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Potential Biological Control Activity and Genetic Diversity of *Pseudomonas syringae* pv. *syringae* Strains

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Abstract

A total of 71 *Pseudomonas syringae* pv. *syringae* strains obtained from herbaceous and woody plant species were evaluated for the *in vitro* inhibition activity against a broad spectrum of bacteria and fungi, and were classified into two distinct groups. The 53 strains of group A inhibited, to a different extent, all the tested micro-organisms: *Rhodotorula pilimanae*, *Bacillus megaterium*, *Salmonella* sp., *Listeria monocytogenes*, *P. syringae*, *Botritys cinerea*, *Penicillium digitatum*, *Alternaria alternata*, *Aspergillus ochraceus*, *Fusarium solani*. The 18 strains of group B inhibited the growth only of *B. megaterium*, *L. monocytogenes* and *P. syringae*. The culture filtrates of strains of group A, unlike those of group B, induced the necrosis of bean pods and showed *in vitro* antimicrobial activity. Strains of group A, on the contrary to those of group B, were moderately to highly virulent when artificially inoculated on citrus fruits. All strains of groups A and B were virulent on bean pods. No strain of the two groups was virulent on apple fruits and grapes. Applications of *P. s.* pv. *syringae* strains reduced postharvest green and blue mould in citrus and apple fruits, respectively. The efficacy of disease control by strains of group A was higher than that of strains of group B. One strain of group A and one from group B showed interesting prerequisites for use as a biocontrol agent towards *P. digitatum* and *P. expansum* on orange and apple fruits, respectively. The *syr*B gene was present in almost all strains. Repetitive-sequence polymerase chain reaction (PCR) using Enterobacterial Repetitive Intergenic Consensus (ERIC) primers and unweighted pair-group method with average linkage (UPGMA) analysis showed that the strains varied genetically and could be grouped into seven clusters.

Introduction

Pathogen resistance to fungicides and the concern for public safety have led to increased interest in alternative methods of plant disease control. This has augmented the interest for the use of micro-organisms or their antimicrobial products to limit the attack caused by phytopathogens. Biological control of postharvest pathogens is an emerging alternative to the use of synthetic chemicals (Wilson et al., 1991). Bacteria have several features that make them attractive candidates in this strategy: their abundance on the surface of plants, the large number of biologically active metabolites they produce and their ability to induce plant resistance.

Strains of *P. syringae* van Hall are effective as biocontrol agents against *Penicillium expansum* and *Botrytis cinerea* on pears (Janisiewicz and Marchi, 1992; Sugar and Spotts, 1999), *Monilinia fructicola* and *Rhizopus stolonifer* on peaches (Zhou et al., 1999), *P. digitatum* on citrus (Bull et al., 1997), *B. cinerea* on grape (Cirvilleri et al., 2000) and *P. expansum* on apples (Janisiewicz and Bors, 1995; Conway et al., 1999). *Pseudomonas syringae* strains produce two groups of cyclic lipodepsipeptides as secondary metabolites, the nonapeptides syringomycins (Segre et al., 1989), which are toxic to a wide range of organisms, including plants (Iacobellis et al., 1992; Lavermicocca et al., 1997), and the larger syringopeptins (Ballio et al., 1991). The production of lipodepsinonapeptides has been correlated with the ability to control plant diseases caused by fungal pathogens. *Pseudomonas syringae* strain ESC-10 is commercially available (BioSave-1000, EcoScience Corporation, Orlando, FL, USA) as a biocontrol agent against moulds of citrus caused by *P. digitatum* and *P. italicum*. This strain produces syringomycin E as it inhibits the *in vitro* growth of *Rhodotorula pilimanae*, *Geotrichum candidum*

and *P. digitatum* (Bull et al., 1998). Syringomycin E may be involved in the biological control of green mould of citrus.

The production of lipodepsinonapeptides can be detected by polymerase chain reaction (PCR) amplification using specific primers to amplify the *syr*B gene encoding these metabolites (Sorensen et al., 1998). Because the toxins are considered the main virulence factor of *P. s.* pv. *syringae*, this analysis can greatly assist the identification and characterization of putative *P. s.* pv. *syringae* strains.

The molecular typing of effective *P. syringae* biocontrol agent is an important prerequisite for possibly tracking their dispersal in the environment. Various molecular methods have been used to assess the genetic diversity of *P. syringae* strains, including restriction fragment length polymorphism (RFLP; Scholz et al., 1994), repetitive-sequences PCR (Louws et al., 1994), random amplified polymorphic DNA (RAPD; Clerc et al., 1998), pulsed-field gel electrophoresis (PFGE; Güven et al., 2004) and amplified fragment length polymorphism (AFLP; Manceau and Brin, 2003).

The objective of this study was to select strains of *P. syringae* with a putative high potential for biological control activity as well as a lack of harmful effects on plants. We have used repetitive-sequence PCR and Enterobacterial Repetitive Intergenic Consensus (ERIC) primer sets (Louws et al., 1994) to type the *P. s.* pv. *syringae* strains obtained from different woody and herbaceous plant species.

Materials and Methods

Bacterial and fungal strains

The *P. s.* pv. *syringae* strains we used are listed in Table 1. Some have been previously characterized and identified as *P. s.* pv. *syringae* (Cirvilleri et al., 2000; Scortichini et al., 2003). Other strains were isolated from lesions on orange (*Citrus sinensis* Osbeck), strelitzia (*Strelitzia reginae* Banks) artichoke (*Cynara scolymus* L) and strawberry (*Fragraia* × *ananassa* Duch.) tissues presumably infected by *P. syringae*. Small pieces of tissue taken from the margins of necrotic lesions were crushed in sterile water and aliquots were plated on medium B of King et al. (1954) (KB). Plates were incubated for 2–3 days at 25–27°C. The resulting fluorescent colonies were purified on nutrient agar (NA; Oxoid, Basingstoke, UK) and subsequently assayed for LOPAT characters (Lelliott et al., 1966) following the procedures described by Lelliot and Stead (1987). Other strains were obtained from bacterial plant disease laboratories or international culture collections. All strains were maintained in 15% glycerol at −80°C and subcultured on KB as needed. The following micro-organisms were used for *in vitro* and *in vivo* antagonistic activity: *R. pilimanae* ATTCC26423 and *Bacillus megaterium* ITM100 (kindly provided by P. Lavermicocca, Istituto di Tossine e Micotossine da Parassiti Vegetali, C.N.R., Bari, Italy); *Salmonella* sp. and *Listeria monocytogenes* (kindly provided by the Istituto Zooprofilattico Regionale, Catania, Italy); *Aspergillus ochraceus* ATCC18641; *P. syringae* 48SR2, isolated from citrus; *Botritys cinerea*, *P. digitatum*, *P. expansum*, *Alternaria alternata*, *Fusarium solani* isolated from diseased plants in our mycological laboratory. Fungal pathogens were stored on potato dextrose agar (PDA; Oxoid) slants at 4°C.

In vitro inhibitory activity of *P. s.* pv. *syringae* strains

The *P. s.* pv. *syringae* strains (Table 1) were tested for their inhibition activity on PDA agar plate assay against *R. pilimanae*, *B. megaterium*, *Salmonella* sp., *L. monocytogenes*, *P. syringae*, *B. cinerea*, *P. digitatum*, *A. alternata*, *A. ochraceus* and *F. solani*. Bacteria and fungi to be tested were grown, respectively, on NA for 48 h at 27°C and on PDA for 7–14 days at 25°C until sporulation. A 20 μl aliquot of the *P. s.* pv. *syringae* strains suspension obtained from 4-day-old cultures on NA (approximately 1×10^9 CFU/ml) was spotted on PDA plates, using two bacterial strains per plate. The plates were incubated for 4 days at 27°C. Then, they were over sprayed with a suspension containing approximately 1×10^6 CFU/ml of bacteria or 1×10^6 conidia/ml of fungi to be tested and incubated at 27°C for 2–4 days. The presence and size of a clear zone around *P. s.* pv. *syringae* colonies, indicating the inhibitory effect, was scored. All tests were repeated twice.

In vitro inhibitory activity of *P. s.* pv. *syringae* culture filtrates

Pseudomonas syringae pv. *syringae* strains were grown overnight in NA + 1% glucose (NAG) and 1 ml of bacterial suspension ($A_{600} = 0.3$) was added to 100 ml of medium IMM (Improved Minimal Medium) (Surico et al., 1988). After 14 days of incubation at 25°C in still culture and centrifugation (9000 ×***g***, 20 min), the supernatant was passed through a 0.22 μm Millipore filter (Millipore, Billerica, MA, USA) to obtain cell-free culture filtrates. In parallel, 500 μl of bacterial (approximately 1×10^7 CFU/ml) or conidial (approximately 1×10^5 CFU/ml) suspension were added to 15 ml of fluid PDA maintained at 45°C for 30 min. After mixing and agar solidification, 20 μl of culture filtrate were spotted onto agar surface (four spot per plate). The area of inhibition of the test organism induced by the culture filtrates of *P. s.* pv. *syringae* strains was measured after 2–4 days of incubation at 27°C. The assay was repeated twice.

Phytotoxicity assay

The phytotoxic activity of cell-free culture filtrates of the *P. s.* pv. *syringae* strains was evaluated on tobacco (*Nicotiana tabacum* L.) leaves and bean (*Phaseolus vulgaris* L.) pods. Cell-free culture filtrates, prepared as previously described, were inoculated into the mesophyll of fully expanded leaves of 4–5-week-old tobacco plants with a hypodermic syringe.

After injection, plants were maintained in the greenhouse at approximately 22°C and symptoms were evaluated 2 days after the inoculation. The bean pods were

Table 1
Original source and main characteristics of *Pseudomonas syringae* pv. *syringae* strains

Strains[a]	Host	Area of origin	Year	Antagonistic group[b]	*syr*B	ERIC group[c]
PVCT 10.2	*Citrus sinensis*	Italy	1990	A	+	5
PVCT 41_2	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 119_2	*Citrus sinensis*	Italy	1990	A	+	6
PVCT 130_1	*Citrus sinensis*	Italy	1990	A	+	5
PVCT 147_1	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 280_2	*Citrus sinensis*	Italy	1990	A	–	5
PVCT 281_1	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 282_1	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 285_1	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 290_2	*Citrus sinensis*	Italy	1990	A	+	7
PVCT 291_1	*Citrus sinensis*	Italy	1990	A	+	3
PVCT 293_1	*Citrus sinensis*	Italy	1990	A	+	7
PVCT 295_1	*Citrus sinensis*	Italy	1990	A	–	2
PVCT 310	*Citrus sinensis*	Italy	1990	A	+	7
PVCT 334	*Citrus sinensis*	Italy	2000	A	+	7
PVCT 335_2	*Citrus sinensis*	Italy	2000	A	+	7
PVCT 337_1	*Citrus sinensis*	Italy	2000	A	–	7
PVCT 337_2	*Citrus sinensis*	Italy	2000	A	+	2
PVCT 339_1	*Citrus sinensis*	Italy	2000	A	+	4
PVCT 48SR1	*Citrus sinensis*	Italy	1990	A	+	5
PVCT 48SR2	*Citrus sinensis*	Italy	1990	A	+	5
PVCT Al513	*Citrus sinensis*	USA (S.E. Lindow)	1986	A	+	6
ISF 242	*Citrus lemon*	Italy	1996	A	+	4
ISF 243	*Citrus reticulata*	Italy	1996	A	+	4
PVCT 23P	*Pirus communis*	Italy	1998	A	+	5
PVCT 26P	*Pirus communis*	Italy	1998	A	+	5
PVCT 46P	*Pirus communis*	Italy	1998	A	+	5
PVCT 76P	*Pirus communis*	Italy	1998	A	+	5
ISF 280	*Pirus communis*	Italy	1996	A	+	4
ISF 281	*Pirus communis*	Italy	1996	A	+	5
ISF 288	*Pirus communis*	Italy	1996	A	+	5
ISF 347	*Pirus communis*	Italy	1996	A	+	3
PVCT 1.2S	*Strelitzia reginae*	Italy	2000	A	+	5
PVCT 1.3S	*Strelitzia reginae*	Italy	2000	A	+	5
PVCT 1.4S	*Strelitzia reginae*	Italy	2000	A	+	3
PVCT B.I.	*Strelitzia reginae*	Italy	2004	A	–	7
ISF 106 = NCPPB 2426	*Prunus avium*	Switzerland		A	+	4
ISF 107 = NCPPB 1093	*Prunus armeniaca*	New Zealand		A	+	7
ISF 231	*Prunus armeniaca*	Italy	1996	A	+	4
ISF 290 = B3A	*Prunus persica*	USA (J.E. De Vay)		A	+	6
ISF 015 = NCPPB3869	*Laurus nobilis*	Italy	1992	A	+	4
ISF 282	*Castanea sativa*	Italy	1996	A	+	4
AID 48	*Fragaria* × *ananassa*	Italy	1988	A	+	1
HRI 1480A	*Pisum sativum*	UK		A	+	5
ISF 292	*Triticum aestivum*	USA (J.E. De Vay)		A	+	4
ISF 300	*Triticum aestivum*	Italy		A	+	4
ISF 304	*Triticum aestivum*	Italy	1996	A	+	4
ISF 309	*Triticum aestivum*	Italy	1996	A	+	4
ISF 310 = NCPPB2612	*Triticum aestivum*	New Zealand		A	+	4
ISF 355	*Hordeum vulgare*	Italy	1996	A	+	4
ISF 356	*Hordeum vulgare*	Italy	1996	A	+	4
ISF 359 = 475A	*Hordeum vulgare*	J.E. De Vay		A	+	4
ISF 293 = B359	*Setaria italica*	Australia (J.E. De Vay)		A	+	2
AID 122A	*Prunus amygdalus*	Italy	1988	B	+	4
ISF 291 = SY12	*Syringa vulgaris*	Japan (D.C. Gross)		B	+	2
AID 24	*Fragaria* × *ananassa*	Italy	1987	B	+	6
AID 33	*Fragaria* × *ananassa*	Italy		B	+	2
AID 76	*Fragaria* × *ananassa*	Italy	1988	B	+	5
PVCT B728a	*Phaseolus vulgaris*	USA (S.E. Lindow)	1986	B	+	6
ISF 286 = Y37	*Phaseolus vulgaris*	UK (G.L. Ercolani)		B	+	4
ISF 332	*Phaseolus vulgaris*	Italy	1996	B	+	4
PVCT 4	*Cynara scolimus*	Italy	1992	B	+	6
PVCT 40	*Cynara scolimus*	Italy	1992	B	+	6
PVCT 106	*Cynara scolimus*	Italy	1992	B	+	1
PVCT 120	*Cynara scolimus*	Italy	1992	B	+	1
PVCT 152	*Cynara scolimus*	Italy	1992	B	+	1
ISF 353 = ISF-PP2	*Capsicum annuum*	Italy	1997	B	+	4

Table 1
Continued

Strains[a]	Host	Area of origin	Year	Antagonistic group[b]	*syr*B	ERIC group[c]
ISF 284 = PSS61	*Triticum aestivum*	USA (D.C. Gross)		B	+	4
ISF 294 = W451	*Triticum aestivum*	USA (D.C. Gross)		B	+	4
ISF 295 = SD202	*Triticum aestivum*	USA (D.C. Gross)		B	+	4
ISF 357	*Hordeum vulgare*	Italy	1996	B	+	4

[a]AID: Agricultural Industrial Development, Catania, Italy; HRI: HRI Warwick, Wellesbourne, UK; ISF: C.R.A. Istituto Sperimentale per la Frutticoltura, Roma, Italy; NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK; PVCT: Plant Pathology, University of Catania, Italy.
[b]See Fig. 1 for antagonistic groups.
[c]See Figs 5 and 6 for ERIC patterns.

surface-sterilized with 2% sodium hypochlorite for 2 min and rinsed with sterile distilled water (SDW). Then, four punctures (four wounds for pod, four pods for strain) 1 mm wide and 3 mm deep, were made with a sterile needle, and 5 μl of culture filtrate was applied with a pipette. SDW alone served as control. After the inoculation, pods were placed on plastic trays and incubated in a humid chamber at 27°C for 6 days.

Pathogenicity tests

Yellow lemon (*C. lemon* Burm) cv. Femminello, orange (*C. sinensis* Osbeck) cv. Tarocco and mandarin (*C. reticulata* L.) cvs Fortuna, Simeto and 08/28/A were harvested at the typical commercial maturity at the experimental fields of the University of Catania. Green lemons cv. Femminello were also harvested and used for pathogenicity tests. Apple (*Malus domestica* Borkh.) cv. Golden Delicious fruit, grape (*Vitis vinifera* L.) cv. Italia berries and bean (*Ph. vulgaris* L.) pods were obtained from local markets. They were used immediately or held at 4°C for no longer than 1 week before use. Fruits were surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite and then rinsed with SDW. Four punctures, 1 mm wide and 3 mm deep, were made on each fruit with a sterile needle. Bacterial suspensions were prepared from cells grown on NA for 4 days at 27°C. The bacterial cultures were suspended in SDW and the concentrations were photometrically adjusted with sterile water to a cell density corresponding at $1–2 \times 10^9$ CFU/ml. A 20 μl drop of the bacterial suspension was placed on each wound and was allowed to dry. Control fruits were treated with SDW only. After the inoculation, fruits were placed on plastic packaging trays. The cavity trays isolate each fruit and prevent contact infections of adjacent fruits. To provide ample humidity for disease development, a wet paper towel was placed on empty cavity trays and the entire box was placed in a plastic bag. The bags were sealed and the fruits were incubated at 20°C. During incubation, the fruits were observed repeatedly and the width of necrosis was measured 7 days after inoculation. Each experiment was repeated twice.

***In vivo* antagonistic activity towards moulds**

All 71 *P. s.* pv. *syringae* strains were tested for the capacity to suppress the growth of *P. digitatum* (green mould) on orange, lemon and mandarin fruits, *P. expansum* (blue mould) on apple fruits and *B. cinerea* (grey mould) on grape berries. Before each experiment, fruits showing no visible wound were carefully hand selected and were washed with tap water, surface-sterilized by dipping for 2 min in 2% of sodium hypochlorite, rinsed with SDW and then air-dried. Fruits were wounded with a sterile needle to make two 2-mm deep and 5-mm wide wounds on their peel at the equatorial region. Four fruits per each strain were used. Grape berries were wounded only once and each treatment involved nine berries. Bacteria were grown for 4 days at 27°C on NA (Bull et al., 1997) and then were suspended in SDW and the concentrations adjusted with SDW to a cell density corresponding to $1–2 \times 10^9$ CFU/ml. Aliquots (20 μl) of cell suspensions were pipetted into each wound and allowed to dry for approximately 4 h. Subsequently, the wounds were inoculated with 20 μl of spore suspension (1×10^6 CFU/ml) of fungal pathogens. Fruits were then placed in plastic cavity packaging trays with 20–25 fruits per tray. The test was repeated four times. Control fruits were treated with SDW only. The number of wounds showing symptoms of infection was counted and incidence of disease was calculated 1 week after the inoculation. Disease severity was evaluated 1 week after the inoculation with an empiric scale (1 = no visible symptoms; 2 = initial soft rot; 3 = presence of mycelium; 4 = sporulation). In all experiments, disease severity data were converted to percentage midpoint values (Campbell and Madden, 1990), where 1 = 0%, 2 = 35%, 3 = 65% and 4 = 90%. The percentages of decayed tissues (incidence of disease) and disease severity ratings were subjected to an arcsine square root transformation before running the ANOVA. Subsequently, one-way ANOVA was performed. The mean values were separated using the Student–Newman–Keul's mean separation test, at $P \leq 0.05$. The percentages shown in the tables are untransformed data.

DNA preparation

Genomic DNAs of 71 *P. s.* pv. *syringae* strains were extracted and purified using the DNA Purification Kit (Puregene M-Medical, Cornaredo, Milano, Italy) following the manufacturers' instructions. Each strain was grown overnight in 5 ml of nutrient broth (Oxoid) at 27°C at 170 g. Cells were collected by centrifugation

at 5000 × ***g***, and washed twice with SDW. The DNA was quantified by electrophoresis on 0.7% agarose gel in the presence of an appropriate marker (lambda DNA/*Hin*d III; Promega, Madison, WI, USA).

Detection of the *syr*B gene

To detect *syr*B gene, the primer B1 (5′-CTTTCCGT-GGTCTTGATGAGG-3′) and B2 (5′-TCGATTTTG-CCGTGATGAGTC-3′), amplifying a 752 bp band, were used. The PCR amplification procedure described by Sorensen et al. (1998) was followed.

Repetitive-sequence PCR

Repetitive-sequence PCR using ERIC primer sets as described by Louws et al. (1994) was followed. The ERIC 1R (5′-ATG TAA GCT CCT GGG GAT TCA C-3′) and ERIC 2 (5′-AAG TAA GTG ACT GGG GTG AGC G-3′) primers were synthesized by Invitrogen-Life Technologies (Invitrogen-Life Technologies, Paisley, UK). The reaction mixture consisted of 1 μl of DNA template, 2.5 μl of 10X reaction buffer, 0.5 μl of dNTP mix (10 mM), 50 pmol of primer ERIC 1R, 50 pmol of primer ERIC 2, 1.25 μl of $MgCl_2$ (50 mM) and 1.5 U of *Taq* polymerase (Invitrogen-Life Technologies) made up to 25 μl with SDW. The reaction mixture was denatured for 7 min at 95°C and then subjected to 30 cycles of denaturation for 1 min at 94°C, annealing for 1 min at 52°C, extension for 8 min at 65°C and a final extension for 16 min at 65°C. The amplification products were separated by electrophoresis on 1.5% agarose (Bio-Rad, Hercules, CA, USA) in Tris-Acetate-EDTA (TAE) 1X buffer at 5 V/cm, over 4 h. The 1-kb DNA ladder (Invitrogen-Life Technologies) was used as a molecular size marker. The gels were stained with ethidium bromide (0.2 μg/ml). The ERIC–PCR patterns were visualized on a UV light transilluminator, photographed and digitalized by PowerShot G2 (Canon Inc, Lake Success, NY, USA). PCR amplification was performed in duplicate. Banding patterns were analysed using GELCOMPAR II software, version II (Applied Maths, Kortrijk, Belgium). Similarity coefficients were determined by using the Dice's coefficient (Dice, 1945). Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA).

Results

Isolation and identification

Eighteen *P. s.* pv. *syringae* strains were obtained from orange and strelitzia tissues. All strains producing fluorescence on KB medium were positive for levan production and hypersensitive reaction (HR) on tobacco leaves, and negative for the presence of oxidase and arginine dihydrolase and for potato rotting (LOPAT group 1A). The strains were further assessed for antagonistic activity, pathogenicity, presence of *syr*B gene and genomic fingerprinting together with the other strains obtained from national and international culture collections.

In vitro inhibitory activity of *P. s.* pv. *syringae* towards micro-organisms

The majority of the *P. s.* pv. *syringae* strains inhibited the growth of fungal and bacterial pathogens and the size of the inhibition zone varied depending on the test bacterium or the target fungus used (Fig. 1). All *P. s.* pv. *syringae* strains inhibiting the growth of *R. pilimanae* and suppressing to a different extent the growth of the others target micro-organisms, including the human pathogen *L. monocytogenes*, were included in the antagonistic group A (Table 1). Strains unable to inhibit *R. pilimanae* also showed differential antagonistic activity towards the tested micro-organisms and they were included in the antagonistic group B. However, strains of group B showed the widest inhibition zones against *B. megaterium*, *L. monocytogenes* and

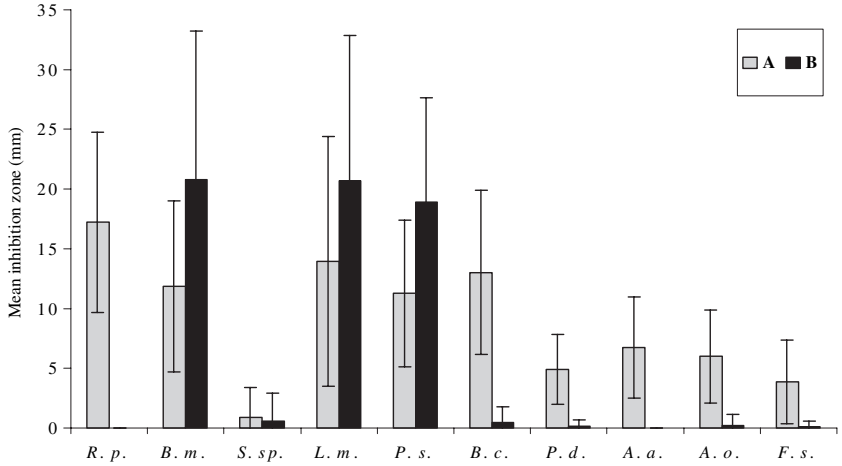

Fig. 1 *In vitro* antagonistic activity recorded as mean inhibition area (mm) showed by *Pseudomonas syringae* pv. *syringae* strains of groups A and B. Columns represent the mean of the zone of growth inhibition (mm) of *Rhodotorula pilimanae* (R.p.), *Bacillus megaterium* (B.m.), *Salmonella* sp. (S.sp.), *Listeria monocytogenes* (L.m.), *Pseudomonas syringae* (P.s.), *Botrytis cinerea* (B.c.), *Penicillium digitatum* (P.d.), *Alternaria alternata* (A.a.), *Al. ochraceus* (A.o.), *Fusarium solani* (F.s.). Vertical bars represent the standard deviation of the mean of four replications for each strain

Table 2
Pathogenicity tests performed with *Pseudomonas syringae* pv. *syringae* strains

Strain	Host	Fruit lesion diameter (mm) after inoculation on:[a]									
		Mandarin cv. Fortuna	Mandarin cv. Simeto	Mandarin cv. 08/28/A	Orange cv. Tarocco	Green lemon[b]	Yellow lemon[b]	Apple[c]	Grape[d]	Bean[e]	Group[f]
PVCT 10.2	*Citrus sinensis*	0.0^{a}	3.0^{a-c}	2.3^{a-d}	3.0^{d}	3.6^{ab}	8^{b-d}	0.0^{a}	0.0^{a}	+ +	A
PVCT 41_2	*Citrus sinensis*	0.0^{a}	1.6^{a-c}	5.6^{c-e}	0.5^{a}	3.0^{ab}	8^{b-d}	0.0^{a}	0.0^{a}	+	A
PVCT 119_2	*Citrus sinensis*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	n.t.	A
PVCT 130_1	*Citrus sinensis*	1.3^{ab}	1.0^{a-c}	8.0^{e}	0.0^{a}	4.3^{ab}	10^{cd}	0.0^{a}	0.0^{a}	+ + +	A
PVCT 147_1	*Citrus sinensis*	1.6^{ab}	1.0^{a-c}	6.6^{de}	3.0^{d}	5.6^{ab}	8^{b-d}	0.0^{a}	0.0^{a}	+ +	A
PVCT 280_2	*Citrus sinensis*	2.0^{ab}	1.3^{a-c}	2.0^{a-d}	4.0^{e}	1.6^{ab}	10^{cd}	0.0^{a}	n.t.	+ +	A
PVCT 281_1	*Citrus sinensis*	1.0^{ab}	1.6^{a-c}	3.6^{a-d}	3.0^{d}	7.6^{ab}	5^{b}	0.0^{a}	0.0^{a}	+ + +	A
PVCT 282_1	*Citrus sinensis*	1.0^{ab}	3.0^{a-c}	4.0^{a-e}	4.0^{e}	7.0^{ab}	5^{b}	0.0^{a}	0.0^{a}	+ +	A
PVCT 285_1	*Citrus sinensis*	3.0^{ab}	3.0^{a-c}	4.6^{b-e}	3.0^{d}	8.0^{b}	10^{cd}	0.0^{a}	0.0^{a}	+ + +	A
PVCT 290_2	*Citrus sinensis*	0.6^{ab}	3.0^{a-c}	3.3^{a-d}	0.0^{a}	2.0^{ab}	10^{cd}	0.0^{a}	0.0^{a}	+ + +	A
PVCT 291_1	*Citrus sinensis*	1.3^{ab}	1.3^{a-c}	4.3^{a-e}	0.0^{a}	2.3^{ab}	9^{b-d}	0.0^{a}	0.0^{a}	+ + +	A
PVCT 293_1	*Citrus sinensis*	0.6^{ab}	2.0^{a-c}	2.6^{a-d}	3.0^{d}	1.0^{ab}	5.0^{b}	0.0^{a}	n.t.	+ + +	A
PVCT 295_1	*Citrus sinensis*	1.3^{ab}	3.0^{a-c}	4.0^{a-e}	0.5^{a}	1.6^{ab}	8^{b-d}	0.0^{a}	n.t.	+ + +	A
PVCT 310	*Citrus sinensis*	1.0^{ab}	0.6^{ab}	4.0^{a-e}	0.0^{a}	2.6^{ab}	10^{cd}	0.0^{a}	n.t.	+ + +	A
PVCT 334	*Citrus sinensis*	2.0^{ab}	2.0^{a-c}	5.3^{d-e}	4.0^{e}	2.3^{ab}	8^{b-d}	0.0^{a}	n.t.	+ + +	A
PVCT 335_2	*Citrus sinensis*	3.0^{ab}	3.0^{a-c}	4.0^{a-e}	1.0^{b}	6.3^{ab}	8^{b-d}	0.0^{a}	n.t.	+ + +	A
PVCT 337_1	*Citrus sinensis*	2.0^{ab}	3.3^{bc}	4.6^{b-e}	0.0^{a}	1.6^{ab}	10^{cd}	0.0^{a}	n.t.	+ + +	A
PVCT 337_2	*Citrus sinensis*	0.6^{ab}	3.0^{a-c}	5.0^{b-e}	4.0^{e}	2.3^{ab}	7.5^{b-d}	0.0^{a}	n.t.	+	A
PVCT 339_1	*Citrus sinensis*	0.6^{ab}	4.0^{c}	4.0^{a-e}	0.0^{a}	1.0^{ab}	7.5^{b-d}	0.0^{a}	n.t.	+	A
PVCT 48SR1	*Citrus sinensis*	3.0^{ab}	3.0^{a-c}	4.0^{a-e}	4.0^{e}	4.3^{ab}	10^{cd}	0.0^{a}	0.0^{a}	+ +	A
PVCT 48SR2	*Citrus sinensis*	3.0^{ab}	3.6^{bc}	4.0^{a-e}	4.0^{e}	4.0^{ab}	10^{cd}	0.0^{a}	0.0^{a}	+ +	A
PVCT Al513	*Citrus sinensis*	1.3^{ab}	0.0^{a}	0.0^{a}	0.0^{a}	3.3^{ab}	0.0^{a}	0.0^{a}	0.0^{a}	+ +	A
ISF 242	*Citrus lemon*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	10^{cd}	0.0^{a}	n.t.	+ + +	A
ISF 243	*Citrus reticulata*	n.t.	n.t.	n.t.	1.0^{b}	n.t.	12^{d}	0.0^{a}	n.t.	+ + +	A
PVCT 23P	*Pirus communis*	3.6^{b}	2.6^{a-c}	4.0^{a-e}	2.0^{c}	2.6^{ab}	8^{b-d}	0.0^{a}	0.0^{a}	+ +	A
PVCT 26P	*Pirus communis*	3.3^{ab}	2.0^{a-c}	2.6^{a-d}	2.0^{c}	0.6^{ab}	10^{cd}	0.0^{a}	0.0^{a}	+ +	A
PVCT 46P	*Pirus communis*	0.0^{a}	2.0^{a-c}	0.0^{a}	2.0^{c}	0.0^{a}	10^{cd}	0.0^{a}	0.0^{a}	+ +	A
PVCT 76P	*Pirus communis*	2.6^{ab}	2.0^{a-c}	3.0^{a-d}	2.0^{c}	0.0^{a}	10^{cd}	0.0^{a}	0.0^{a}	+ +	A
ISF 280	*Pirus communis*	n.t.	n.t.	n.t.	4.0^{e}	n.t.	9^{b-d}	0.0^{a}	n.t.	+ + +	A
ISF 281	*Pirus communis*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	5^{b}	0.0^{a}	n.t.	+ + +	A
ISF 288	*Pirus communis*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	6.5^{bc}	0.0^{a}	n.t.	+ +	A
ISF 347	*Pirus communis*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	10^{cd}	0.0^{a}	n.t.	+ + +	A
PVCT 1.2S	*Strelitzia reginae*	3.0^{ab}	0.6^{ab}	5.0^{a-e}	1.0^{b}	1.3^{ab}	5^{b}	0.0^{a}	0.0^{a}	+ +	A
PVCT 1.3S	*Strelitzia reginae*	0.6^{ab}	2.0^{a-c}	1.3^{a-c}	0.0^{a}	2.0^{ab}	8.5^{b-d}	0.0^{a}	0.0^{a}	+ +	A
PVCT 1.4S	*Strelitzia reginae*	2.0^{ab}	3.6^{bc}	2.0^{a-c}	2.0^{c}	2.6^{ab}	5.5^{bc}	0.0^{a}	0.0^{a}	+ +	A
PVCT B.I.	*Strelitzia reginae*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	–	A
ISF 106 = NCPPB 2426	*Prunus avium*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ + +	A
ISF 107 = NCPPB 1093	*Prunus armeniaca*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ + +	A
ISF 231	*Prunus armeniaca*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ +	A
ISF 290 = B3A	*Prunus persica*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ + +	A
ISF 015 = NCPPB3869	*Laurus nobilis*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	12^{d}	0.0^{a}	n.t.	+ +	A
ISF 282	*Castanea sativa*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	8^{b-d}	0.0^{a}	n.t.	+ + +	A
AID 48	*Fragaria × ananassa*	2.3^{ab}	2.3^{a-c}	2.0^{a-c}	1.0^{b}	4.6^{ab}	12^{d}	0.0^{a}	0.0^{a}	+ +	A
HRI 1480A	*Pisum sativum*	1.0^{ab}	2.3^{a-c}	1.0^{ab}	1.0^{b}	1.3^{ab}	7.0^{bc}	0.0^{a}	0.0^{a}	+ +	A
ISF 292	*Triticum aestivum*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ +	A
ISF 300	*Triticum aestivum*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	7.0^{bc}	0.0^{a}	n.t.	+ + +	A
ISF 304	*Triticum aestivum*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ + +	A
ISF 309	*Triticum aestivum*	n.t.	n.t.	n.t.	0.5^{a}	n.t.	8^{b-d}	0.0^{a}	n.t.	+ + +	A
ISF 310 = NCPPB2612	*Triticum aestivum*	n.t.	n.t.	n.t.	1.0^{b}	n.t.	5.5^{bc}	0.0^{a}	n.t.	+ + +	A
ISF 355	*Hordeum vulgare*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	5.0^{b}	0.0^{a}	n.t.	+ +	A
ISF 356	*Hordeum vulgare*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	5.0^{b}	0.0^{a}	n.t.	+ +	A
ISF 359 = 475A	*Hordeum vulgare*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	12^{d}	0.0^{a}	n.t.	+ + +	A
ISF 293 = B359	*Setaria italica*	n.t.	n.t.	n.t.	2.0^{c}	n.t.	8^{b-d}	0.0^{a}	n.t.	+ + +	A
AID 122A	*Prunus amygdalus*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	+ +	B
SY12	*Syringa vulgaris*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	0.0^{a}	n.t.	+ +	B
AID 24	*Fragaria × ananassa*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	–	B
AID 33	*Fragaria × ananassa*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	+	B
AID 76	*Fragaria × ananassa*	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	0.0^{a}	+	B
PVCT B728a	*Phaseolus vulgaris*	1.3^{ab}	0.3^{ab}	0.0^{a}	0.0^{a}	6.3^{ab}	4.0^{b}	0.0^{a}	0.0^{a}	+ +	B
ISF 286 = Y37	*Phaseolus vulgaris*	n.t.	n.t.	n.t.	0.0^{a}	n.t.	0.0^{a}	3.0^{a}	n.t.	+ + +	B

Table 2
Continued

Strain	Host	Fruit lesion diameter (mm) after inoculation on:[a] Mandarin cv. Fortuna	Mandarin cv. Simeto	Mandarin cv. 08/28/A	Orange cv. Tarocco	Green lemon[b]	Yellow lemon[b]	Apple[c]	Grape[d]	Bean[e]	Group[f]
ISF 332	*Phaseolus vulgaris*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B
PVCT 4	*Cynara scolimus*	0.0[a]	0.0[a]	0.7[ab]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	++	B
PVCT 40	*Cynara scolimus*	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	++	B
PVCT 106	*Cynara scolimus*	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	++	B
PVCT 120	*Cynara scolimus*	0.0[a]	0.0[a]	0.7[ab]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	++	B
PVCT 152	*Cynara scolimus*	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	++	B
ISF 353	*Capsicum annuum*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B
ISF 284 = PSS61	*Triticum aestivum*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B
ISF 294 = W451	*Triticum aestivum*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B
ISF 295 = SD202	*Triticum aestivum*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B
ISF 357	*Hordeum vulgare*	n.t.	n.t.	n.t.	0.0[a]	n.t.	0.0[a]	0.0[a]	n.t.	++	B

The number refers to the mean diameter of lesion recorded 7 days after artificial inoculation of *Citrus* spp., apple, grape and bean fruits.
[a]Mean values are based on four replications per experiment. Each experiment was repeated twice. Number in column followed by the same letter are not significantly different using the Student–Newman–Keul's mean separation test at $P \leq 0.05$. Lesion diameter after 7 days at 22–24°C in wounded citrus, lemon, mandarin, apple and grape fruits inoculated with *P. syringae* strains.
[b]Lemon cv. Femminello.
[c]Apple cv. Golden Deliciosus.
[d]Grape cv. Italia.
[e] −, no sign of progressive lesion; +, lesion associated with inoculated sites; ++, progressive necrotic lesion; +++, progressive necrotic lesion and red halo on bean pods.
[f]Antagonistic group.

P. syringae (Fig. 1). Only 10 strains belonging to the groups A and B showed antagonistic activity against *Salmonella* sp.

Phytotoxic and antimicrobial activity of culture filtrates

Culture filtrates of the strains of group A inhibited the *in vitro* growth of *R. pilimanae*, *B. megaterium*, *B. cinerea*, *P. digitatum* and *F. solani*, but were phytotoxic to tobacco and bean pods. On bean pods they caused symptoms of different intensity, sometimes similar to those caused by the whole cells. Culture filtrates of group B strains did not cause necrosis on tobacco and bean pods, and did not show any *in vitro* inhibitory activity against all the target micro-organisms tested.

Pathogenicity tests

The results of pathogenicity tests (Table 2) showed that 33 and 44 of the 53 *P. s.* pv. *syringae* strains belonging to the antagonistic group A were pathogenic on orange and lemon fruits, respectively. Twenty-three, four and nine *P. s.* pv. *syringae* strains of group A were not pathogenic on orange, green and yellow lemons, respectively. By contrast, only one of 18 of group B strains induced necrosis on lemon fruits. The mean size of lesions caused by the strains of group A, ranging between 0.5 and 4 mm on orange, 0.6 and 8 mm on green lemon, 5 and 12 mm on yellow lemon, were 1.4, 2.9 and 6.9 mm, respectively (Fig. 2). Concerning mandarin, all except two of the 51 strains of group A incited necrotic symptoms, and lesions varied among the three

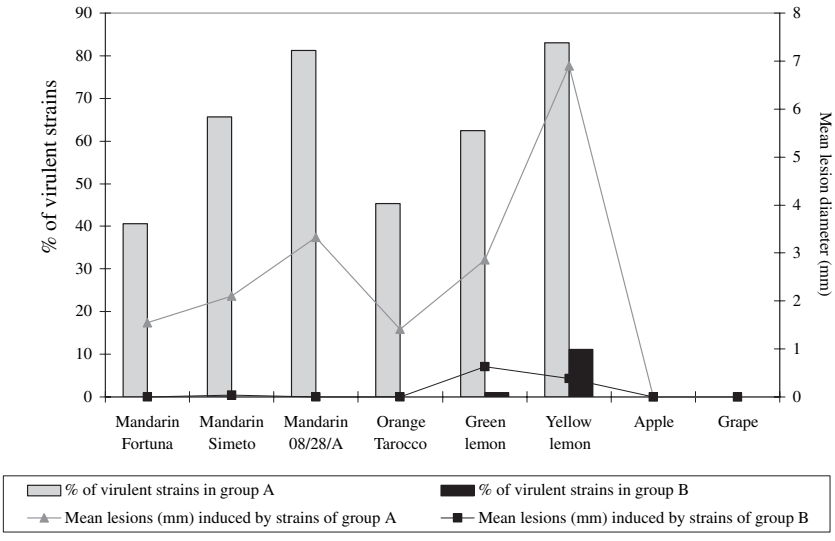

Fig. 2 Percentage of *Pseudomonas syringae* pv. *syringae* strains belonging to antagonistic groups A and B showing virulence recorded as mean diameter of lesions (mm) on fruits

cultivars tested. The group B strains were weakly or non-pathogenic. None of the strains of either group was pathogenic to apple fruits and grape berries, whereas almost all strains induced necrotic lesions on bean pods (Table 2). Strain PVCT 119_2 of group A and seven strains of group B did not incite any symptoms on any of the fruits used (Table 2). One strain of group B (AID 24) also did not cause any symptom on bean pods.

***In vivo* antagonistic activity towards moulds**

Several *P. s.* pv. *syringae* strains from both groups showed activity in controlling decay of lemon, orange, mandarin and apple fruits caused by *P. digitatum* and *P. expansum*, respectively (Fig. 3). The incidence of disease (percentage of infected wounds) observed on citrus and apple fruits was significantly lower than the control ($P < 0.05$). By contrast, no effect was observed on grape berries and so was similar to that of the control. The *P. s.* pv. *syringae* strains of group A showed an antagonistic activity to green mould significantly higher than that showed by group B strains (Fig. 3). The lemon, orange and mandarin fruits inoculated with *P. digitatum* and subsequently treated with group A strains showed a mean incidence of disease (percentage of infected wounds) of 44.5%, whereas treatments with group B strains resulted in a mean incidence of disease of 76.4%. Only 28.7% and 27.2% of lesions on apples were decayed after treatment with strains of groups A and B, respectively, thus demonstrating the high antagonistic activity of both groups.

Approximately 63% of *P. s.* pv. *syringae* strains belonging to groups A and B completely inhibited *P. digitatum* on lemon (zero infected wounds; Fig. 4), whereas 25% reduced disease incidence (≤50% of infected wounds) and 11% were totally ineffective (100% of infected wounds). About 24% of strains completely inhibited *P. digitatum* on orange, 22% reduced the incidence of disease (≤49%) and 53% caused a disease incidence between 46% and 100%. No strain of either group totally inhibited *P. digitatum* on mandarin and only 21% reduced the incidence of disease (≤33% of infected wounds); 53% completely inhibited *P. expansum* on apples and 39% reduced the incidence of disease. Only five strains were inactive. No strain was active against *B. cinerea* on grape.

On lemon wounds the addition of the *P. s.* pv. *syringae* strains significantly reduced disease severity compared with the control which was inoculated only with *P. digitatum* (sporulation after 7 days; Table 3). In fact, 45 *P. s.* pv. *syringae* strains showed zero disease severity (no symptoms), and 17 strains strongly reduced the severity of disease that ranged between 17.5% and 45% (the infected wounds showed soft rot). Six strains weakly reduced the severity of disease that ranged between 68.1% and 80.6% (infected wounds with mycelium), and only two strains were totally ineffective

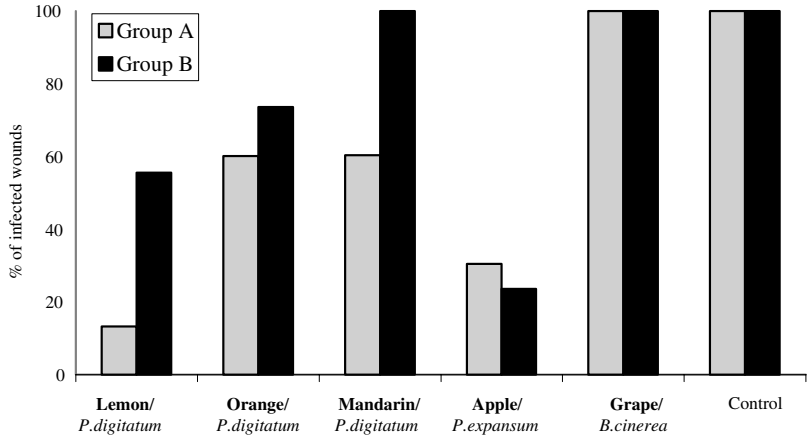

Fig. 3 Incidence of disease (percentage of infected wounds) on *Citrus* spp., apple fruits and grape berries after inoculating with *Penicillium digitatum*, *P. expansum* and *Botrytis cinerea*. The wounds were subsequently treated with *Pseudomonas syringae* pv. *syringae* strains. Values are mean of four replicates, two replicates for experiment

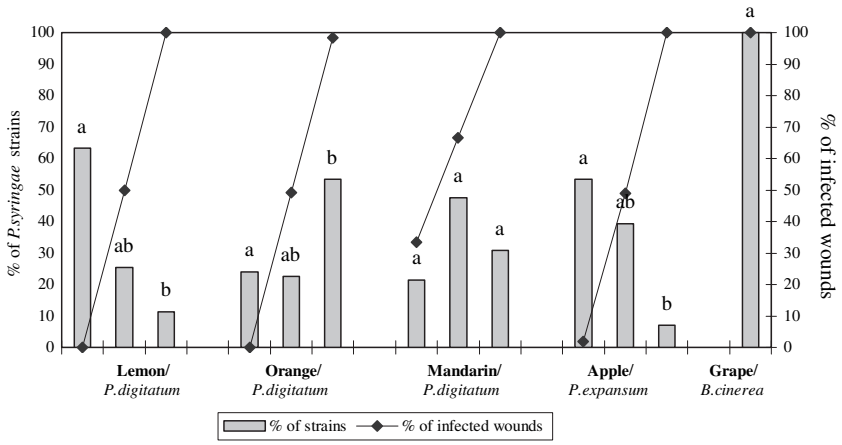

Fig. 4 Percentage of *Pseudomonas syringae* pv. *syringae* strains with antagonistic activity and corresponding incidence of disease (percentage of decayed wounds) on *Citrus* spp., apple and grape fruits. Columns with the same letter are not significantly different using the Student–Newman–Keul's mean separation test at $P \leq 0.05$. Mean values of four replications and four fruits per replication

Table 3
Disease severity of green (*Penicillium digitatum*), blue (*P. expansum*) and grey (*Botrytis cinerea*) mould 7 days after artificial inoculation of *Citrus* spp., apple and grape fruits and subsequently treated with *Pseudomonas syringae* pv. *syringae* strains

Strains	Host	Disease severity[a] (%)				
		Lemon/ *P. digitatum*	Orange/ *P. digitatum*	Mandarin/ *P. digitatum*	Apple/ *P. expansum*	Grape/ *B. cinerea*
Control		90[e]	90[f]	90[a]	90[h]	90[f]
PVCT 10.2	*Citrus sinensis*	0.0[a]	17.5[ab]	60.0[a]	0.0[a]	n.t.
PVCT 41_2	*Citrus sinensis*	45.0[b-d]	35.0[b-e]	60.0[a]	8.1[a]	72.2[b-e]
PVCT 119_2	*Citrus sinensis*	0.0[a]	8.8[a]	30.0[a]	0.0[a]	n.t.
PVCT 130_1	*Citrus sinensis*	0.0[a]	35.0[b-e]	60.0[a]	0.0[a]	68.3[a-e]
PVCT 147_1	*Citrus sinensis*	45.0[b-d]	24.4[a-c]	60.0[a]	8.1[a]	69.4[a-e]
PVCT 280_2	*Citrus sinensis*	0.0[a]	17.5[ab]	30.0[a]	0.0[a]	n.t.
PVCT 281_1	*Citrus sinensis*	0.0[a]	17.5[ab]	60.0[a]	0.0[a]	71.4[b-e]
PVCT 282_1	*Citrus sinensis*	0.0[a]	0.0[a]	60.0[a]	0.0[a]	73.9[c-e]
PVCT 285_1	*Citrus sinensis*	0.0[a]	24.4[a-c]	60.0[a]	4.4[a]	63.8[a-d]
PVCT 290_2	*Citrus sinensis*	0.0[a]	0.0[a]	60.0[a]	0.0[a]	64.8[a-d]
PVCT 291_1	*Citrus sinensis*	0.0[a]	24.4[a-c]	60.0[a]	0.0[a]	64.4[a-d]
PVCT 293_1	*Citrus sinensis*	0.0[a]	0.0[a]	60.0[a]	0.0[a]	n.t.
PVCT 295_1	*Citrus sinensis*	0.0[a]	0.0[a]	30.0[a]	0.0[a]	n.t.
PVCT 310	*Citrus sinensis*	0.0[a]	0.0[a]	60.0[a]	21.3[a-d]	n.t.
PVCT 334	*Citrus sinensis*	0.0[a]	35.0[b-e]	60.0[a]	8.1[a]	n.t.
PVCT 335_2	*Citrus sinensis*	0.0[a]	41.9[b-e]	30.0[a]	21.3[a-f]	n.t.
PVCT 337_1	*Citrus sinensis*	0.0[a]	35.0[b-e]	30.0[a]	30.0[a-f]	n.t.
PVCT 337_2	*Citrus sinensis*	32.5[a-c]	35.0[b-e]	30.0[a]	0.0[a]	n.t.
PVCT 339_1	*Citrus sinensis*	0.0[a]	17.5[ab]	30.0[a]	0.0[a]	n.t.
PVCT 48SR1	*Citrus sinensis*	0.0[a]	0.0[a]	60.0[a]	4.4[a]	90.0[f]
PVCT 48SR2	*Citrus sinensis*	0.0[a]	24.4[a-c]	30.0[a]	0.0[a]	57.2[a]
PVCT Al513	*Citrus sinensis*	45.0[b-d]	24.4[a-c]	90.0[a]	0.0[a]	59.2[ab]
ISF 242	*Citrus lemon*	0.0[a]	30.6[b-d]	n.t.	0.0[a]	n.t.
ISF 243	*Citrus reticulata*	0.0[a]	35.0[b-e]	n.t.	26.9[a-d]	n.t.
PVCT 23P	*Pirus communis*	0.0[a]	0.0[a]	60.0[a]	30.6[a-f]	n.t.
PVCT 26P	*Pirus communis*	0.0[a]	0.0[a]	60.0[a]	0.0[a]	n.t.
PVCT 46P	*Pirus communis*	0.0[a]	62.5[d-f]	60.0[a]	39.4[a-f]	n.t.
PVCT 76P	*Pirus communis*	0.0[a]	0.0[a]	60.0[a]	24.4[a-d]	n.t.
ISF 280	*Pirus communis*	0.0[a]	35.0[b-e]	n.t.	47.5[b-g]	n.t.
ISF 281	*Pirus communis*	0.0[a]	0.0[a]	n.t.	12.5[a-c]	n.t.
ISF 288	*Pirus communis*	77.5[de]	0.0[a]	n.t.	31.9[a-f]	n.t.
ISF 347	*Pirus communis*	17.5[ab]	65.0[d-f]	n.t.	8.8[ab]	n.t.
PVCT 1.2S	*Strelitzia reginae*	0.0[a]	35.0[b-e]	60.0[a]	40.0[a-f]	n.t.
PVCT 1.3S	*Strelitzia reginae*	0.0[a]	56.9[d-f]	30.0[a]	0.0[a]	n.t.
PVCT 1.4S	*Strelitzia reginae*	0.0[a]	17.5[ab]	60.0[a]	0.0[a]	n.t.
PVCT B.I.	*Strelitzia reginae*	0.0[a]	71.3[ef]	90.0[a]	0.0[a]	90.0[f]
ISF 106 = NCPPB 2426	*Prunus avium*	45.0[b-d]	8.8[a]	n.t.	15.6[a-c]	n.t.
ISF 107 = NCPPB 1093	*Prunus armeniaca*	0.0[a]	67.5[ef]	n.t.	27.5[a-d]	n.t.
ISF 231	*Prunus armeniaca*	77.5[de]	59.4[d-f]	n.t.	16.3[a-c]	n.t.
ISF 290 = B3A	*Prunus persica*	0.0[a]	0.0[a]	n.t.	0.0[a]	n.t.
ISF 015 = NCPPB3869	*Laurus nobilis*	0.0[a]	17.5[ab]	n.t.	0.0[a]	n.t.
ISF 282	*Castanea sativa*	0.0[a]	17.5[ab]	n.t.	25.6[a-e]	n.t.
AID 48	*Fragaria × ananassa*	35.6[a-c]	35.0[b-e]	90.0[a]	48.8[c-h]	60.0[a-c]
HRI 1480A	*Pisum sativum*	0.0[a]	38.8[b-e]	60.0[a]	30.6[a-f]	n.t.
ISF 292	*Triticum aestivum*	0.0[a]	53.1[de]	n.t.	0.0[a]	n.t.
ISF 300	*Triticum aestivum*	0.0[a]	35.0[b-e]	n.t.	32.5[a-f]	n.t.
ISF 304	*Triticum aestivum*	0.0[a]	90.0[f]	n.t.	4.4[a]	n.t.
ISF 309	*Triticum aestivum*	38.8[a-c]	90.0[f]	n.t.	53.8[c-h]	n.t.
ISF 310 = NCPPB2612	*Triticum aestivum*	0.0[a]	35.0[b-e]	n.t.	68.1[f-h]	n.t.
ISF 355	*Hordeum vulgare*	80.6[de]	21.9[a-c]	n.t.	31.9[a-f]	n.t.
ISF 356	*Hordeum vulgare*	0.0[a]	35.0[b-e]	n.t.	86.9[h]	n.t.
ISF 359 = 475A	*Hordeum vulgare*	0.0[a]	53.8[de]	n.t.	80.6[gh]	n.t.
ISF 293 = B359	*Setaria italica*	0.0[a]	0.0[a]	n.t.	52.5[d-h]	n.t.
AID 122A	*Prunus amygdalus*	80.6[de]	90.0[f]	90.0[a]	67.5[e-h]	77.5[d-f]
SY12	*Syringa vulgaris*	90.0[e]	0.0[a]	n.t.	0.0[a]	n.t.
AID 24	*Fragaria × ananassa*	90.0[e]	90.0[f]	90.0[a]	53.8[c-h]	77.2[d-f]
AID 33	*Fragaria × ananassa*	45.0[b-d]	63.1[d-f]	90.0[a]	17.5[a-d]	71.9[b-e]
AID 76	*Fragaria × ananassa*	35.6[a-c]	74.4[ef]	90.0[a]	13.1[a-c]	74.6[de]
PVCT B728a	*Phaseolus vulgaris*	0.0[a]	38.8[b-e]	90.0[a]	0.0[a]	n.t.
Y37	*Phaseolus vulgaris*	0.0[a]	0.0[a]	n.t.	0.0[a]	n.t.
ISF 332	*Phaseolus vulgaris*	45.0[b-d]	0.0[a]	n.t.	8.8[ab]	n.t.
PVCT 4	*Cynara scolimus*	28.1[ab]	68.1[ef]	90.0[a]	0.0[a]	90.0[f]
PVCT 40	*Cynara scolimus*	35.6[a-c]	63.1[d-f]	90.0[a]	23.8[a-d]	90.0[f]
PVCT 106	*Cynara scolimus*	45.0[b-d]	52.5[de]	90.0[a]	0.0[a]	90.0[f]
PVCT 120	*Cynara scolimus*	80.6[de]	58.8[d-f]	90.0[a]	67.5[e-h]	69.7[a-e]

Table 3
Continued

Strains	Host	Disease severity[a] (%)				
		Lemon/ *P. digitatum*	Orange/ *P. digitatum*	Mandarin/ *P. digitatum*	Apple/ *P. expansum*	Grape/ *B. cinerea*
PVCT 152	*Cynara scolimus*	45.0^{b-d}	74.4^{ef}	90.0^{a}	15.6^{a-c}	79.2^{ef}
ISF 353	*Capsicum annuum*	32.5^{a-c}	0.0^{a}	n.t.	0.0^{a}	n.t.
ISF 284 = PSS61	*Triticum aestivum*	17.5^{ab}	90.0^{f}	n.t.	0.0^{a}	n.t.
ISF 294 = W451	*Triticum aestivum*	0.0^{a}	21.3^{ab}	n.t.	0.0^{a}	n.t.
ISF 295 = SD202	*Triticum aestivum*	68.1^{c-e}	73.8^{ef}	n.t.	0.0^{a}	n.t.
ISF 357	*Hordeum vulgare*	45.0^{b-d}	49.4^{c-e}	n.t.	0.0^{a}	n.t.

[a]Mean values of four replications and four fruits per replication. Numbers in columns followed by the same letter are not significantly different using the Student–Newman–Keul's mean separation test at $P \leq 0.05$. In all replicated experiment disease severity ratings were converted to percentage midpoint values, where 0% = no symptoms; 35% = soft rot; 65% = mycelium; 90% = sporulation. Before running ANOVA, percentages of infection were subjected to an arcsine square root transformation. The percentages shown in the table are untransformed data. One-way ANOVA was performed.

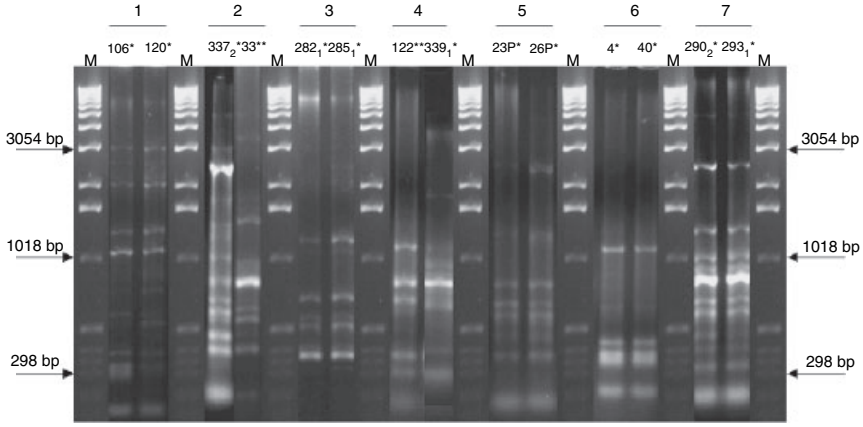

Fig. 5 Repetitive-sequence polymerase chain reaction (PCR) using Enterobacterial Repetitive Intergenic Consensus (ERIC) primer patterns of *Pseudomonas syringae* pv. *syringae* strains representing the seven main cluster. Amplification with ERIC primers (ERIC 1R and ERIC 2) yielded 6–10 distinct PCR products, ranging in size from approximately 150 to over 3000 bp and allowed differentiation of strains. Ladder used (M) was 1 kb (Invitrogen-Life Tecnologies). *PVCT strains; **AID strains

and sporulation occurred on infected wounds. On orange wounds, 17 strains showed zero disease severity (no symptoms) and 21 strains strongly reduced the severity of disease that ranged between 8.8% and 35% (only soft rot was observed). Eighteen *P. s.* pv. *syringae* strains weakly reduced the severity of disease to between 52.9% and 90% (mycelium development was observed). Only five strains failed to reduce the severity of disease, and sporulation was observed on infected wounds. On mandarin 08/28/A, nine strains strongly reduced (30% of severity) and 30 weakly reduced (60% of severity) the severity of disease compared with the control. On apple wounds 32 strains totally inhibited the disease, 27 strains strongly reduced the disease severity that ranged between 8.1% and 32.5%, seven strains reduced the severity of disease (from 39.4% to 53.8%) and only for five strains the average of severity was weakly reduced (between 67.5% and 86.9%). On grape, addition of antagonists did not consistently reduce the severity of disease; all of the tested strains showed a disease severity higher than 59.0%.

Detection of the *syr*B gene

PCR amplification with primers B1 and B2 gave rise to a 752 bp band indicating that the *syr*B gene was present. Only five strains failed to show amplification and hence did not possess the *syr*B gene (Table 1).

Repetitive-sequence PCR

The PCR amplification performed with primers ERIC 1R and ERIC 2 showed the presence of several PCR products with molecular weights ranging from 150 to over 3000 bp. The fingerprints revealed a high degree of genetic diversity among the *P. s.* pv. *syringae* strains analysed. A representative gel is illustrated in Fig. 5. A total of 10 reproducible, clearly resolved bands were used for cluster analysis. UPGMA analysis pointed out seven main clusters (Fig. 6). Two clusters (2 and 7) included strains all belonging to the antagonistic group A, two clusters (3 and 7) included 75% of strains obtained from *Citrus* spp. The other clusters included strains obtained from different host plant species and belonging to either the antagonistic groups A or B. There was no correlation between the strains and the host plant from where they were originally isolated.

Discussion

The *P. s.* pv. *syringae* strains evaluated in this study showed differential *in vitro* antagonistic activity towards the tested micro-organisms. Strains showing a broader activity were placed in the group A, whereas those with reduced antagonistic activity were included in group B. The application to wounds on citrus and apple fruits inoculated with *P. digitatum* and *P. expan-*

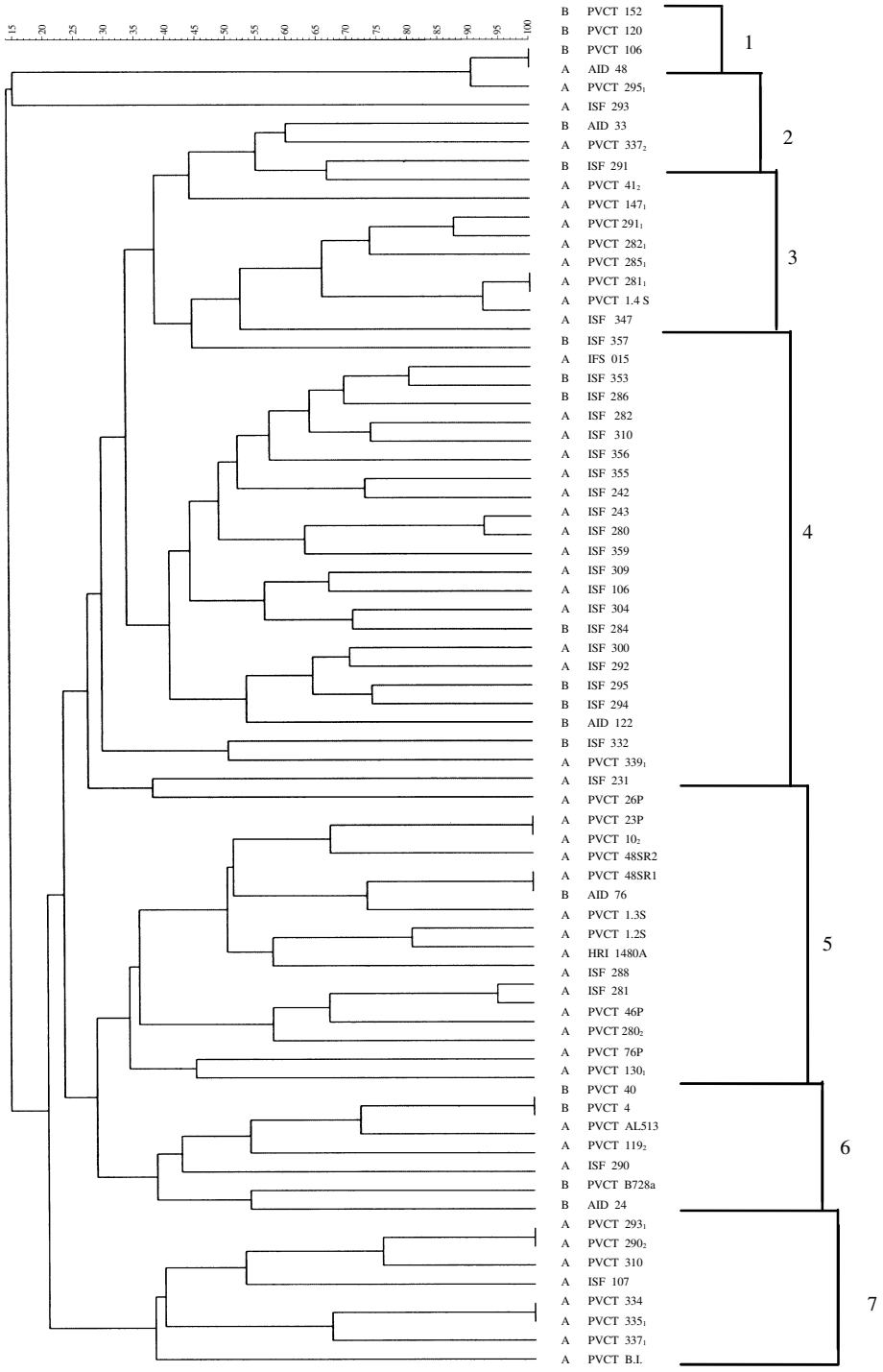

Fig. 6 Dendrogram of relationships among *Pseudomonas syringae* pv. *syringae* strains obtained using repetitive-sequence polymerase chain reaction (PCR) and Enterobacterial Repetitive Intergenic Consensus (ERIC) primer sets. PCR products in the range of 150–3000 bp were compared by numerical analysis using GELCOMPAR II software. Similarity between fingerprints was calculated with the Dice's coefficients using the unweighted pair-group method with average linkages (UPGMA)

sum of strains of both *P. s.* pv. *syringae* groups was effective in reducing the incidence and severity of disease. However, the antagonistic action on the A and B strains appeared different. Thus, strains of group A showed antagonistic activity against the above pathogens either *in vitro* or *in vivo* whereas strains of group B were active only in *in vivo* assays.

Establishing a correlation between *in vitro* inhibition of the target pathogens and biological control *in vivo* is the first step for demonstrating the involvement of antibiotic(s) in biological control (Handelsman and Parke, 1989). Inhibition zones of different areas on PDA, a medium conductive to toxin production by *P. syringae* strains, were observed among the tested strains. This probably confirms that regulation of toxin production is a variable feature in a bacterial species, as previously observed (Bultreys and Gheysen, 1999). Lipodepsipeptides inhibited the growth of a broad spectrum of bacteria and fungi (Lavermicocca et al., 1997). *Rhodotorula pilimanae* allows the detection of low concentration of both syringomycins and syringopeptins, whereas *B. megaterium* allows the detection of syringopeptins but not syringomycins (Lavermicocca et al., 1997). As both

toxins are simultaneously produced by field strains investigated so far (Vassilev et al., 1996), the zones of inhibition we observed in the biological tests should most likely be attributed to a mixture of lipodepsipeptides.

Wounds on fruits, the primary infection site for moulds, are readily colonized by *P. syringae* strains that survive and more easily colonize wounds than the fruit surface. In fact, *P. syringae* ESC-10 survived well in wounds on orange and lemon fruits and reduced the incidence of green and blue moulds and quickly became undetectable on the surface of fruits (Bull et al., 1997).

Because some *P. s.* pv. *syringae* strains are pathogenic to *Citrus* spp. and other plant species (Schaad et al., 2001), biological control strains should be approved only after the evaluation of their virulence on a large range of plants. The pathogenicity of strains precludes their use as biological control agents, as previously reported with the antagonistic strain *P. s.* pv. *syringae* 485-10 (Bull et al., 1997). Consequently, the purpose of this study was also to investigate the pathogenicity of several strains of *P. s.* pv. *syringae* selected as putative effective biological control agents. The ability of some strains to control green and blue mould of citrus and apple fruits was not associated with significant virulence to fruits, and, very often, only small brown lesions were observed at the inoculation sites. The development of brown areas at the inoculation sites probably indicates a plant defence reaction involving oxidation of phenolic compounds, as previously reported with antagonistic strains of *P. syringae* and *P. cepacia* (Huang et al., 1993; Burkowicz and Rudolph, 1994).

Pseudomonas syringae strains obtained from lesions can occasionally be virulent pathogens on non-host plants (Schaad et al., 2001). In the present study, differently sized brown lesions were observed on mandarin, orange and lemon fruits after inoculation with some *P. s.* pv. *syringae* strains from *Citrus* spp., pear, strelitzia (antagonistic group A), whereas strains isolated from almond, strawberry, bean and artichoke (antagonistic group B) did not produce any lesions on fruits.

Although our tests showed that some strains are effective biological control agents under laboratory conditions, full-scale commercial evaluation is needed to demonstrate the possible value of these agents to the citrus and apple industry. Further testing of the best strains here selected is needed under commercial conditions, including assessment of biological control efficacy, population dynamics of these antagonists on fruits, and observations of the quality of treated fruits.

The commercially available *P. syringae* strains ESC-10 and ESC-11 (Bull et al., 1997) do not control green mould on citrus and blue mould on apple fruits to the same extent. In fact, the HR-positive, weak pathogen (ESC-10) was less effective on apple but controlled green mould on citrus better than the HR-negative, non-pathogenic strain (ESC-11).

A characteristic unique to almost all *P. s.* pv. *syringae* strains is that they have genes homologous to *syr*B and *syr*D that are responsible for the production and export of syringomycins and syringopeptins toxins, respectively (Quigley and Gross, 1994). These genes are conserved as single copies in the bacterial genome of the toxin-producing *P. s.* pv. *syringae* strains (Quigley and Gross, 1994). PCR for the detection of *syr*B and *syr*D genes have been applied to identify *P. syringae* strains that produce lipodepsipeptides (Sorensen et al., 1998; Bultreys and Gheysen, 1999; Scortichini et al., 2003). In this study, the majority of *P. syringae* strains, either producing and not producing *in vitro* compound(s) inhibiting *R. pilimanae*, *B. megaterium*, *P. digitatum* and *B. cinerea*, showed DNA sequences homologues to the *syr*B gene, suggesting that the majority of the *P. syringae* strains we used belong to *P. s.* pv. *syringae*. PCR amplification of the *syr*B fragment could be exploited for further applications such as the detection and quantification of lipodepsipeptides-producing bacteria in host plants.

Repetitive-sequence PCR using ERIC primer sets allowed the typing of the *P. s.* pv. *syringae* strains evaluated for the antagonistic activity. This technique enables the characterization of bacterial species at the strain level (Louws et al., 1994) and also seems useful for possibly tracking the behaviour of the biocontrol agents in the environment. Such a capability would result very important to verify the presence of the biocontrol agent in the long-term period under commercial conditions. The possibility that the traditional techniques (i.e. isolation and biochemical tests) could not properly detect other pseudomonads colonizing the fruits under storage conditions cannot be eliminated. In addition, the present study revealed relevant genetic diversity among the 71 *P. s.* pv. *syringae* strains and, as previously observed using repetitive-sequence PCR and BOX primer (Scortichini et al., 2003), no clear relationship was demonstrated between a strain and the host plant from where it was originally obtained.

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