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Monitoring the colonisation of olive tissues by *Verticillium dahliae* and its interaction with the beneficial root endophyte *Pseudomonas fluorescens* PICF7

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Abstract: The colonisation process of olive by an enhanced yellow fluorescent protein (EYFP)-tagged *Verticillium dahliae* defoliating (D) pathotype derivative (VDAT-36I), and its *in planta* interaction with an enhanced green fluorescent protein (EGFP)-tagged derivative of *Pseudomonas fluorescens* PICF7 have been monitored on/in olive tissues using a nongnotobiotic system, vibratome-tissue sectioning, and confocal laser scanning microscopy (CLSM). Isolate VDAT-36I 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. Early and localised root surface and root endophytic colonisation by *P. fluorescens* PICF7 is needed to impair full progress of verticillium wilt in olive.

Key words: Biological control, confocal microscopy, plant-microbe interaction, *Verticillium* wilt

Introduction

Among all biotic constraints affecting olive cultivation, *verticillium* wilt of olive (VWO) is the most serious threat for this commodity worldwide. So far, no efficient control measure is available. A promising element in a preventive strategy is the protection of pathogen-free planting material from early *Verticillium dahliae* infection during plant propagation and/or at transplanting. This could be accomplished by the use of biocontrol agents (BCAs), an approach fulfilling sustainable agriculture requirements for diseases control. Previous studies have revealed that diverse *Pseudomonas* spp. strains native to olive roots can antagonize *V. dahliae in vitro*, and effectively suppress VWO caused by the most virulent (defoliating, [D]) pathotype (Mercado-Blanco *et al*., 2004). Recently, we have also shown that *Pseudomonas fluorescens* strain PICF7 can endophytically colonise the intercellular spaces of the root cortex (Prieto and Mercado-Blanco, 2008). The development of confocal laser scanning microscopy (CLSM) has overcome most of the problems associated with the *in situ* localisation of microorganisms in the rhizosphere. In this study we aimed to: i) monitor by CLSM the infection and colonisation of the entire olive plant by an enhanced yellow fluorescent protein (EYFP)-tagged transformant of a *V. dahliae* D pathotype isolate; ii) assess the biocontrol activity of an enhanced green fluorescent protein (EGFP)-tagged *P. fluorescens* PICF7 derivative in young olive plants (cv. Arbequina); and iii) analyse the interaction between the EYFP-tagged *V. dahliae* isolate and the *P. fluorescens* EGFP-tagged PICF7 strain on/in olive roots.

Material and methods

Bacterial and fungal culture conditions and inocula production

Culture conditions and inoculum preparation of *P. fluorescens* PICF7(pMP4655) have been previously described (Prieto and Mercado-Blanco, 2008). Single-spore *V. dahliae* isolate V937I (Collado-Romero *et al*., 2006), representative of the olive D pathotype, and its EYFP-tagged derivative transformant (VDAT-36I, see below) were used in this study. Fungal cultures were reactivated on 2% (w/v) water agar amended with chlortetracycline ($0.3gl^{-1}$) and further subcultured on potato dextrose agar. 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 diverse culturing media for 25 days in the dark, at 22, 25 or 28°C. Growth experiments were carried out three times. V937I and VDAT-36I inocula were prepared from conidial suspensions produced by cultures grown in potato-dextrose broth. Cultures were incubated on an orbital shaker (125rpm) at 25°C in the dark for 7 days. Conidia were obtained by filtering through layers of sterile cheesecloth, counted with a haemo-cytometer, and the inoculum concentrations adjusted as necessary.

Construction and characterization of a* Verticillium dahliae *EYFP-tagged transformant

Tagging of *V. dahliae* V937I with the *eyfp* reporter gene was achieved by *Agrobacterium*-mediated transformation (Mullins *et al*., 2001; Dobinson *et al.* 2004) with vector pSK1035 (Klimes *et al*., 2008). Geneticin-resistant transformants were single-spore purified as done by Dobinson (1995), and checked as described in Klimes *et al*., (2008). The transformant finally selected for use in this study (VDAT-36I) was compared to the parental strain V937I at the morphological, physiological and pathological levels.

Olive*-Pseudomonas fluorescens-Verticillium dahliae *bioassays: disease assessment and olive colonisation studies by CLSM

Four-month-old olive plants cv. Arbequina were used. One bioassay was carried out to asses the aggressiveness on 'Arbequina' plants of isolates V937I and VDAT-36I. A second bioassay was conducted to evaluate whether *P. fluorescens* PICF7(pMP4655) suppressed VWO caused by isolate VDAT-36I. A number of additional plants were included to monitor the olive colonisation process by the EYFP-tagged transformant, as well as to study its *in planta* interaction with the BCA. Manipulation of plants and transplanting, bacterial treatment of olive root systems, *V. dahliae* infestation of an artificial sandy soil with conidia (final inoculum density 5×10^6 conidia g^{-1} soil), and bioassay conditions have been previously described in detail (Mercado-Blanco *et al*., 2004; Prieto and Mercado-Blanco, 2008). *Verticillium* wilt development was assessed as described before (Mercado-Blanco *et al*., 2004). The EYFP-tagged *V. dahliae* transformant VDAT-36I was used to monitor the infection and colonisation process of an entire olive plant by CLSM, sampling inoculated plants at defined time-points over a period of 60 days after inoculation (DAI) (30 inoculated plants were examined). A second group of 'Arbequina' plants (30) was also examined to assess both the colonisation of VDAT-36I in the presence of *P. fluorescens* PICF7 (EGFP-tagged), and the *in planta* interaction between the two microorganisms. 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 (roots, stems and petioles) samples for microscopic studies and confocal analysis were done as previously described (Prieto and Mercado-Blanco, 2008). EGFP-tagged bacterial cells were exposed to 488nm Argon laser light (detection at 500-520nm), and the EYFP-tagged *V. dahliae* to 514nm Argon laser light (emission 530-620nm). Data were recorded and analysed and final figures processed as described previously (Prieto and Mercado-Blanco, 2008).

Results and discussion

An isolate (VDAT-36I) of the *V. dahliae* D pathotype was successfully transformed and tagged with the *eyfp* reporter gene. Presence of the *eyfp* gene was confirmed by DNA-DNA blot hybridisation. Moreover, VDAT-36I did not show any difference in growth rate, morphology and number of microsclerotia (MS) produced in diverse culture media and temperatures and pathogenicity when compared with its parental wild-type (V937I). Results from bioassay showed that strain PICF7(pMP4655) was effective in suppressing VWO caused by isolate VDAT-36I in 'Arbequina' plants. The final disease intensity index and standardised area under the disease progress curve parameters were significantly different ($P<0.05$) between the two treatments at the end of the bioassay (60 days after VDAT-36I-inoculation).

The use of fluorescent tagging in conjunction with 3-D tissue sectioning and CLSM detection made it possible to differentially detect both *V. dahliae* and the BCA *in planta* and *in vivo*. VDAT-36I conidia were readily detected on the root surface just one DAI. Profuse VDAT-36I colonisation of the entire root surface was found during the first 1-2 days without differences among the different root zones. However, the differentiation and the elongation zones were more abundantly colonised by VDAT-36I than was the meristematic zone at three DAI. Most of the samples from this time-point showed a non-specific pathogen growth pattern, resulting in a complicated network of hyphae covering the root epidermis. Development of VDAT-36I MS was detected on the olive root surface as early as six DAI. The formation of MS pointed to pathogen preparation for survival. Transverse and longitudinal root vibratome sections were prepared routinely to assess internal root colonisation by VDAT-36I. We concluded that inner colonisation of olive root tissues by *V. dahliae* takes place by means of a passive entrance mechanism through either micro- or macro-breakages. Internal proliferation of pathogen hyphae was observed within the root cortex nine DAI, both inter and intracellularly. Hyphal ramification, swelling and appressoria were consistently observed. The first observation of xylem vessel colonisation was also made at this time, and proliferation of pathogen hyphae within the xylem vessel cells took place both longitudinally and transversally. A progressive reduction in detectable VDAT-36I was observed at 15 DAI-20 DAI. From this time-point on, fluorescent hyphae were increasingly detected within the secondary xylem vessel cells of the main stem, lateral branches, and petioles of both fallen and still-attached leaves.

The olive root rhizoplane was rapidly colonised by PICF7. Intracellular colonisation of root hairs by tagged-bacteria has been repeatedly detected, demonstrating that root hairs are a preferred site for penetration into olive roots by PICF7. Although we did not detect root penetration of PICF7 cells through sites other than root hair cells, passive entrance through root breakages could not be discarded. PICF7 microcolonies were finally observed in the intercellular spaces of the cortex as previously reported (Prieto and Mercado-Blanco, 2008).

P. fluorescens PICF7 and *V. dahliae* VDAT-36I were simultaneously detected on olive root surface at one-two DAI (Figure 1). The presence of PICF7 did not completely impair VDAT-36I conidia germination on the examined tissue, but less geminating conidia or elongating hyphae were detected when the BCA was present. Moreover, direct contact between PICF7 cells and VDAT-36I hyphae was observed at early stages (one to five DAI), and colonisation of hyphae by bacterial cells was evident. In addition, our results suggested that once *P. fluorescens* PICF7 had endophytically colonised specific areas within the cortex, *V. dahliae* inner root colonisation was impaired to some degree. Therefore, effective root colonisation by PICF7, including endophytic establishment, seemed to be necessary for controlling VWO under our experimental conditions. However, PICF7 did not completely prevent VDAT-36I colonisation and further disease

development in some plants. In conclusion, we have provided evidence that effective control of VWO requires that intact roots be persistently colonised with *P. fluorescens* PICF7, at both superficial and endophytic levels, prior to exposure to *V. dahliae*.

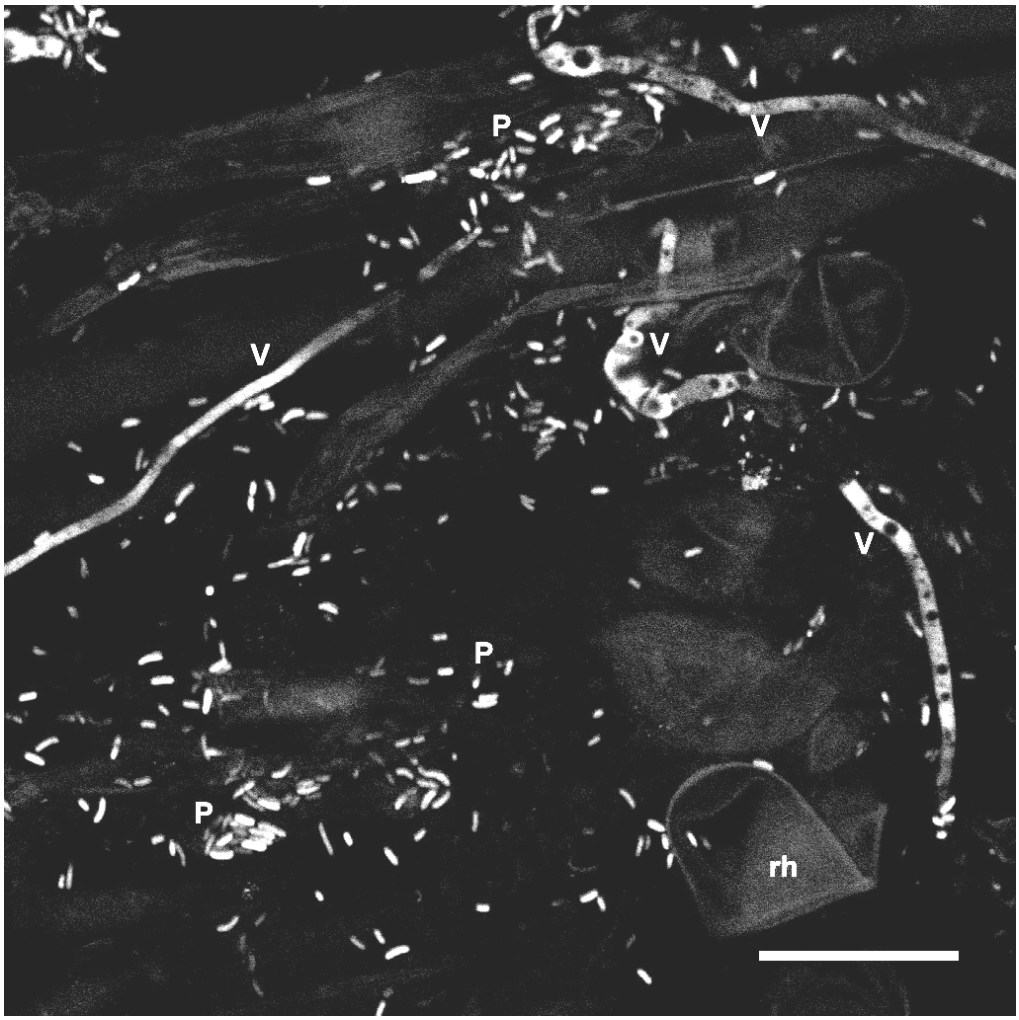

Figure 1. Expression of *eyfp* and *egfp* genes in VDAT-36I and PICF7 strains, respectively, on the differentiation zone of the olive root surface at two days after inoculation. V, *Verticillium dahliae* VDAT-36I (EYFP-tagged) hyphae; P, *Pseudomonas fluorescens* PICF7 (EGFP-tagged) cells; rh, root hair. Scale bar represents 20µm.

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