RESEARCH ARTICLE

Endophytic colonization of olive roots by the biocontrol strain *Pseudomonas fluorescens* PICF7

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Abstract

Confocal microscopy combined with three-dimensional olive root tissue sectioning was used to provide evidence of the endophytic behaviour of *Pseudomonas fluorescens* PICF7, an effective biocontrol strain against *Verticillium* wilt of olive. Two derivatives of the green fluorescent protein (GFP), the enhanced green and the red fluorescent proteins, have been used to visualize simultaneously two differently fluorescently tagged populations of *P. fluorescens* PICF7 within olive root tissues at the single cell level. The time-course of colonization events of olive roots cv. Arbequina by strain PICF7 and the localization of tagged bacteria within olive root tissues are described. First, bacteria rapidly colonized root surfaces and were predominantly found in the differentiation zone. Thereafter, microscopy observations showed that PICF7-tagged populations eventually disappeared from the root surface, and increasingly colonized inner root tissues. Localized and limited endophytic colonization by the introduced bacteria was observed over time. Fluorescent-tagged bacteria were always visualized in the intercellular spaces of the cortex region, and no colonization of the root xylem vessels was detected at any time. To the best of our knowledge, this is the first time this approach has been used to demonstrate endophytism of a biocontrol *Pseudomonas* spp. strain in a woody host such as olive using a nongnotobiotic system.

Introduction

A large number of soil-inhabiting bacterial genera can internally colonize root plant tissues without causing any deleterious effect, establishing themselves as beneficial endophytes (Chanway, 1996; Rosenblueth & Martínez-Romero, 2006). This internal colonization process may induce positive effects for both partners. It is not clear whether becoming endophytic is advantageous for the bacteria (Rosenblueth & Martínez-Romero, 2006), although it is assumed that, once endophytic, bacteria are protected against (a)biotic stresses (Hallmann *et al.*, 1997). The benefits induced by endophytic (or simply surface root-associated) bacteria for the plant are growth promotion and/or protection against a broad range of phytopathogens and pests, which offers great potential for their exploitation in agriculture (Glick, 1995; Hallmann *et al.*, 1997; Sturz *et al.*, 2000; Gerhardson, 2002; Compant *et al.*, 2005a, b).

Nondeleterious, root-associated *Pseudomonas* spp. strains have long been known to be beneficial to plants either because of their plant-growth promotion (PGP) effect or their potential as biological control agents (BCAs). Some of them may also establish endophytically, and have been identified and/or isolated from a wide range of plant species (Mercado-Blanco & Bakker, 2007, and references therein). PGP exerted by endophytic *Pseudomonas* spp. (probably a consequence of a combined action with other endophytic bacterial genera) has been reported, for example, in oilseed rape (*Brassica napus* L.) and tomato (*Lycopersicon esculentum* L.) (Nejad & Johnson, 2000), rice (*Oryza sativa* L.) (Adhikari *et al.*, 2001) and or soybean (*Glycine max* L.) (Kuklinsky-Sobral *et al.*, 2004). In addition, PGP induced by endophytic *Pseudomonas* spp. can also be a consequence of controlling phytopathogens by means of mechanisms such as antibiosis, competition for nutrients and/or induction of systemic resistance. Although *in planta* endophytic biocontrol activity

is not always easy to demonstrate, there are some reports showing true biocontrol activity promoted by endophytic pseudomonads (Brooks *et al.*, 1994; Bell *et al.*, 1995; Chen *et al.*, 1995; Adhikari *et al.*, 2001; Grosch *et al.*, 2005).

Appraisal for endophytic behaviour of *Pseudomonas* spp. populations may come from both culture- and culturing-independent identification methods (Elvira-Recuenco & van Vuurde, 2000; Garbeva *et al.*, 2001; Sessitsch *et al.*, 2002; Reiter *et al.*, 2003; Cankar *et al.*, 2005). However, a definitive assessment of true endophytism by a given bacterial species should go beyond isolation from surface-disinfected plant tissues, and must be supported by microscopic evidence and by the capacity of the putative endophyte to reinfect disinfected seedlings (Reinhold-Hurek & Hurek, 1998a; Rosenblueth & Martínez-Romero, 2006).

Although reports are not abundant, endophytic populations of *Pseudomonas* spp. have been detected in diverse tissues of different woody plants. Examples are the xylem sap of poplar trees (*Populus trichocarpa* x *deltoides* cv. Hoogvorst) (Germaine *et al.*, 2004), stems and roots of elm trees (*Ulmus* spp.) (Mocali *et al.*, 2003), meristematic bud tissues of Scots pine (*Pinus sylvestris* L.) (Pirttilä *et al.*, 2004), seeds of coffee (*Coffea arabica* L.) (Vega *et al.*, 2005) or Norway spruce (*Picea abies* L. Karst) (Cankar *et al.*, 2005), and stems of orange [*Citrus sinensis* (L.) Osbeck] (Lacava *et al.*, 2006). It has also been demonstrated that endophytic *Pseudomonas* spp. control plant diseases in woody hosts; for example, a *Pseudomonas corrugata* isolate effectively controlled the population of the causal agent of grapevine (*Vitis vinifera* L.) crown gall disease (*Agrobacterium vitis*) (Bell *et al.*, 1995), or endophytic isolates of *Pseudomonas denitrificans* and *Pseudomonas putida* from oak (*Quercus fusiformis* Small.) reduced oak wilt disease caused by *Ceratocystis fagacearum* (Brooks *et al.*, 1994). Additionally, PGP exerted by endophytic *Pseudomonas* spp. has been reported in hybrid spruce (*Picea glauca* x *engelmannii*) (Chanway *et al.*, 2000).

Pseudomonas fluorescens PICF7 is a native colonizer strain of olive (*Olea europaea* L.) roots, which, upon artificial introduction, has been demonstrated to control *Verticillium* wilt of olive effectively (Mercado-Blanco *et al.*, 2004). This disease, caused by the soil-borne fungal phytopathogen *Verticillium dahliae* Kleb., has become one of the most important biotic menaces for olive cultivation worldwide (Jiménez-Díaz *et al.*, 1998). The use of antagonistic bacteria with good root colonization, PGP and biocontrol capabilities is one of the control measures that are currently under study. Furthermore, the exploitation of beneficial traits from indigenous, endophytic olive-root bacteria offers the additional advantage of exploiting PGP/BCA candidates that are ecologically adapted to the target niche (i.e. olive root tissues). Thus far, there is no definitive evidence that *P. fluorescens* PICF7 as a true olive-root endophyte. Thus, it has been simply regarded as a native, root-associated bacterium (Mercado-Blanco *et al.*, 2004). In the present work, we present microscopic evidence of the endophytic behaviour of strain PICF7 in olive roots using vibratome tissue sectioning (VTS; Prieto *et al.*, 2007), confocal laser scanning microscopy (CLSM) and fluorescent bacterial tagging. The time-course of colonization events of olive roots by strain PICF7 and the distribution of introduced tagged bacteria populations within olive root tissues are shown.

Materials and methods

Bacterial strains, culture conditions and inoculum production

Pseudomonas fluorescens PICF7 was originally isolated from roots of nursery-propagated olive plants cv. Picual (Mercado-Blanco *et al.*, 2004). This strain has been shown to colonize and to persist in olive roots as well as to be effective in controlling *Verticillium* wilt of olive, caused by the so-called defoliating, highly virulent pathotype of *V. dahliae* in artificial inoculation bioassays (Mercado-Blanco *et al.*, 2004). Fresh cultures of strain PICF7 were obtained by incubation at 25 °C on modified (Geels & Schippers, 1983) King's medium B agar (KBA) (King *et al.*, 1954). Fresh cultures of PICF7 transformants (harbouring either plasmid pMP4655 or plasmid pMP4662) (Bloemberg *et al.*, 2000) were obtained on Luria–Bertani (LB) agar (Miller, 1972). *Escherichia coli* strain DH5α harbouring plasmids pMP4655 or pMP4662 were grown in LB broth. Tetracycline (Sigma, St Louis, MO) was added at 20 μg mL^{-1} when required. All bacterial cultures were cryopreserved with 30% glycerol at −80 °C.

Inocula of *P. fluorescens* strains PICF7(pMP4655) and PICF7(pMP4662) were prepared from the bacterial biomass grown on LB agar plates at 25 °C for 48 h, scraped from the medium with a sterile glass rod, and suspended in 10 mM $MgSO_4 \cdot 7H_2O$. Bacterial cell density of the suspensions was determined by measuring $A_{600\,nm}$ using standard curves for each transformant derivative and by culturing viable cells in a serial dilution series. The densities of each strain present in the final inoculum mix used for colonization of olive roots was determined to be 1.4×10^8 CFU mL^{-1} for PICF7(pMP4655) and 2.0×10^8 CFU mL^{-1} for PICF7(pMP4662).

Tagging of *P. fluorescens* PICF7

Plasmids pMP4655 [harbouring the enhanced green fluorescent protein (EGFP) marker] and pMP4662 [harbouring the red fluorescent protein (RFP) marker] (Bloemberg *et al.*, 2000) were purified from *E. coli* DH5α host cells using the Plasmid Mini Purification Kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Aliquots (40-μL) of ice-thawed, previously prepared electrocompetent cells (Miller & Nickoloff, 1995) of *P. fluorescens* PICF7 were mixed with 2 μL of each undiluted, purified plasmid DNA

(0.2–0.5 mg mL^{-1}) in prechilled 1.5-mL microfuge tubes. The plasmids were electroporated into *P. fluorescens* PICF7 (Micropulser™, Bio-Rad, Hercules, CA; with a single pulse using the default conditions 'Ec2' of the electroporation unit 2.5 kV; 12.5 kV cm^{-1}; 25 μF; 200 Ω; pulse time: 4–5 ms). Immediately after the pulse, 1 mL of KB broth (King *et al.*, 1954) was added to the electroporation cuvette, and the cells were resuspended by gentle pipetting, transferred to polypropylene tubes (17 mm × 100 mm) and shaken (225 r.p.m.) at 28 °C for 2 h. Finally, 100-μL aliquots of the cells suspensions were plated onto LB agar plates amended with tetracycline and incubated at 28 °C. Four individual tetracycline-resistant (Tc^R) colonies from each plasmid electroporation experiment were randomly chosen. The presence of the plasmids pMP4655 or pMP4662 was checked by plasmid DNA purification using the Plasmid Mini Purification Kit (Qiagen), and by subsequent EcoRI restriction analysis. One colony from each transformation experiment was selected and used as fluorescently tagged strains [named as PICF7(pMP4655) and PICF7(pMP4662)] in olive-root colonization experiments. Selected transformants were cryopreserved with 30% glycerol at − 80 °C.

Olive–*P. fluorescens* PICF7 time-course of colonization bioassay

A bioassay was conducted to verify the ability of fluorescently tagged *P. fluorescens* PICF7 transformant derivatives to colonize olive roots endophytically. Olive plants used in this bioassay were nursery-propagated by rooting of leafy stem cuttings under mist conditions in plastic tunnels. Four-month-old olive plants cv. Arbequina (30 plants) purchased from a commercial nursery in Córdoba province (southern Spain) were carefully uprooted from the original substrate, their roots thoroughly washed in tap water without intentional wounding, and dipped for 15 min in a bacterial suspension containing a mixture of *P. fluorescens* strains PICF7(pMP4655) and PICF7(pMP4662) prepared as indicated above. For the control treatment, eight 'Arbequina' plants were treated similarly except that the root systems were dipped in 10 mM $MgSO_4 \cdot 7H_2O$. Plants were then individually transplanted 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). Plants (randomly distributed over 1 m^2 clean surface) were incubated in a growth chamber adjusted to 23 ± 1 °C, 60–90% relative humidity and a 14-h photoperiod of fluorescent light at 360 μE m^{-2} s^{-1} for 80 days. Plants were watered as needed and fertilized weekly with 50 mL per pot with Nipofol-K Plus 12-4-36 plus micronutrients (1 g L^{-1}) (Fercampo, Málaga, Spain).

Root samples were analysed on the confocal microscope over 80 days after bacterial inoculation (DAB) to monitor *P. fluorescens* PICF7 colonization of root tissues. Twenty-two inoculated plants were sequentially taken: one plant per day from 1 to 10 DAB; one plant every 5 days until 40 DAB; and finally, one plant at 50, 60, 70 and 80 DAB. Two plants were also sampled at 10 and 20 DAB to assess experimental variability among different plants. Finally, noninfected, control plants were sampled on days 1, 10, 40 and 80 to check for possible fluorescent native bacteria or cross-contamination.

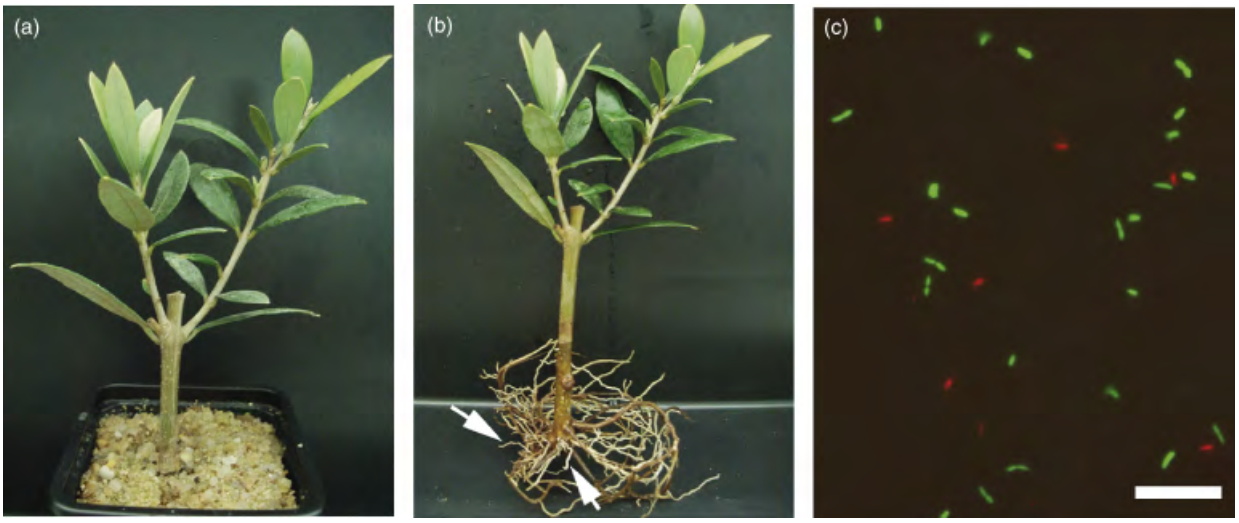


Fig. 1. Appearance of a bacterially inoculated 4-month-old olive plant cv. Arbequina before being uprooted from the sandy substrate (a), and once it was uprooted, clean and ready for microscopy analysis (b). Pictures correspond to a plant sampled 80 days after bacterial inoculation. Arrows show examples of the root type taken for confocal analysis. (c) Expression of *rfp* and *egfp* genes in PICF7(pMP4662) and PICF7(pMP4655) cells, respectively. Image was taken by epifluorescence analysis from an aliquot of a bacterial suspension containing a mixture of both transformant derivatives (see text). Scale bar = 20 μm.

Colonization of olive roots by EGFP- and RFP-tagged bacteria: root sectioning and CLSM

Olive plants were carefully uprooted from pots and the root systems were washed by dipping them in tap water. Owing to the sandy nature of the substrate used, sand particles were easily removed by gently shaking the roots within water. Therefore, clean and nearly intact root systems were obtained for microscopic studies (Fig. 1b). Roots from each plant were exhaustively analysed by confocal microscopy and those about 3–4 cm long were chosen for sectioning. Olive roots were sectioned under distilled water using a Vibratome Series 1000plus (TAAB Laboratories Equipment Ltd, Aldermarston, UK). Root longitudinal sections were *c.* 20 μm thick, contained two to three cell layers and were placed on multiwell slides (ICN Biomedicals Inc., Costa Mesa, CA). The slides were pretreated by washing in 3% Decon (Decon Laboratories Ltd, Sussex, UK) for at least 1 h, coating with 2% (v/v) 3-aminopropyl triethoxy silane (APTES; Sigma), rinsed thoroughly with distilled water and allowed to air-dry. Root longitudinal sections were analysed using a confocal microscope.

We collected confocal optical stacks using an Axioskop 2 MOT microscope (Carl Zeiss Inc., Jena GmbH, Germany) equipped with a krypton and an argon laser, controlled by Carl Zeiss Laser Scanning System LSM5 PASCAL software (Carl Zeiss Inc.). EGFP-tagged bacterial cells were excited with the 488-nm Ar laser line and were detected in the 500–520-nm window. RFP-tagged bacterial cells were excited with the 568-nm laser line and detected in the 580–620-nm window. The microscope data were recorded and then transferred for analysis to Zeiss LSM Image Browser version 4.0 (Carl Zeiss Inc.).

Bacterial colonization of olive roots was analysed from three-dimensional (3D) confocal data stacks. Projections from adjacent confocal optical sections were made for building up the images shown in Figs 2 and 3. Final figures were processed with PHOTOSHOP 4.0 software (Adobe Systems Inc., San Jose, CA).

Isolation of bacteria from plant roots

To determine bacterial population sizes present in and on root tissue of bacterially infected olives, randomly selected plants were carefully uprooted from pots and root systems were washed as indicated above. Roots were then dried with sterile filter paper, and for each plant, samples of 1 g of root tissues were ground in 10 mL of 10 mM $MgSO_4 \cdot 7H_2O$ using an autoclaved pestle and mortar. Serial dilutions of the macerates were plated onto LB agar plates (three plates per dilution) amended with tetracycline (20 μg mL^{-1}) and incubated at 25 °C for at least 48 h. Then, Tc^R bacterial colonies were counted and bacterial populations were expressed as CFU g^{-1} of fresh root tissues. Bacterial populations were checked at two time-points during the time-

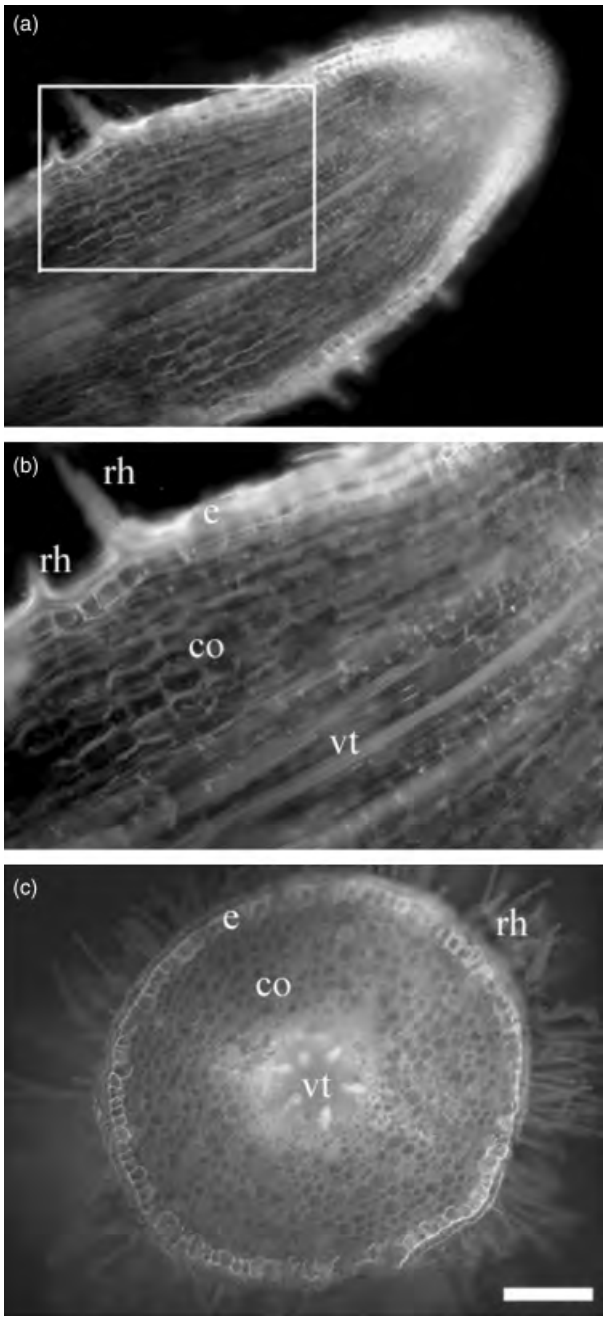


Fig. 2. Confocal images of vibratome root sections from olive cv. Arbequina. A 6-cm-long representative root fragment from an uninoculated plant was analysed by confocal microscopy on day 5 of the experiment to show root morphology. (a) Projection of three adjacent confocal optical sections of a vibratome longitudinal tissue section. A central column of vascular tissue is shown. The focal step size between adjacent optical sections was 1 μm. (b) Single confocal image of the area inset in (a). (c) Projection of three adjacent confocal optical sections of a vibratome transversal olive root section from the differentiation zone. co, cortical cells; e, epidermal cells; rh, root hair; vt, vascular tissue. Scale bar = 200 μm in (a) and (c) and 100 μm in (b).

course of the bioassay: 21 and 51 DAB. For each sampling time-point, three infected plants and one control (uninfected) plant were analysed.

Results and discussion

Pseudomonas fluorescens PICF7 was previously identified as naturally inhabiting olive plant roots. Until now it could only be demonstrated that strain PICF7 is a root-associated bacterium of 3-month-old 'Picual', nursery-propagated olives (Mercado-Blanco *et al.*, 2004), as well as able to colonize and persist in roots of 18–24-month-old 'Cornicabra', 'Hojiblanca', 'Lechín', 'Manzanilla' and 'Picual' olives (E. Tejedor-González, unpublished data). In addition, it has been demonstrated that strain PICF7 is an effective BCA against *Verticillium* wilt of olive although the biocontrol mechanism(s) involved still remain(s) to be elucidated (Mercado-Blanco *et al.*, 2004). Previous assessment of this strain as an olive root-associated inhabitant was merely based upon isolation procedures from thoroughly surface-disinfected olive roots. These results could not therefore be considered as proof of endophytism (Rosenblueth & Martínez-Romero, 2006). Here, we provide evidence that strain PICF7 internally colonizes olive root tissues.

Preliminary experiments demonstrated that a sandy substrate artificially prepared for culturing young olive plants was appropriate for supporting olive growth during the time-course of the bioassay (up to 80 days). Indeed, healthy olive plants were observed throughout the experiment (Fig. 1a), exhibiting an appearance similar to that observed when a different growing substrate was previously employed (Mercado-Blanco *et al.*, 2004). In addition, this sandy substrate facilitated the uprooting of intact, clean olive root pieces (Fig. 1b) perfectly suitable for subsequent tissue sectioning and CLSM observation.

Transformation of strain PICF7 by electroporation with plasmids pMP4655 (EGFP) and pMP4662 (RFP) (Bloemberg *et al.*, 2000) was successful. The expected fluorescence of *P. fluorescens* PICF7 transformants harbouring each of these plasmids was confirmed by epifluorescence observation of cell suspensions prepared from bacterial cultures grown on LB agar plates (Fig. 1c). Therefore, fluorescently tagged *P. fluorescens* PICF7 derivatives were validated for use as tools to visualize olive root colonization by this strain.

Among the diverse methods available for assessing the occurrence and localization of endophytic bacteria, CLSM combined with fluorescent tagging of bacteria offers several advantages. On the one hand, there is no need for plant tissue manipulation as CLSM provides an optical section of the sample, avoiding plant structure disruption. On the other hand, the use of nondiffusible fluorescent markers to tag bacterial cells also allows their *in situ* localization without the need to carry out extensive manipulation techniques. Thus, the use of CLSM and fluorescently tagged bacteria has previously been used to assess the endophytic behaviour of *Pantoea* sp. and *Ochrobactrum* sp. in deep-water rice (Verma *et al.*, 2004), *Pseudomonas chlororaphis* in barley (*Hordeum vulgare* L.) seeds (Tombolini *et al.*, 1999), *Klebsiella pneumoniae* in maize (*Zea mays* L.) (Chelius & Triplett, 2000), *Herbaspirillum* sp. in *Oryza offcinalis* seedlings (Elbeltagy *et al.*, 2001), *Azoarcus* sp. in rice roots (Egener *et al.*, 1998), and *Bacillus megaterium* C4 in maize and rice roots (Liu *et al.*, 2006). Also, *P. fluorescens* WCS365 populations tagged with up to three different autofluorescent proteins (including EFGP and RFP) could be differentially visualized by CLSM (Bloemberg *et al.*, 2000). However, these latter authors only detected bacterial microcolonies on the root surface of tomato seedlings and no endophytic colonization was reported.

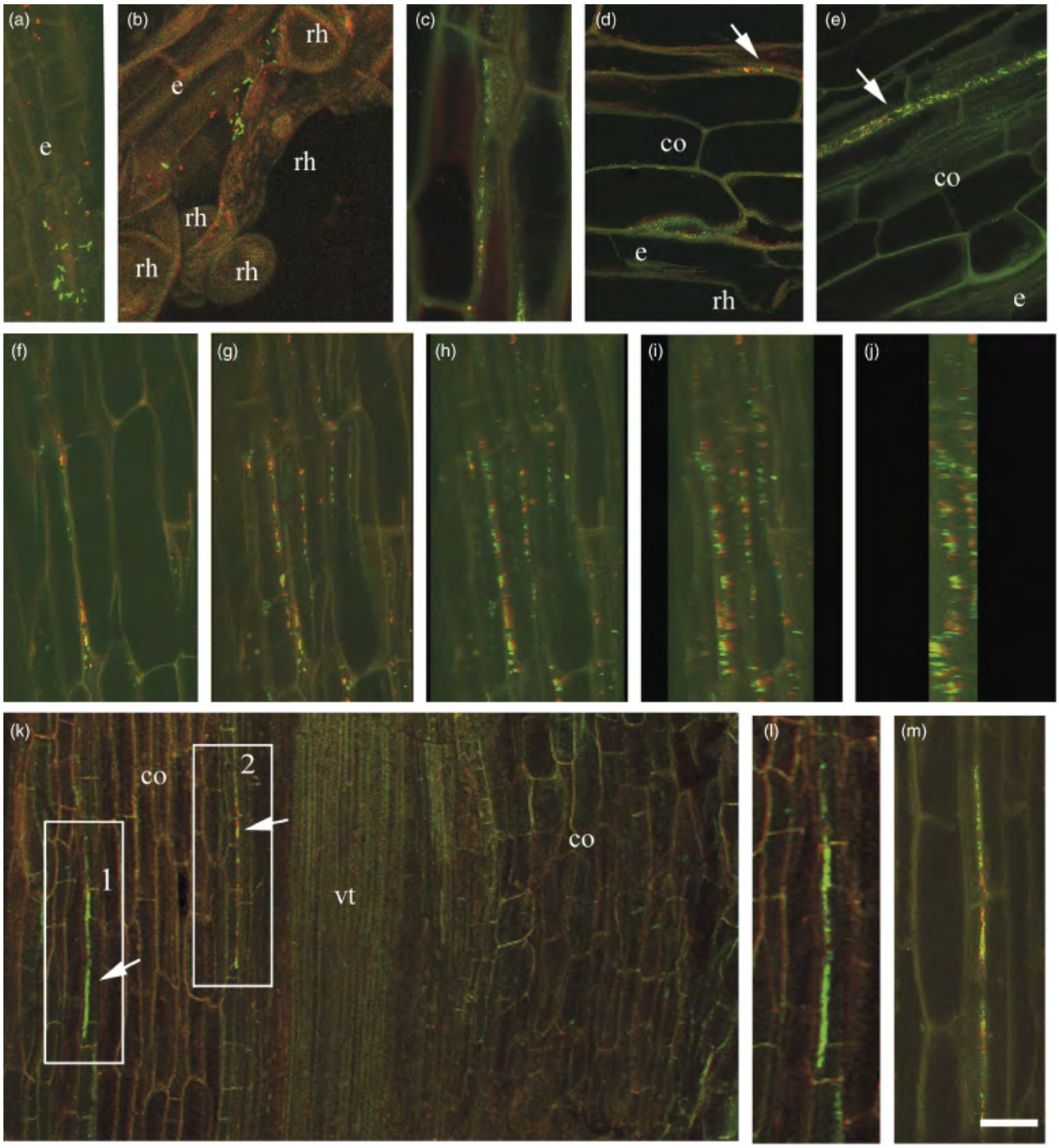
Profound colonization of plant tissues by bacterial endophytes is difficult to demonstrate and visualize even with the use of CLSM, due to the limitations on obtaining images deeper that 100 μm. To increase accessibility to inner cell layers dissection or sectioning must be used. VTS is a simple way to produce relatively thick sections (20–50 μm), which can be imaged to reveal the structure of the underlying tissues. It has the advantage of preserving 3D structure well, so that subcellular organization and identification of cell types in the tissue context can be reliably imaged. For example, the use of CLSM on 3D VTS has been crucial to describe nuclear organization and chromosome association in cereals (Martínez-Pérez *et al.*, 2003; Prieto *et al.*, 2004a, b). Therefore, for the present study, we decided to carry out CLSM on 3D vibratome sections of olive roots.

Investigation in to whether the fluorescently tagged bacterial strains could achieve their putative endophytic status when plants were not in a highly artificial environment was pursued. The majority of previous studies to assess endophytism used gnotobiotic test systems, very young plants or seedlings, and culturing conditions greatly facilitating plant colonization by the presumed endophyte under study, i.e. mostly in the absence of additional native microbial communities. In this present study, nursery-propagated young olive plants were employed, which have been grown in commercial substrates and most likely carry an abundant, native, and largely unknown rhizospheric and endophytic microbial communities. Therefore, a nongnotobiotic system has been used to test the putative endophytic capability of *P. fluorescens* PICF7, representing a more ecologically relevant study system.

An additional novelty of the present study is the examination of bacterial endophytism in a woody host. The majority of studies demonstrating true endophytism in bacteria were conducted on herbaceous plant hosts, and there are just a few examples indicative of endophytic (including *Pseudomonas* spp.) colonization of woody hosts tissues by GFP-tagged bacteria, although none using CLSM (Germaine *et al.*, 2004, 2006; Compant *et al.*, 2005b). In conclusion, and considering the previous premises, here we

present for what we believe is the first time microscopic (CLSM) evidence of deep endophytic colonization of young olive roots by *P. fluorescens*-tagged cells with two different autofluorescent markers (EGFP and RFP), employing VTS and using a nongnotobiotic system.

The use of VTS has been crucial in revealing detailed structures of internal plant cells that are not accessible to confocal microscopy on intact plant tissues. We developed a whole mount sectioning method for olive roots using a vibratome, as has previously been used for other plant species (Prieto *et al.*, 2004a, b, 2007). As far as we are aware, this is the first study in which VTS has been applied to a woody species such as olive. Each root section was *c.* 20 μm thick and contained about two to three cell layers. Using longitudinal root sections, we examined the morphology of the apical region of a representative 'Arbequina' olive root



(Fig. 2). Low-magnification confocal images of a 50 μm-thick longitudinal section along an olive root showed the meristematic region. All internal root tissues were visible and well preserved. It was possible to identify clearly the vascular tissue, as the central columns of cells, the cortex and the epidermis, with root hair cells in the differentiation zone (Fig. 2a and b). Detailed transverse sections of olive roots were also obtained (Fig. 2c).

Under the growth conditions assayed, 'Arbequina' olive roots were colonized by the introduced, fluorescently tagged bacteria immediately after inoculation, and bacterial populations persisted in roots throughout the duration of the bioassay. However, the population size of introduced bacteria decreased over time. At 21 DAB, the number of Tc^R colonies recovered from macerated root tissue of three plants varied from plant to plant (1×10^6, 4×10^6, 1×10^7 CFU g^{-1}). At 51 days, the number decreased to 2×10^5 (in one sampled plant) and 9×10^5 CFU g^{-1} (in two plants). This decline in the population size of introduced bacteria was in accordance with previous strain PICF7 colonization results obtained for 'Picual' olives of similar characteristics (age, plant propagation procedure, phenology, etc.) (Mercado-Blanco *et al*., 2004). No Tc^R colonies were recovered from control (uninoculated) plants sampled at 21 or 51 days.

Confocal microscope images of olive roots inoculated with a mixture of two *P. fluorescens* PICF7 fluorescently tagged populations showed that *rfp* and *egfp* genes are expressed in the olive rhizosphere, and that both are suitable markers for visualizing introduced bacteria in olive roots at the single cell level (Fig. 3a). Bacteria carrying either pMP4662 or pMP4655 plasmids were detected on the surface of root epidermis just 1 day after olive root bacterial inoculation (Fig. 3a). Olive roots of uninoculated control plants did not show any fluorescent bacteria at any time during the experiment (data not shown). Two to 3 days after inoculation, single bacteria from both PICF7-tagged populations were observed predominantly among root hairs (Fig. 3b). However, bacteria occasionally were also observed on the elongation zone at that time. The colonization pattern of introduced bacteria was similar during the next few days, but both PICF7(pMP4662) and PICF7(pMP4655) were only found on the surface of the differentiation zone and around root hairs. This may suggest that the latter zone is a preferential site for olive root surface colonization by strain PICF7. This root region might be favoured by either being a more protective microenvironment for the bacteria or a better source of nutrients and/or bacterial chemoattractants. Alternatively, preferential attachment sites (defined by specific proteins and/or structures) for this bacterial strain could be present on the olive root hairs.

Internal colonization of olive root tissues by both PICF7(pMP4662) and PICF7(pMP4655) cells was first detected at 9 DAB (Fig. 3c). Bacterial microcolonies were usually a heterogeneous mix of both fluorescently tagged populations, although we also found microcolonies where either PICF7(pMP4665) or PICF7(pMP4662) was the prevalent population. Either single bacterial cells or bacterial microcolonies were predominantly localized in the intercellular spaces in the cortex of the differentiation zone (Fig. 3d) and, in some cases, in the elongation zone. Bacterial colonization of the intercellular spaces of the division zone was not observed at any time. This suggests that internal colonization takes place where root surface colonization has occurred previously. Therefore, a possible route of primary colonization by strain PICF7 is the elongation and differentiation zones at the root tip, as has been previously reported for different microorganisms (Reinhold-Hurek & Hurek, 1998b; James, 2000). However, the mechanism of entry remains to be elucidated. On the other hand, the highly localized and limited distribution of introduced bacteria in these zones in all plants sampled and throughout the bioassay indicated that there was no bacteria translocation to other root zones. Finally, at no time during the bioassay and in any sampled plant either were fluorescently tagged bacteria observed as intracellular colonizers. Similarly, no apparent plant cell damage caused or induced by the introduced fluorescently tagged PICF7 was detected.

Fig. 3. CLSM images of the time-course colonization events of olive cv. Arbequina roots by *Pseudomonas fluorescens* PICF7 fluorescently tagged cells. Confocal analysis was performed on whole representative roots to show surface PICF7(pMP4662) and PICF7(pMP4655) colonization (a and b). Vibratome root sections were made (see text) for confocal analysis to show endophytic bacteria colonization (c–m). Images are projections of 12 adjacent confocal optical sections in all the panels except in (a) and (g–j), where projections were made of five and 25 sections, respectively. The focal step size between confocal optical sections was 1 μm. Expression of *rfp* and *egfp* genes in PICF7(pMP4662) and PICF7(pMP4655) strains, respectively on the root surface (a) 1 day and (b) 3 days after bacterial inoculation. Intercellular colonization of the cortical tissue of the differentiation root zone by a mixed population of PICF7(pMP4662) and PICF7(pMP4655) observed at (c) 9, (d) 14 and (e) 21 days after bacterial inoculation. (f) Detection of PICF7(pMP4662) and pICF7(pMP4655) cells in the intercellular spaces in the cortex of the differentiation zone 35 days after bacterial inoculation. (g–j) 3D rotations of the same root region shown in (f). The 3D rotations (0, 30, 60 and 90° in g, h, i and j, respectively) are generated from the projections of 25 adjacent optical sections to show two to three root cell layers. (k) Vibratome longitudinal section of a representative 1.5-mm-thick root from a plant sampled 37 days after bacterial inoculation. The image shows a near complete view of the root thickness and the localized distribution of the introduced bacteria-tagged cells. Bacterial microcolonies are visible in the intercellular spaces of the cortex region either close to the epidermis (inset 1) or close to the vascular vessels (inset 2). The image is a projection of three adjacent optical sections. (l, m) Confocal analysis of the areas inset 1 and 2 in (k). co, cortical cells; e, epidermal cells; rh, root hairs; vt, vascular tissue. Scale bar = 20 μm in all except, 30 μm in (a) and (b) and 100 in (k).

An increasing number of bacteria, larger microcolonies and the same endophytic localization pattern was imaged until 21 DAB (Fig. 3e). From this time-point on, no significant changes in sampled plants were observed up to the end of the bioassay (80 DAB). Indeed, bacteria were always visualized in the intercellular spaces within the cortex region (Fig. 3f–j), either close to the epidermis or close to the xylem vessels but never inside them (Fig. 3k–m). Furthermore, no PICF7 cells were detected in new emergent roots during the bioassay. This indicated that only roots that have been dipped directly in the bacterial suspension were susceptible to be endophytically colonized. This may suggest that once bacteria colonize a defined root area, they multiply in it and apparently do not translocate to other zones. The absence of *P. fluorescens* PICF7 in the vascular tissue or in new emergent roots may also be explained by the possible presence of native competitors impairing PICF7 colonization. It must be taken into account that the olive plants employed in this study originated from a commercial nursery, and that diverse fungal and bacterial genera are common inhabitants of olive roots (Mercado-Blanco *et al.*, 2001; J. Mercado-Blanco, unpublished observations). Nevertheless, persistence of PICF7 over time was demonstrated, indicating that it is a good endophytic olive root colonizer and supporting our previous findings on persistence in 'Picual' roots (Mercado-Blanco *et al.*, 2004). In this previous study, *P. fluorescens* PICF7 was not identified as one of the best olive root colonizers compared with several other indigenous pseudomonad strains assayed. However, it was one of the best effective biocontrol strains against *Verticillium* wilt of olive caused by the highly virulent, defoliating pathotype of *V. dahliae*. It might well be that the biocontrol effect induced may not require high bacterial populations within olive root tissues. In fact, our results have shown that PICF7 seems to colonize only very defined zones of the olive root, and that spread to distant areas simply does not take place or is impaired given that vascular colonization was never observed. One interesting question arising from this study is whether PICF7 biocontrol activity could be exerted once it is established endophytically in root tissues. Although nothing is yet known about the *in planta* biocontrol mechanism(s) involved, it has been previously demonstrated that strain PICF7 produces pseudobactins and salicylic acid *in vitro* (Mercado-Blanco *et al.*, 2004). Whether these and/or other PICF7-produced metabolites could be implicated in *Verticillium* wilt of olive suppression, or whether the endophytic persistence of PICF7 favours or enhances its biocontrol activity remains to be demonstrated. Moreover, to what extent the introduction of PICF7 may influence the indigenous microbiota structure in and on olive root tissues, and how different (a)biotic factors (i.e. olive cultivar, soil characteristics, temperature, etc.) (Berg *et al.*, 2006; Costa *et al.*, 2006) may affect the establishment and persistence of PICF7, as well as its antagonistic effect towards *V. dahliae*, will be exciting matters of future investigation.

In conclusion, this is the first time that fluorescent bacterial tagging, VTS and CLSM imaging have been used to demonstrate root endophytism of a biocontrol *Pseudomonas* spp. strain in a woody host such as olive and using a nongnotobiotic test system. Confocal microscopy combined with vibratoming sections can be a powerful tool for analysing the formation, localization and function of beneficial microbial populations when endophytically interacting with plants.

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