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Antimicrobial activity of secondary metabolites produced by different pathovars of *Pseudomonas syringae* and by strains of *P. avellanae*

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Summary. Several species in the genus *Pseudomonas* produce secondary metabolites showing phytotoxic and antimicrobial activity. Thirty-three strains of nine pathovars of *P. syringae* and four strains of *P. avellanae* were tested for antibacterial and antifungal activity using *Bacillus megaterium* and *Rhodotorula pilimanae* as assay organisms. The assay was carried out on nine different media. Inhibitory behaviour was found with almost all the media tested. Medium 523 favoured the production of antimicrobial substances against *B. megaterium*. Medium King B was the optimal substrate, allowing 95% of the strains to inhibit *R. pilimanae* growth. Medium IMMA appeared the best substrate for inducing lipodepsipeptide production. Dendrograms drawn on the basis of the activity of each pathovar or species vs *B. megaterium* or *R. pilimanae* gave a representation of non-homogenous clusters. A dendrogram combining pathovar data from tests against both *B. megaterium* and *R. pilimanae* gave homogenous clusters which showed a similarity between the pathovars. The strains of pv. *aptata*, *lachrymans* and *syringae* were lipodepsinonapeptide and syringopeptin producers. The toxigenic activity of these bacteria may improve the diagnosis and identification of *P. syringae* pathovars.

Key words: lipodepsipeptides, phytotoxins, *Bacillus megaterium*, *Rhodotorula pilimanae*.

Introduction

Several species of the genus *Pseudomonas* produce various phytotoxic compounds (Bender *et al.*, 1999) which on susceptible plants cause a range of symptoms including leaf spots, chlorosis, necrosis, blight and galls. Many strains of *P. syringae* pv. *syringae* are known to produce cyclic lipodepsipeptides (LDPs) as secondary metabolites. LDPs are amphipathic molecules with a polar peptide head and an apolar fatty acid tail, and they are produced by most strains of some *P. syringae* pathovars (*syringae, aptata, atrofaciens* and *lachrymans*), and by *P. fuscovaginae* (Stewart, 1971; Ballio *et al.,* 1991, 1994a, 1994b; Fukuchi *et al.,* 1992; Vassilev, 1996; Greco *et al.,* 1998). These metabolites consist of small forms, the lipodepsinonapeptides with 9 amino acids such as the syringomycins (SRs), syringostatins, pseudomycins and syringotoxins; and of large forms, the syringopeptins (SPs) consisting of either 22 or 25 amino-acid residues.

LDPs are thought to be plant virulence factors and antifungal agents. They affect plant plasmalemma activity, protoplast permeability, vacuoles, plasma membrane vesicles, the chloroplast membrane, mitochondria (Di Giorgio *et*

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al., 1994), lysosomes (Camoni *et al*., 1995), stomata (Di Giorgio *et al*., 1996) and the membrane permeability of human red blood cells (Szabo *et al*., 2002). The amphipathic character of these phytotoxins allows them to reach and insert themselves into the lipid bilayers, forming channels that are freely permeable by a series of monovalent and divalent cations (Hutchinson *et al*., 1995). Channel formation facilitates the rapid and sustained influx of Ca^{2+} ions, which activates a series of reactions associated with cellular signalling in plants, such as phosphorylation of the membrane proteins and incorporation of 1,3 β-callose into the plant cell walls (Kauss *et al*., 1991). Transmembrane channels lead to the extrusion of nutrient substances into the intercellular spaces of the host tissues (Hutchinson *et al*., 1995), as well as to the alkalisation of the intercellular fluid, thereby creating more favourable conditions for bacterial growth (Che *et al*., 1992). Due to their characteristics, these molecules exhibit potent biosurfactant activity lowering the interfacial tension of water, and thus favouring the adhesion of bacterial cells and their subsequent colonisation of leaf surfaces.

LDPs act as antibiotics against a great number of gram-positive bacteria and fungi. The lipodepsinonapeptides are especially active against filamentous fungi (Lavermicocca *et al.,* 1997) and they have a potential for practical application. Encouraging results have been obtained using SRs to control fungi such as *Penicillium digitatum,* the agent of post-harvest green mould of oranges (Bull *et al.,* 1998), and *Candida* spp. which cause clinical illnesses in human beings (Sorensen *et al*., 1998); while the SPs are also active against various fungi and bacteria (Lavermicocca *et al*., 1997).

The structure of LDPs is still being studied, but it is mainly their biological activity that is of interest. The work described in this paper aimed to assess the toxigenicity of strains of *P. syringae* pathovars and strains of *P. avellanae* and their LDPs production.

Materials and methods

Bacterial strains

The pathovars of *P. syringae* and the strains of *P. avellanae* tested in this work are listed in Table 1. Before being used as inoculum, the purity of each strain was checked by streaking on nutrient broth-sucrose-agar (NSA) dishes (Lelliot and Stead, 1987) and incubating at 25°C for 48 h. Pure cultures were kept in glass tubes containing 5 ml of nutrient-broth-glycerol-agar (NGA) (Lelliot and Stead, 1987) and maintained at 4°C.

Stationary cultures of bacterial strains

Initially the 37 bacterial strains were tested for antimicrobial activity through growth inhibition trials against two micro-organisms, *Bacillus megaterium* de Bary (strain ITM 100, Collezione dell'Istituto di Scienze delle Produzioni Alimentari, CNR, Bari, Italy), sensitive to SPs (Lavermicocca *et al.,* 1997), and *Rhodotorula pilimanae* Bulmer (strain ATCC 26423, American Type Culture Collection, Rockville, MD, USA) sensitive to both SPs and lipodepsinonapeptides (Lavermicocca *et al.,* 1997). Each strain was grown on nine substrates: 1. NGA; 2. NSA; 3. potato-dextrose-agar (PDA, potato-dextrose-broth [Difco, Sparks, MD, USA] pH 6, gelified with 16 g l^{-1}); 4. PDA supplemented with 4% casamino acids (PDCA); 5. King B medium (King *et al*., 1954); 6. SRM (Gross, 1985); 7. Woolley's medium (WM) (Woolley *et al*., 1955); 8. 523 medium (Kodo and Heskett, 1970); and 9. IMMA medium, a modification of IMM medium (Surico *et al*., 1988): L-histidine-HCl 4 g l^{-1}, Mannitol 10 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.2 g l^{-1}, KH_2PO_4 0.8 g l^{-1}, K_2HPO_4 0.8 g l^{-1}, $CaCl_2 \cdot 2H_2O$ 0.1 g l^{-1} and $FeSO_4 \cdot 7H_2O$ 0.02 g l^{-1} stabilised in citric acid (2 g of citric acid dissolved in 100 ml distilled water before adding 2 g of $FeSO_4 \cdot 7H_2O$; 1 ml of this solution was then added to one litre of substrate before sterilisation). The last four substrates were gelified with 16 g l^{-1} Bacto Agar (Difco).

Antimicrobial test

An agar-spot test was used to detect antimicrobial activity. A sterile toothpick was immersed in a colony grown for 48 h on NSA, and was then used to spot-seed a Petri dish, containing one of the nine growth media mentioned above. Each dish was spotted in three equidistant places corresponding to the three points of a triangle. After 5 days at 25°C, the dishes were nebulised with a cell suspension of each of the two indicator micro-organisms, prepared by dissolving a loopful of 48-h-old culture streaked on PDA in 10 ml of sterile water. The dishes were left to dry in a sterile laminar flow

Table 1. Characteristics of *Pseudomonas syringae* pathovars and *P. avellanae* strains used in this study.

Bacterial strain	Host	Origin	Source
P. syringae			
pv. *actinidiae*			
020	Actinidia	Italy	MS[a]
193 NA	Actinidia	Italy	MS
pv. *aptata*			
NCPPB 872	Sugar beet	USA	NSI[b]
NCPPB 2664	Sugar beet	Italy	NSI
NCPPB 871	Sugar beet	USA	NSI
pv. *japonica*			
NCPPB 3093	Barley	Japan	NSI
pv. *lachrymans*			
S91	Melon	Italy	MS
pv. *panici*			
NCPPB 3955	Wheat	Italy	NSI
pv. *papulans*			
B485	Apple	Italy	NSI
NCPPB 1015	Apple	Italy	NSI
pv. *persicae*			
NCPPB 2324	Peach	France	NSI
NCPPB 2761	Peach	France	NSI
pv. *pisi*			
895A race 4	Pea	USA	NSI
F2 race 6	Pea	Italy	NSI
NCPPB 1366 race 2	Pea	Canada	NCPPB[c]
NCPPB 2222 race 1	Pea	Italy	NCPPB
NCPPB 3430 race 1	Pea	New Zealand	NSI
NCPPB 3431 race 3	Pea	USA	NCPPB
NCPPB 3492 race 6	Pea	UK	NCPPB
NCPPB 3496 race 3	Pea	USA	NSI
NCPPB 3498 race 5	Pea	USA	NCPPB
NCPPB 3503 race 4	Pea	UK	NCPPB
pv. *syringae*			
B3A	Peach	Italy	GS[d]
B359	Millet	Italy	GS
B362-2	Bean	Italy	GS
B366	Sugar beet	USA	NSI
B382-4	Hazel	Italy	GS
B426	Walnut	Italy	GS
B459	Orange	USA	NSI
N23	Bean	South Africa	GS
SC F2	Yellow nectarine	Italy	GS
Y27	Bean	USA	NSI
Y37	Bean	USA	NSI
P. avellanae			
ISPAVE 038	Halznut	Italy	MS
ISPAVE 063	Halznut	Italy	MS
ISPAVE 064	Halznut	Italy	MS
ISPAVE 066	Halznut	Italy	MS

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[b] N.S. Iacobellis, Dipartimento di Biologia, Difesa e Biotecnologie Agro-forestali, Università della Basilicata, Potenza, Italy.
[c] NCPPB, National Culture of Plant Pathogenic Bacteria, Harpenden, UK.
[d] G. Surico, Dipartimento di Biotecnologie Agrarie, Università degli Studi, Firenze, Italy.

cabinet. After 48 h incubation at 25°C the diameter (mm) of the inhibition zone or halo formed around the colonies was measured (Lops, 2001). The assays were repeated twice, with three replicates in each.

Elaboration of the data and their graphic representation as a dendrogram was done with the Systat 5.1 computer package which followed cluster analysis that grouped the data, on the basis of the similarity or distance between them, into mutually exclusive clusters consisting of related objects.

Toxin production

After testing each bacterial strain with the antimicrobial assay, the following 16 strains were chosen for LDP production: pv. *aptata* strains NCPPB 871, NCPPB 872 and NCPPB 2664; pv. *syringae* strains B359, Y27, Y37, B382-4, B366, B426, N23, B362-2 and B459; pv. *actinidiae* strain 020; pv. *pisi* strain F2 race 6; pv. *lachrymans* strain S91 and pv. *japonica* strain NCPPB 3093. Each strain was grown in 1 l Roux flasks containing 100 ml of modified IMM liquid medium (pH 5.5) as stationary cultures at 25°C in darkness (Lops, 2001). For each strain 3 flasks were inoculated each with an aliquot of 100 μl of a suspension made in sterile water with a 24–48-h-old bacterial culture grown on NGA (the inoculum was approximately 10^8 cfu ml^{-1}). The experiment was repeated three times.

After 5 days, the bacterial culture was acidified to pH 2 with 6N HCl. Cold acetone was added in the ratio 1:1 (v:v) and stored overnight at 4°C. The bacterial culture was then centrifuged at 9,000 *g* for 20 min. The pellet was discarded and the acetone layer was evaporated from the supernatant under reduced pressure. The term acetone extract will henceforth be used to indicate the liquid watery solution resulting from the acidified bacterial culture treated with acetone and from which the solvent has been removed. The LDP titre from the acetone extract, brought to initial volume by adding sterile distilled water, was determined by 7 serial dilutions (in the ratio 1:1). Twofold serial dilutions of acetone extract from each bacterial culture (10 μl) were spotted onto the surface of Petri dishes containing 7 ml of PDA. The dishes were dried under a sterile laminar flow cabinet and sprayed with a cell suspension of *B. megaterium* by dissolving a loopful of 48-h-old culture streaked on PDA in 10 ml of sterile water, or with a suspension of *R. pilimanae* obtained in the same manner. After 24–48 h of incubation at 25°C, the growth inhibition of the indicator micro-organism was expressed as arbitrary units per ml of initial culture broth (AU ml^{-1}): for instance indicating as 100 the LDP concentration in an end-point dilution which completely inhibited the growth of the test micro-organism in the area of application of a 10 μl droplet.

LPD extraction and purification

Purification, fractioning, chemical characterisation of the LDPs in the stationary culture of each bacterial strain were conducted at the Dipartimento di Scienza degli Alimenti dell'Università di Napoli "Federico II" as previously reported (Fogliano *et al*., 1999; Gallo *et al*., 2000; Monti *et al*., 2001).

Results

Comparison between media for antibacterial activity

The antibacterial activity of the 33 strains from 9 pathovars of *P. syringae* and of 4 strains of *P. avellanae* on 9 media are summarised in Fig. 1 and Table 2. Of the strains grown on 523 medium, 89.2% inhibited *B. megaterium*. Various inhibition diameters were observed, ranging from 3 to 31 mm. On the PDA and NGA media, 81.1% of strains exhibited antibacterial activity. The inhibition zones were up to 77 mm wide on PDA, while on NGA they were narrower, with a width between 6 and 31 mm. The percentage of bacterial strains producing an inhibitory reaction was lower on NSA (78.4%) and on PDCA (75.7%), with mean inhibition zones of 52 and 46 mm respectively. On King B 70.3% of strains inhibited bacterial growth (mean diameter of the inhibition zone from 5 to 25 mm) while on SRM 64.9% of the strains inhibited bacterial growth (mean diameter of inhibition zone from 2 to 29 mm).

IMMA and WM stimulated antimicrobial activity to a less extent. On IMMA, 40.5% of strains produced inhibition zones, while on WM only 30% of strains did so, with inhibition zone diameters ranging from 2 to 29 mm on IMMA and from 2 to 18 mm on WM.

Comparison between media for antifungal activity

The 9 media used for the assay against *B. megaterium* were also tested for their inhibitory

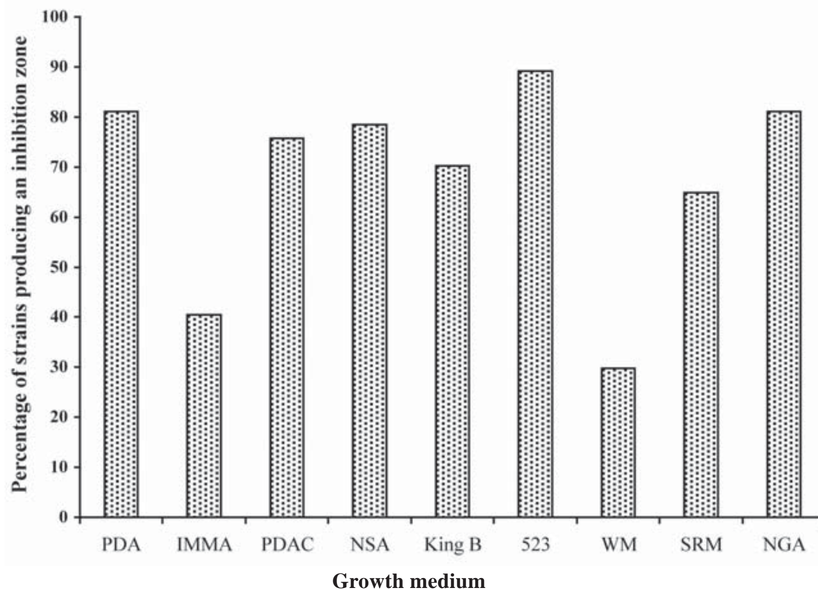

Fig. 1. Inhibition zone against *Bacillus megaterium* produced by 33 pathovars of *Pseudomonas syringae* and 4 strains of *P. avellanae* on 9 different substrates growth media.

action against *R. pilimanae*. Figure 2 and Table 3 show the results of these tests. When the growth media are compared for these results it can easily be seen that King B favoured the formation of inhibition zones in 94.6% of the *Pseudomonas* strains tested, with inhibition zone diameters ranging from 9 to 47 mm. Medium 523 and NGA also favoured an inhibitory reaction, but the percentage of strains inducing fungal growth inhibition was lower than it was with King B (70.3% with 523 and 62.2% with NGA), and the inhibition zone diameter ranged from 2 to 28 mm with 523, and from 7 to 35 mm with NGA. PDCA medium inhibited 51.2% of strains, with inhibition zone diameters of up to 52 mm. The other media were less inhibitory, with inhibition of 45.9% on PDA and 43.2% on NSA, and inhibition zone diameters from 3 to 57 mm and from 2 to 31 mm respectively. IMMA also did not enhance antifungal activity, with only 37.8% of strains showing inhibitory activity, and zone diameters from 7 to 64 mm. On SRM 35.1% of strains gave a positive reaction, with zone diameters from 6 mm to 63 mm. The WM medium was the least favourable substrate, stimulating only 29.7% of strains, with inhibition zones from 3 to 22 mm.

Antibacterial activity elicited by each bacterial strain

Table 2 shows the mean diameters of the inhibition zones produced by 33 strains of 9 pathovars of *P. syringae*, and 4 strains of *P. avellanae*. The two strains of the pv. *actinidiae* caused wider inhibition zones on PDA and on PDCA, but little or no antibacterial activity on the other substrates. All 9 substrates stimulated production of active metabolites against the bacterium from the three strains of pv. *aptata,* but PDA gave the greatest inhibition zone diameter with these strains. Pathovar *japonica* NCPPB 3093 produced an inhibition zone on PDA, PDCA, NSA, 523, NGA, IMMA and SRM. The inhibition zone on PDA was the widest, whereas those on IMMA and SRM were the narrowest.

Pathovar *lachrymans* S91 possessed high antibacterial activity on IMMA, NGA, NSA, SRM and 523, weak activity on King B and PDA, and no inhibitory activity on PDCA and WM. Pathovar *panici* NCPPB 3955 caused inhibition zones on all the tested media except IMMA and SRM. The strongest inhibitory reaction with this strain was on PDA.

The two strains of pv. *papulans* showed a pos-

Table 2. Diameter (mm) of the inhibition zone produced by 33 strains of 9 pathovars of *Pseudomonas syringae* and 4 strains of *P. avellanae* tested on 9 media for antimicrobial activity against *Bacillus megaterium*.

Strain	Growth medium								
	PDA	IMMA	PDCA	NSA	King B	523	WM	SRM	NGA
P. syringae									
pv. *actinidiae*									
193 NA	49	0	36	2	0	0	0	0	0
020	53	0	36	2	0	0	0	9	7
pv. *aptata*									
NCPPB 871	26	12	13	16	23	17	4	5	16
NCPPB 872	44	10	30	24	17	9	2	7	16
NCPPB 2664	36	16	3	18	15	16	13	14	13
pv. *japonica*									
NCPPB 3093	58	2	33	18	0	19	0	2	28
pv. *lachrymans*									
S91	2	29	0	23	7	17	0	21	26
pv. *panici*									
NCPPB 3955	60	0	43	27	20	21	8	0	23
pv. *papulans*									
B485	43	0	27	12	12	3	0	0	25
NCPPB 1015	38	0	23	14	18	19	0	0	19
pv. *persicae*									
NCPPB 2324	26	0	25	0	0	5	0	0	6
NCPPB 2761	47	0	31	0	5	0	0	3	6
pv. *pisi*									
895A race 4	41	14	0	22	7	11	0	11	0
F2 race 6	59	19	39	37	14	13	0	11	20
NCPPB 1366 race 2	59	19	39	35	10	11	0	11	0
NCPPB 2222 race 1	24	0	30	0	0	14	0	10	0
NCPPB 3430 race 1	0	0	10	0	0	12	0	7	0
NCPPB 3431 race 3	39	0	0	26	10	10	0	11	17
NCPPB 3492 race 6	60	0	30	34	11	13	0	12	18
NCPPB 3496 race 3	0	0	0	19	12	12	0	0	18
NCPPB 3498 race 5	43	0	32	52	0	11	0	11	0
NCPPB 3503 race 4	64	14	46	31	0	17	0	12	0
pv. *syringae*									
B3A	35	0	8	17	16	31	3	19	18
B359	6	21	9	11	12	20	14	24	8
B362-2	75	0	27	24	25	12	0	.	31
B366	10	26	14	17	15	14	18	13	18
B382-4	30	0	6	13	19	9	5	0	11
B426	46	22	19	24	16	7	5	5	28
B459	47	0	15	8	14	13	0	0	14
N23	77	0	31	28	11	8	0	29	15
SC F2	19	29	9	13	13	7	8	22	6
Y27	2	16	2	12	14	7	0	16	6
Y37	0	29	0	20	19	24	7	7	20
P. avellanae									
ISPAVE 038	0	0	0	0	0	0	0	0	23
ISPAVE 063	0	0	0	0	11	18	0	0	8
ISPAVE 064	0	0	0	0	0	12	0	0	18
ISPAVE 066	0	0	0	0	0	5	0	0	21

Table 3. Diameter (mm) of the inhibition zone produced by 33 strains of 9 *Pseudomonas syringae* pathovars and 4 strains of *P. avellanae* tested on 9 media for inhibition of *Rhodotorula pilimanae*.

Strain	Growth medium								
	PDA	IMMA	PDCA	NSA	King B	523	WM	SRM	NGA
P. syringae									
pv. *actinidiae*									
020	19	26	20	0	23	0	0	0	0
193 NA	6	7	4	0	18	0	0	0	0
pv. *aptata*									
NCPPB 871	6	30	7	2	43	28	4	8	28
NCPPB 872	13	33	9	20	43	19	0	11	22
NCPPB 2664	12	46	4	10	36	23	18	25	19
pv. *japonica*									
NCPPB 3093	3	0	4	0	20	0	0	0	0
pv. *lachrymans*									
S91	25	54	2	6	24	14	0	36	32
pv. *panici*									
NCPPB 3955	12	0	10	3	40	25	0	0	19
pv. *papulans*									
B485	0	0	2	9	31	11	0	0	19
NCPPB 1015	0	0	2	17	24	10	0	0	9
pv. *persicae*									
NCPPB 2324	0	0	0	0	11	6	0	0	7
NCPPB 2761	0	0	0	0	9	2	0	0	10
pv. *pisi*									
895A race 4	0	0	0	0	24	7	0	0	0
F2 race 6	0	0	0	0	25	13	0	0	10
NCPPB 1366 race 2	0	0	0	0	20	10	0	0	9
NCPPB 2222 race 1	0	0	0	0	17	0	0	0	0
NCPPB 3430 race 1	0	0	0	0	0	0	0	7	0
NCPPB 3431 race 3	0	0	0	0	19	10	0	0	0
NCPPB 3492 race 6	0	0	0	0	24	0	0	0	0
NCPPB 3496 race 3	0	0	0	18	22	10	0	0	10
NCPPB 3498 race 5	0	0	0	0	11	0	0	0	0
NCPPB 3503 race 4	0	0	0	0	13	0	0	0	0
pv. *syringae*									
B3A	14	27	15	3	26	28	7	7	10
B359	16	51	17	17	25	20	21	36	17
B362-2	0	0	0	0	27	5	0	0	0
B366	13	58	19	12	38	18	16	22	21
B382-4	14	12	4	0	47	13	9	6	14
B426	30	54	37	16	30	8	10	12	18
B459	34	0	52	31	23	11	0	29	35
N23	0	0	0	0	20	8	3	0	0
SC F2	57	64	43	29	25	13	5	63	33
Y27	7	49	6	14	30	12	22	23	12
Y37	10	55	4	14	40	24	15	21	16
P. avellanae									
ISPAVE 038	0	0	0	0	0	0	0	0	0
ISPAVE 063	0	0	0	0	18	0	0	0	0
ISPAVE 064	0	0	0	0	24	11	0	0	20
ISPAVE 066	0	0	0	0	24	0	0	0	27

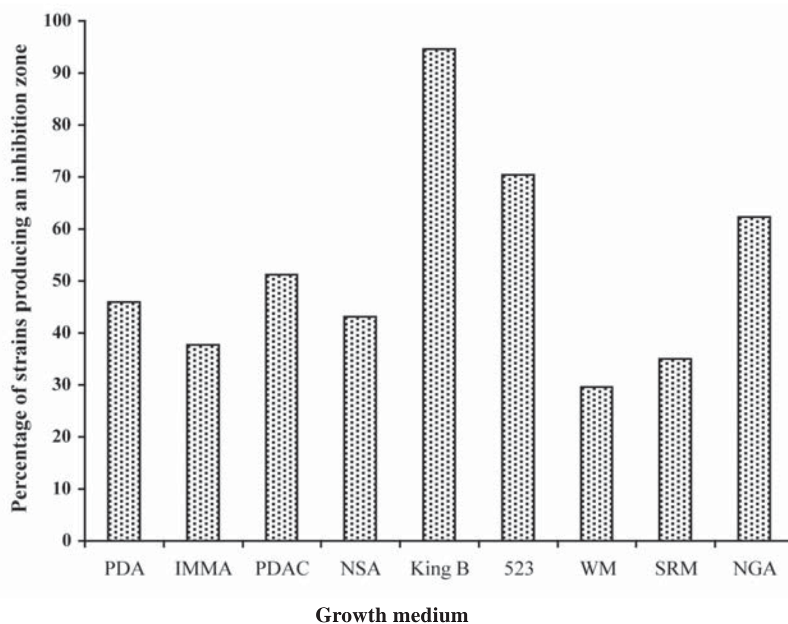

Fig. 2. Inhibition zone against *Rhodotorula pilimanae* produced by 33 pathovars of *Pseudomonas syringae* and 4 strains of *P. avellanae* on 9 different growth media.

itive inhibitory reaction on PDA, PDCA, NGA, NSA, King B and 523, but no reaction on IMMA, WM or SRM. The widest inhibition zone was formed by strain B485 on PDA.

Pathovar *persicae* NCPPB 2324 was inhibitory on PDA, PDCA, 523 and NGA, but not on IMMA, NSA, King B, WM or SRM. Pathovar *persicae* strain NCPPB 2761 was positive on PDA, PDCA, King B, SRM and NGA, but negative on IMMA, NSA, 523 and WM.

All ten strains of pv. *pisi* again caused inhibition zones on various substrates. All these strains inhibited *B. megaterium* on 523, nine were inhibitory on SRM, eight on PDA and NSA, seven on PDCA, six on King B, but only four on NGA and IMMA. None of the strains was inhibitory on WM. The widest inhibition zones were mostly on PDA.

The pv. *syringae* strains were the most active of all strains. All these strains showed antimicrobial activity on NSA, King B, 523 and NGA, ten were active on PDA and PDCA, eight on SRM, seven on WM, but only six on IMMA. The widest inhibition zones were on PDA.

Strains of *P. avellanae* inhibited *B. megaterium* to varying extent only on three substrates King B, 523 and NGA: strain ISPAVE 063 was inhibitory on King B, 523 and NGA, ISPAVE 064 and 066 on 523 and NGA, and strain ISPAVE 038 only on NGA.

Antifungal activity elicited by each bacterial strain

Table 3 shows the antifungal activity of all bacterial strains against *R. pilimanae*. The two strains of pv. *actinidiae* produced inhibition zones only on PDA, IMMA, PDCA and King B, but not on NSA, 523, WM, SRM or NGA. Strain 020 was the best producer of antifungal substances. All the substrates tested stimulated antifungal substance production by the three strains of pv. *aptata* except WM for strain NCPPB 872 . The three pv. *aptata* strains formed the largest inhibition zones on IMMA and King B. Pathovar *japonica* was a weak producer of antifungal substances, and did so only on PDA, PDCA and King B. Pathovar *lachrymans* S91 was positive on eight media and negative on WM. Its widest inhibition zone (54 mm) was on IMMA. Pathovar *panici* NCPPB 3955 formed inhibition zones only on NSA, PDCA, PDA, NGA, 523 and King B. Both strains of pv. *papulans* formed

inhibition zones on PDCA, NSA, King B, 523 and NGA, but not on the other media. The two strains of pv. *persicae* formed small inhibition zones on King B, 523 and NGA, but not on any of the other media.

All the pv. *pisi* strains failed to form inhibition zones on PDA, IMMA, PDCA or WM. Strains NCPPB 3430 race 1 and NCPPB 2222 race 1 were inhibitory only on SRM and King B respectively. NCPPB 1366 race 2 was inhibitory on King B, 523 and NGA; NCPPB 3496 race 3 on NSA, King B, 523 and NGA; NCPPB 3431 race 3 on King B and 523; and F2 race 6 on King B, 523 and NGA.

The pv. *syringae* strains adapted well to the different substrates. All these strains formed inhibition zones when spotted on King B and on 523; nine formed such zones on PDA, PDCA, WM, SRM and NGA; and eight on NSA and IMMA. All the pv. *syringae* isolates except strains B382-4, B362-2, B459 and N23 produced inhibition zones on all the media, with the largest diameter on IMMA. The two strains B362-2 and N23 were similar, with little or no inhibitory activity on most of the substrates. They formed an inhibition zone of some size only on King B (27 mm for B362-2, 20 mm for N23). Strain B459 was most inhibitory when spotted on PDCA, but showed no inhibitory activity on IMMA and WM. Strain B382-4 also produced inhibition zones on most of the substrates (NSA), but the zones were not very wide, except on King B, where the inhibition zone had a mean diameter of 47 mm.

The strains of *P. avellanae* were on the whole weak producers of antimicrobial substances: strain ISPAVE 038 did not show any inhibitory activity at all, three of the other strains were inhibitory on King B, two on NGA, and only one (ISPAVE 064) on 523.

Numerical analysis of the phenotypic features (antibacterial or antifungal activity) of strains of *P. syringae* pathovars or of *P. avellanae*

Schematic representations were created based on the inhibition data of the *Pseudomonas* pathovars/strains against *B. megaterium* (Fig. 3A), against *R. pilimanae* (Fig. 3B) and against *B. megaterium* and *R. pilimanae* combined (Fig. 3C). The inhibition zones produced by each strain on each medium and referred to the single indicator micro-organism, were very dispersed and did not constitute homogeneous subsets. The third representation, which combined the data on antibacterial and antifungal activity, was more indicative of the activity of the bacterial strains because the clusters formed included strains from the same pathovars.

Assay of organic extracts of bacterial cultures

Pathovar *syringae* B366, pv. *aptata* NCPPB 2664 and pv. *lachrymans* S91 were the greatest producers of secondary metabolites in liquid culture, and were active against both *B. megaterium* and *R. pilimanae*. Antifungal activity (3200 AU ml^{-1}) was always greater than antibacterial activity (1600 AU ml^{-1}). Of the pv. *aptata* strains, NCPPB 871 inhibited only *R. pilimanae,* whereas NCPPB 872 and NCPPB 2664 inhibited both indicator organisms. Pathovar *japonica* NCPPB 3093 also inhibited both indicator organisms but its activity was weak. Pathovar *syringae* strain B359, Y27, Y37, B382-4 and B426 showed moderate inhibition of the indicator organisms. Pathovar *syringae* B362-2, B459 and N23, pv. *actinidiae* 020 and pv. *pisi* F2 race 6 were not inhibitory on either the bacterium or the fungus (Table 4).

LDP content in liquid cultures of *P. syringae* pathovars

Table 5 shows the data on the chemical, spectrometric and immunological analyses carried out at the Dipartimento di Scienza degli Alimenti dell'Università di Napoli "Federico II" in order to ascertain whether any LDPs were contained in the culture filtrates of 5 strains of pv. *syringae*, 3 strains of pv. *aptata*, 1 strain of pv. *japonica* and 1 strain of pv. *lachrymans,* all of which had given a positive reaction in the antimicrobial activity assay.

Both lipodepsinonapeptides and SPs were produced by all the strains of pv. *aptata*, pv. *syringae* and pv. *lachrymans*. Only pv. *japonica* strain NCPPB 3093 did not produce any LDP.

Discussion

The preliminary antimicrobial assay, carried out on all 37 strains, allowed research to start and provided indications about the ability of the bacterial strains to produce toxic metabolites, including LDPs. Comparative medium screening demonstrated that individual bacterial strains were adapted to particular substrates and caused inhib-

itory reactions against both micro-organisms tested. On medium 523 89% of strains inhibited *B. megaterium* to varying extent, and on King B 95% of strains were inhibitory against *R. pilimanae*. The inhibitory effect of PDA, most commonly used in tests of this type (Gross and De Vay, 1977; Young and Triggs, 1994; Völksch and Weingart, 1998; Bultreys and Gheysen, 1999), was less than that of 523 for antibacterial activity and less than that of King B for antifungal activity. But it should be

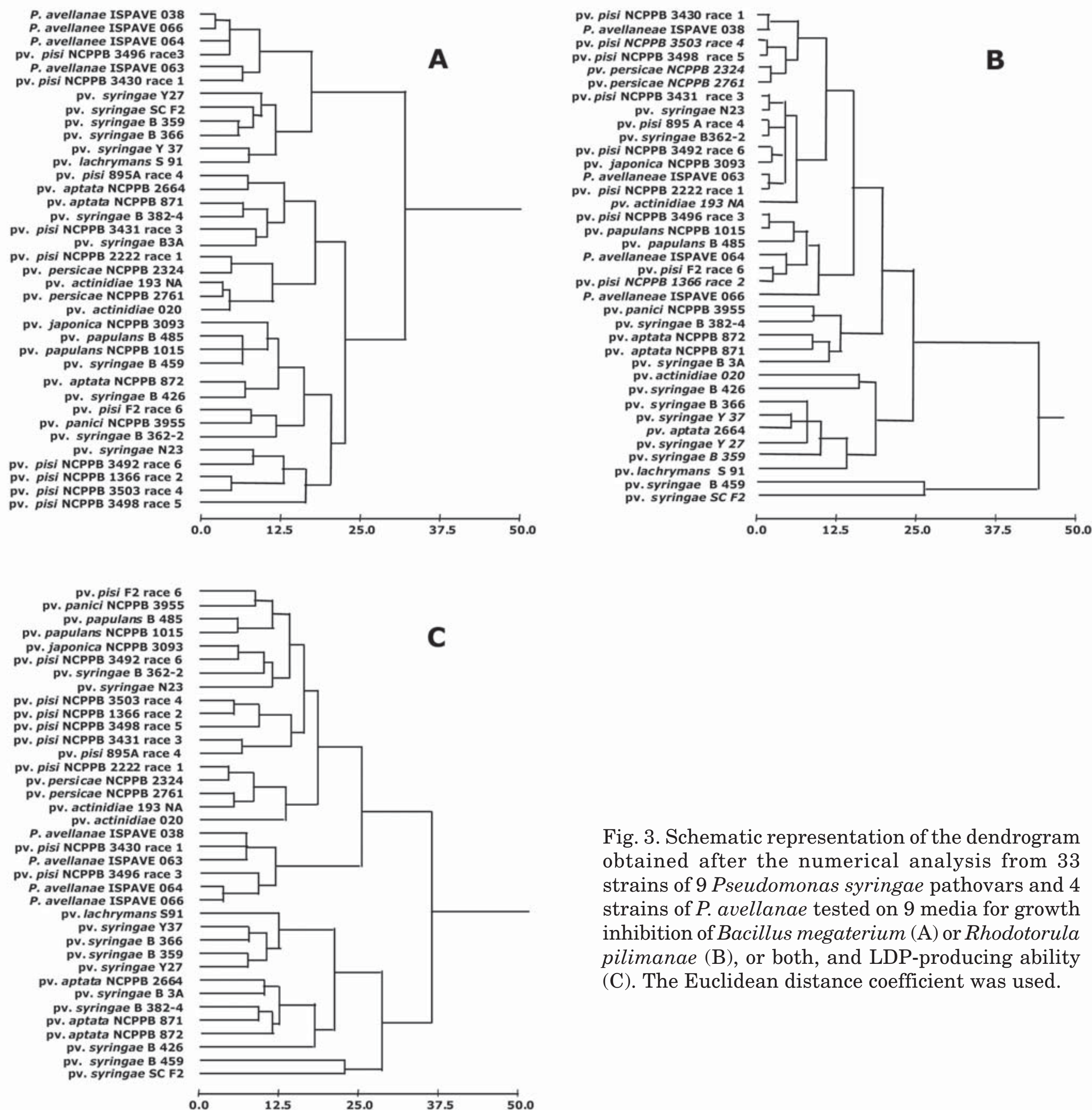

Fig. 3. Schematic representation of the dendrogram obtained after the numerical analysis from 33 strains of 9 *Pseudomonas syringae* pathovars and 4 strains of *P. avellanae* tested on 9 media for growth inhibition of *Bacillus megaterium* (A) or *Rhodotorula pilimanae* (B), or both, and LDP-producing ability (C). The Euclidean distance coefficient was used.

Table 4. Antimicrobial activity, expressed as arbitrary units (AU ml^{-1}), against *Bacillus megaterium* and *Rhodotorula pilimanae*, produced by acetone extract solutions of 16 strains of 5 *Pseudomonas syringae* pathovars grown in modified IMM liquid medium.

Strain	Indicator micro-organism	
	B. megaterium	*R. pilimanae*
pv. a*ctinidiae*		
020	0	0
pv. *aptata*		
NCPPB 871	0	200
NCPPB 872	200	400
NCPPB 2664	1600	3200
pv. *japonica*		
NCPPB3093	100	200
pv. *lachrymans*		
S91	1600	3200
pv. *pisi*		
F2 race 6	0	0
pv. *syringae*		
B359	400	1600
Y27	200	800
Y37	100	400
B382-4	250	800
B426	1600	800
B366	1600	3200
B362-2	0	0
B459	0	0
N23	0	0

Table 5. Lipodepsipeptide production by four pathovars of *Pseudomonas syringae*.

Strain	Lipodepsipeptides	
	Lipodepsinonapeptides	Syringopeptins
pv. *aptata*		
NCPPB 871	+	+
NCPPB 872	+	+
NCPPB 2664	+	+
pv. *japonica*		
NCPPB 3093	–	–
pv. *lachrymans*		
S91	+	+
pv. *syringae*		
B426	+	+
B359	+	+
B366	+	+
B382-4	+	+
Y27	+	+
Y37	+	+

noted that PDA favoured the inhibition zone which was always wide. King B could be used together with a peptone-glucose-NaCl medium, in antifungal testing of pv. *P. syringae* for the production of LPDs against *R. pilimanae* as recently proposed by Bultreys and Gheysen (1999), both these substrates contain peptone but not iron, so that any antifungal activity they have is more likely to be due to the occurrence of siderophores than to the production of LPDs.

All LPD-producing strains examined in this work, with the exception of pv. *syringae* 382-4, and those strains reported to be LDP-producers by other authors, such as strains SCF2 and B3A of pv. *syringae* (Surico *et al*., 1988), presented their strongest inhibitory activity against *R. pilimanae* when assayed on IMMA. On this medium the inhibition zones were wider than on PDA, in agreement with Bultreys and Gheysen (1999). The fact that LDPs were isolated from the culture filtrates of strains that produced a wide inhibition zone on IMMA supported the theory that the haloes were wider because of the LDPs produced. On IMMA the inhibitory activity against *R. pilimanae* was always greater than that against *B. megaterium*. These findings suggest that two classes of LPDs are produced, the lipodepsinonapeptides and SPs. The lipodepsinonapeptides and SPs together, or a high concentration of SPs, inhibit *R. pilimanae*, while the SPs are sufficient by themselves to reduce *B. megaterium* (Lavermicocca *et al*. 1997).

The LDP-producing strains inhibited *B. megaterium* to different degrees depending on whether they were grown on PDA or IMMA. On IMMA the widest inhibition zones were formed by pv. *lachrymans* strain S91, and by pv. *syringae* strains B359, B366, Y37 and Y27, whereas on PDA the inhibition zones were widest with the three strains of pv. *aptata* and with pv. *syringae* isolates B382-4 and B426. The difference in behaviour between these two groups of LDP-producers may be due to the occurrence of other metabolites, already reported but as yet unidentified (Völksch and Weingart, 1998).

Tests at the Dipartimento di Scienza degli Alimenti dell'Università di Napoli "Federico II" (V. Fogliano, personal communication) using MALDI-TOF, HPLC/ESI-MS and immuno-assay methods revealed both cyclic lipodepsipeptides, lipodepsinonapeptides and SPs, in the liquid cultures

of pv. *aptata* (NCPPB 871, NCPPB 872, NCPPB 2664), pv. *lacrymans* (S91) and pv. *syringae* (B426, B359, B366, B382-4, Y27, Y37). These data confirmed some of the results previously reported by Gallo *et al*. (2000), Monti *et al*. (2001) and Grgurina *et al*. (2002).

Subsequent tests revealed that pv. *syringae* strains N23, B459 and B362-2 and pv. *japonica* strain NCPPB 3093 were not LPD producers. Though these strains formed wide inhibition zones against the bacterium on PDA, and against the fungus on King B, they displayed little or no inhibitory activity against the indicator micro-organisms when spotted on IMMA.

Pathovar *pisi* F2 race 6 and pv. *actinidiae* strain 020 produced inhibition zones against one or both indicator micro-organisms on IMMA, but they did not produce LPDs; and in any case, the behaviour of these two strains on the other substrates was different from that of the LPD producers, so that the statistical analysis of the data relating to both activities (Fig. 3C) placed these strains in different groups from those containing the LPD producing strains.

The Systat 5.1 computer package was used for statistical analysis of the antimicrobial activity of the strains of *P. syringae* pathovars against *B. megaterium* and *R. pilimanae,* expressed as inhibition zones on Petri dishes. This package also made it possible to examine similar behaviour within groups of strains belonging to individual pathovars, and the behaviour of strains from different pathovars. In the dendrogram, the 37 isolates were initially subdivided into 2 groups: toxigenic and non-toxigenic. The strains of LPD-producing pathovars such as pv. *syringae,* pv. *lachrymans* and pv. *aptata* fell into adjacent clusters.

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