

# Biocontrol of Avocado *Dematophora* Root Rot by Antagonistic *Pseudomonas fluorescens* PCL1606 Correlates With the Production of 2-Hexyl 5-Propyl Resorcinol

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A collection of 905 bacterial isolates from the rhizospheres of healthy avocado trees was obtained and screened for antagonistic activity against *Dematophora necatrix*, the cause of avocado *Dematophora* root rot (also called white root rot). A set of eight strains was selected on the basis of growth inhibitory activity against *D. necatrix* and several other important soilborne phytopathogenic fungi. After typing of these strains, they were classified as belonging to *Pseudomonas chlororaphis*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. The eight antagonistic *Pseudomonas* spp. were analyzed for their secretion of hydrogen cyanide, hydrolytic enzymes, and antifungal metabolites. *P. chlororaphis* strains produced the antibiotic phenazine-1-carboxylic acid and phenazine-1-carboxamide. Upon testing the biocontrol ability of these strains in a newly developed avocado-*D. necatrix* test system and in a tomato-*F. oxysporum* test system, it became apparent that *P. fluorescens* PCL1606 exhibited the highest biocontrol ability. The major antifungal activity produced by strain *P. fluorescens* PCL1606 did not correspond to any of the major classes of antifungal antibiotics produced by *Pseudomonas* biocontrol strains. This compound was purified and subsequently identified as 2-hexyl 5-propyl resorcinol (HPR). To study the role of HPR in biocontrol activity, two Tn5 mutants of *P. fluorescens* PCL1606 impaired in antagonistic activity were selected. These mutants were shown to impair HRP production and showed a decrease in biocontrol activity. As far as we know, this is the first report of a *Pseudomonas* biocontrol strain that produces HPR in which the production of this compound correlates with its biocontrol activity.

*Additional keywords:* *Persea americana*.

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Nucleotide sequence data reported are available in the GenBank database under accession numbers DQ073039 for 16S rDNA, DQ073040 and DQ073041 for the interrupted gene in mutant PCL1613 and DQ073042 and DQ073043 for the mutant PCL1614.

Avocado (*Persea americana* Mill.) is a crop plant that was relatively recently introduced into Europe. European avocado orchards are mainly located in Spain and Portugal, where root fungal diseases have proved to be the major threat to plant health.

*Dematophora necatrix* (teleomorph: *Rosellinia necatrix*), the agent that causes *Dematophora* root rot in avocado and in many other crops (this disease is also called white root rot), has a very wide host-range, including 170 plant species or varieties in 63 genera and 30 families of dicotyledonous angiosperms. This fungal pathogen is destructive to many fruit tree crops, including tropical and subtropical species such as avocado and mango (*Mangifera indica* L.), which are particularly susceptible (Sztejnberg and Madar 1980).

Disease development in avocado trees infected by *D. necatrix* is usually rapid, killing the trees within a few weeks of the first foliar symptoms. The pathogen is capable of surviving for many years and remaining active for long periods on residues of crops susceptible to *D. necatrix*, such as olive (*Olea europaea* L.), grapevine (*Vitis vinifera* L.), and almond (*Prunus amygdalus* Batsch.) that are common in the region. The high susceptibility of cv. Topa-Topa, the rootstock most frequently used in avocado orchards in this area, may have favored the increase in disease incidence (López-Herrera et al. 1998).

Control of avocado *Dematophora* root rot poses difficulties, because any preplanting treatment must have a long-term effect and any postplanting treatment must not adversely affect the crop (Sztejnberg et al. 1987). Several approaches have been used over the past two decades for the control of *D. necatrix* before planting, such as i) soil fumigation using methyl bromide (Sztejnberg et al. 1983), ii) soil solarization (López-Herrera et al. 1998), which may induce disease suppressive activity of the soil by increasing microbial activities, resulting in protection of the plants (Greenberger et al. 1987), and iii) biological control using the antagonistic fungus *Trichoderma harzianum* (Sztejnberg et al. 1987). The use of bacterial strains as biocontrol agents against avocado soilborne phytopathogens has not been reported. Biological control of avocado diseases has not been investigated, although recently, an approach for isolating bacterial biocontrol strains for the control of avocado root rot caused by *Phytophthora cinnamomi* was reported (Yin et al. 2004).

Microbial mechanisms by which rhizobacteria may protect plants against soilborne pathogens include competition for

niches (Paulitz 2000), antibiosis (O'Sullivan and O'Gara 1992), and the induction of systemic resistance (Pieterse et al. 1996). In recent years, several plant root-colonizing *Pseudomonas* spp. have been shown to be potent microbiological control agents in various plant-pathogen systems. The use of such plant growth-promoting rhizobacteria is considered an addition to and an alternative for chemical pesticides, several of which are a threat to our health and environment (de Weger et al. 1995; Lugtenberg et al. 1994).

Antifungal compounds produced by *Pseudomonas* spp. biocontrol agents that are correlated with biological effectiveness include phenazine-1-carboxylic acid (PCA) (Thomashow and Weller 1988), phenazine-1-carboxamide (PCN) (Chin-A-Woeng et al. 1998), 2,4-diacetylphloroglucinol (DAPG) (Keel et al. 1992), pyrrolnitrin (PRN) (Howell and Stipanovic 1979), pyoluteorin (PLT) (Howell and Stipanovic 1980), hydrogen cyanide (HCN) (Voisard et al. 1989), siderophores (Becker and Cook 1988), and some hydrolytic enzymes such as proteases (Sacherer et al. 1994).

In this sense, *Pseudomonas* spp. produce many other antifungal substances (Budzikiewicz 1993), and many of them have not yet been related to the biocontrol activity of the microorganisms. This is the case with the antimicrobial activity of alkylresorcinol and especially the antifungal compound 2-hexyl 5-propyl resorcinol (HPR), which is produced by *Pseudomonas* spp. (Kanda et al. 1975; Kitahara and Kanda 1975; Nowak-Thompson et al. 2003.)

In this paper, we report the screening of rhizosphere bacteria isolated from healthy avocado for in vitro antagonistic activity towards the fungal phytopathogen *D. necatrix* as well as against several other soilborne phytopathogenic fungi. The strongest antagonistic isolates against fungi belonged to the genus *Pseudomonas* and appeared to be able to suppress *Dematophora* root rot substantially in a novel avocado-*Dematophora* in vitro biocontrol test system, using plants from germinated avocado embryos. In addition, the strains were also active against tomato foot and root rot caused by *Fusarium oxysporum*

f. sp. *radicis-lycopersici*. The antifungal metabolite produced by the best protective strain, *P. fluorescens* PCL1606, was structurally identified after purification, and its role in biocontrol was analyzed.

## RESULTS

### Identification and antagonistic activity of selected rhizobacterial isolates.

The numbers of culturable bacterial cells present on the avocado root samples were estimated after isolation and growth on tryptic soy agar (TSA) and King's B (KB) agar (King et al. 1954) plates. The values for total culturable bacteria ranged from 5.3 to 6.5 log CFU per gram of fresh weight avocado roots. In all root samples, the presence of pseudomonads was indicated by growth of colonies on PI (*Pseudomonas* isolation) medium and ranged from 2.8 to 4.5 log CFU per gram of fresh weight of roots.

A total of 905 bacterial isolates were selected, based on abundance and differences in colony morphology, and were tested for their antagonistic ability against highly virulent *Dematophora necatrix* 400, using an agar plate assay. Antagonistic strains were isolated from all root samples, types of soil, and on all media used for isolation. The total percentage of antagonistic bacterial strains growing on TSA or KB agar plates, or both, was 8.7% (62 antagonistic bacterial isolates from 712 tested), most of which were fluorescent on KB medium, whereas the percentage of antagonistic *Pseudomonas*-like strains isolated from colonies growing on PI medium was 57.5% (111 antagonistic isolates from 193 tested). The antagonistic strains were subsequently tested against other soilborne fungi phytopathogenic to avocado and other plants, resulting in eight *Pseudomonas*-like isolates with broad antagonistic activity (Table 1).

Identification of these rhizobacterial isolates was performed by the API20NE and BIOLOG tests, combined with homology analyses of nucleotide sequences of polymerase chain reaction

**Table 1.** Identification and in vitro antagonistic ability of the selected strains from avocado rhizosphere against plant pathogenic fungi

Bacterial strain	Characterization	Antagonistic activity on King's B agar plates against <sup>a</sup>					
		<i>Dematophora necatrix</i> (n = 5) <sup>b</sup>	<i>Phytophthora cinnamomi</i> (n = 3) <sup>c</sup>	<i>Rhizoctonia solani</i>	<i>Pythium ultimum</i>	<i>Sclerotium rolfsii</i>	<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i>
PCL1601	<i>P. chlororaphis</i>	++	++	(+)	(+)	++	++
PCL1602	<i>P. fluorescens</i>	+	+	-	-	+	(+)
PCL1603	<i>P. putida</i>	++	+	+	(+)	+	(+)
PCL1604	<i>P. chlororaphis</i>	++	++	+	+	+	++
PCL1606	<i>P. fluorescens</i>	++	++	++	++	++	++
PCL1607	<i>P. chlororaphis</i>	++	(+)	++	+	++	++
PCL1609	<i>P. putida</i>	+	+	+	-	+	-
PCL1611	<i>P. chlororaphis</i>	++	++	++	+	++	++

<sup>a</sup> ++ = diameter of the growth inhibition zone >10 mm; + = diameter of the growth inhibition zone between 3 to 10 mm; (+) = diameter of the growth inhibition zone <3 mm; and - = no antifungal activity observed.

<sup>b</sup> Five strains of *Dematophora necatrix* (400, 397, 358, CECT2817, and CECT2818, respectively), were tested and gave all similar results.

<sup>c</sup> Three strains of *Phytophthora cinnamomi* (344, 301, and 273, respectively) were tested and gave similar results.

**Table 2.** Production of hydrolytic activities and antifungal metabolites by the isolated *Dematophora* antagonistic bacterial strains

Production of	Strains <sup>a</sup>							
	PCL 1601	PCL 1602	PCL 1603	PCL 1604	PCL 1606	PCL 1607	PCL 1609	PCL 1611
Protease	+	+	+	+	+	+	+	+
Lipase	+	-	-	+	+	+	-	+
β-Glucanase	-	-	-	-	-	-	-	-
Cellulase	-	-	-	-	-	-	-	-
HCN	+	(+)	(+)	+	-	+	(+)	+
Antifungal metabolite <sup>b</sup>	PCA, PCN	-	-	PCA, PCN	HPR	PCA, PCN	-	PCA, PCN

<sup>a</sup> - = absence; + = presence; (\*) = very weak detection; NT = no tested.

<sup>b</sup> PCA = phenazine-1-carboxylic acid, PCN = phenazine-1-carboxamide, and HPR = 2-hexyl 5-propyl resorcinol.

(PCR)-amplified 16S rDNA fragments. These studies revealed that the selected rhizobacterial strains belong to the species *Pseudomonas chlororaphis*, *P. fluorescens*, and *P. putida* (Table 1).

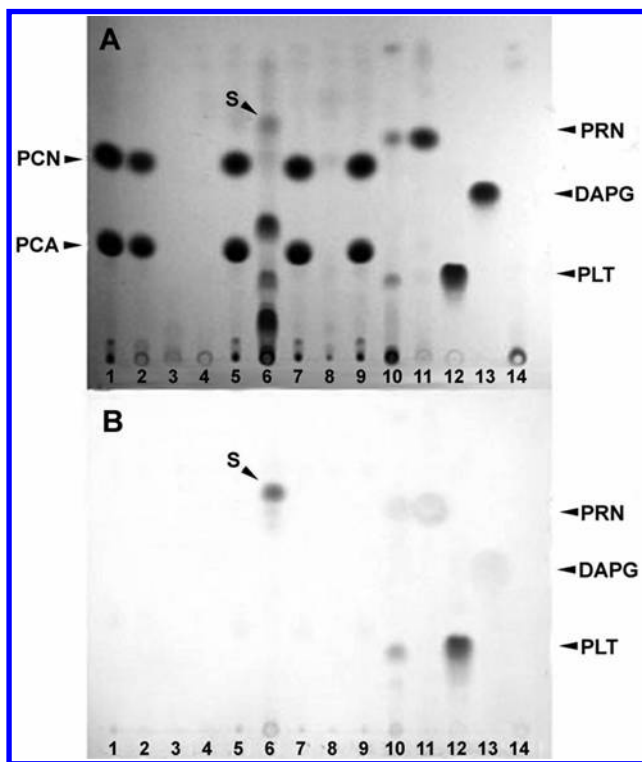
*P. fluorescens* PCL1606 and the four strains of *P. chlororaphis* (PCL1601, PCL1604, PCL1607, and PCL1611) showed the strongest antagonistic activity against nearly all the fungi tested (Table 1). None of the rhizobacterial isolates showed clear antifungal activity against the pathogen *Verticillium dahliae* (data not shown).

The sequence of the 16S DNA of PCL1606, the strain on which further studies focused, was submitted to the GenBank database under accession number DQ073039.

### Preliminary analysis of antifungal activities produced by the isolated

#### *Dematophora*-antagonistic *Pseudomonas* spp. strains.

The eight selected antagonistic strains were tested for the production of exoenzymatic activities (Table 2). All the rhizobacterial strains selected produced proteases. Lipase activity was produced by all *P. chlororaphis* strains and by *P. fluorescens* PCL1606. No  $\beta$ -glucanase or cellulase activities were detected as produced by the *Pseudomonas* strains. Strong HCN production was detected for *P. chlororaphis* strains, whereas very weak production was detected for *P. fluorescens* PCL1602 and *P. putida* strains PCL1603 and PCL1609 (Table 2).



**Fig. 1.** Silica thin-layer chromatography analysis of organic solvent extracts of cell-free culture supernatants of *Pseudomonas* strains from the rhizosphere of avocado. Compounds were visualized **A**, under ultraviolet light of 254 nm and **B**, under visible light after spraying with diazotized sulfanilic acid. Lane 1: *P. chlororaphis* PCL1391; lane 2: *P. chlororaphis* PCL1601; lane 3: *P. fluorescens* PCL1602; lane 4: *P. putida* PCL1603; lane 5: *P. chlororaphis* PCL1604; lane 6: *P. fluorescens* PCL1606; lane 7: *P. chlororaphis* PCL1607; lane 8: *P. putida* PCL1609; lane 9: *P. chlororaphis* PCL1611; lane 10: *P. fluorescens* Pf5; lanes 11 through 13 contain standards of pyrrolnitrin (PRN, lane 11), pyoluteorin (PLT, lane 12), and 2, 4-diacetylphloroglucinol (DAPG, lane 13); lane 14: *P. fluorescens* WCS365. The spot “S” corresponds to an unidentified antifungal compound, with an  $R_f$  value of 0.86, which is different from the  $R_f$  values of the major families of antifungal compounds and which was identified as described in this paper as 2-hexyl 5-propyl resorcinol.

For preliminary identification of antibiotics with antifungal properties, different extractions with organic solvents were carried out from cell-free supernatants of KB liquid cultures of the eight selected strains. Extracts were separated using silica thin-layer chromatography (TLC), and the purified antifungal compounds, PCA and PCN, PRN, PLT, and DAPG were used as standards for comparison. All *P. chlororaphis* strains and the control strain *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998) produced compounds with the same  $R_f$  values as PCA ( $R_f = 0.45$ ) and PCN ( $R_f = 0.78$ ). After observation under UV light and development of the TLC with diazotized sulfanilic acid (DASA), no compounds with similar  $R_f$  values to PRN, PLT, and DAPG were detected for any of the *Pseudomonas* spp. strains tested, and only the control strain *P. fluorescens* Pf5 produced PRN and PLT as well as very small amounts of DAPG. No TLC spots were detected in the extracts of *P. fluorescens* PCL1602 or *P. putida* PCL1603 and PCL1609 or the control strain *P. fluorescens* WCS365 (Fig. 1) (Simons et al. 1996). Strain *P. fluorescens* PCL1606 showed different spots under ultraviolet light, but only one of these was very reactive with DASA (Fig. 1, indicated with “S”). This spot with  $R_f = 0.86$  was the only spot able to inhibit the growth of *D. necatrix* 400 in plate tests after extraction of all detectable spots from TLC plates. In addition, this spot inhibited the growth of *F. oxysporum* in a TLC bioassay.

#### Biocontrol ability of the isolated *Pseudomonas* strains.

Based on the method of germination of avocado embryos (Pliego-Alfaro et al. 1987), sufficient numbers of avocado seedlings with very well-developed root systems were obtained, which showed homogeneous characteristics in biocontrol experiments. These avocado seedlings were used to develop a novel avocado-*Dematophora* biocontrol test system using growth substrates infected with *D. necatrix*.

The isolates *P. chlororaphis* PCL1601 and PCL1611 and *P. fluorescens* PCL1606, which formed the largest growth-inhibition zone (Table 1), appeared to possess the best biocontrol activity in the avocado-*Dematophora necatrix* test system when vermiculite was used as inert substrate (Fig. 2A). In these tests, the previously isolated PCN-producing *P. chlororaphis* PCL1391 and the presumed inducer of systemic resistance *P. fluorescens* WCS365 were included as additional strains. Of the isolated antagonistic strains, *P. fluorescens* PCL1606 and *P. chlororaphis* PCL1601 caused the largest reduction in the number of diseased plants. Reduction in the number of diseased plants was correlated with a large reduction in disease severity. When no bacteria were applied to the avocado roots, the plants growing in vermiculite infected with *D. necatrix* showed a disease index of 74%, whereas bacterization with the wild-type biocontrol strains *P. chlororaphis* PCL1601 and *P. fluorescens* PCL1606 reduced the disease indexes of the plants to 37 and 44%, respectively (Fig. 2A). Application of the bacterial strains in substrate without added pathogenic fungi did not result in disease or any other visible response of the plant (data not shown).

When potting soil was used as a complex substrate in the biocontrol experiments, with the avocado-*Dematophora* system using the same strains and procedures, similar results were obtained (Fig. 2B) but the disease levels were slightly lower than when vermiculite was used (66% diseased plants when no test bacteria were applied). Again, strains *P. fluorescens* PCL1606 and *P. chlororaphis* PCL1601 were the most protective strains against *Dematophora* root rot of avocado in potting soil (22 and 29% disease index, respectively). The control strains *P. chlororaphis* PCL1391 and *P. fluorescens* WCS365 also showed biocontrol activity in this system, resulting in disease incidences of 40 and 39%, respectively.

The selected rhizobacterial strains were also tested in a tomato-*F. oxysporum* f. sp. *radicis-lycopersici* biocontrol system (Fig. 3). When no bacteria were applied to the tomato seeds, more than 75% of the plants were diseased after 16 days of growth in soil infested with *F. oxysporum*, whereas coating with cells of the most protective strains in this test system, *P. chlororaphis* PCL1601 and *P. fluorescens* PCL1606, reduced disease incidence to 30 and 26%, respectively (Fig. 3). Other strains also significantly reduced the disease symptoms, with nearly all the *Pseudomonas* strains showing disease indexes below 60% and being significantly different from the uninoculated control (Fig. 3).

#### Structural analysis of the antifungal compound produced by *P. fluorescens* PCL1606.

To structurally identify the antifungal metabolite produced by *P. fluorescens* PCL1606, KB medium of a 4-day culture supernatant was extracted with chloroform/methanol (9:1). Antifungal activity of the spot migrating on TLC with an  $R_f$  value of 0.86 was extracted and tested for activity in the *D. necatrix* microtiter plate bioassay and by the *F. oxysporum* TLC bioassay. Subsequently, the activity was purified by high-performance liquid chromatography (HPLC) analysis, showing that the activity eluted from the HPLC column after 31 min as a single

peak (data not shown). In total, 10 mg of the antifungal compound was purified and used for structural analyses.

Accurate mass measurement of the HPLC-purified antifungal component of PCL1606, obtained using electron ionization on a magnetic sector mass spectrometer gave an  $m/z$  of 236.177, suggesting  $C_{15}H_{24}O_2$  as the most likely elemental composition; this has a double-bond equivalence of 4. Nuclear magnetic resonance (NMR) studies suggested the structure to be 2-hexyl-5-propylbenzene-1,3-diol (also referred to as HPR) (Table 3). The mass spectrometric data collected on this sample support this assignment (Fig. 4).

$^1H$ -NMR spectra of the purified HPLC fraction eluting at 31 min (Fig. 4) at 700 MHz produced eight resolvable features, which were then attributed to discrete carbon resonances via appropriate heteronuclear multiple quantum coherence (HMQC) spectra (Table 3). Notably, double quantum-filtered chemical shift-correlated spectroscopy measurements revealed the unambiguous presence of a propyl group in the isolated fraction (protons k, l, and m). The unique singlet of area 2 at  $\delta$  6.05 suggested the presence of a pair of equivalent aromatic protons ( $^{13}C$  for CH at  $\delta$  107) with both long- and short-range HMQC measurements confirming the presence of three further carbon signals at  $\delta$  141,  $\delta$  156, and  $\delta$  113 in this C6-based ring; none of the other carbons contained directly bound protons.

Examination of the proton spectrum (Table 2) revealed a triplet at  $\delta$  2.53 for a group that was adjacent to the ring carbon resonating at  $\delta$  113. This group forms part of a second ring substituent. A total of 11 proton resonances remained unaccounted for in the proton spectrum at this stage. A simple distortionless enhancement by polarization transfer experiment located the associated carbon resonances as four separate  $CH_2$  signals at  $\delta$  22.8,  $\delta$  29.4,  $\delta$  29.6, and  $\delta$  32.1 with a  $CH_3$  signal at  $\delta$  13.2. These proved to be characteristic of a hexyl group bound directly to the aromatic ring. These NMR spectra also contained an OH resonance at around  $\delta$  4.8 that disappeared on shaking with  $D_2O$ , suggesting that the product is the simple symmetrical diol 2-hexyl-5-propylbenzene-1,3-diol (Table 2).

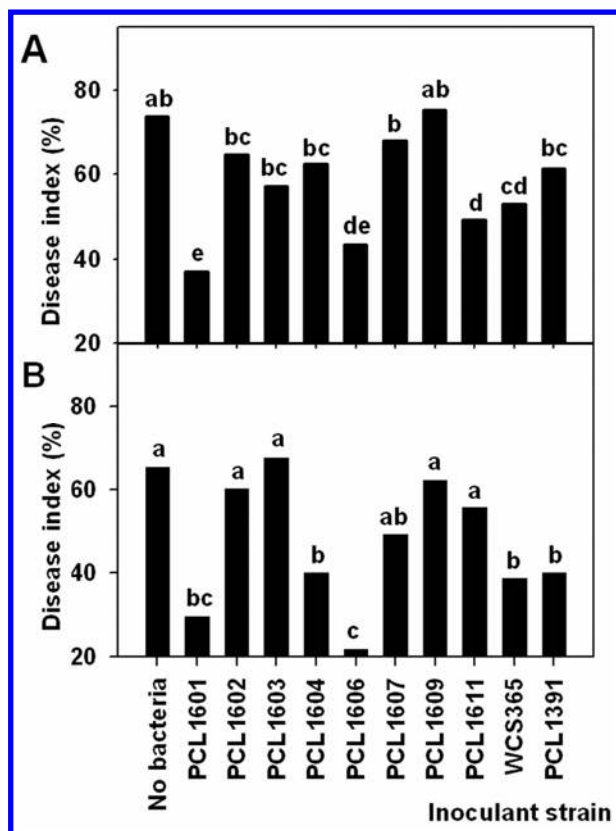


Fig. 2. Biocontrol of *Dematophora* root rot in avocado caused by *Dematophora necatrix* (teleomorph *Rosellinia necatrix*) by *Pseudomonas* strains isolated from the avocado rhizosphere. Eight strains isolated from the avocado rhizosphere as well as the previously described tomato foot and root rot biocontrol strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 were tested in a *D. necatrix*-avocado test system **A**, in vermiculite and **B**, in potting soil. Roots of avocado seedlings were inoculated with the different strains before transferring them to the different growth substances infested with *D. necatrix*. Plants were scored as sick or healthy after 18 to 21 days of growth after bacterization. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ( $P = 0.05$ ). Values of bars with different letter indications denote a statistically significant difference.

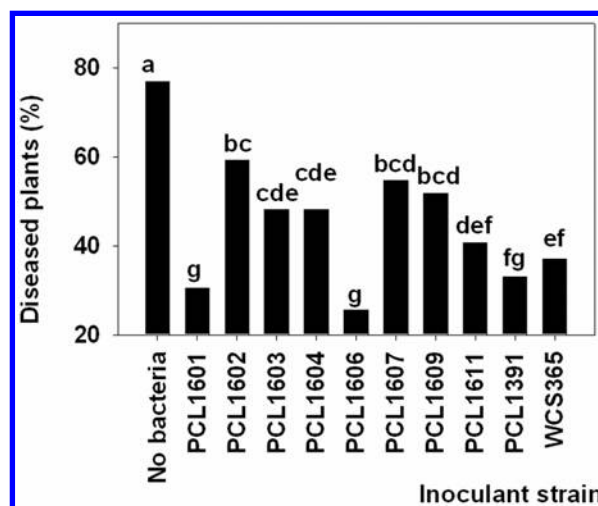


Fig. 3. Biocontrol of tomato foot and root rot caused by *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Eight *Pseudomonas* strains isolated from the avocado rhizosphere as well as the biocontrol strains *P. fluorescens* WCS365 and *P. chlororaphis* PCL1391 were tested in a *F. oxysporum*-tomato test system using potting soil. Bacteria were coated onto tomato seeds prior to sowing in soil infested with *Fusarium* spores. Plants were scored as healthy or sick after 21 days of growth. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ( $P = 0.05$ ). Values of bars with different letter indications represent a statistically significant difference.

Gas chromatographic-mass spectrometry (GC-MS) analysis of the component eluting at 31 min showed a single major peak. The very simple electron ionization mass spectrum of the component giving rise to that peak contained a signal for the  $M^+$  ion at  $m/z$  236 and only one major fragment ion at  $m/z$  165 (data not shown). This would be consistent with the loss of  $C_5H_{11}$  leaving a highly stable fragment ion with four resonance structures.

Electrospray ionization collision-induced dissociation tandem mass spectra ( $MS^1$ ) were collected using an ion trap mass spectrometer. In positive ion mode, the  $MH^+$  ion  $m/z$  237 was isolated and fragmented in the trap, to give three major fragment ions ( $m/z$  195, 153, and 111) that can be assigned to the loss of either or both of the alkyl side chains (Fig. 4). Spraying the sample from deuterated solvent showed peaks in the mass spectrum at  $m/z$  237, 238, 239, and 240, suggesting the presence of two exchangeable protons plus the charge-bearing species. Isolation and fragmentation of the  $m/z$  240 ion gave supporting evidence for this interpretation. Similarly  $MS^2$  spectra obtained in the negative ion mode from  $^1H$ - and  $^2H$ -containing solvents were also consistent with the parent ion at  $m/z$  235 having the proposed structure.

To confirm the presence of the two  $-OH$  groups, the sample was permethylated and the product was analyzed using the ion trap mass spectrometer. It would be expected that both of these

groups would be methylated, resulting in a total increase in mass of 28 Da. The positive ion mode mass spectrum showed an  $m/z$  265 ion, and the fragmentation pattern of that ion confirms the incorporation of the methyl groups onto the benzyl  $-OH$  groups, with major fragment ions at  $m/z$  139, 181, and 223.

#### Isolation of *P. fluorescens* PCL1606 derivatives impaired in antifungal metabolite production.

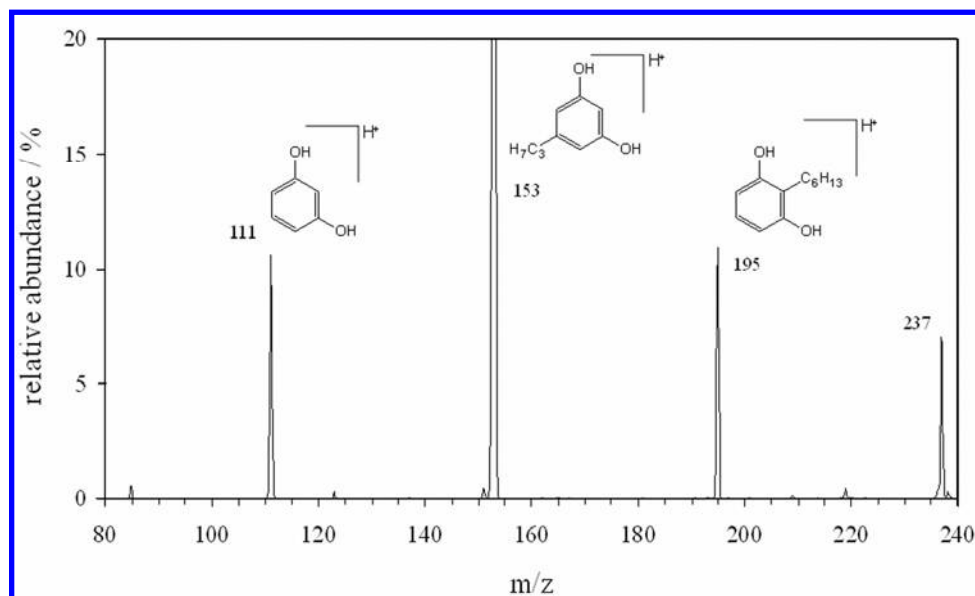
A PCL1606 Tn5-*luxAB* transposon library consisting of more than 5,000 transposants was screened for decreased antagonistic activity against *D. necatrix* in the agar plate assay and subsequently for HPR production. From this library, two mutants, PCL1613 and PCL1614, were identified that were impaired in antagonistic activity. PCL1613 showed much reduced inhibition of *D. necatrix* and *F. oxysporum*. For mutant PCL1614, no fungal inhibition was observed. HPR production by the mutants was analyzed using TLC of chloroform and methanol extracts of spent growth medium. PCL1613 showed a very small HPR spot, whereas PCL1614 showed no detectable HPR spot (data not shown). PCL1613 and PCL1614 were not altered in exoenzymatic production and growth rate in comparison with the wild-type strain (data not shown).

The chromosomal insertion site of the transposon in mutants PCL1613 and PCL1614 was determined after reisolation of

**Table 3.** Nuclear magnetic resonance (NMR) spectroscopy analysis and proposed structure of the purified antifungal compound produced by *Pseudomonas fluorescens* PCL1606<sup>a</sup>

Center	<sup>1</sup> H-NMR (700 MHz)		<sup>13</sup> C-NMR (176.0 MHz)		Structure
	δ	Area, mult, $J_{HH}/Hz$	δ	Area	
a	0.91	3H, t, 8	13.2	1 CH <sub>3</sub>	
b	{ 1.31	{ 6H, m	22.8	1 CH <sub>2</sub>	
c			32.1	1 CH <sub>2</sub>	
d			29.6	1 CH <sub>2</sub>	
e	1.47	2H, m	29.4	1 CH <sub>2</sub>	
f	2.53	2H, t, 8	23.0	1 CH <sub>2</sub>	
g			113.0	1 C	
h	4.80	2H, s	156.0	2 C	
i	6.05	2H, s	107.0	2 CH	
j			141.0	1 C	
k	2.37	2H, t, 8	38.0	1 CH <sub>2</sub>	
l	1.57	2H, sex, 8	24.6	1 CH <sub>2</sub>	
m	0.94	3H, t, 8	13.5	1 CH <sub>3</sub>	

<sup>a</sup> NMR signals from 2-hexyl 5-propyl resorcinol (HPR). Structure of HPR deduced from <sup>1</sup>H-NMR and <sup>13</sup>C-NMR signals is shown in the last column.



**Fig. 4.** Positive ion mode electrospray ion trap collision-induced dissociation mass spectral analysis and fragment ion assignment of precursor ion  $m/z$  237.

the transposon with adjacent chromosomal parts, which were sequenced and subsequently analyzed for the presence of open reading frames (ORF) and sequence homologies. The transposon in PCL1613 appeared to be inserted in an ORF that showed highest homology with predicted hydrolases or acyl-transferases (alpha/beta hydrolase superfamily) of *P. fluorescens* PfO-1 (73% identity at the amino acid level; protein identification number ZP 00262402). For PCL1614, it was shown that the transposon had inserted in an ORF with high homology to *gcvT* (89% identity at the amino acid level with *GcvT* of *P. fluorescens* PfO-1; protein identification number ZP 00264790). Chromosomal sequences adjacent to the Tn5 inserted in PCL1613 and PCL1614 have been submitted to the GenBank database under accession numbers DQ073040, DQ073041, DQ073042, and DQ073043, respectively.

#### Biological control of HPR mutants of *P. fluorescens* PCL1606.

Using the avocado-*D. necatrix* test system, it was shown that wild-type *P. fluorescens* PCL1606 is able to reduce the white root rot disease index from 73, in the untreated control, to 46% (Fig. 5A). Strains PCL1613 and PCL1614 did not suppress disease formation (Fig. 5A).

When the tomato-*F. oxysporum* foot and root rot test system was used, it was shown that wild-type *P. fluorescens* PCL1606 is able to reduce the number of diseased plants from 75, in the untreated control, to 29% (Fig. 5B). The derivative strain PCL1613 significantly suppressed disease formation but to a significantly

higher value (43% diseased plants) when compared with its wild-type strain PCL1606. For the derivative strain PCL1614, the disease level recorded was higher (52%) compared with PCL1613 but was still a statistically significant difference from the disease value observed in the untreated control.

## DISCUSSION

The isolation of rhizobacterial strains from the rhizosphere of healthy avocado trees present in an area affected by soil-borne phytopathogenic fungi represents a strategy for selecting microorganisms with antagonistic ability (Schroth and Hancock 1982). In this study, a set of eight *Pseudomonas* strains with high antagonistic ability against *D. necatrix* and other soil-borne fungi was isolated (Table 1). Various typing methods, including BIOLOG, API20NE, and comparisons of the (partial) 16S rDNA sequence provided consistent results in characterizing these strains (Table 1). Our results indicate that pseudomonads are one of the dominant culturable bacteria associated with the roots of avocado.

The selected antagonistic *Pseudomonas* spp. showed a very broad range of antagonistic activity against many phytopathogenic fungi tested, including *Phytophthora cinnamomi*, an oomycete that causes the most serious fungal disease on avocado plants worldwide (Erwin and Ribeiro 1996) and *D. necatrix*, which causes most of the crop losses in the Mediterranean area. The antagonistic activity of the isolated *Pseudomonas* spp. against the five strains of *D. necatrix* tested was generally high. The percentage of isolated antagonistic *Pseudomonas* sp. strains was similar to the percentage (1%) previously observed for isolation of antagonistic bacteria from the tomato rhizosphere of plants grown in southern Spain (Chin-A-Woeng et al. 1998).

Antifungal compounds produced by the *D. necatrix*-antagonistic *Pseudomonas* spp. were analyzed. Strains of *P. chlororaphis* (PCL1601, PCL1604, PCL1607, and PCL1611) produce PCA as well as PCN (Table 2 and Fig. 1), and these metabolites have been shown to be involved directly in the suppression of take-all disease of wheat, caused by *Gaeumannomyces graminis* var. *tritici* (Thomashow and Weller 1988) and in the inhibition of tomato foot and root rot caused by *F. oxysporum* f. sp. *radicis-lycopersici* (Chin-A-Woeng et al. 1998), and they can also enhance the biocontrol of non-PCA producing strains (Huang et al. 2004). *P. fluorescens* PCL1606 produced proteases, lipases, and an antifungal metabolite (Fig. 1, S) that is different from a range of defined compounds used as standards. Purification followed by structural analysis (Table 3, Fig. 4) showed that this antifungal metabolite is HPR, a compound with antimicrobial properties that could be related to PRN (Gaffney et al. 1994; Nowak-Thompson et al. 2003).

The *P. putida* strains PCL1603 and PCL1609 and *P. fluorescens* PCL1602 do not produce antifungal antibiotics but only a very small amount of HCN (Table 2), which could be responsible for the moderate antagonistic activity against the fungi studied (Table 1).

Some strains did not show biocontrol activity in either of the two biocontrol systems (Fig. 3), despite their antifungal activity on agar plate tests (Table 1). This lack of biocontrol activity in the rhizosphere environment in vitro by antagonistic strains has been reported before (Chin-A-Woeng et al. 1997) and could be due to poor colonization of the root system and, therefore, poor delivery of antifungal metabolites along the root system (Chin-A-Woeng et al. 2000; Lugtenberg et al. 2001). Alternatively, the production of antifungal metabolites, which is very sensitive to environmental conditions (Duffy and Defago 1999) may be reduced or not occur in the root environment (Fiddaman and Rossall 1994).

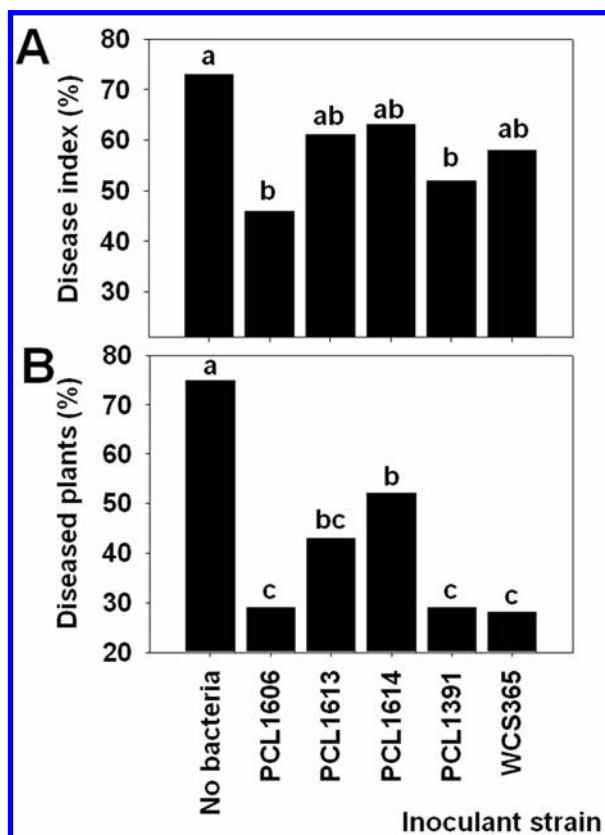


Fig. 5. Biocontrol ability of mutant derivatives of *Pseudomonas putida* PCL1606 impaired in antagonistic activity. Strains were tested on potting soil in **A**, an avocado-*D. necatrix* system and **B**, a tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* system. Biocontrol strains *Pseudomonas fluorescens* WCS365 and *P. chlororaphis* PCL1391 were tested as controls. Data were analyzed for significance after arcsine square root transformation with analysis of variance, followed by Fisher's least significant difference test ( $P = 0.05$ ). Values with different letter indications represent a statistically significant difference.

The strains *P. chlororaphis* PCL1601 and *P. fluorescens* PCL1606 appeared to have the strongest activity against *D. necatrix* of the eight avocado rhizobacterial *Pseudomonas* antagonistic strains (Fig.2). These strains were found to have a significant ability to biocontrol *Dematophora* root rot of avocado when grown in inert substrate vermiculite (Fig. 2A) as well as in complex substrate potting soil (Fig. 2B). The slightly lower levels of disease observed when using potting soil as a substrate could be due to the complex interactions of *D. necatrix* with either the substrate, other microorganisms, or both, as described for the phytopathogenic oomycete *P. cinnamomi* in the presence of organic material (Downer et al. 2002). The degree of biocontrol activity of the new isolates *P. chlororaphis* PCL1601 and *P. fluorescens* PCL1606 results in a lower disease index than that of the control strains *P. chlororaphis* PCL1391 (Chin-A-Woeng et al. 1998) and *P. fluorescens* WCS365 (Dekkers et al. 2000) (Figs. 2 and 3). This shows that these latter two strains, which are efficient in the tomato-*Fusarium* test system and which control tomato foot and root rot by different mechanisms, are not specific in controlling a particular disease but may be broadly applicable.

This is the first report of an avocado-*D. necatrix* biocontrol test system, used with the purpose of selecting plant growth-promoting rhizobacteria to be used as biocontrol agents.

The use of the tomato-*Fusarium* test system revealed that most of the newly isolated strains described in this paper gave a significant reduction in the disease symptom levels (Fig. 3). This observation suggests a higher sensitivity of the tomato-*Fusarium* test system to biocontrol than the avocado-*Dematophora* test system, but it shows that the utilization of the avocado-*Dematophora* test system is necessary to select good biocontrol strains for *D. necatrix*. The new isolates *P. chlororaphis* PCL1601 and *P. fluorescens* PCL1606 also appeared to be the best biocontrol strains in this tomato-*Fusarium* test system, with disease indices of 77% in plants without bacterial treatment and 26 and 30% in plants treated with *P. fluorescens* PCL1606 and *P. chlororaphis* PCL1601, respectively (Fig. 3), suggesting that these two strains can also be applied against different fungal pathogens and that their biocontrol activity is not specific.

Strain PCL1606, the most active of the antagonistic isolates selected, was found to have a significant biocontrol activity against *Dematophora* root rot of avocado (Figs. 2 and 5A) and foot and root rot of tomato (Figs. 3 and 5B). The antibiotic pro-

**Table 4.** Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Reference or source
<b>Bacterial strains</b>		
DH5 $\alpha$	General-purpose <i>Escherichia coli</i> host strain; donor strain of pRL1063a in conjugation experiments	Boyer and Roulland-Dussoix 1969
K12 HB101	General-purpose <i>E. coli</i> host strain; helper strain harboring the plasmid pRK2013, used in conjugation experiments	Figurski and Helinski 1979
PCL1391	Wild-type <i>Pseudomonas chlororaphis</i> , producing phenazine-1-carboxamide and biocontrol strain of tomato foot and root rot caused by <i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> .	Chin-A-Woeng et al. 1998
PCL1601	Wild-type <i>P. chlororaphis</i> , isolated from Spanish avocado rhizosphere	This study
PCL1602	Wild-type <i>P. fluorescens</i> , isolated from Spanish avocado rhizosphere	This study
PCL1603	Wild-type <i>P. putida</i> , isolated from Spanish avocado rhizosphere	This study
PCL1604	Wild-type <i>P. chlororaphis</i> , isolated from Spanish avocado rhizosphere	This study
PCL1606	Wild-type <i>P. fluorescens</i> , isolated from Spanish avocado rhizosphere; nal <sup>r</sup>	This study
PCL1607	Wild-type <i>P. chlororaphis</i> , isolated from Spanish avocado rhizosphere	This study
PCL1609	Wild-type <i>P. putida</i> , isolated from Spanish avocado rhizosphere	This study
PCL1611	Wild-type <i>P. chlororaphis</i> , isolated from Spanish avocado rhizosphere	This study
PCL1613	PCL1606 derivative in which a promoterless Tn5 <i>luxAB</i> has been inserted.	This study
PCL1614	PCL1606 derivative in which a promoterless Tn5 <i>luxAB</i> has been inserted.	This study
Pf5	Wild-type <i>P. fluorescens</i> , producing DAPG, PLT, and PRN, and biocontrol strain of cotton damping off caused by <i>Rhizoctonia solani</i> .	Howell and Stipanovic 1979
WCS365	Wild-type <i>Pseudomonas fluorescens</i> , efficient competitive colonizer of roots and biocontrol strain of tomato foot and root rot caused by <i>F. oxysporum</i> f. sp. <i>radicis-lycopersici</i>	Geels and Schippers, 1983; Simons et al. 1996
<b>Plasmids</b>		
pRL1063a	Plasmid harboring Tn5 transposon containing promoterless <i>luxAB</i> ; km <sup>r</sup>	Wolk et al. 1991
pRK2013	Contains RK12 genes; helper plasmid for mobilization on non-self transmissible plasmids; km <sup>r</sup>	Figurski and Helinski 1979

<sup>a</sup> nal<sup>r</sup> = nalidixic acid resistant, DAPG = 2,4-diacetylphloroglucino, PLT = pyoluteorin, PRN = pyrrolnitrin, and km<sup>r</sup> = kanamycin resistant.

**Table 5.** Fungal strains used in this study

Strain designation	Relevant characteristics <sup>a</sup>	Reference or source <sup>b</sup>
<b><i>Dematophora necatrix</i></b>		
400	Wild-type, isolated from Drr; High virulence	Pérez-Jiménez 1997
397	Wild-type, isolated from Drr; medium virulence	Pérez-Jiménez 1997
358	Wild-type isolated from Drr; low virulence	Pérez-Jiménez 1997
CECT2817	Isolated from avocado pear rot	CECT, Valencia, Spain
CECT2818	Isolated from avocado pear rot	CECT, Valencia, Spain
<b><i>Phytophthora cinnamomi</i></b>		
344	Wild-type, isolated from Prr; Low virulence	Pérez-Jiménez 1997
301	Wild-type, isolated from Prr; medium virulence	Pérez-Jiménez 1997
273	Wild-type, isolated from Prr; High virulence	Pérez-Jiménez 1997
<i>Pythium ultimum</i> LBOP17	Can cause avocado root rot, pathogen to many plants	IPO-DLO, Wageningen, The Netherlands
<i>Rhizoctonia solani</i> 3R4FNA	Can cause <i>Rhizoctonia</i> seed and root rot of avocado; pathogen to many plants	IPO-DLO, Wageningen, The Netherlands
<i>Sclerotium rolfsii</i> 151.31	Can cause avocado seedling blight, pathogen to many plants	CBS, Utrecht, The Netherlands.
<i>Verticillium dahliae</i> CECT2694	Can cause <i>Verticillium</i> wilt of avocado; pathogen to many plants.	CECT, Valencia, Spain.
<i>Fusarium oxysporum</i> f. sp. <i>radicis-lycopersici</i> ZUM2407	Causes crown and foot rot of tomato	IPO-DLO Wageningen, The Netherlands

<sup>a</sup> Drr = Avocado trees with *Dematophora* root rot; Prr = Avocado trees with *Phytophthora* root rot.

<sup>b</sup> CECT = Spanish type culture collection; IPO-DLO = Institute for Plant Protection-Agriculture Research Department; CBS = Fungal Biodiversity Center.

duced by *P. fluorescens* PCL1606 was shown to be HPR, an antifungal compound reported previously to be produced by *Pseudomonas* spp. with antimicrobial activity (Kanda et al. 1975; Kitahara and Kanda 1975; Nowak-Thompson et al. 2003). The role in biocontrol of HPR was studied by using derivative strains impaired in HPR production. The reduced biocontrol ability of HPR derivative mutants PCL1613 with reduced HPR production and PCL1614, a HPR-negative mutant, in the avocado-*D. necatrix* and tomato-*F. oxysporum* biocontrol test systems strongly suggests that HPR forms an essential determinant of the biocontrol mechanism of *P. fluorescens* PCL1606 (Fig 5). As far as we know, this is the first report of a *Pseudomonas* strain that produces HPR in which the production of this compound is shown to be crucial for biocontrol activity. Functions of the mutated genes or downstream genes present in the inserted operon in the production of HPR are being clarified in more detail in a follow-up study. The predicted gene product of the mutated gene of PCL1613 shows similarity to enzymes with acyltransferase activity, which could fit into a putative synthetic pathway of HPR, which contains acyl chains bound to the aromatic resorcinol group. Previous studies have described incorporation of acetate into the alkylresorcinol moiety (Achenbach et al. 1982; Sankawa et al. 1981).

For PCL1614, the mutated gene shows high homology with a glycine cleavage protein system, with an aminomethyl transferase activity, which can catalyze the transfer of an aminomethyl or methyl moiety from the substrates to ring-based molecules, such as H4folate (Masai et al. 2004), and could fit into a putative synthetic pathway of HPR by adding C atoms to the precursor molecule.

The disrupted genes in PCL1613 and PCL1614 are different from those described for the biosynthetic pathways of HPR in *Pseudomonas aurantiaca* (Nowak-Thompson et al. 2003), suggesting a new biosynthetic pathway (or a branch) for HPR synthesis in *P. fluorescens* PCL1606.

In both biocontrol systems, the disease suppression produced by the derivative mutants is lower than that obtained with the wild-type strain, although disease levels do not reach those of untreated plants. This could mean that other traits could also be involved in the biocontrol activity of PCL1606, such as colonization or induction of defense systems (Lugtenberg et al. 2001).

## MATERIALS AND METHODS

### Microorganisms, culture, and conditions.

The bacterial strains used in this study are listed in Table 4. KB was used routinely for culturing *Pseudomonas* spp. strains at 24°C. Luria-Bertani medium (LB) (Bertani 1951) was used for culturing *Escherichia coli* strains at 37°C. The bacterial strains were stored for preservation at -80°C in 15% glycerol. Media were supplemented with kanamycin to a final concentration of 50 µg/ml when required.

The fungal strains used in this study (Table 5) were grown at 24°C on potato dextrose agar (PDA) (Difco Laboratories, Detroit) or KB and were stored at 4°C in water. They were routinely replicated every six months on PDA to test viability.

### Isolation of bacteria from avocado rhizosphere.

Rhizobacterial strains were isolated from the rhizosphere of healthy avocado trees from areas affected by root pathogens. Roots were sampled from 20-year-old healthy avocado trees located in different orchards in Algarrobo, Fuengirola, and Vélez-Málaga (Málaga, Spain). The experimental sites were naturally affected by *Dematophora* root rot. In each sampling orchard, samples were taken from different types of soil (slate, clay, and sandy soils). Roots were collected from avocado trees at a distance of 1 m from the trunk and 10 cm from the

soil surface. The root samples were gently shaken to remove loosely adhering soil and were aseptically transferred to storage bags. The roots were maintained on ice and were transported to the laboratory, where they were immediately stored at -80°C before processing.

A total of 24 root samples were analyzed. Root samples were washed twice in tap water and were weighed and homogenized in a lab blender for 3 min with 10 ml of sterile phosphate buffered saline (PBS), pH 7.2, per gram of fresh root material. Suspensions were diluted 10-fold. In order to evaluate the amount of culturable bacteria, suspensions were plated on TSA (Oxoid, Perth, U.K.) amended with cycloheximide (100 µg/ml) to prevent fungal growth and on KB with cycloheximide and PI (Difco Laboratories, Detroit) to recover *Pseudomonas*-like strains. Plates were incubated at 24°C for 48 to 72 h. After incubation, colony numbers and morphology characteristics were recorded. The most abundant colonies with different morphology were plated on TSA and KB agar.

### Antagonistic activity.

Antagonistic activity of rhizobacterial isolates was tested as described previously (Geels and Schippers 1983). Initial screening for in vitro antifungal activity on KB and PDA agar plates against *D. necatrix* 400 was performed by placing on the agar in the center of a petri dish a 0.6-cm diameter mycelium disk from a 5-day-old fungal culture grown at 24°C, followed by inoculation of the bacterial strains at a distance of about 3 cm from the fungus. Bacterial strains inhibiting mycelial growth after five days of growth, as judged by a growth inhibition zone, were selected for further characterization. For the selected bacterial isolates, a second trial of in vitro inhibition of fungi was carried out against a number of other avocado and plant fungal pathogens (Table 5). These assays were performed in the same way as described above.

### Characterization of bacterial isolates.

A number of phenotypic and physiologic tests were performed to characterize the selected antagonistic bacterial isolates by using the API20NE (BioMerieux, Mercy L'etoyle, France) and BIOLOG (Biolog Inc., Hayward, CA, U.S.A.) systems. Further characterization was performed by analysis of the 16S rDNA sequence. For this, colony PCR was performed on the selected strains, using the primers 41F (5'-GCT CAG ATT GAA CGC TGG CG-3') and 1486-N (5'-GCT ACC TTG TTA CGA CTT CAC CCC-3'), described by Stackebrandt and Goodfellow (1991). A single colony from a 24- to 48-h culture on a KB plate was suspended in 20 µl of sterile water. Subsequently, 1.0 ml of a 0.05 M NaOH and 0.25% SDS (sodium dodecyl sulphate) solution was added and the mixture was incubated at 95°C for 15 min. Ten microliters of a 10-fold dilution of this suspension in sterile water was used as a template in a PCR reaction with a hot start for 120 s at 95°C, followed by 10 cycles consisting of 40 s at 94°C, 60 s at a decreasing temperature gradient of 1°C per cycle starting from 60°C, 120 s at 72°C, and subsequently, 25 cycles consisting of 40 s at 94°C, 60 s at 50°C, 120 s at 72°C, and a final extension of 72°C for 120 s. The resulting PCR fragment was purified (QIAquick PCR purification kit 50; Westburg, Leusden, The Netherlands) and was used directly for sequencing (Baseclear, Leiden, The Netherlands). The sequence was analyzed using DNAMAN software (Lynnon Biosoft, Quebec, Canada). Homology studies were carried out using National Center for Biotechnology Information GenBank Blast software (Bethesda, MD, U.S.A.).

### Characterization of antifungal metabolites.

The selected *Pseudomonas* strains were tested for production of HCN (Castric 1975), protease, lipase, β-glucanase, and



cellulase by general procedures described previously (Gerhardt 1994). Detection of antifungal metabolites was performed by silica TLC.

Analysis of phenazines was carried out after extraction of 2-day-old culture supernatants (KB and LB medium) with acetonitrile (Chin-A-Woeng et al. 1998). RP-18F<sub>2548</sub> TLC plates (Merck AG, Darmstadt, Germany) were developed with chloroform/acetone (9:1, vol/vol) for 2 h. The presence of spots with  $R_f$  values of 0.45 and 0.78 under UV light (254 nm) was indicative of the presence of PCA and PCN, respectively.

For detection of the antibiotics DAPG, PRN, and PLT, extractions of 4-day-old supernatant cultures were carried out with chloroform/methanol (2:1, vol/vol). Extracted material was fractionated by silica TLC using chloroform/acetone (9:1, vol/vol). After drying, the chromatogram was observed under UV light and was sprayed with DASA (Whistler et al. 2000). Antibiotics were preliminarily identified by their characteristic color and  $R_f$  values, corresponding to those of authentic standards: PLT,  $R_f$  = 0.32, brown; PRN,  $R_f$  = 0.81, maroon; and DAPG,  $R_f$  = 0.64, yellow.

To analyze the antifungal activity of the different fractions after chromatographic analysis, TLC plates were run in duplicate, and the different spots were located on one TLC plate while the other TLC plate was used to recover the fractions by extraction. To extract the spots, silica regions were scratched off the plate and were extracted overnight with acetonitrile/water (70:30 vol/vol). The extract was concentrated by evaporation, and its activity was tested against *D. necatrix* as follows. Extract (10  $\mu$ l) was placed on paper 0.5-cm diameter filter disks, which were dried and placed on a 12-well plate (Lucron Bioproducts, Gennep, The Netherlands) containing a layer of 250  $\mu$ l of KB agar on the bottom to avoid dehydration. Finally, a disk of mycelium was placed on the top of each disk. After three days, visual inhibition of fungal growth was recorded. To test the antifungal activity of the extracted fractions against *F. oxysporum*, a TLC bioassay was carried out as described previously (Chin-A-Woeng et al. 1998).

#### Growth of avocado plants for biocontrol tests.

To develop a new avocado-*D. necatrix* biocontrol test system, avocado plants were obtained by growing seedlings from avocado embryos (Pliego-Alfaro et al. 1987). Briefly, avocado seeds (cvs. Reed and Topa-Topa) were disinfected by immersion in a solution containing 0.5% sodium hypochlorite and 0.1% Tween 20. Subsequently, they were split into two separate cotyledons, excising the plumule-radicle axes together with 1- to 2-cm-thick cotyledon sections, and were transferred in sterile nutrient tubes containing Murashige and Skoog medium (Murashige and Skoog 1962), the pH of which was adjusted to 5.7 before adding 0.6% agar. Each 25  $\times$  150-mm tube contained 25 ml of nutrient medium. The tubes were capped with polypropylene stoppers (Bellco Glass, Inc., Vineland, NJ, U.S.A.) and were autoclaved. One plumule-radicle axis was placed in each tube, which was incubated in a growth chamber at 24°C with 16 h of daily light exposure.

After four weeks, the seedling shoots were removed from the tubes, the roots were washed with tap water and were placed in pots containing 30 g of wet perlite (Agra-perlite, Maasmond Westland B.A., Rijnsburg, The Netherlands). The seedlings were maintained in a growth chamber for 20 to 30 days in order to harden the roots, after which the avocado plants were ready to be used in biocontrol experiments.

#### Avocado-*Dematophora* biocontrol test.

*Dematophora necatrix* inoculum was produced on wheat seeds (Freeman et al. 1986). Briefly, the seeds were soaked for 12 h in 250-ml Erlenmeyer flasks filled with distilled water.

The flasks, each containing 100 ml of seeds, were subsequently autoclaved, after excess water had been drained off. After sterilization, three fungal disks of a 2-week-old culture of *D. necatrix* grown on KB were placed aseptically in each flask. Flasks were subsequently incubated at 24°C in the light for two weeks, unless otherwise stated, and were shaken every two to three days to avoid clustering of seeds.

Plants with hardened roots were removed from the perlite, and the roots were washed with tap water in order to remove remaining perlite. The roots of the seedlings were surface disinfected by immersion in 0.1% NaOCl for 20 min and were washed.

The roots were then bacterized, following a similar method to that previously described (Lugtenberg et al. 1994). Roots of avocado seedlings were immersed in a suspension of the bacterial isolate ( $10^9$  CFU/ml) or in sterile tap water as a control for 20 min. Excess suspension was allowed to drip off, and the seedlings were placed in pots containing 30 g of wet vermiculite or potting soil (Jongkind Grond B.V., Aalsmeer, The Netherlands) infected with *D. necatrix* using wheat grains (four infected grains per pot).

Fifty plants were tested per treatment in series of 10 plants each. Seedlings were grown in a growth chamber at 24°C, 70% relative humidity, and 16 h of daylight. Plants were watered twice per week from above (25 ml each time). The number of diseased plants was determined when a substantial number of the plants (>60%) in the untreated control were diseased, usually between 14 and 21 days after the bacterization.

Because of the difficulty of observing the symptoms on the hardened avocado roots and because of the broad development of *D. necatrix*, we calculated the disease index percentage of foliar symptoms. Disease index percentage was recorded after evaluation of aerial symptoms, with values ranging from 0 (healthy plant), 1 (yellowing and wilting of the leaves), 2 (overall drying of the plant), to 3 (dead plant), using the following previously described formula (Teixeira de Sousa 1985).

$$DI = 100 \times \frac{(a \times 0) + (b \times 1) + (c \times 2) + (d \times 3)}{3 \times n}$$

where  $a$ ,  $b$ ,  $c$ , and  $d$  correspond to the number of plants showing evaluation disease values of 0, 1, 2, and 3, respectively, and  $n$  is the total number of plants tested.

#### Biocontrol of tomato foot and root rot.

Tomato-*Fusarium oxysporum* f. sp. *radicis-lycopersici* bioassays were performed as described previously (Chin-A-Woeng et al. 1998). One third of a 10-day-old PDA plate culture of *F. oxysporum* f. sp. *radicis-lycopersici* was homogenized and inoculated in 200 ml of Czapek-Dox medium in a 1-liter Erlenmeyer flask. After growth for three days at 28°C under aeration (110 rpm), the fungal material was placed on top of sterile glass wool and the filtrate was adjusted to a concentration of 5.7 log spores per milliliter. For inoculation, spores were mixed thoroughly with potting soil to a final concentration of 6.5 log spores per kilogram.

Tomato (*Lycopersicon esculentum* Mill.) seeds (cv. Carmello) were coated with bacteria by dipping the seeds in a mixture of 1% (wt/vol) methylcellulose (Sigma, St. Louis) and 9 log CFU bacteria per milliliter in PBS buffer. Coated seeds were dried in a sterile stream of air. In a pot containing 25 g of soil, one seed was sown per pot, at a depth of approximately 1.5 cm. Ten series of 10 plants were tested per treatment. Seedlings were grown in a greenhouse at 24°C, 70% relative humidity, and 16 h of daylight. Plants were watered from the bottom. The number of diseased plants was determined when a substantial number of the plants (>60%) in the untreated control were diseased, usually 18 days after sowing. Plants were

removed from the soil and were washed, and the plant roots were examined for crown and root rot indicated by root browning and lesions. Roots without any disease symptoms were classified as healthy.

### Statistical methods.

Data were statistically analyzed using analysis of variance (Sokal and Rohlf 1986) followed by Fisher's least significant difference test ( $P = 0.05$ ), using SPSS software (SPSS Inc., Chicago). All experiments were performed at least twice.

### Purification of the antifungal compound produced by *P. fluorescens* PCL1606.

For the purification of the antifungal compound produced by *P. fluorescens* PCL1606, the antifungal spot was extracted from TLC as described above and was fractionated by HPLC performed with an Alltech Econosphere C8 column (5  $\mu\text{m}$ , 250  $\times$  4.6 mm) (Alltech Associates, Inc., Deerfield, IL, U.S.A.). Samples were eluted isocratically with 50% acetonitrile in water with a flow rate of 1 ml/min. UV detection was performed with a Pharmacia RSD 2140 diode array detector (Pharmacia, Uppsala, Sweden) with wave-length detection from 190 to 400 nm, and 1.0-ml fractions were collected for testing antifungal activity as previously described. Fractions exhibiting antifungal activity were dried and collected for mass spectrometry and NMR analysis.

### Mass spectrometric and NMR analyses.

NMR measurements were carried out on a Jeol ECA 700, using an inverse geometry 5-mm  $^1\text{H}$  and  $^{13}\text{C}$  probe (Jeol, Tokyo, Japan) and a Bruker AMX 500, using a Protasis microcoil probe. A Hewlett Packard (Palo Alto, CA, U.S.A.) 5890 MSD electron impact instrument was used for GC-MS analysis. Sample solution (1  $\mu\text{l}$ ) in methanol was injected using spitless injection, and separation was performed using a DB-5 MS column (0.32  $\times$  30m; J & W Scientific, Folsom, CA, U.S.A.), with helium as the carrier gas and the following temperature program: 40°C (held 1 min), followed by a linear temperature gradient at 6° per min to 240°C (held isothermal for 10 min). The accurate mass measurement was carried out on a VG Autospec (VG Analytical, Manchester, U.K.) standard magnetic sector instrument with voltage scanning and perfluorokerosene as calibrant, using electron ionization. Electrospray ionization tandem mass spectra were collected from samples dissolved in protonated or deuterated (following NMR) methanol containing 0.1% formic acid using a LCQ DECA XP Plus (Thermo Electron, Hemel Hempstead, U.K.) ion trap mass spectrometer. The electrospray voltage used was 4,500 V, the temperature of the heated inlet capillary was 185°C, and the sample solution was infused at 1 to 2  $\mu\text{l min}^{-1}$ . Permethylolation was carried out using the method of Ciucanu and Kerek (1984) as described by Boone and associates (1999).

### Isolation of PCL1606 mutants impaired in antagonistic activity.

A Tn5 transposon library of *P. fluorescens* PCL1606 was generated with pRL1063a harboring a promoterless Tn5-*luxAB* transposon (Wolk et al. 1991), as described by Kragelund and associates (1995). Conjugation experiments were carried out using *E. coli* DH5 $\alpha$  pRL1063a as donor strain, *E. coli* K12 HB101 harboring plasmid pRK2013 as helper, and *P. fluorescens* PCL1606 as recipient, selecting the transposants on TSA supplemented with nalidixic acid (15  $\mu\text{g ml}^{-1}$ ) and kanamycin (30  $\mu\text{g ml}^{-1}$ ). Antifungal mutants were identified by loss of antagonistic activity on a plate assay and loss of the antifungal spot as detected by the TLC detection analysis. The selected mutants were characterized for uniformity with the wild-type

strain by using the BIOLOG and API tests. Growth of these selected mutants in rich and minimal media was monitored, and the resistance or sensitivity to different concentrations of the antibiotics rifampicin (from 0 to 100  $\mu\text{g ml}^{-1}$ ), lysozyme (from 0 to 10,000  $\mu\text{g ml}^{-1}$ ), and erythromycin (from 0 to 1,000  $\mu\text{g ml}^{-1}$ ) was surveyed to avoid mutants with altered membrane permeability (López-Solanilla et al. 1998).

To isolate the DNA region flanking the Tn5, total genomic DNA was isolated and digested with *EcoRI*, which does not cut pRL1063a. Digested genomic DNA fragments were recircularized and were selected for kanamycin resistance, resulting in plasmids containing genomic DNA regions flanking the Tn5-*luxAB*. All DNA techniques were performed as described in Sambrook and Russel 2001.

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