Analysis of *Pseudomonas syringae* Populations and Identification of Strains as Potential Biocontrol Agents Against Postharvest Rot of Different Fruits

G. Cirvilleri[1], G. Scuderi[1], A. Bonaccorsi[1], and M. Scortichini[2]

Abstract A collection of Pseudomonas syringae strains was evaluated for in vitro and in vivo growth inhibition of a broad spectrum of bacteria and fungi, for production of hydrolytic enzymes, presence of syrB gene and for pathogenicity on detached fruits and then they were genetically characterized by ARDRA, ERIC-PCR and fAFLP.

P. syringae strains and their culture filtrates inhibited a wide range of phytopathogenic bacteria and fungi *in vitro* and the inhibition depended on the bacterial and fungal strain. The cell-wall degradation enzymes *N*-acetyl-β-D-glucosaminidase (NAGase), β-glucosidase, cellobiohydrolase, cellulase and protease were detected in culture filtrates of the majority of strains while only a few strains showed chitinase and glucanase activities. The antagonistic activity *in vivo* of *P. syringae* strains – evaluated on wounded lemon, orange and mandarin fruits against *Penicillium digitatum*, on apples against *P. expansum*, on grape berries against Botrytis cinerea – was apparent for some of the strains evaluated. *P. syringae* strains isolated from *Citrus* spp., pear and strelitzia were moderately to highly pathogenic on tested detached citrus fruits.

All *P. syringae* strains showed the presence of *syr*B gene and their molecular characterization with ARDRA, ERIC-PCR and fAFLP allowed their clustering in distinct groups. A partial correlation between groups delineated on the basis of antagonistic activity *in vitro* and genomic fingerprints was also apparent. Fluorescent AFLP analysis also produced characteristic profiles for each strains useful for risk assessment, monitoring and identification of released antagonistic strains.

Keywords *Pseudomonas syringae*, antagonistic activity, ARDRA, ERIC-PCR, fAFLP

[1] Dipartimento di Scienze e Tecnologie Fitosanitarie- Sezione Patologia Vegetale, Università degli Studi di Catania, Via S. Sofia 100, 95100 Catania, Italy

[2] C.R.A.-Istituto Sperimentale per la Frutticoltura, Via di Fioranello, 52, I-00040 Ciampino aeroporto, Roma, Italy

Author for correspondence: Gabriella Cirvilleri; e-mail: gcirvil@unict.it

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1 Introduction

Fruits and vegetables suffer significant losses after harvest due to fungal diseases (Snowdon, 1990, 1991). Traditionally, postharvest diseases are controlled by postharvest application of synthetic fungicides (Eckert and Ogawa, 1988). However, the development of resistance to fungicides and the concern for public safety have led to increase interest in alternative and effective natural methods of postharvest diseases control.

Biological control of postharvest pathogens using microbial antagonist has been considered an emerging alternative to the use of synthetic chemicals (Janisiewicz, 1988; Wilson et al., 1991; Janisiewicz and Korsten, 2002). Among bacteria, a number of Gram positive and Gram negative bacteria have been studied as Biological Control Agents (BCAs). *Bacillus subtilis* (Obagwu and Korsten, 2003), *Pantoea agglomerans* (Nunes et al., 2001, 2002), *Serratia plymuthica* (Meziane et al., 2006), *Pseudomonas cepacia* (Janisiewicz and Roitman, 1988) and *P. syringae* (Janisiewicz and Jeffers, 1997) were reported as effective BCAs against postharvest diseases of different fruit crops.

The bacterial species *Pseudomonas syringae* includes both strains responsible of diseases, for which the mechanism of action is actively investigated, and biocontrol agents already applied in agriculture at a commercial level. Strains of *P. syringae* have been reported to be effective as biocontrol agents against *Penicillium expansum* and *Botrytis cinerea* on pear fruits (Janisiewicz and Marchi, 1992; Sugar and Spotts, 1999; Northover and Zhou, 2002), *Monilinia fructicola* and *Rhizopus stolonifer* on peaches (Zhou et al., 1999), *P. digitatum* on *Citrus* spp. (Bull et al., 1998; Cirvilleri et al., 2000, 2005), *P. expansum* on apple fruits (Janisiewicz and Bors, 1995; Conway et al., 1999; Zhou et al., 2001; Vinale et al., 2005), *E. coli* O157:H7 on apple wounds (Janisiewicz et al., 1999). *P. syringae* strains ESC-10 and ESC-11 were registered in 1995 and developed by EcoScience Corporation in the Bio-Save product line for the suppression of postharvest diseases of *Citrus* spp., pome, stone fruit as well as tubers.

Pseudomonas spp. strains produce a wide spectrum of lipodepsipeptides (LDPs) (Bender et al., 1999) showing antimicrobial activity against a broad range of fungi, including yeast and human pathogens (Sorensen et al., 1996; Lavermicocca et al., 1997). Production of the lipodepsinonapeptide syringomycin E by *P. syringae* strains ESC-10 and ESC-11 has been correlated with the ability of these strains to control postharvest diseases of citrus and pome fruits caused by *P. digitatum, P. italicum, P. expansum, B. cinerea* and *Geotricum candidum* (Bull et al., 1998).

Molecular typing of *P. syringae* strains is an important prerequisite in risk assessment analysis for tracking their dispersal and fate in the environment when used as BCAs and various molecular techniques have been used to characterize them. They include Pulsed-Field Gel Electrophoresis (PFGE) (Grothues and Rudolph, 1991), Restriction Fragment Length Polymorphism (RFLP) (Scholz et al., 1994), Random Amplified Polymorphic DNA (RAPD) (Clerc et al., 1998), repetitive-

sequence PCR (rep-PCR) (Little et al., 1998; Scortichini et al., 2003), Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA) (Scortichini et al., 2001) and Amplified Fragment Length Polymorphism (AFLP) (Clerc et al., 1998; Manceau and Brin, 2003; Cirvilleri et al., 2006). Presently, AFLP analysis can be considered one of the most discriminating genomic methods to distinguish among bacterial strains (Vos et al., 1995).

This study was conducted to explore criteria for selecting *P. syringae* antagonistic strains using both biological and molecular methods. Strains of *P. syringae* and their culture filtrates were evaluated for the antagonistic activities either *in vitro* against a broad spectrum of bacteria and fungi, for pathogenicity on fruit, or *in vivo* on citrus, apple and grapes, for production of hydrolytic enzymes, for presence of *syr*B gene, and were genetically characterized by ARDRA, ERIC-PCR and fAFLP.

2 Materials and Methods

2.1 Biochemical Characterization

P. syringae strains used in this study were isolated from different woody and herbaceous host plants whereas some other were obtained from international culture collections (Table 1). All the strains were evaluated for LOPAT characters following the procedures described by Schaad et al. (2001).

2.2 In Vitro Antagonistic Activity

All *Pseudomonas syringae* strains were tested for the growth inhibition activity on Potato Dextrose Agar plate (PDA, Oxoid) of microorganisms listed in Table 2 by following the technique described elsewhere (Cirvilleri et al., 2005). The presence and size of a clear zone around *P. syringae* colonies, indicating the growth inhibitory effect, were scored after 2–4-day incubation.

2.3 Antimicrobial and Phytotoxic Activity of Culture Filtrates

Cell-free culture filtrates of *P. syringae* strains in IMM (Surico et al., 1988) were evaluated for the antimicrobial activity on PDA as previously described (Cirvilleri et al., 2005). The phytotoxic activity of cell-free culture filtrates was evaluated on tobacco leaves and bean pods and the necrosis scored after 2 and 6 days, respectively.

Table 1 Original source and main characteristic of *Pseudomonas syringae* strains

Strains[a]	Host	Geographic origin	Year	Antagonistic group[b]	*syr*B	Pathogenicity on lemon
PVCT 10.2	*Citrus sinensis*	Italy	1990	A	+	8.0[b–d]
PVCT 40_2	”	Italy	1990	A	+	n.t.
PVCT 41_2	”	Italy	1990	A	+	8.0[b–d]
PVCT 119_2	”	Italy	1990	A	+	0.0[a]
PVCT 130_1	”	Italy	1990	A	+	10.0[cd]
PVCT 147_1	”	Italy	1990	A	+	8.0[b–d]
PVCT 280_2	”	Italy	1990	A	–	10.0[cd]
PVCT 281_1	”	Italy	1990	A	+	5.0[b]
PVCT 282_1	”	Italy	1990	A	+	5.0[b]
PVCT 285_1	”	Italy	1990	A	+	10.0[cd]
PVCT 287_1	”	Italy	1990	A	+	n.t.
PVCT 290_2	”	Italy	1990	A	+	10.0[cd]
PVCT 291_1	”	Italy	1990	A	+	9.0[b–d]
PVCT 293_1	”	Italy	1990	A	+	5.0[b]
PVCT 295_1	”	Italy	1990	A	–	8.0[b–d]
PVCT 310	”	Italy	1990	A	+	10.0[cd]
PVCT 334	”	Italy	2000	A	+	8.0[b–d]
PVCT 335_2	”	Italy	2000	A	+	8.0[b–d]
PVCT 337_1	”	Italy	2000	A	–	10.0[cd]
PVCT 337_2	”	Italy	2000	A	+	7.5[b–d]
PVCT 339_1	”	Italy	2000	A	+	7.5[b–d]
PVCT 342_1	”	Italy	2000	A	+	n.t.
PVCT 48SR1	”	Italy	1990	A	+	10.0[cd]
PVCT 48SR2	”	Italy	1990	A	+	10.0[cd]
PVCT 40SR4	”	Italy	1990	A	+	n.t.
PVCT Al513	”	USA (S.E. Lindow)	1986	A	+	0.0[a]
ISF 242	*Citrus lemon*	Italy	1996	A	+	10.0[cd]
ISF 243	*Citrus reticulata*	Italy	1996	A	+	12.0[d]
PVCT 23P	*Pyrus communis*	Italy	1998	A	+	8.0[b–d]
PVCT 26P	”	Italy	1998	A	+	10.0[cd]

PVCT 46P	”	Italy	1998	A	+	10.0[cd]
PVCT 76P	”	Italy	1998	A	+	10.0[cd]
ISF 280	”	Italy	1996	A	+	9.0[b–d]
ISF 281	”	Italy	1996	A	+	5.0[b]
ISF 288	”	Italy	1996	A	+	6.5[bc]
ISF 347	”	Italy	1996	A	+	10.0[cd]
PVCT 1.1S	*Strelitzia reginae*	Italy	2000	A	+	n.t.
PVCT 1.2S	”	Italy	2000	A	+	5.0[b]
PVCT 1.3S	”	Italy	2000	A	+	8.5[b–d]
PVCT 1.4S	”	Italy	2000	A	+	5.5[bc]
PVCT B.I.	”	Italy	2004	A	–	0.0[a]
ISF 106 = NCPPB 2426	*Prunus avium*	Switzerland		A	+	0.0[a]
ISF 107 = NCPPB 1093	*Prunus armeniaca*	New Zealand		A	+	0.0[a]
ISF 231	”	Italy	1996A	+		0.0[a]
ISF 290 = B3A	*Prunus persicae*	USA (J.E. De Vay)		A	+	0.0[a]
ISF 015 = NCPPB3869	*Laurus nobilis*	Italy	1992	A	+	12.0[d]
ISF 282	*Castanea sativa*	Italy	1996	A	+	8.0[b–d]
AID 48	*Fragaria* x *ananassa*	Italy	1988	A	+	12.0[d]
HRI 1480A	*Pisum sativum*	UK		A	+	7.0[bc]
ISF 292	*Triticum aestivum*	USA (J.E. De Vay)		A	+	0.0[a]
ISF 300	”	Italy		A	+	7.0[bc]
ISF 304	”	Italy	1996	A	+	0.0[a]
ISF 309	”	Italy	1996	A	+	8.0[b–d]
ISF 310 = NCPPB2612	”	New Zealand		A	+	5.5[bc]
ISF 355	*Hordeum vulgare*	Italy	1996	A	+	5.0[b]
ISF 356	”	Italy	1996	A	+	5.0[b]
ISF 359 = 475A	”	(J.E. De Vay)		A	+	12.0[d]
ISF 293 = B359	Setaria italica	Australia (J.E. DeVay)		A	+	8.0[b–d]
PVCT 7NC	*Corylus avellana*	Italy	2005	A	n.t.	n.t.
PVCT 44NC	”	Italy	2005	A	n.t.	n.t.
AID 122A	*Prunus amygdali*	Italy	1988	B	+	0.0[a]
ISF 291 = SY12	*Syringa vulgaris*	Japan (D.C. Gross)		B	+	0.0[a]
AID 24	*Fragaria x ananassa*	Italy	1987	B	+	0.0[a]
AID 33	”	Italy		B	+	0.0[a]

(continued)

Table 1 (continued)

AID 76	”	Italy	1988	B	+	0.0[a]
AID 88	”	Italy	1988	B	+	n.t.
PVCT B728a	*Phaseolus vulgaris*	USA (S.E. Lindow)	1986	B	+	4,0[b]
ISF 286 = Y37	”	USA (D.C. Gross)		B	+	0.0[a]
ISF 332	”	Italy	1996	B	+	0.0[a]
PVCT 4	*Cynara scolimus*	Italy	1992	B	+	0.0[a]
PVCT 14	”	Italy	1992	B	+	n.t.
PVCT 29	”	Italy	1992	B	+	n.t.
PVCT 40	”	Italy	1992	B	+	0.0[a]
PVCT 74	”	Italy	1992	B	+	n.t.
PVCT 96	”	Italy	1992	B	+	n.t.
PVCT 98	”	Italy	1992	B	+	n.t.
PVCT 106	”	Italy	1992	B	+	0.0[a]
PVCT 113	”	Italy	1992	B	+	n.t.
PVCT 120	”	Italy	1992	B	+	0.0[a]
PVCT 133	”	Italy	1992	B	+	n.t.
PVCT 141	”	Italy	1992	B	+	n.t.
PVCT 152	”	Italy	1992	B	+	0.0[a]
PVCT 169	”	Italy	1992	B	+	n.t.
ISF 353 = ISF-PP2	*Capsicum annuum*	Italy	1997	B	+	0.0[a]
ISF 284 = PSS61	*Triticum aestivum*	USA (D.C. Gross)		B	+	0.0[a]
ISF 294 = W451	”	USA (D.C. Gross)		B	+	0.0[a]
ISF 295 = SD202	”	USA (D.C. Gross)		B	+	0.0[a]
ISF 357	*Hordeum vulgare*	Italy	1996	B	+	0.0[a]
PVCT 38NC	*Corylus avellana*	Italy	2005	B	n.t.	n.t.

[a] AID: Agricultural Industrial Development, Catania, Italy; HRI: Horticulture Research International, Wellesbourne, United Kingdom; ISF: C.R.A.: Istituto Sperimentale per la Frutticoltura, Roma, Italy; NCPPB: National Collection of Plant Pathogenic Bacteria, York, United Kingdom; PVCT: Plant Pathology, University of Catania, Italy

[b] Antagonistic groups (see also Cirvilleri et al., 2005)

[c] Pathogenicity tests performed with *P. syringae* strains. The number refers to the mean diameter of lesion recorded seven days after artificial inoculation of lemon cv. Femminello fruits. Mean 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 $P \leq 0.05$ (see also Cirvilleri et al., 2005).

Table 2 Antagonistic activity *in vitro* of *Pseudomonas syringae* strains recorded as mean inhibition zones (mm)

	Inhibition zone (mm) ± SD[a]	
Target microorganism	Antagonistic group A	Antagonistic group B
Rhodotorula pilimanae ATCC26423	17.5 ± 7.3	–
Bacillus megaterium ITM100	12.0 ± 7.4	23.8 ± 10.9
Salmonella spp[b]	1.0 ± 2.7	1.1 ± 4.3
Listeria monocytogenes[b]	14.2 ± 10.5	23.3± 10.9
Pseudomonas syringae PVCT48SR2	11.4 ± 6.3	21.0 ± 7.7
Botrytis cinerea[c]	13.4 ± 6.7	–
Penicillium digitatum[c]	4.8 ± 2.9	–
Alternaria alternata[c]	6.5 ± 4.2	–
Aspergillus ochraceus ATCC18641	6.0 ± 4.0	–
Fusarium solani 1A[c]	3.6 ± 3.5	–

[a] Inhibition zones ± standard deviation (SD) of the mean of four replications for each strain

[b] Kindly provided by the Istituto Zooprofilattico Regionale, Catania, Italy

[c] From mycologial laboratory of Plant Pathology, University of Catania, Italy

2.4 Pathogenicity Tests

Pathogenicity tests were performed at 20°C on lemon (*Citrus lemon* Burm) cv. Femminello, orange (*Citrus sinensis* Osbeck) cv. Tarocco, mandarin (*Citrus reticulata* L.) cv. Fortuna, apple (*Malus domestica* Borkh.) cv. Golden Delicious fruits, grape (*Vitis vinifera* L.) cv. Italia berries and bean pods (*Phaseolus vulgaris* L.) as elsewhere described (Cirvilleri et al., 2005). Fruits were observed repeatedly and the width of necrosis was measured 7 days after inoculation.

2.5 In Vivo Antagonistic Activity

P. syringae strains were tested for the ability to inhibit the growth of *P. digitatum* on lemon, orange and mandarin fruits, of *P. expansum* on apple fruits and of *B. cinerea* on grape berries. Fruits were co-inoculated with *P. syringae* suspensions (1×10^9 cfu/ml) and fungal spore suspension (1×10^6 cfu/ml). Incidence and severity of disease were calculated one week after inoculation as previously described (Cirvilleri et al., 2005).

2.6 Enzymatic Activity

N-acetyl-β-D-glucosaminidase (NAGase), β-glucosidase, cellobiohydrolase, cellulase, protease, chitinase and glucanase activity were detected in cell free culture filtrates as previously described (Nielsen and Sorensen, 1997; Madesen and de Neergard, 1999; Campisano et al., 2001).

2.7 Molecular Characterization

The presence of *syr*B gene, Amplified 16S Ribosomal DNA Restriction Analysis (ARDRA), repetitive-sequence PCR (rep-PCR) using ERIC-PCR primers, and fluorescent Amplified Fragment Length Polymorphism (fAFLP) were performed as described elsewhere (Cirvilleri et al., 2005, 2006; Manceau and Brin, 2003; Scortichini et al., 2001). Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA). Similarity coefficients were determined using the Dice's coefficient (Dice, 1945) and the robustness of the tree was assessed by bootstrap analysis (1000 repeated samplings) (Felsenstein, 2004).

3 Results

3.1 In Vitro Antagonistic Activity

Pseudomonas syringae strains evaluated in this study inhibited *in vitro* the growth of a wide range of phytopathogenic bacteria and fungi and the inhibition rate depended on bacterial and fungal strains. *P. syringae* strains were divided into two distinct groups A and B on the basis of *in vitro* inhibitory activity (Table 2). In particular, *P. syringae* strains inhibiting to a different extent the growth of *R. pilimanae* and of all the others target microorganisms were included in the antagonistic group A whereas strains unable to inhibit *R. pilimanae* and the majority of the target microorganisms were included in the antagonistic group B. Strains of the latter group showed the widest inhibition zones against *B. megaterium*, *L. monocytogenes* and *P. syringae*.

3.2 Antimicrobial and Phytotoxic Activity of Culture Filtrates

Culture filtrates of strains belonging to group A were phytotoxic on tobacco and caused bean pods necrosis with an intensity sometimes similar to those caused by the pathogen strain inoculation. Cell-free culture filtrates of strains of group B did not cause necrosis on tobacco and on bean pods (data not shown).

3.3 Pathogenicity Tests

P. syringae strains belonging to the antagonistic group A and isolated from *Citrus* spp., pear and strelitzia were moderately to highly virulent on lemon, orange and mandarin fruits (Table 3). Mean size of lesions ranged between 5 and 12 mm on

Table 3 Percentage of *Pseudomonas syringae* strains from different hosts and belonging to antagonistic groups A and B causing lesions (mm) on inoculated fruits

Host of origin	Antagonistc groups	Fruits											
		Lemon		Orange		Mandarin		Apple		Grape		Bean pods	
		Strains (%)	Lesions (mm)[a]	Strains (%)	Lesions (mm)[a]	Strains (%)	Lesions (mm)[a]	Strains (%)	Lesions (mm)[a]	Strains (%)	Lesions (mm)[a]	Strains (%)	Pathogenicity[b]
Citrus spp.	A	100	5–12	47	0.5–4	94	0.6–3	100	0	100	0	100	+
Pyrus communis		100	5–10	71	2–4	43	2.6–3.6	100	0	100	0	100	++
Strelitzia reginae		100	5–8.5	33	1–2	66	0.6–3	100	0	100	0	100	++
Prunus armeniaca		100	0	0	0	0	0	100	0	100	0	100	++
Laurus nobilis		100	12	0	0	0	0	100	0	100	0	100	++
Castanea sativa		100	8	100	2	0	0	100	0	100	0	100	+++
Fragaria x ananassa		100	12	0	0	100	2.3	100	0	100	0	100	++
Pisum sativum		100	7	0	0	0	0	100	0	100	0	100	++
Triticum aestivum		50	5–8	25	0.5–2	0	0	100	0	100	0	100	++
Hordeum vulgare		100	5–12	100	2	0	0	100	0	100	0	100	++
Setaria italica		100	8	100	2	0	0	100	0	100	0	100	+++
Corylus avellana		100	0	100	0	0	0	100	0	100	0	100	+++
Fragaria x ananassa	B	100	0	100	0	100	0	100	0	100	0	100	+
Triticum aestivum		100	0	100	0	100	0	100	0	100	0	100	++
Hordeum vulgare		100	0	100	0	100	0	100	0	100	0	100	++
Corylus avellana		100	0	100	0	100	0	100	0	100	0	100	++
Syringa vulgaris		100	0	100	0	100	0	100	0	100	0	100	++
Phaseolus vulgaris		100	4	100	0	100	0	100	0	100	0	100	++
Cynara scolymus		100	0	100	0	100	0	100	0	100	0	100	++

[a] Diameter of lesions (mm) recorded 7 days after artificial inoculation with 1×10^9 CFU ml^{-1} and incubated at 20°C. Mean values are based on four replication per experiment and each experiment was repeated twice.

[b] +, lesion associated with inoculated sites; ++, progressive necrotic lesion; +++, progressive necrotic lesion and red halo on bean.

Table 4 Disease severity on lemon, orange, mandarin, apple fruits and grape berries 7 days after artificial inoculation with *Penicillium digitatum*, *P. expansum* and *Botrytis cinerea* and subsequently treated with *Pseudomonas syringae* strains

	P. digitatum/lemon[a]		*P. digitatum*/orange[a]		*P. digitatum*/mandarin[a]		*P. expansum*/apple[a]		*B. cinerea* grape[a]	
	Strains (%)	Disease severity (%)	Strains (%)	Disease severity (%)	Strains (%)	Disease severity (%)	Strains (%)	Disease severity (%)	Strains (%)	Disease severity (%)
Antagonistic group										
A	79%	0	21%	0	0%	0	30%	0	0%	0
(60 strains)	11%	22–45	56%	17–45	93%	30–60	51%	4–45	0%	45
	10%	77–90	23%	53–90	7%	90	19%	47–90	100%	57–90
B	14%	0	7%	0	0%	0	57%	0	0%	0
(29 strains)	50%	17–45	14%	21–38	0%	30–60	29%	13–45	0%	45
	36%	68–90	79%	49–90	100%	90	14%	52–90	100%	49–90

[a] Disease severity was evaluated with an empiric scale and ratings was converted to percentage midpoint values, where 0% = no visible symptoms, 35% = initial soft rot, 65% = presence of mycelium, 90% = sporulation. Mean values are based on four replications and four fruits per replication

lemon, 0.5 and 4 mm on orange and 0.6 and 3 mm on mandarin. By contrast, group B strains did not induce necrosis on lemon, orange and mandarin fruits. None of the strains of either group was virulent on apple fruits and grape berries whereas almost all strains induced necrotic lesions on bean pods.

3.4 In Vivo Antagonistic Activity

Several strains belonging to group A prevented (disease severity 0%) the growth of *P. digitatum* on lemon and orange fruits (respectively 79% and 21% of strains) and of *P. expansum* on apple fruit (30% of strains) whereas only a few strains of group B prevented the growth of *P. digitatum* on lemon and orange (14% and 7% of strains, respectively) and *P. expansum* on apple fruits (57% of strains) (Table 4). None of the strains of groups A and B totally controlled *P. digitatum* on mandarin fruits and *B. cinerea* on grapes. One strain of group A (PVCT 119_2) and two strains of group B (ISF284 and ISF294), that did not induce symptoms on artificially inoculated fruits, totally inhibited the growth of *P. digitatum* and *P. expansum* on lemon and apple fruits.

3.5 Enzymatic Activity

The cell-wall degradation enzymes NAGase, b-glucosidase, cellobiohydrolase, cellulase and protease were detected in culture filtrates of the majority of strains while only a few strains showed chitinase and glucanase activities (Table 5). At least one of the seven tested enzymatic activities was detected in the cell-free culture filtrates of *P. syringae* strains, irrespective of the antagonistic group and of the host of origin. NAGase, β-glucosidase and cellobiohydrolase (up to 10, 30 and 7 mM *p*-nitrophenol ml^{-1} min^{-1} respectively) and cellulase and protease activity were detected in culture filtrates of the most part of the strains. Chitinase and glucanase activities were almost undetectable in the majority of the strains.

Table 5 Enzymatic activity of *Pseudomonas syringae* strains

		Production of[a]						
	% of strains	NAGase	β-gluc	cellbio	pro	cell	chi	glu
Group								
A	36	–	–	–	+	+	–	–
	21	+	+	+	+	+	–	–
	29	+	+	–	+	+	–	–
	14	+	–	–	+	+	–	–
B	19	+	+	+	+	–	–	–
	56	+	–	+	+	+	–	–
	25	–	–	+	–	–	–	–

[a]NAGase = *N*-acetyl-β-D-glucosaminidase; β-gluc = β-glucosidase; cell-bio = cellobiohydrolase; pro = protease; cell = endocellulase; gluc = β-glucanase; chi = chitinase; glu = glucanase

3.6 Molecular Characterization

All *P. syringae* strains belonging to both group A and B had the *syr*B gene. ARDRA analysis was performed with many representative *P. syringae* strains. P0 and P6 primers amplified an approximately 1600 bp fragments (Fig. 1), and restriction analysis with *Alu*I (Fig. 2a) revealed two different patterns: pattern I, comprising all strains belonging to the antagonistic group A, and pattern II, comprising all strains belonging to the group B. The same analysis with *Hae*III revealed a single pattern with all *P. syringae* strains (Fig. 2b). ARDRA analysis did not permit to distinguish *P. syringae* strains within the two groups A and B.

PCR amplification with ERIC primers yielded six to ten distinct PCR products, ranging in size from approximately 150 to over 3000 bp. A representative gel is illustrated in Fig. 3. Fingerprints showed high degree of genetic diversity among the strains and UPGMA analysis revealed seven main clusters (Fig. 4). The clusters included strains obtained from different host plants species and belonging to either the antagonistic groups A or B, with the exception of two clusters (2 and 7) which included strains all belonging to antagonistic group A, and two clusters (3 and 7) which included the majority of strains isolated from *Citrus* spp.

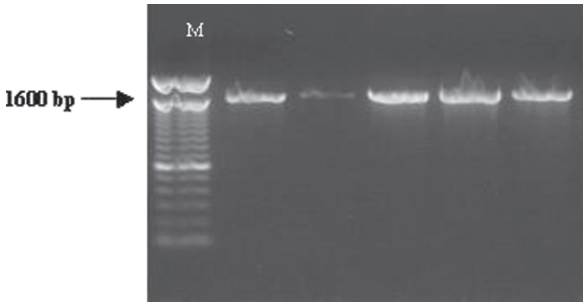

Fig. 1 ARDRA-16S PCR with P0 and P6 primer. All Pseudomonas syringae strains showed a fragment of about 1600 bp. Ladder used (M) was 100 bp (Invitrogen-Life Technologies, Paisley, UK)

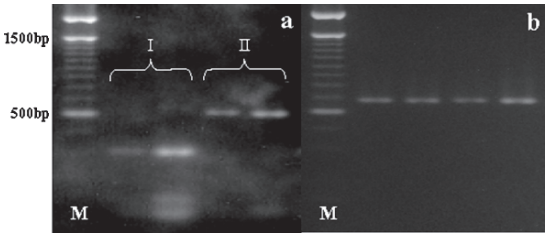

Fig. 2 ARDRA analysis with restriction enzymes *Alu*I and *Hae*III. Restriction performed with *Alu*I (a) showed two different patterns (I, II) corresponding to the groups (A, B) previously identified. Restriction permormed with *Hae*III (b) showed single pattern group. Ladder used (M) was 100 bp (Invitrogen-Life Technologies, Paisley, UK)

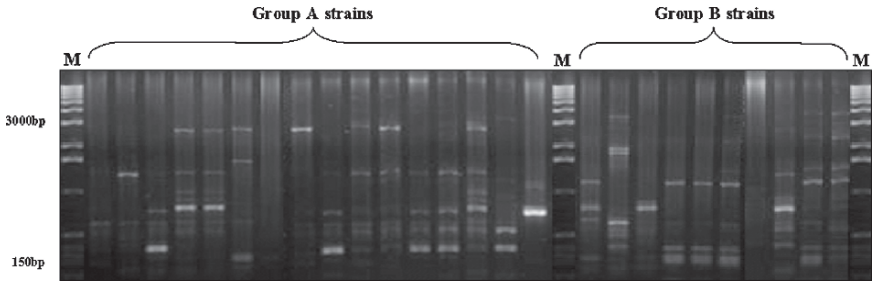

Fig. 3 Representative ERIC-PCR patterns of *Pseudomonas syringae* strains used in this study. Amplification with ERIC primers (ERIC 1R and ERIC 2) yielded six to ten distinct PCR products, ranging in size from approximately 150 bp to over 3000 bp and allowed differentiation of strains. Ladder used (M) was 1 Kb Invitrogen-Life Technologies, Paisley, UK (From Circvilleri et al., 2005 partially modified)

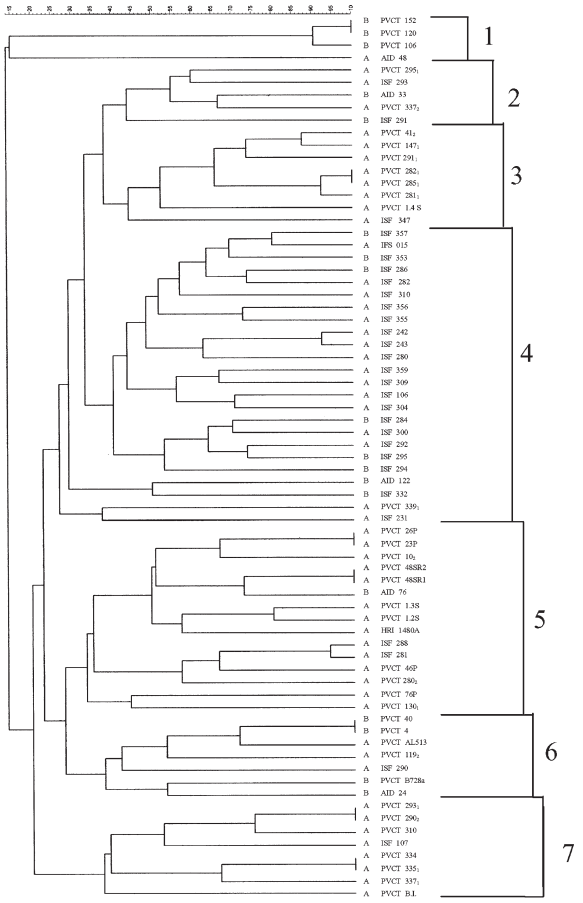

Fig. 4 Dendrogram of relationships among *Pseudomonas syringae* strains obtained using repetitive-sequence PCR and ERIC primer sets. PCR products in the range of 150–3000 bp were compared by numerical analysis using GelCompar II software. Similarity between fingerprints was calculated with the Dice's coefficients using the unweighted pair-group method with average linkages (UPGMA) (From Circvilleri et al., 2005)

fAFLP analysis generated 29–43 fragments sized within 1 bp upon amplification with *Mse*I/*Eco*RI primer set. fAFLP analysis allowed to distinguish between groups of strains isolated from different hosts and between strains isolated from the same host. Eight distinct fAFLP clusters were identified on the basis of host origin (Fig. 5).

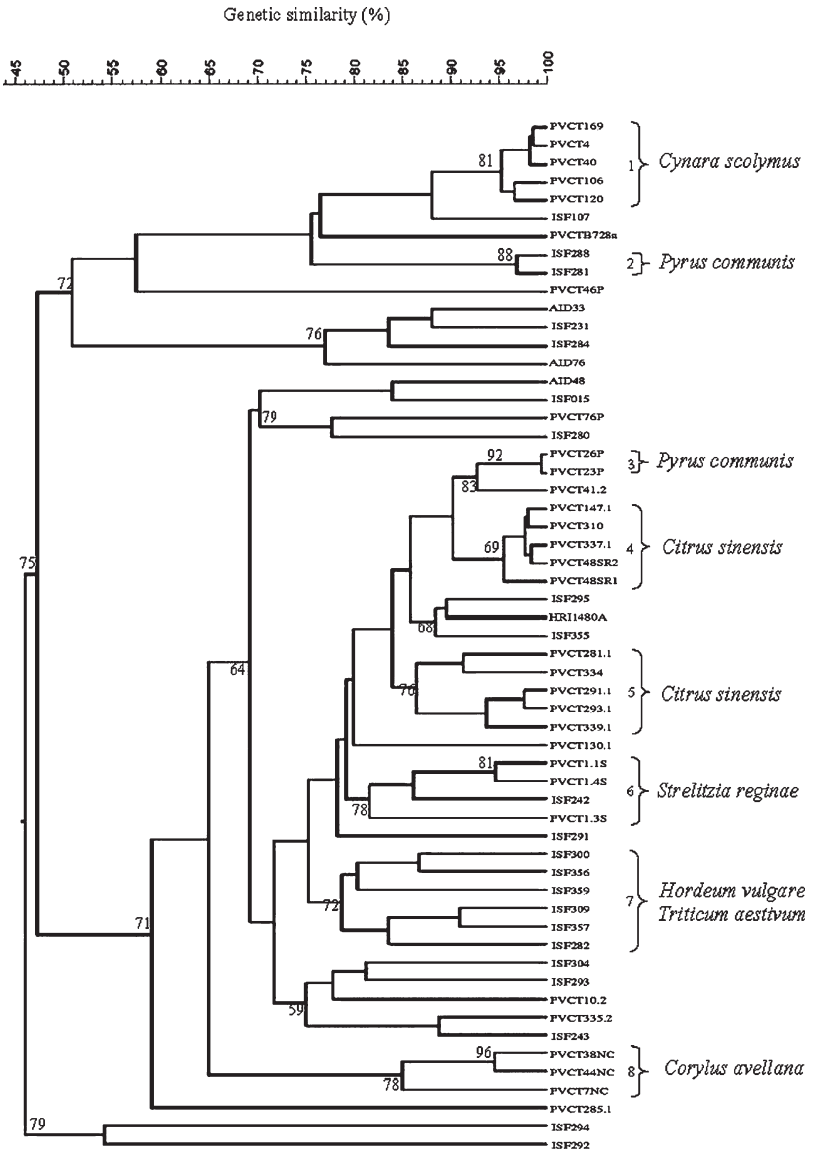

Fig. 5 Dendrogram of *Pseudomonas syringae* strains analysed by fluorecent AFLP. Profiles in the range of 100–700 bp were compared by numerical analysis using Phylip 3.6 software. Similarity between fingerprints was calculated with unweighted pair-group method with average linkages (UPGMA) and the robustness of the tree was assessed by bootstrap analysis (1000 repeated samplings)

Table 6 fAFLPs characteristic fragments in selected *Pseudomonas syringae* strains

		Fragments (bp)[a]							
Host	Strains	113	217	242	246	317	415	555	583
Cynara scolymus	PVCT4, PVCT40, PVCT106, PVCT120, PVCT169	–	–	+	–	–	+	+	–
Corylus avellana	PVCT7NC, PVCT38NC, PVCT44NC	–	–	+	–	–	–	–	+
Strelitzia reginae	PVCT1.1S, PVCT1.3S, PVCT1.4S	–	–	+	–	+	–	–	–
Citrus sinensis	PVCT10.2, PVCT 337_1, PVCT48SR1, PVCT48SR2	–	+	+	–	–	–	–	–
Hordeum vulgare	ISF356, ISF357, ISF359	+	–	+	–	–	–	–	–
Fragaria x ananassa	AID 33, AID 76	–	–	+	+	–	–	–	–

[a] The presence or absence of differential fragments is indicated: +, a fragment characteristically present in fAFLP profiles of that strain; –, characteristic absence of fAFLP fragment from that profiles

The eight clusters included strains belonging to either the antagonistic groups A or B. Bootstrap values depicted the robustness of the dendrogram and almost all nodes showed moderate consistency in clustering.

Analysis of electropherograms showed that fifteen fragments were shared by most of strains and a 242 bp fragment was common to 90% of strains (Table 6). Strains isolated from *Cynara scolymus* showed two specific fragments of 415 and 555 bp, strains isolated from *Corylus avellana* showed a specific fragment of 583 bp, strains isolated from *Strelitzia reginae* showed a specific fragment of 317 bp and some strains isolated from *Citrus* spp. (PVCT 10.2, PVCT 337_1, PVCT $48SR_1$ and PVCT $48SR_2$) showed a specific fragment of 217 bp. In addition, three strains isolated from *Hordeum vulgare* (ISF356, ISF357, ISF359) and two from *Fragaria x ananassa* (AID33, AID76) showed specific fragments of 113 and 246 bp, respectively.

4 Discussion

Pseudomonas syringae strains and the relative culture filtrates evaluated in this study showed an antagonistic activity *in vitro* towards a variety of microorganisms. Strains showing a broader *in vitro* activity and those with reduced antagonistic activity were placed in the group A and group B, respectively.

Screening tests useful for minimizing the risk of introducing noxious organisms into the environment should be an important part of any effort to develop biological control antagonists and this is particularly true with pseudomonads which contain plant pathogens. Like other plant pathogenic pseudomonads, *P. syringae* strains isolated from lesions are virulent on either on the original host or occasionally on other non-host plants (Schaad et al., 2001).

In the present study, some *P. syringae* strains isolated from *Citrus* spp., *Pyrus communis*, *Strelitzia reginae* and belonging to antagonist group A produced necrotic lesions of different size on lemon, orange and mandarin fruits but not on apple and grape ones. On the contrary, strains isolated from *Prunus amygdali*, *P. armeniaca*, *Corylus avellana*, *Fragaria* x *ananassa*, *Phaseolus vulgaris* and *Cynara scolimus* (antagonistic groups A and B) did not produce any lesions on all fruits tested.

The consequences of necrotic lesions caused by antagonistic *P. syringae* strains evaluated in this study on the quality of the fruit might be of minor importance under commercial conditions. Moreover, the relative large and fresh wounds employed in laboratory may not simulate many of those inflicted during typical harvest and subsequent handling.

As observed in previous studies (Smilanick et al., 1996), the ability of antagonistic *P. syringae* strains ESC-10 to control postharvest green mold was not associated with virulence to citrus fruits when 10^6–10^7 CFU ml^{-1} was used whereas 10^8 CFU ml^{-1} caused small necrotic lesions on lime fruits. In the same studies, *P. syringae* strain 485–10 able to control green mould better that ESC-10 (Smilanick et al., 1996), caused cosmetically unacceptable black pit symptoms on fruit and blast symptoms on shoots and foliage. Light brown and dark brown lesions have been also reported as reactions associated, respectively, with virulent and non virulent *P. syringae* strains (Burkowicz and Rudolph, 1994), and dark brown reactions were supposed to represent a more vigorous plant defence reaction involving oxidation of phenolic compounds.

In this study we showed the ability of some *P. syringae* strains to completely or strongly control green and blue mould and this was not associated with significant alteration of citrus and apple fruits. One strain of group A (PVCT 119_2) and two strains of group B (ISF284 and ISF294), that did not induce symptoms on artificially inoculated citrus and apple fruits, totally inhibited *P. digitatum* and *P. expansum* on lemon and apple fruits even if high inoculum doses were used (10^9 CFU ml^{-1}) in both tests. Moreover, several strains belonging to group A controlled green and blue mould significantly better than PVCT 119_2, but produced large lesions (5–12 mm) on citrus fruits, thus precluding their potential use as biological control agents.

Strains of *P. syringae* groups A and B were effective to a different extent in reducing the incidence and severity of disease on lemon, orange, mandarin and apple fruits artificially inoculated with *P. digitatum*, *P. expansum* and *B. cinerea*. However, the antagonistic action of the A and B strains appeared different. Thus, strains of group A showed antagonistic activity against the above pathogens either *in vitro* and *in vivo*, whereas strains of group B were active only in *in vivo* assays. Two strains of group B showed interesting prerequisite for use as biocontrol agents towards *P. digitatum* and *P. expansum* on orange and apple fruits, respectively.

The mechanism of biocontrol ability of *P. syringae* strains ESC-10 and ESC-11 has been proposed to be due to competition for sites and nutrients in wounds since their application to wounds before or shortly after the introduction of the pathogen (Smilanick and Denis-Arrue, 1992; Smilanick et al., 1999), and large and metabolically active populations are required for optimum efficacy (Bull et al., 1997; Janisiewicz and Marchi, 1992). Bull et al. (1998) also found that ESC-10 and ESC-11 produced syringomycin E in culture at quantities sufficient to inhibit growth of *P. digitatum in vitro*, and that purified syringomycin E controlled green mold of lemons to levels similar to application of the producing strains but since syringomycin E or other antibiotics have not been detected in fruits treated with ESC-10 and ESC-11 it may not be important in the biocontrol activity. Thus the role of syringomycin E as well as of other LDPs produced *in vitro* by the majority of *P. syringae* strains is still uncertain and remain to be clarified. *P. syringae* strains are also able to produce enzymes such as chitinases and glucanases which degrade fungal cell walls, and recently Fogliano et al. (2002) demonstrated that LDPs and cell-wall degrading enzymes can act synergistically in the biocontrol of different pathogens. Two *P. syringae* strains, HRI 1480A and PVCT 46P, isolated respectively from pea and pear and with the behaviour of the strains belonging to group A, were able to control *P. expansum* on apple fruits also at 10^7 and 10^6 CFU ml^{-1} (Vinale et al., 2005) and showed considerable level of glucanase and chitinase activity They were not able to produce LDPs antibiotics and this suggest that ability to produce LDPs *in vitro* is not related to biocontrol activity of these strains.

The genetic characterization of *P. syringae* by ERIC-PCR and fAFLP pointed out an high variability among strains from the same host plant, as well as among strains within the same antagonistic group or with similar pathogenic feature. This variability was in agreement with other investigations performed on the same strains using BOX analysis (Scortichini et al., 2003), or on other strains of *P. syringae* using ERIC (Little et al., 1998) or AFLP analysis (Clerc et al., 1998; Manceau and Brin, 2003). The latter analyses revealed that there was no strict correlation between the ERIC-PCR and fAFLP profiles and the host of origin. Clusters of *P. syringae* strains defined with ERIC-PCR, with antagonistic groups and pathogenicity, were partially revealed with fAFLP clustering.

fAFLP fingerprints seems useful for tracking the biocontrol agents in the environment. Such a capability would be very important to verify the presence of the biocontrol agent in the long-term period under commercial conditions. Moreover, strain-specific discriminative amplicons, typically detected in strains isolated from the same host, may be useful for rapid identification of biocontrol agents strains and for epidemiological studies.

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 is needed under commercial conditions, including assessment of biological control efficacy, population dynamics of these antagonists on fruit and observation of the quality of treated fruit.

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