′ and Neo-R: 5′-AAGCGGACATTTTCAATC-3′ (Klimes et al., 2008). PCR amplifications contained 1× PCR buffer [160 mM (NH₄)₂SO₄, 670 mM Tris-HCl (pH 8.8 at 25°C), 0.1% Tween-20], 2 mM MgCl₂, 1 mM of each dNTP, 400 µM of each primer, 0.75 U BioTaq DNA polymerase (Bioline, USA, Taunton, MA), and plasmid DNA template (1–5 ng). PCR conditions were: an initial denaturation step (2 min at 94°C) followed by 30 cycles of denaturation (45 s at 94°C), annealing (45 s at 60°C), and elongation (1 min at 72°C), followed by a final elongation step (1 min at 72°C).

Plant material. P. fluorescens and V. dahliae plant inoculation, olive growth conditions, and disease assessment

Two bioassays were conducted using 4-month-old olive plants of the VWO susceptible cv. Arbequina (López-Escudero et al., 2004) that had been purchased from a commercial nursery in Córdoba province (southern Spain). These plants were propagated by rooting of leafy stem cuttings under mist conditions in plastic tunnels. A DAB experiment was carried out to assess the aggressiveness of ‘Arbequina’ plants of V. dahliae D isolate V937I and its EYFP-tagged derivative VDAT-36I. A BCB experiment was conducted to evaluate whether the EYFP-tagged derivative of P. fluorescens PICF7 suppressed VWO caused by isolate VDAT-36I, as it has been previously demonstrated for PICF7 in ‘Picual’ plants of similar phenology (Mercado-Blanco et al., 2004). In this experiment, a number of additional plants were included to monitor the olive colonization process by the yellow fluorescent protein-tagged transformant, as well as to study its in planta interaction with the BCA.

In both experiments, olive plants were carefully uprooted from the original substrate, and their roots thoroughly washed in tap water without intentional wounding. For the DAB experiment, washed plant root systems were dipped into 10 mM MgSO₄·7H₂O for 15 min. Plants were then transplanted to soil infested with enough volume of a suspension of V937I or VDAT-36I conidia in sterile water to reach a final inoculum density of 5 × 10⁸ conidia g⁻¹ soil. Control plants were planted in non-infested soil to which the same volume of sterile water was added. For this bioassay, 12 plants per treatment were inoculated (36 plants in total).

For the BCB experiment, the entire washed root systems of the plants were dipped for 15 min in a P. fluorescens PICF7(pMP4655) suspension (1 × 10⁶ cfu ml⁻¹) in 10 mM MgSO₄·7H₂O, prepared as previously described (Prieto and Mercado-Blanco, 2008). Roots of non-bacterized and non-inoculated plants (control treatment) were dipped into 10 mM MgSO₄·7H₂O for 15 min. Plants were then transplanted into VDAT-36I conidia-infested soil as described above. For disease and biocontrol assessment, 15 plants per treatment (non-bacterized/non-inoculated treatment (control), PICF7(pMP4655)-treated/VDAT-36I-inoculated treatment, and non-bacterized/VDAT-36I-inoculated treatment) were used (45 plants in total). In addition, 35 PICF7(pMP4655)-treated plants and 35 non-bacterized plants were planted into VDAT-36I infested soil. These latter two groups of plants were used to monitor the colonization process (see below), and to determine PICF7(pMP4655) population sizes in plant roots during the bioassay, as previously described (Prieto and Mercado-Blanco, 2008). An additional 15 plants were treated similarly except that they were not bacterized, and were planted in non-infested soil. These plants served as control plants for CLSM observations, to check for the presence of native autofluorescent microflora. In both bioassays, all plants were individually transplanted after treatment into 7 × 7 × 8 (cm) polypropylene pots filled with autoclaved (121°C, 1 h, twice on consecutive days) sandy substrate (1–10 mm sand grain sizes, thoroughly mixed) (Prieto and Mercado-Blanco, 2008). Plants were randomly distributed over one square meter clean surface per treatment, and incubated in a growth chamber adjusted to 23 ± 1°C, 60–70% relative humidity, and a 14 h photoperiod with fluorescent light (360 µE m⁻² s⁻¹) for 60 days. Plants were watered as needed, and fertilized weekly with 50 ml per pot Nipofol-K Plus 12-4-36 + microelements (1 g l⁻¹) (Fercampo, Málaga, Spain).

Verticillium wilt development was assessed by scoring the severity of disease symptoms, using a 0–4 rating scale according to the percentage of affected leaves and twigs (0 = no symptoms, 1 = 1–33%, 2 = 34–66%, 3 = 67–100% and 4 = dead plant). Disease symptoms were scored at weekly intervals after inoculation. Data were subjected to analysis of variance. Data on disease severity were used to calculate the following: (i) a DII (ranging 0–1) determined as DII = (Σ S𝑖 × Ni)/(4 × Nf), where S𝑖 is the symptom severity, Ni is the number of plants with S𝑖 symptom severity, and Nf is the total number of plants; and (ii) the SAUDPC of DII plotted

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Verticillium dahliae and P. fluorescens olive colonization studies: tissue sectioning and CLSM

The EYFP-tagged V. dahliae transformant VDAT-36I was used to monitor the infection and colonization process of an entire olive plant by the V. dahliae D pathotype. As described in the previous section, 35 non-bacterized olive plants were inoculated with VDAT-36I, and the colonization of root and above-ground tissues (stems and leaf petioles) samples was monitored by CLSM (see below) over the 60 day post-inoculation period. Inoculated plants were sampled as follows: one plant per day from 1 to 15 DAI (15 plants), and one plant every 5 days from 20 until 60 DAI (nine plants). Finally, to assess experimental variation among plants, an extra plant was sampled at each 1, 5, 10, 15, 20 and 30 DAI. In total 30 out of 35 inoculated plants were examined by CLSM.

A second group of 35 olive plants cv. Arbequina was inoculated as described in the previous section to examine the colonization of VDAT-36I in the presence of P. fluorescens PICF7 (EGFP-tagged), and to assess the in planta interactions between the microorganisms. Thirty inoculated plants were sequentially taken during the period from 1 to 60 DAI, using the sampling strategy described above. Lastly, non-inoculated control plants were sampled on days 1, 10, 40 and 60, in order to check for the presence of fluorescent, native microorganisms.

Preparation of olive tissue samples for microscopic studies was done as previously described (Prieto and Mercado-Blanco, 2008). Briefly, olive roots, stems and petioles were sectioned under distilled water using a Vibratome Series 1000plus (TAAB Laboratories Equipment, Aldermaston, UK). Longitudinal and transversal sections (approximately 20 μm thick, two to three plant cell layers) were placed on multi-well slides (ICN Biomedicals, Costa Mesa, CA) that had been pre-treated as described previously (Prieto et al., 2007), and analysed by CLSM. At least three different roots per plant were completely sectioned and exhaustively examined by CLSM. Similarly, to analyse the colonization pattern of the above-ground tissues by isolate VDAT-36I, three continuous longitudinal plus three continuous transversal stem regions, as well as three petioles were vibratome-sectioned completely. From all these vibratome sections we collected single confocal optical sections using an Axioplan 2 MOT microscope (Carl Zeiss, Jena GmbH, Germany) equipped with a Krypton and an Argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss). Enhanced green fluorescent protein-tagged bacterial cells were exposed to 488 nm Argon laser light (detection at 500–520 nm), and the EYFP-tagged V. dahliae to 514 nm Argon laser light (emission 530–620 nm). Data were recorded and the images transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss). Confocal stacks were mounted and analysed to assess colonization of olive roots, branches and petioles by P. fluorescens and V. dahliae (images shown in Figs 2–4 were generated from projections of adjacent confocal optical sections). Final figures were processed with PhotoShop 10.0 software (Adobe Systems, San Jose, CA, USA).

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