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Suppression of Verticillium wilt in olive planting stocks by root-associated fluorescent *Pseudomonas* spp.

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Abstract

Protection of pathogen-free olive planting material from infection by *Verticillium dahliae* during plant propagation and/or at planting would help in the management of Verticillium wilt of olive. In this study, 8 isolates of *Pseudomonas fluorescens* (6) and *Pseudomonas putida* (2) obtained from roots of olive plants were tested for suppression of Verticillium wilt in nursery-produced olive planting stocks under controlled conditions. All tested bacteria produced the green fluorescent siderophore pseudobactin in vitro but only some *P. fluorescens* isolates produced either salicylic acid in succinate medium or HCN in vitro assays. The antagonistic activity of *P. fluorescens* and *P. putida* isolates from olive against defoliating (D) and nondefoliating (ND) *V. dahliae* pathotypes varied with culture media. On PDA, isolates of *P. putida* were more inhibitory to the pathogen than those of *P. fluorescens*. In planta bioassays were conducted either under growth chamber or greenhouse conditions, by inoculating bacterial-treated and -nontreated 3- to 4-month-old, own-rooted or micropropagated plants of susceptible olive cv. Picual with the highly virulent D *V. dahliae*. Results from three experiments indicated that root treatment with some of *P. fluorescens* isolates significantly delayed the onset of symptoms, and reduced the final disease incidence and severity by 31–82% and 73–96%, respectively, compared with the nontreated controls, under conditions of severe Verticillium wilt. In addition, those bacteria counteracted the deleterious effects caused by the pathogen infection through enhancement of plant growth. Our results indicate that root treatment of olive plants with selected *P. fluorescens* isolates during nursery propagation can help in the biocontrol of D *V. dahliae* in olive. No correlation was found between efficacy of tested bacterial isolates for in vitro antagonism of the pathogen and in planta suppression of Verticillium wilt.

Keywords: *Olea europaea*; *Pseudomonas fluorescens*; *Pseudomonas putida*; *Verticillium dahliae*; Biological control; Rhizobacteria; Hydrogen cyanide; Pseudobactin; Salicylic acid; Siderophores

1. Introduction

Verticillium wilt, caused by the soilborne fungus *Verticillium dahliae* Kleb., is one of the most serious diseases affecting olive (*Olea europaea* L.) worldwide (Jiménez-Díaz et al., 1998), and may cause severe losses and plant death (Levin et al., 2003; Thanassoulopoulos et al., 1979). In Spain, which grows some 2.3 million ha of olive accounting for near 24% of the olive world acreage (Civantos, 2001), Verticillium wilt has spread throughout the main olive-growing areas of the country during the last 20 years (Blanco-López et al., 1984; Sánchez-Hernández et al., 1998; R.M. Jiménez-Díaz, unpublished). This increasing spread of the disease may result from the establishment of new orchards in infested soils or close to affected crops, the use of infected planting material, and the establishment of high-tree-density, irrigated orchards (Rodríguez-Jurado et al., 1993; Thanassoulopoulos, 1993). Severity of Verticillium wilt in olive is strongly influenced by virulence (i.e., the amount of disease caused in a host genotype) of the pathogen isolates (Rodríguez-Jurado, 1993). *V. dahliae* infecting olive can be classified as defoliating (D) or nondefoliating (ND) pathotypes according to their ability to defoliate or not the plant, respectively. This differential virulence shown by *V.*

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dahliae from olive also occurs in upland cotton (*Gossypium hirsutum* L.), and isolates from cotton show cross-virulence in olive plants (Rodríguez-Jurado et al., 1993; Schnathorst and Sibbett, 1971). While infections by the D pathotype can be lethal to the plant, olive plants infected by the ND pathotype can show complete remission from symptoms (Jiménez-Díaz et al., 1998; Rodríguez-Jurado, 1993).

Management of Verticillium wilt in olive is primarily by means of an integrated disease management strategy, involving the application of control measures before and after planting (Tjamos, 1993). Some elements of such a strategy applicable before planting would include the choice of planting sites with low inoculum levels of *V. dahliae* and free from the D pathotype, the use of pathogen-free planting material (Mercado-Blanco et al., 2001a, 2002), and the protection of this material from early infections by *V. dahliae* during plant propagation and/or at planting. An interesting option for protecting olive planting material from *V. dahliae* would be the use of bacterial endophytes as biocontrol agents (Tjamos et al., 2000). Bacterial endophytes can be effective as antagonists of plant pathogens or by eliciting plant defense mechanisms through microbially mediated induced resistance (Tuzun and Kloepper, 1995). Furthermore, use of bacterial endophytes as biocontrol agents will satisfy requirements in modern, sustainable agriculture of environmentally friendly disease control measures.

A few bacterial strains antagonists of *V. dahliae* have been identified, some of which provided a degree of Verticillium wilt suppression in different host plants (for a recent review see Pegg and Brady, 2002, chapter 10, and references therein). Thus, *Bacillus subtilis* was shown effective in the biological control of Verticillium wilt in strawberries (Jordan and Tarr, 1978). Similarly, rhizosphere strains of *Bacillus*, *Erwinia*, *Flavobacterium*, *Pantoea*, *Pseudomonas*, *Serratia*, *Sphingomonas*, *Stenotrophomonas*, and *Streptomyces* from *Brassica* spp., *Capsella* spp., and *Fragaria* spp. produced substances inhibitory of *V. dahliae* and suppressed Verticillium wilt of oilseed rape and strawberry (Berg, 1996; Berg et al., 1994, 2000). Those latter authors proposed that siderophore, lytic enzymes or antibiotics production might be responsible of Verticillium wilt suppression by those strains. Comparatively, the use of bacteria for the biological control of vascular wilt diseases in trees has been investigated in few cases and had variable or limited success (O'Brien et al., 1984; Scheffer, 1983). Hall et al. (1986) suggested that strains of *B. subtilis* that are natural colonizers of maple stem tissue can suppress Verticillium wilt, and observed that in vitro antagonism against the pathogen was not correlated with the degree of disease suppression.

The objectives of this research were: (i) to determine whether root-associated *Pseudomonas* isolated from olive can be effective in protecting nursery-produced olive planting stocks from infection by the highly virulent D *V. dahliae* and (ii) to identify bacterial metabolites that may be involved in the inhibition of *V. dahliae*.

2. Materials and methods

2.1. Bacterial isolates and inoculum production

Six isolates of *Pseudomonas fluorescens*, namely isolates PICF1, PICF3, PICF4, PICF6, PICF7, and PICF8 and two of *Pseudomonas putida*, namely isolates PICP2, and PICP5, were used in this research. These bacteria were isolated from roots of nursery-propagated olive plants cv. Picual, and characterized to biovars 2 (PICF1 and PICF6), 4 (PICF4), 5 (PICF3 and PICF7), 6 (PICF8), and biovar A (PICP2 and PICP5) in previous studies (Mercado-Blanco et al., unpublished). In this present study, these bacteria were further characterized and typed by BOX-PCR assay to determine genetic similarity of genotypes (see below). Also, *P. fluorescens* Q2-87 and *Pseudomonas* sp. CHAO kindly provided by D.M. Weller (USDA-ARS, Pullman, WA, USA), and *P. fluorescens* WCS374 and *P. putida* WCS358 kindly provided by P.A.H.M. Bakker (Utrecht University, Utrecht, The Netherlands), were used for comparison. *P. fluorescens* Q2-87 suppresses Take-all of wheat (Raaijmakers and Weller, 1998), *P. fluorescens* WCS374 and *P. putida* WCS358 suppress Fusarium wilt diseases of radish (Leeman et al., 1995) and carnation (Duijff et al., 1994), respectively, and *Pseudomonas* sp. CHAO has biocontrol activity against different fungi in diverse host plants (Stutz et al., 1986). For bacterial isolations, olive roots were washed under running tap water, surface-disinfested in NaClO (0.5% available chlorine) for 3 min, rinsed thoroughly in sterile water, and ground with an autoclaved pestle and mortar in 10 mM $MgSO_4 \cdot 7H_2O$. Aliquots of macerates were plated on modified (Geels and Schippers, 1983) King's medium B agar (KBA) (King et al., 1954) and incubated at 25 °C for 48 h. Single bacterial colonies were transferred to Luria–Bertani (LB) broth (Miller, 1972), and cultures were cryopreserved with 30% glycerol at −80 °C. Isolates were characterized to species according to Palleroni (1984) and Stanier et al. (1970).

Mutants of *P. fluorescens* and *P. putida* from olive resistant to rifampicin (Rf) (Sigma, St. Louis, MO) were selected by culturing wild-type isolates on KBA amended with 25, 50, or 100 μg Rf ml^{-1} at 28 °C for 5 days. Rf-resistant colonies were selected on the basis of their morphology and growth being similar to those of the wild-type isolates on KBA. Three individual Rf-resistant colonies were selected per *Pseudomonas* isolate and Rf-resistance was further re-confirmed by subculturing individual colonies on KBA amended with 100 μg Rf ml^{-1}. The Rf-resistant isolates thus obtained were labeled as

"Rf" after the isolate code number and cryopreserved in LB broth amended with 50 μg Rf ml^{-1} and 30% glycerol at −80 °C.

Inocula of *Pseudomonas* wild-type and Rf-mutant isolates were grown on KBA at 25 °C for 48 h, scrapped from the medium with a sterile glass rod, and suspended in 10 mM $MgSO_4 \cdot 7H_2O$. Bacterial suspensions were centrifuged (10,500*g*, 20 min) twice in sterile distilled water to remove residual metabolites and trace of nutrients and resuspended in 10 mM $MgSO_4 \cdot 7H_2O$. Bacterial concentration in the suspension was determined by measuring absorbance at 600 nm using standard curves for each isolate.

2.2. Fungal isolates and inoculum production

Monoconidial *V. dahliae* isolates V4I and V138I representatives of the ND and D pathotypes, respectively, were used in this study. Those isolates originate from diseased cotton plants in the Guadalquivir Valley, southern Spain and were characterized in previous studies using olive plants as a host (Mercado-Blanco et al., 2001a, 2002; Rodríguez-Jurado et al., 1993). The isolates are deposited in the culture collection of Departamento de Protección de Cultivos, Instituto de Agricultura Sostenible, (CSIC), Córdoba, Spain. Isolates were stored axenically by covering cultures on plum-extract agar (Talboys, 1960) with liquid paraffin (Bejarano-Alcázar et al., 1996) at 4 °C in the dark. Active cultures of isolates were obtained as described by Mercado-Blanco et al. (2001a, 2002). For experiments, inoculum consisted of conidial suspensions from cultures in potato–dextrose broth (PDB) incubated at 125 rpm on an orbital shaker (Adolf Kühner AG, Birsfelden, Switzerland) at 24 °C in the dark for 7 days. Conidia in the liquid cultures were filtered through eight layers of sterile cheesecloth and inoculum concentration was adjusted as needed using a haemocytometer.

2.3. In vitro inhibition of V. dahliae hyphal growth by bacteria

Wild-type and Rf-mutants derivatives of *Pseudomonas* isolates from olive were tested for their ability to inhibit the hyphal growth of *V. dahliae* isolates V4I and V138I in vitro. Assays were performed in petri plates containing 20 ml of PDA, KBA, or KBA supplemented with 100 μM $FeCl_3 \cdot 6H_2O$ (KBA + Fe^{3+}). Bacterial suspensions in 10 mM $MgSO_4 \cdot 7H_2O$ were adjusted to a final concentration of 10^8 cells ml^{-1}. Two 5 μl droplets of a bacterial suspension were spotted per plate, 1 cm from the opposite edges of a plate and opposite each other. Bacteria were incubated at 28 °C for 48 h and a suspension of 5×10^4 conidia ml^{-1} of either *V. dahliae* V4I or V138I was sprayed over the plates. The bacterial and fungal isolates were plated separately as controls. Cultures were incubated at 24 °C for 48 h, and the antagonistic activity by bacteria was assessed by the presence of a zone without fungal growth surrounding the bacterial colony. Each combination of microorganisms, controls, and culture media were replicated three times in a randomized complete block design and the experiment was repeated once.

2.4. Production of siderophores by bacteria

Isolates of *Pseudomonas* spp. from olive, *P. fluorescens* WCS374, and *P. putida* WCS358 were grown on the universal siderophore detection medium CAS agar to determine production of siderophores (Schwyn and Neilands, 1987). Droplets (5 μl) of overnight KB cultures were spotted onto CAS agar plates, incubated at 28 or 37 °C and the relative halo size ([halo diameter − colony diameter]/halo diameter]) was determined after 1–3 days of growth. A siderophore-producer colony chelates iron from the medium so that a shift from blue (chelated CAS) to orange (unchelated CAS) occurs. There were four replicated plates per isolate and incubation temperature, and the experiment was repeated once. Means were compared using the least significant difference (LSD) test at $P = 0.05$.

2.5. In vitro production of salicylic acid and pseudobactins by bacteria

Production of salicylic acid (SA) by bacteria was determined at low iron availability in liquid standard succinic medium (SSM; pH 7.0) (Meyer and Abdallah, 1978). Isolates of *Pseudomonas* spp. from olive, *P. fluorescens* WCS374, and *P. putida* WCS358 were grown in SSM at 28 °C for 3 days. *P. fluorescens* WCS374 and *P. putida* WCS358 were used as SA-producer and SA-nonproducer (Leeman et al., 1996) controls, respectively. Bacterial concentration in the cultures was determined for each isolate as previously described. The amount of pseudobactins produced by bacteria was assessed by measuring the absorbance of cell-free culture centrifugates (10,500*g*, 20 min), pH 7.0, at 400 nm (Leeman et al., 1996). After that, SA in the supernatants was determined according to Leeman et al. (1996) and Meyer et al. (1992). To determine low levels of SA production, the procedure was conducted as previously described (Mercado-Blanco et al., 2001). Production of SA and pseudobactins was measured twice in each of two, repeated experiments. Means were compared using the LSD test at $P = 0.05$.

2.6. Production of cyanide by bacteria

Bacterial isolates were tested for cyanide (HCN) production with the "Aquaquant 14417 Cyanid-Test" (Merck, Darmstadt, Germany) according to manufacturer's instructions, using cell-free centrifugates

(10,500*g*, 20 min) of cultures of *Pseudomonas* spp. from olive grown in KB broth at 28 °C for 72 h. A positive reaction (formation of cyanogen chloride) was revealed by the presence of a violet polymethine pigment. Bacteria positive for production of HCN were further tested for the HCN synthase-encoding *hcnBC* genes by PCR assay.

2.7. DNA extraction and PCR assays

Total genomic DNA was extracted from bacteria using the "DNeasy Tissue Handbook" kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. Approximately 2×10^9 cells from overnight cultures on KB broth at 28 °C were used. Purity of the extracted DNA was determined by electrophoresis in ethidium-bromide stained agarose gels (0.7% w/v in TAE buffer) (Sambrook et al., 1989), and DNA concentration was determined spectrophotometrically in a BioPhotometer (Eppendorf AG, Hamburg, Germany). For PCR assays, concentration of the bacterial DNA was set at 20 ng/μl.

Bacteria were typed by BOX-PCR using the BOX A1R element (5′-CTACGGCAAGGCGACGCTGAC G-3′) as described by Rademaker and De Bruijn (1992). For each *Pseudomonas* isolate, BOX-PCR assays were carried out with DNA from two colonies and reactions were repeated at least once. PCR assays for detection of the HCN synthase-encoding *hcnBC* genes were carried out using the forward ACa (5′-ACTGCCAGGGGCG GATGTGC-3′) and the reverse ACb (5′-ACGATGTG CTCGGCGTAC-3′) primer pair (Ramette et al., 2003). PCR conditions were as described by those authors, except that annealing temperature was raised to 65 °C, and 100 ng of DNA template were used. Total genomic DNA from *Pseudomonas* sp. CHAO was used as a positive control for presence of *hcnBC* genes. All primers used were synthesized by Proligo France SAS. PCRs were performed in a Perkin–Elmer 2400 (Perking–Elmer, Norwalk, CT) or a PTC-100 Progammable Thermal Controller (MJ Research, Watertown, MA) thermocyclers. PCR products were separated by gel electrophoresis and visualized according to standard protocols (Sambrook et al., 1989).

2.8. Root colonization assay

An experiment was conducted to determine the ability of *Pseudomonas* spp. isolates from olive and their Rf-mutants to colonize olive roots. Three-month-old olive plants cv. Picual kindly provided by Agromillora Catalana S.A. (Barcelona, Spain) were carefully uprooted from the substrate, their roots thoroughly washed in tap water without intentional wounding, and dipped in a bacterial suspension (5×10^8 cells ml^{-1}) for 10 min. For the control treatment, plants were treated similarly except that roots were dipped in 10 mM $MgSO_4 \cdot 7H_2O$. Plants were then transplanted (one per pot) into 15-cm-diameter clay pots filled with an autoclaved (121 °C, 1 h, twice on consecutive days) soil mixture (sand/loam, 2:1, vol/vol). There were four replicated plants for each bacterial treatment in a randomized complete block design. Plants were incubated under greenhouse conditions at 25 ± 10 °C for 3 months. Plants were watered as needed, and fertilized weekly with a hydro-sol fertilizer 20-5-32 + microelements (Haifa Chemicals, LTD, Haifa, Israel).

To determine colonization of root tissue by bacteria, plants were uprooted delicately from pots and the root systems were thoroughly washed under running tap water, dried with sterile filter paper, and cut into 1-cm-long pieces. For each plant, samples of 2 g of root pieces were surface-deinfested in 1% NaOCl for 3 min, washed three times in sterile distilled water, and ground in 10 ml of 10 mM $MgSO_4 \cdot 7H_2O$ using an autoclaved pestle and mortar. This deinfestation procedure was shown robust enough to kill surface bacteria in previous studies (Mercado-Blanco et al., 2001a, 2002; Rodríguez-Jurado, 1993). Serial dilutions of the macerates were plated onto modified KB and modified KB amended with 100 μg Rf ml^{-1} and incubated at 25 °C for 48 h. Then, bacterial colonies were counted and bacterial populations were expressed as colony-forming units (cfu) g^{-1} of fresh root tissue. Data were subjected to analysis of variance. Means were compared using Fisher's protected LSD test at $P = 0.05$.

2.9. Suppression of Verticillium wilt of olive by bacteria

Three experiments (I–III) were conducted to determine the ability of *Pseudomonas* spp. isolates to suppress Verticillium wilt of olive caused by the D *V. dahliae* pathotype. Nursery-propagated, own-rooted olive plants cv. Picual were used. 'Picual' olives were shown to be more susceptible to D *V. dahliae* than to ND *V. dahliae* in previous studies (Rodríguez-Jurado et al., 1993).

For experiment I, 4-month-old olive plants kindly provided by Agromillora Catalana S.A. (Barcelona, Spain) were used. These plants were propagated by rooting of leafy stem cuttings under mist conditions in plastic tunnels. Isolates *P. fluorescens* bv 4 PICF4, bv 2 PICF6, bv 5 PICF7, and bv 6 PICF8, and *P. putida* bv A PICP2 and PICP5 were tested as biocontrol agents. Plants were root-dip inoculated with a bacterial suspension (5×10^8 cells ml^{-1}) or root dipped in 10 mM $MgSO_4 \cdot 7H_2O$ (controls) as described above for the root colonization assay. Then, inoculated and control plants were transplanted into 15-cm-diameter clay pots (one plant per pot) filled with the autoclaved soil mixture infested with conidia of the pathogen. The soil mixture was infested by thoroughly mixing 100 ml of a conidial

suspension (2×10^7 conidia ml^{-1}) of *V. dahliae* isolate V138I with 1 kg of the mixture to obtain a final concentration of 2×10^6 conidia g^{-1} of soil. There were four replicated blocks in a randomized complete block design, each block comprising three pots. Plants were incubated in a greenhouse at 25 ± 10 °C for 70 days. Experiment II was conducted using 3-month-old olive plants kindly provided by Cotevisa S.A. (Valencia, Spain). These plants were micropropagated from axillary olive buds under axenic conditions, then grown in the greenhouse for 3 months. Isolates *P. fluorescens* bv 4 PICF4, bv 2 PICF6, bv 5 PICF7, bv 6 PICF8, and Q2-87, and *P. putida* bv A PICP2 and PICP5 were tested as biocontrol agents. Plants were inoculated with a bacterial suspension and transplanted into the soil mixture infested with conidia of *V. dahliae* V138I as described for experiment I. There were four replicated blocks in a randomized complete block design, each block comprising five pots (one plant per pot). Plants 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 $\mu E\, m^{-2}\, s^{-1}$ for 98 days. Experiment III was conducted as described for experiment II, except that each of the four replicated blocks comprised four pots (one plant per pot), and plants were incubated in the growth chamber for 72 days. For all experiments, plants were watered as needed and fertilized weekly with 100 ml of the hydro-sol fertilizer as previously described. Upon termination of experiments, plants were excised at the soil level and the diameter at the stem base, total stem length, and fresh shoot weight were determined for each plant.

2.10. Disease assessment and data analyses

Disease reactions were assessed by the incidence (percentage) and severity of symptoms on 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) at weekly intervals after inoculation with the pathogen. Upon termination of experiment II, isolations of the fungus on chlorotetracycline-amended (30 mg L^{-1}) water agar (CWA) were made from roots and stems segments of symptomless and diseased plants to determine vascular infection by *V. dahliae*. The whole root system and stem (without leaves and bark) of a plant were washed thoroughly under tap water, surface-disinfested in 0.5% NaClO for 1.5 min (stems) or 2 min (roots), and then washed twice with sterile water (Rodríguez-Jurado, 1993). The disinfested tissues were cut into 5-mm-long pieces and four stem pieces representative of the total length stem, and four root pieces arbitrarily chosen were plated onto CWA and incubated at 24 °C in the dark for at least 9 days (Rodríguez-Jurado, 1993).

Data were subjected to analysis of variance. Percentage values were arcsine transformed $(Y/100)^{1/2}$ before analyses. Data on disease severity were used to calculate the following: (i) a disease intensity index (*DII*) determined as $DII = (\sum Si \times Ni)/(4 \times Nt)$, where *Si* is the symptoms severity, *Ni* is the number of plants with *Si* symptoms severity, and *Nt* is the total number of plants; (ii) the incubation period (*IP*) established as the number of days from inoculation with the pathogen until $DII > 0$; (iii) the final disease incidence (percentage of affected plants) (final *DI*); and (iv) the standardized area under the disease progress curve of *DII* plotted over time (days) (*SAUDPC*) calculated according to Campbell and Madden (1990). Analyses of variance were made using Statistix (NH Analytical Software, Roseville, MN). Treatment means were compared with those of the control using the Dunnett's test at $P = 0.05$.

3. Results

3.1. Molecular characterization of Pseudomonas isolates from olive

Box-primed PCR assays were carried out to molecularly differentiate *Pseudomonas* isolates from olive. The PCR products obtained yielded electrophoretic profiles with enough DNA bands to differentiate the eight *Pseudomonas* isolates used in this study. Results indicated that *P. fluorescens* bv 4 PICF3 and PICF7 were identical in DNA bands profile, and same occurred between *P. putida* bv A PICP2 and PICP5 (Fig. 1). In addition, electrophoretic profiles of *P. fluorescens* bv 2 PICF1 and PICF6 differentiated in several polymorphic DNA (Fig. 1). On the other hand, BOX-PCR patterns were clearly different among biovars of *P. fluorescens*, and between *P. fluorescens* and *P. putida* isolates (data not shown). Based on these results, as well as on those of in vitro antagonism, metabolite production, and root colonization assays (see below), we chose only one isolate per each of *P. fluorescens* biovars and the two *P. putida* bv A isolates to test biocontrol activity against infection by D *V. dahliae* in olive.

3.2. In vitro inhibition of V. dahliae hyphal growth by bacteria

The isolates of *Pseudomonas* spp. from olive varied in the ability to inhibit hyphal growth of D and ND *V. dahliae* on PDA (Table 1). While *P. putida* bv A PICP2 and PICP5 were highly inhibitory to both *V. dahliae* pathotypes, isolates of *P. fluorescens* bv 2 (PICF1 and PICF6) and bv 4 (PICF4) inhibited fungal growth, but those of bv 5 (PICF3 and PICF7) and bv 6 (PICF8) did not. Also, isolates of *P. putida* inhibited the pathogen to a larger extent compared with that by the inhibitory

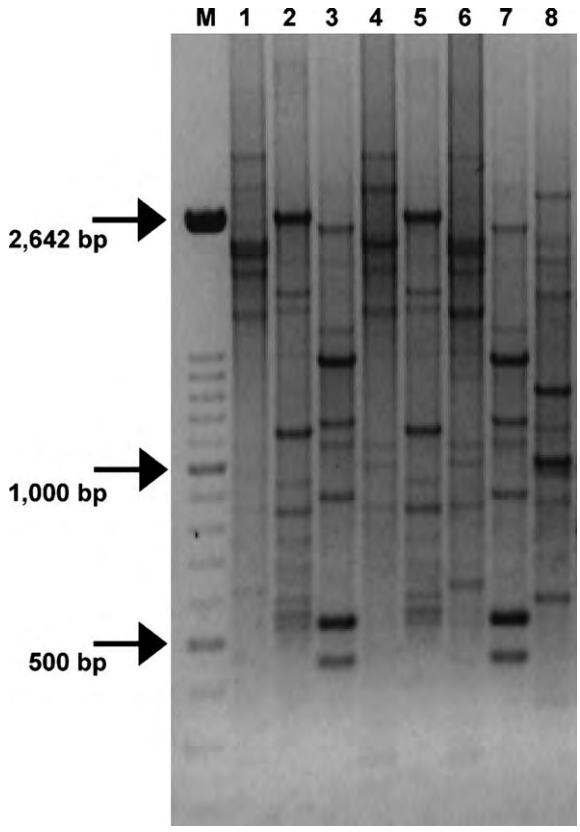

Fig. 1. Genomic BOX-PCR profiles of *Pseudomonas* spp. isolates using the BOX A1R element (Rademaker and De Bruijn, 1992) (see text for details). *P. fluorescens* isolates: PICF1 (lane 1), PICF3 (lane 3), PICF4 (lane 4), PICF6 (lane 6), PICF7 (lane 7), and PICF8 (lane 8). *P. putida* isolates: PICP2 (lane 2) and PICP5 (lane 5). M: molecular weight marker ladder.

isolates of *P. fluorescens*. Conversely, the ability of bacterial strains to inhibit growth of *V. dahliae* on PDA was not influenced by the nature of pathotype (Table 1). In general, Rf-mutants of the *Pseudomonas* isolates were as inhibitory of in vitro *V. dahliae* hyphal growth as their respective parents, except for *P. fluorescens* bv 2 PICF1-Rf which lost the inhibitory activity compared with the wild-type isolate. Inhibition of *V. dahliae* hyphal growth was also demonstrated on KBA and KBA + Fe^{3+} (Table 1). Inhibition of fungal growth on KBA should be a consequence of production of siderophores by the *Pseudomonas* isolates. Indeed, a green-yellow halo clearly fluorescent under UV light developed around a bacterial colony by 24 h of incubation of dual cultures. Interestingly, the siderophore-mediated growth inhibition of D *V. dahliae* V138I was stronger compared with that of ND *V. dahliae* V4I. Thus, siderophore-mediated inhibition of isolate V138I occurred for isolates *P. fluorescens* bv 2 PICF1 and PICF6, and bv 5 PICF7, and *P. putida* bv A PICP2 and PICP5; but that of V4I took place only for *P. fluorescens* bv 2 PICF6 and bv 5 PICF7, and *P. putida* bv A PICP5. Siderophore-mediated inhibition was not detected when production of siderophore was repressed by amending KBA with Fe^{3+}.

Finally, an overall impairment of *V. dahliae* growth mediated by isolates *P. fluorescens* bv 2 PICF1 and PICF6 and *P. fluorescens* bv 6 PICF8 was also detected on KBA + Fe^{3+} and to a lesser extent on KBA (Table 1). While the fungal growth was not completely inhibited by 48 h of incubation, colonies of *V. dahliae* were sparse and much smaller compared with the control. The pattern of the observed fungal growth inhibition suggested that a volatile compound, such as HCN, could be involved. Actually, when supernatants of KB cultures of the *Pseudomonas* isolates were tested for HCN production, the supernatants of *P. fluorescens* bv 2 PICF1 and PICF6, and bv 6 PICF8 were positive for HCN while those of other *Pseudomonas* isolates were not. In addition, PCR assays carried out to demonstrate the presence of HCN biosynthetic genes yielded a single, expected product of 590 bp for HCN^+ isolates *P. fluorescens* bv 2 PICF1 and PICF6 as well as for *Pseudomonas* sp. CHAO used as positive control (data not shown). However, the 590-bp product was not amplified from DNA of HCN^+ *P. fluorescens* bv 6 PICF8, that rather yielded a single product of 1200 bp. Interestingly, the 590-bp product was also amplified from DNA of *P. fluorescens* bv 4 PICF4 (data not shown), although this isolate did not produce HCN on KB broth supernatants and did not inhibit *V. dahliae* hyphal growth (Table 1).

3.3. In vitro production of pseudobactin and salicylic acid by bacteria

All *Pseudomonas* spp. isolates tested produced an orange halo on CAS agar. After 72 h of incubation at 28 °C, which is optimum temperature for growth of *Pseudomonas* isolates, and at 37 °C, which represses pseudobactin production (Marugg et al., 1985), only isolates of *P. fluorescens* bv 5 PICF7 and bv 6 PICF8 produced significantly larger halos at 28 °C than at 37 °C. Thus, at 28 °C mean relative size of halo for isolates PICF7 and PICF8 were 0.61 ± 0.01 (mean $\pm$ SD) and 0.63 ± 0.01, respectively, compared with 0.31 ± 0.01 for isolate PICF7 and 0.31 ± 0.06 for isolate PICF8 at 37 °C. This would indicate either enhanced production of pseudobactin(s) by these two isolates or production of additional siderophores other than pseudobactin(s). Production of additional siderophores was indicated by siderophore activity detected in all isolates at 37 °C. Production of the green fluorescent siderophore pseudobactin by the *Pseudomonas* spp. isolates was confirmed in liquid SSM. There was no significant difference among amount of pseudobactin produced by the bacterial isolates (Table 2).

Only *P. fluorescens* bv 5 PICF3 and PICF7, and *P. fluorescens* bv 4 PICF4 produced detectable levels of SA in vitro (Table 2). However, production of SA by those

Table 1
Hyphal growth inhibition of defoliating *V. dahliae* V138I and nondefoliating *V. dahliae* V4I isolates on PDA, KBA, and KBA + Fe^{3+} media by *Pseudomonas* spp. isolated from olive and their rifampicin mutants (Rf)

Bacteria[a]	Growth inhibition of *V. dahliae*[b]					
	PDA		KBA		KBA + Fe^{3+}	
	V4I	V138I	V4I	V138I	V4I	V138I
P. fluorescens bv 2						
PICF1	+	+	+/–	+ (*)	(*)	(**)
PICF1-Rf	–	–	+/–	+ (*)	(*)	(**)
PICF6	+	+	+	+ (*)	(*)	(**)
PICF6-Rf	+	+	+	+ (*)	(*)	(**)
P. fluorescens bv 4						
PICF4	+	+	+/–	+/–	–	–
PICF4-Rf	+	+	+/–	+/–	–	–
P. fluorescens bv 5						
PICF3	–	–	+/–	+/–	–	–
PICF3-Rf	–	–	+/–	+/–	–	–
PICF7	–	–	+	+	–	–
PICF7-Rf	–	–	+	+	–	–
P. fluorescens bv 6						
PICF8	–	–	+/–	(*)	(**)	(**)
PICF8-Rf	–	–	+/–	(*)	(**)	(**)
P. putida bv A						
PICP2	++	++	+/–	+	–	–
PICP2-Rf	++	++	+/–	+	–	–
PICP5	++	++	+	+	–	–
PICP5-Rf	++	++	+	+	–	–

[a] Suspensions of *Pseudomonas* spp. isolates and their Rf-mutants in 10 mM $MgSO_4 \cdot 7H_2O$ were spotted (5 μl droplets) onto media, incubated at 28 °C for 48 h, and then sprayed with conidia of *V. dahliae* isolates V4I of V138I. Plates without bacteria were used as control. There were three replicated plates for each bacterium–fungal isolate-culture medium combination and the experiment was repeated once.

[b] (-) No sharp zone of hyphal growth inhibition; +/–, only a faint zone of hyphal growth inhibition surrounding the bacterial colony; +, a sharp circular zone of hyphal growth inhibition (halo) less than 20 mm in diameter around the bacterial colony; ++, a halo larger than 20 mm in diameter around the bacterial colony; (*) Fungal growth was impaired but not inhibited throughout the medium in a plate giving rise to sparse fungal colonies smaller than those on control plates. (**) Strong impairment of fungal growth or near complete hyphal growth inhibition throughout the medium in a plate. In some instances, both inhibition haloes and sparse fungal colonies occurred together in a plate.

isolates was much lower compared with that of the previously characterized SA-producer *P. fluorescens* WCS374 (Leeman et al., 1996). Production of SA by isolates of *P. putida* was not detected in culture supernatants.

3.4. Root colonization assay

There was no bacterial growth when root extracts from nontreated (control) plants or from plants treated with the wild-type bacterial isolates were plated onto modified KBA supplemented with Rf (100 μg ml^{-1}) (Table 3). Only the introduced Rf-mutants were recovered on the growth medium containing Rf. Bacterial population sizes in roots treated with the bacterial Rf-mutants determined using KBA were not significantly different from those assessed with Rf-amended KBA. Mean population sizes of *P. fluorescens* bv 2 PICF1-Rf and PICF6-Rf, *P. putida* bv A PICP2-Rf, and *P. fluorescens* bv 4 PICF4-Rf, were significantly higher than those of *P. putida* bv A PICP5-Rf, *P. fluorescens* bv 5 PICF7-Rf, and *P. fluorescens* bv 6 PICF8-Rf. The population size of the *P. fluorescens* bv 5 PICF7-Rf mutant was the lowest ($P < 0.05$) among the bacterial isolates of study.

The population sizes of total fluorescent pseudomonads determined on modified KBA were significantly higher in roots treated with the *Pseudomonas* spp. isolates or their respective Rf-mutants compared with that in roots of control plants, except for *P. fluorescens* bv 5 PICF7-Rf which population size was not significantly different from that of bacteria in the control plants. The highest ($P < 0.05$) population sizes of fluorescent pseudomonads occurred in roots treated with either *P. fluorescens* bv 2 PICF1 or PICF6, and *P. fluorescens* bv 4 PICF4. In general, the bacterial populations in roots treated with the wild-type strains were significantly higher than those in roots treated with their respective Rf-mutant, except for *P. putida* bv A PICP2 and PICP5.

3.5. Suppression of Verticillium wilt of olive by bacteria

Symptoms developed neither in noninoculated controls nor in plants treated with the bacterial isolates

Table 2
Production of salicylic acid and pseudobactins by isolates of *Pseudomonas* spp. at low iron availability in liquid standard succinic medium

Bacteria[a]	Salicylic acid ($\mu g\,ml^{-1} \pm SD$)[b,d]	Pseudobactins (OD_{400}/OD_{600})[c,d]
P. fluorescens		
bv 2 PICF1	0	2.00 ± 1.26
bv 5 PICF3	0.55 ± 0.12	2.30 ± 0.34
bv 4 PICF4	1.57 ± 0.26	2.78 ± 0.12
bv 2 PICF6	0	2.27 ± 1.23
bv 5 PICF7	0.59 ± 0.35	1.72 ± 1.12
bv 6 PICF8	0	4.08 ± 1.02
WCS374	35.45 ± 1.50	1.75 ± 1.23
P. putida		
bv A PICP2	0	2.82 ± 0.24
bv A PICP5	0	2.07 ± 0.60
WCS358	0	2.16 ± 0.41

[a] Isolates of *Pseudomonas* spp. were grown in standard succinic medium at 28 °C for 3 days. *P. fluorescens* WCS374 and *P. putida* WCS358 were used as salicylic acid (SA)-producer and SA-nonproducer (Leeman et al., 1996) controls, respectively.

[b] Production of SA by bacteria was determined in culture supernatants by spectrophotometric detection of the purple iron–SA complex at 527 nm (see Section 2).

[c] Production of pseudobactins by bacteria was determined in culture supernatants by absorbance at 400 nm referred to the culture optical cell density (OD_{600}) (see Section 2).

[d] Data are means of two measurements in each of duplicate experiments.

Table 3
Population sizes of isolates of *Pseudomonas fluorescens* and *P. putida* recovered from root tissues of olive cv. Picual after root-dip inoculation with bacterial suspensions

Bacteria[a]	Bacterial population ($\log CFU\,g^{-1}$ of fresh root)[b,c]	
	KBA	KBA + Rf ($100\,\mu g\,ml^{-1}$)
P. fluorescens bv 2		
PICF1	5.74 a	—
PICF1-Rf	4.81 bc	4.91 a
PICF6	5.75 a	—
PICF6-Rf	4.89 b	4.90 a
P. fluorescens bv 4		
PICF4	5.92 a	—
PICF4-Rf	5.07 b	5.17 a
P. fluorescens bv 5		
PICF7	4.80 bc	—
PICF7-Rf	3.21 f	2.80 c
P. fluorescens bv 6		
PICF8	4.32 d	—
PICF8-Rf	3.75 e	3.81 b
P. putida bv A		
PICP2	5.10 b	—
PICP2-Rf	4.92 b	4.84 a
PICP5	4.24 d	—
PICP5-Rf	4.43 cd	4.21 b
Control (no bacteria)	3.15 f	—

[a] The root system of 3-month-old olive plants were dipped in 5×10^8 cells ml^{-1} in 10 mM $MgSO_4 \cdot 7H_2O$ bacterial suspension for 10 min. Control plants were dipped in a sterile solution of 10 mM $MgSO_4 \cdot 7H_2O$. Plants were transplanted into autoclaved soil and grown in a greenhouse for 3 months.

[b] Populations of *Pseudomonas* spp. were determined on modified King's medium B agar (KBA) or on KBA amended with rifampicin (see Section 2).

[c] Data are means of four 2-g fresh root samples (one sample per plant). Means in a column followed by the same letter are not significantly different according to Fisher's protected LSD test ($P = 0.05$).

grown in noninfested soil in experiments I–III. In experiment I, treatment of roots of 4-month-old, rooted olive stem cuttings with *Pseudomonas* spp. isolates influenced the development of Verticillium wilt in cv. Picual (Table 4). Nontreated, inoculated plants grown in D *V. dahliae* V138I-infested soil showed symptoms characteristic of those caused by the D pathotype in olive cv. Picual (Rodríguez-Jurado et al., 1993). In those plants, first symptoms developed by 33 days after inoculation reaching a final *DI* of 91.7% (Table 4). Prior bacterization with *P. fluorescens* bv 5 PICF7 significantly delayed the *IP* by 14 days and reduced the final *DI* by 82%, the final *DII* by 96%, and *SAUDPC* by 97%, compared with the control. Treatment of "Picual" plants with *P. fluorescens* bv 2 PICF6 or *P. fluorescens* bv 6 PICF8 also reduced significantly the final *DII* and *SAUDPC* compared with the control, though to a lesser extent than reduction by isolate PICF7. Compared with the control, treatment with *P. fluorescens* bv 4 PICF4 significantly reduced *SAUDPC* by 50%, but reduction of the final *DI* and *DII* was not significant. At the end of the experiment, 70 days after inoculation with the pathogen, treatment of inoculated plants with *P. fluorescens* bv 5 PICF7 significantly increased the diameter at the base of the stem by 16%, the total stem length by 24%, and the fresh shoot weight by 79%, compared with those of nontreated, inoculated control plants (Table 4). Treatment with *P. fluorescens* bv 6 PICF8 also increased the fresh shoot weight significantly compared with that of nontreated, inoculated control plants, though to a lesser extent than isolate PICF7 (Table 4).

In experiment II, treatment of 3-month-old micropropagated plants with *Pseudomonas* spp. isolates did not significantly influenced development of Verticillium wilt in cv. Picual (Table 5). In control plants grown in soil infested with the pathogen, first symptoms developed by 29 days after inoculation and the disease developed slowly to reach a final *DI* of 68.7% (Table 5). Also, foliar symptoms affected less than 50% of aerial plant part by 98 days after inoculation, which resulted in the final *DII* and *SAUDPC* of 0.15 and 0.13, respectively (Table 5). Prior treatment of 'Picual' plants with either *P. fluorescens* bv 4 PICF4, *P. fluorescens* bv 6 PICF8, or *P. putida* bv A PICP5 reduced the final *DI* by 16–25%, the final *DII* by 53%, and *SAUDPC* by 38–61%, compared with the control, but those differences were not

Table 4
Effect of treatment of rooted olive stem cuttings with isolates of *Pseudomonas* spp. from olive on development of Verticillium wilt and growth of olive cv. Picual in soil infested with defoliating *V. dahliae*

Treatment [a]	Disease assessment[b]				Plant growth[b]		
	IP (days)	*SAUDPC*	Final *DII*	Final *DI* (%)	Diameter at the stem base (in mm)	Total stem length (in cm)	Fresh shoot weight (in g)
Control	33.2	0.38	0.49	91.7	3.1	40.7	6.2
P. fluorescens							
bv 4 PICF4	31.6	0.19*	0.28	75.0	3.3	45.0	8.2
bv 2 PICF6	41.5	0.07*	0.11*	50.0	3.2	42.1	7.5
bv 5 PICF7	47.9*	0.01*	0.02*	16.7*	3.6*	50.4*	11.1*
bv 6 PICF8	36.8	0.10*	0.15*	58.3	3.4	43.1	9.5*
P. putida							
bv A PICP2	31.5	0.21	0.30	75.0	3.3	44.2	7.2
bv A PICP5	38.5	0.20	0.36	87.5	2.8	35.3	6.0

[a] The root systems of 4-month-old, own-rooted plants were dipped in 5×10^8 cells ml^{-1} bacterial suspensions in 10 mM $MgSO_4 \cdot 7H_2O$ or a sterile 10 mM $MgSO_4 \cdot 7H_2O$ solution (control) for 10 min, then transplanted into autoclaved soil artificially infested with 2×10^6 conidia g^{-1} of defoliating *V. dahliae* V138I and grown in the greenhouse at 25 ± 10 °C for 70 days.

[b] A disease intensity index (*DII*) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 7-day-intervals. *DII* values were plotted over time to develop curves of disease increase. *IP*, incubation period (number of days until $DII > 0$). *SAUDPC*, standardized area under the curves of *DII* increase over time. *DI*, disease incidence (%). Data are the average of four blocks each with three replicated pots (one plant per pot). Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. Means in a column followed by an asterisk are significantly different to the mean for the control treatment according to Dunnett's test ($P = 0.05$).

statistically significant (Table 5). Also, *P. fluorescens* bv 5 PICF7 reduced the final *DII* and *SAUDPC* by 46 and 54%, respectively, but not the final *DI*. As for experiment I, treatment of inoculated plants with *P. fluorescens* bv 5 PICF7 significantly increased the diameter at the stem base by 25%, the total stem length by 41%, and the fresh shoot weight by 47%, compared with the nontreated, inoculated control plants (Table 5). Treatment with *P. fluorescens* bv 2 PICF6, bv 6 PICF8, or Q2-87, and *P. putida* bv A PICP5 also increased the diameter at the stem base by 11–14%, the total stem length by 30–34% and the fresh shoot weight by 23–35%, compared with the control (Table 5). However, only the increase in fresh shoot weight induced by isolate PICF8 was statistically significant from the control. A moderate level of disease developed in the nontreated, inoculated control plants by 98 days after the inoculation. To confirm infection by the pathogen, isolations of the fungus were carried out from the stems and roots of symptomless and diseased plants. Results indicated that positive isolation of the fungus did correlate with severity of symptoms in the plant (data not shown).

Severe incidence and severity of Verticillium wilt of 'Picual' olive developed in experiment III, as indicated by 100% final *DI* and 0.83 final *DII*. Only treatment with *P. fluorescens* bv 4 PICF4 significantly delayed the *IP* by 8.5 days and reduced the final *DI* by 31%, the final *DII* by 73% and *SAUDPC* by 76%, compared with the control (Table 5). In addition, prior treatment with most of the tested bacteria significantly influenced development of disease either by reducing the final *DII* by 45–71% and *SAUDPC* by 58–71% (i.e., *P. fluorescens* bv 2 PICF6, bv 5 PICF7, Q2-87; and *P. putida* bv A PICP5), or only *SAUDPC* by 45% (i.e., *P. putida* bv A PICP2 and *P. fluorescens* bv 6 PICF8), compared with the control (Table 5). Most of the tested bacteria increased the fresh shoot weight of the inoculated plant compared with the nontreated, inoculated control, either significantly by 157–185% (i.e., *P. fluorescens* bv 4 PICF4, *P. fluorescens* bv 2 PICF6, or *P. putida* bv A PICP5), or not by 57–100% (i.e., *P. fluorescens* bv 5 PICF7, bv 6 PICF8, Q2-87; and *P. putida* bv A PICP2).

4. Discussion

Management of Verticillium wilts of woody hosts such as olive is difficult and should be based on an integrated strategy (Tjamos, 1993; Tjamos and Jiménez-Díaz, 1998). Exploiting the potential of microbial antagonists for the protection of olive planting material would be a desirable pre-planting measure for the integrated management of Verticillium wilt. The main objective of the present work was to determine the ability of olive root-associated *Pseudomonas* spp. to protect planting material of the highly susceptible olive cv. Picual against Verticillium wilt caused by the highly virulent D pathotype of *V. dahliae*. Our reasoning was that bacteria adapted to occupy the plant infection court might be good candidates to prevent or reduce efficiency of the pathogen to invade the plant and cause disease. To the best of our knowledge this is the first report on

Table 5
Effect of treatment of micropropagated olive plants with isolates of *Pseudomonas* spp. from olive on development of Verticillium wilt and growth of olive cv. Picual in soil infested with defoliating *V. dahliae*

Experiment	Treatment	Disease assessment[c]				Plant growth		
		IP (days)	*SAUDPC*	Final *DII*	Final *DI* (%)	Diameter at the stem base (in mm)	Total stem length (in cm)	Fresh shoot weight (in g)
II[a]	Control	28.7	0.13	0.15	68.7	2.8	10.8	1.7
	P. fluorescens							
	bv 4 PICF4	47.2	0.08	0.07	55.0	2.8	11.8	1.8
	bv 2 PICF6	36.7	0.08	0.12	68.7	3.2	14.1	2.2
	bv 5 PICF7	40.2	0.06	0.08	61.2	3.5*	15.2*	2.5*
	bv 6 PICF8	36.7	0.05	0.07	51.2	3.1	14.5	2.3*
	Q2-87	38.5	0.11	0.13	66.2	3.1	14.1	2.1
	P. putida							
	bv A PICP2	36.7	0.09	0.11	72.5	2.8	10.2	1.5
	bv A PICP5	36.7	0.06	0.07	57.5	3.2	14.3	2.2
III[b]	Control	26.5	0.38	0.83	100.0	2.2	10.9	0.7
	P. fluorescens							
	bv 4 PICF4	35.0*	0.09*	0.22*	68.7*	2.6	11.9	2.0*
	bv 2 PICF6	31.7	0.16*	0.45*	81.2	3.1*	13.3	1.8*
	bv 5 PICF7	28.2	0.16*	0.42*	81.2	2.4	10.9	1.4
	bv 6 PICF8	33.0	0.21*	0.54	93.7	2.2	9.7	1.1
	Q2-87	29.5	0.12*	0.30*	100.0	2.3	8.6	1.4
	P. putida							
	bv A PICP2	30.0	0.21*	0.55	93.7	2.7	11.9	1.3
	bv A PICP5	31.0	0.11*	0.24*	75.0	2.8	11.3	2.0*

[a] The root system of 3-month-old plants were dipped in 5×10^8 cells ml^{-1} bacterial suspensions in 10 mM $MgSO_4 \cdot 7H_2O$ or in a sterile 10 mM $MgSO_4 \cdot 7H_2O$ solution (control) for 10 min, then transplanted into autoclaved soil artificially infested with 2×10^6 conidia g^{-1} of defoliating *V. dahliae* V138I and grown in the 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 98 days.

[b] The experiment was conducted as described for experiment II (a) except that plants were incubated in the growth chamber for 72 days and there were four blocks each with four replicated pots (one plant per pot).

[c] A disease intensity index (*DII*) ranging 0–1 was calculated with data on incidence and severity of symptoms recorded at 7-day-intervals. *DII* values were plotted over time to develop curves of disease increase. *IP*, incubation period (number of days until $DII > 0$). *SAUDPC*, standardized area under the curves of *DII* increase over time. *DI*, disease incidence (%). Data are the average of four blocks each with five (experiment II) or four (experiment III) replicated pots (one per pot). Control plants grown in noninfested soil were free from symptoms and were not included in statistical analyses. Percentage values were transformed into arcsine $(Y/100)^{1/2}$ for analyses of variance. For each experiment, means in a column followed by an asterisk are significantly different from the mean of the control treatment according to Dunnett's test ($P = 0.05$).

the use of root-associated bacteria for control of Verticillium wilt in a woody host such as olive.

The *Pseudomonas* spp. isolates used in this work were isolated from internal root tissues of nursery-produced olive plants, and their ability to thoroughly colonize the olive root system was confirmed by root colonization assays of 'Picual' olive planting stocks from different sources using the *Pseudomonas* wild-type isolates and their Rf-mutant derivatives. Indigenous *Pseudomonas* spp. were isolated from root tissues of the olive plants inoculated with the selected bacteria. However, the population sizes of total pseudomonads and Rf-resistant mutants recovered from treated roots were significantly higher than those of nontreated, control plants, thus making possible to distinguish the introduced *Pseudomonas* isolates. No attempts were made in this work to further determine whether these isolates can colonize above-soil plant tissues, including vascular ones. Therefore, no claim can be made at this time that the *Pseudomonas* isolates are true endophytes (Wilson, 1995). Since Rf resistance mutation apparently affected the root colonization (and/or persistence) ability of the introduced *Pseudomonas* isolates, the wild-type isolates were subsequently used for Verticillium wilt suppression experiments. By doing so, we used indigenous, root-associated, nonmodified *Pseudomonas* isolates as biocontrol agents in its natural host plant, which would fit into environmentally friendly, sustainable disease management strategy.

Isolates of *Pseudomonas* spp. varied in their ability to suppress Verticillium wilt of olive under conditions favorable for severe disease. Thus, *P. fluorescens* bv 4 PICF4, bv 2 PICF6, bv 5 PICF7, and bv 6 PICF8 were effective in suppressing the disease in young, nursery-produced olive plants cv. Picual to a degree, with isolates PICF7 and PICF4 being the most effective ones.

Those latter isolates significantly reduced the final *DI* and *DII* by 31–82% and 73–96%, respectively, under conditions that resulted in 92–100% *DI* and 0.49–0.83 *DII* in the nontreated controls. Isolates of *P. putida* bv A PICP2 and PICP5 were less effective than those of *P. fluorescens*, or no effective at all. Also, the degree of Verticillium wilt suppression by *P. fluorescens* isolates varied among experiments.

Lack of consistency in the performance of *Pseudomonas* spp. and *Bacillus* spp. as biocontrol agents under field conditions has often been one of the factors limiting the use of them in commercial agriculture (Raaijmakers et al., 2002). Much of that inconsistency has been attributed to variability in the physical and chemical properties within the niches occupied by biocontrol agents, as well as the plant, that affect both colonization and expression of biocontrol mechanisms (Ownley et al., 2003; Smith and Goodman, 1999). In this present study, different environmental conditions (greenhouse and controlled growth chamber), propagation procedures (rooted stem cuttings and micropropagated plants), and plant sources, were used in the experiments that may contribute with variability factors referred above and would explain differences in disease suppression ability found. By doing so, we aimed to challenge the biocontrol efficacy of the tested isolates under conditions similar to those used by the olive-nursery industry. For example, *P. fluorescens* bv 5 PICF7 was the most effective isolate in Verticillium wilt suppression for all disease parameters scored, when bacteria were used on own-rooted, 4-month-old plants under greenhouse conditions. Conversely, when bacteria were tested on micropropagated plants under growth chamber conditions, *P. fluorescens* bv 4 PICF4 was the most effective isolate in disease suppression. Nevertheless, it was interesting to note that significant disease suppression was achieved when severe Verticillium wilt developed in nontreated, control plants regardless environmental conditions (experiments I and III). In contrast, Verticillium wilt development in experiment II was not significantly influenced by the same *Pseudomonas* spp. isolates tested on the same olive plant material and culture conditions as for in experiment III. It is possible to speculate that potential for biocontrol activity by those bacteria was not revealed because of low disease pressure in experiment II. Nevertheless, for most tested bacteria lower *SAUDPC*, final *DII*, and final *DI* values occurred in the treated plants compared with the untreated control. Although these differences were not statistically significant they indicate a clear tendency for biocontrol. This trend would support the effective disease suppression scored in experiments I and III, where disease pressure was higher than in experiment II. On the other hand, treatment of olive plants with isolate *P. fluorescens* bv 5 PICF7 significantly increased the diameter at the stem base, total stem length, and fresh shoot weight compared with untreated, inoculated plants in experiments I and II. None of the tested bacteria promoted plant growth in noninfested soil (data not shown). Therefore, the enhanced growth of *V. dahliae*-inoculated olive plants by some of the tested bacteria could just be attributable to counteracting of deleterious effects caused by the pathogen in infected plants, rather than actual stimulation of the plant growth. Moreover, no significant differences in plant growth promotion were found among plants treated with the different bacteria tested in this study, as well as between treated and untreated plants in the absence of the pathogen (data not shown).

Several mechanisms have been proposed for disease suppression mediated by nonpathogenic *Pseudomonas*: production of antibiotics, siderophores, HCN, and lytic enzymes, competition for nutrients and suitable niches on a root surface, and induction of systemic resistance (Lemanceau and Alabouvette, 1993; O'Sullivan and O'Gara, 1992; van Loon et al., 1998). The mechanism(s) that could be involved in the suppression of Verticillium wilt of olive by some of the tested bacteria are not known yet and their precise role can only be analyzed using deficient mutants. However, as a first step to assess capabilities by the bacteria, we were able to detect in vitro production of pseudobactin (and probably other siderophores) and HCN, which showed inhibitory activity against D and ND *V. dahliae*. In addition SA, which also has siderophore activity, was produced by three of the tested bacteria including the most effective ones in the suppression of Verticillium wilt, i.e., *P. fluorescens* bv 4 PICF4 and bv 5 PICF7. Bacterial SA has an important role in inducing resistance in plants against pathogen infection in addition to siderophore activity (De Meyer et al., 1999, Maurhofer et al., 1998). Production of HCN was demonstrated for *P. fluorescens* bv 2 PICF1 and PICF6 and bv 6 PICF8 because: (i) enhanced fungal inhibition was observed in KBA + Fe^{3+} plates, and induction of cyanogenesis in *Pseudomonas* required the presence of Fe^{3+} as reported by Voisard et al. (1989); (ii) HCN was detected in culture supernatants of those bacteria, and (iii) putative *hcnBC* genes were amplified when using the ACa/ACb specific primer pair for HCN biosynthesis genes (Ramette et al., 2003). However, it should be noted that while the expected PCR product of 590 bp was amplified from DNA templates of *P. fluorescens* bv 2 PICF1 and PICF6, PCR assays using DNA template from HCN producer *P. fluorescens* bv 6 PICF8 yielded a much larger amplification product. That circumstance, as well as the reason why HCN nonproducer *P. fluorescens* bv 4 PICF4 amplified the *hcnBC*-containing amplicon, remain to be investigated.

In vitro experiments showed that the *P. putida* isolates tested in this study were highly inhibitory of *V. dahliae* on PDA. Possibly, this was due to production of

an unidentified antibiotic compound(s). However, those bacteria showed little or no ability of suppressing Verticillium wilt of olive in our experiments. On the contrary, *P. fluorescens* bv 5 PICF7, one of the most successful biocontrol agent in this present work, showed antagonistic effect neither on PDA nor on Fe^{3+}-amended KBA. Only siderophore-mediated in vitro antagonism against *V. dahliae* was found for that latter bacterium. Those results indicate that there was no correlation between in vitro antagonism against *V. dahliae* and in vivo suppression of Verticillium wilt of olive by the tested bacteria. This lack of correlation between antagonism under in vitro conditions and efficacy in disease suppression is not uncommon in plant disease biocontrol, and has been reported elsewhere (Fravel, 1988; Paulitz et al., 1992).

In summary, this present study demonstrate the potential of some native, root-associated bacteria from olive as effective biocontrol agents against D *V. dahliae* in nursery-produced olive planting stocks. The efficacy of some of those bacteria (when introduced either alone or in combination) for biocontrol of Verticillium wilt in adult olive plants, as well as studies on the mechanism(s) involved in disease suppression are scopes of future research.

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