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Pseudomonas populations causing pith necrosis of tomato and pepper in Argentina are highly diverse

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Pseudomonas species causing pith necrosis symptoms on tomato and pepper collected in different areas of Argentina were identified as *Pseudomonas corrugata*, *P. viridiflava* and *Pseudomonas* spp. Their diversity was analysed and compared with reference strains on the basis of their phenotypic characteristics, copper and antibiotic sensitivity tests, serology, pathogenicity, DNA fingerprinting and restriction fragment length polymorphism (RFLP) analysis of a 16S rRNA gene fragment. All *P. corrugata* strains tested were copper-resistant while *P. viridiflava* strains were more variable. Numerical analysis of phenotypic data showed that all *P. corrugata* strains formed a single phenon that clustered at a level of about 93%, while all the *P. viridiflava* strains clustered in a separated phenon at a level of 94%. Genomic analysis by repetitive (rep)-PCR and 16S rRNA-RFLP fingerprinting and serological analysis showed that the two species contained considerable genetic diversity. Inoculations of tomato and pepper plants with strains from both hosts caused similar pith necrosis symptoms. Strains of both *P. corrugata* and *P. viridiflava* were grouped according to their geographical origin and not according to the original host. This is the first report of *Pseudomonas viridiflava* causing pith necrosis on pepper.

Keywords: copper resistance, pathogenicity, pepper, *Pseudomonas corrugata*, *Pseudomonas viridiflava*, tomato pith necrosis

Introduction

Symptoms of tomato pith necrosis (TPN) in glasshouse tomatoes have been reported to be caused by several different bacterial pathogens, such as *Pseudomonas corrugata* (Scarlett *et al.*, 1978), *P. cichorii* (Wilkie & Dye, 1974), *P. viridiflava* (Malathrakis & Goumas, 1987; Goumas & Chatzaki, 1998), *Erwinia carotovora* ssp. *carotovora* (Speights *et al.*, 1967; Dhanvanthari & Dirks, 1987, Malathrakis & Goumas, 1987), *E. carotovora* ssp. *atroseptica* (Malathrakis & Goumas, 1987), and *P. fluorescens* biotype I (Malathrakis & Goumas, 1987). Also, fluorescent *Pseudomonas* spp., closely related to *P. corrugata*, have been identified as causing TPN in France, Spain and Italy (Catara *et al.*, 1997; Sutra *et al.*, 1997), Greece (Aliviazatos, 1984) and Canada (Dhanvanthari, 1990).

Pseudomonas corrugata is considered to be a ubiquitous bacterium with a broad host range, causing pith necrosis mainly on tomato, but also on pepper (Lopez *et al.*, 1994) and chrysanthemum (Fiori, 1992) with the same symptomatology. It has also been isolated from symptomless alfalfa roots (Lukezic, 1979), rice grains (Van Outryve *et al.*, 1992) and soil (Scortichini, 1989). Results of studies producing biochemical and serological characteristics (Catara *et al.*, 1997; Sutra *et al.*, 1997), lipopolysaccharide patterns (LPS) (Siverio *et al.*, 1993; Catara *et al.*, 1997), fatty acid profiles (Lopez *et al.*, 1994) and protein profiles (Catara *et al.*, 1997) showed *P. corrugata* to be highly heterogeneous.

Pseudomonas viridiflava also produces pith necrosis and other diseases in several plants. It has been reported to cause pith necrosis and basal stem rot of tomato (Malathrakis & Goumas, 1987), fruit rot on tomato (Goumas *et al.*, 1999), discoloured pith on chrysanthemum (Goumas & Chatzaki, 1998), bacterial blight of sweet onion (Gitaitis *et al.*, 1991), root and crown rot of alfalfa (Lukezic *et al.*, 1983), bacterial canker of poinsettia (Suslow & McCain, 1981), blossom blight of kiwifruit (Conn *et al.*, 1993), necrosis of melon, blite, eggplant (Goumas & Chatzaki, 1998) and basil (Little *et al.*, 1994). It has been reported as a secondary invader (Billing, 1970) and an epiphyte (Mariano & McCarter, 1993).

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Tomato pith necrosis caused by *P. corrugata* has became widespread in Argentina since the introduction of hybrid tomato crops grown under plastic greenhouse conditions in the green belt area of La Plata (Alippi *et al.*, 1993). *Pseudomonas viridiflava* has been only reported in Argentina as the cause of basil leaf spot (Alippi *et al.*, 1999).

Because a variety of bacterial species have been reported to cause similar symptoms on tomato and pepper, this work was initiated to identify the bacteria causing TPN, and determine the diversity within native populations isolated from tomato and pepper in Argentina. The purpose of this study was to collect strains from tomato and pepper and characterize them using phenotypic and DNA techniques. The results demonstrated that several pseudomonads cause pith necrosis, including *P. corrugata*, *P. viridiflava* and unidentified *Pseudomonas* spp., and considerable diversity was revealed within these organisms.

Materials and methods

Bacterial collection

A total of 15 newly isolated bacteria and 27 reference strains were divided into six groups (Table 1). The first group included nine strains isolated in different areas of Argentina from diseased tomato and pepper plants and identified as *P. corrugata* based on standard phenotypic criteria (Catara *et al.*, 1997). A second group included nine known *P. corrugata* strains obtained from international culture collections. Four *P. viridiflava* strains isolated in Argentina from tomato and pepper exhibiting symptoms of pith necrosis were studied along with nine *P. viridiflava* strains from culture collections, and two *P. viridiflava* strains isolated from basil in Argentina (Alippi *et al.*, 1999). These strains were used as reference strains for the restriction fragment length polymorphism (RFLP) analysis and included in the numerical analysis. A fifth group included two *Pseudomonas* spp. strains isolated in Argentina from tomato exhibiting symptoms of pith necrosis, and finally single known strains of *P. cichorii*, *P. fluorescens*, *P. syringae* pv. *syringae*, *Ralstonia solanacearum* and *Erwinia carotovora* ssp. *carotovora* were used as reference strains for the numerical analysis.

Isolations from tomato and pepper showing pith necrosis were made on peptone–yeast–glucose agar (PYGA) Lopez *et al.* (1994) and King's medium (KB) (King *et al.*, 1954) according to the procedure described by Lopez *et al.* (1994). The strains were maintained on nutrient agar (NA) at 4°C and stored in liquid KB plus 20% glycerol (v/v) at –80°C and in sterile tap water at room temperature.

Characterization

Fifteen strains isolated from tomato and pepper in Argentina were characterized by the following tests as described by Lelliot *et al.* (1966) or Klement *et al.* (1990): Gram stain reaction, accumulation of poly-β-hydroxybutyrate inclusions (PBH), production of fluorescent pigment on KB, levan formation, presence of oxidase, potato rot, hypersensitive reaction (HR) on tobacco leaves, arginine dihydrolase activity, catalase production, oxidative/fermentative metabolism of glucose, ability to reduce nitrates to nitrites, amylase activity with soluble starch, utilization of Tween 80, ability to liquefy gelatin, growth at 4 and 37°C, tyrosinase activity, litmus test milk, and growth on 0·05% tetrazolium chloride agar (TTC). The accumulation of PBH was also tested on Nile blue medium using Nile blue as a fluorescent stain (Pierce & Schroth, 1994).

Colony appearance was determined on sucrose peptone agar (SPA) (Hayward, 1960), NA + 1% glucose (NGA), KB and PYGA. Pigment production and fluorescence were tested on glucose–peptone agar (GPA) as described by Lukezic (1979). Production of yellow pigment on PYGA, SPA and NGA (Siverio *et al.*, 1993), and growth on semiselective T-5 medium (Gitaitis *et al.*, 1997) was also evaluated. Presence of flagella and cell morphology were determined by electron microscopy using staining bacterial suspensions in double distilled water on carbon-coated 400-mesh grid with 1·5% phosphotungstic acid (pH 7·2) (Schaad, 2001).

Utilization of D-arabinose, L-arabinose, D-celobiose, D-galactose, D-glucose, inositol, maltose, mannitol, L-rhamnose, sucrose, sorbitol and D-trehalose was tested by using the method of Dye (1962). Sugars were filter-sterilized and added to a final concentration of 1%. Utilization of citrate, malate, malonate, propionate, pyruvate and *meso*-tartrate was tested using the OY medium of Dye (1968) supplemented with 0·2% (w/v) organic acid sodium salts. In addition, the strains were tested on API 20E strips (Biomerieux, 00144 Rome, Italy).

Resistance to copper, oxytetracycline, and streptomycin

Copper sensitivity of strains was determined by the agar dilution method using casitone–yeast–extract–glycerol medium (CYEG), a low nutrient medium with limited copper ion binding capacity (Andersen *et al.*, 1991). CYEG was supplemented with different concentrations of copper (as $CuSO_4 \cdot 5H_2O$) in order to determine the minimal inhibitory concentration (MIC) values, expressed as $\mu g\ mL^{-1}$ of cupric sulphate per mL. The lowest concentration of copper preventing bacterial growth is defined as the MIC. For the controls, CYEG without copper was used. Concentrations of copper tested were 40, 80, 180, 200, 240, 280 and 320 $\mu g\ mL^{-1}$. Ten drops (15 μL) of an aqueous bacterial suspension of each strain containing $\sim 3 \times 10^6$ cells mL^{-1} (OD = 0·2 at a wavelength of 620 nm) were placed on the surface of the culture medium (dried and supplemented with copper) using an automatic micropipette. This procedure was repeated twice for each strain and each cupric sulphate concentration. After the bacterial suspensions were absorbed, the plates were incubated in an inverted position at 28°C. After 48 h incubation, bacterial growth was observed and the MIC

Table 1 Bacterial strains used in this study

Species	Strain	Other designation	Host	Origin	Source[a]
Pseudomonas corrugata	Pc 1	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 3	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 4	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 5	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 15	–	Tomato	Argentina	2
Pseudomonas corrugata	Pc 16	–	Pepper	Argentina	2
Pseudomonas corrugata	Pc 22	NCPPB 2445	Tomato	U.K.	3
Pseudomonas corrugata	Pc 23	536·7	Tomato	Spain	4
Pseudomonas corrugata	Pc 24	592·4	Pepper	Spain	4
Pseudomonas corrugata	Pc 25	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 26	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 27	PD 1394	Tomato	Turkey	5
Pseudomonas corrugata	Pc 28	–	Tomato	Argentina	1
Pseudomonas corrugata	Pc 29	CFBP 10883	Tomato	France	6
Pseudomonas corrugata	Pc 30	CFBP 10146	Tomato	France	6
Pseudomonas corrugata	Pc 31	CFBP 10900	Tomato	France	6
Pseudomonas corrugata	Pc 32	CFBP 10904	Tomato	France	6
Pseudomonas corrugata	Pc 33	CFBP 10906	Tomato	France	6
Pseudomonas spp. (TPN associated)	Ps 2	–	Tomato	Argentina	1
Pseudomonas spp. (TPN associated)	Ps 17	–	Tomato	Argentina	1
Pseudomonas cichorii	Pcich1	–	Celery	Argentina	7
Pseudomonas fluorescens bv. 5	ATCC 11253	NRRL B–1244	NDA	USA	8
Pseudomonas fluorescens bv. 2	Fr 2	C.TR212	Soil	France	9
Pseudomonas syringae pv. *syringae*	Pss	ATCC 12273	Lilac	NDA	8
Pseudomonas viridiflava	Pvir 8	–	Pepper	Argentina	1
Pseudomonas viridiflava	Pvir 9	–	Pepper	Argentina	1
Pseudomonas viridiflava	Pvir 11	–	Tomato	Argentina	1
Pseudomonas viridiflava	Pvir 14	–	Tomato	Argentina	1
Pseudomonas viridiflava	PV 1	ATCC 43557	Tomato	USA	10
Pseudomonas viridiflava	PV 2	–	Pepper	USA	10
Pseudomonas viridiflava	PV 11	–	Poinsettia	USA	10
Pseudomonas viridiflava	PV 16	–	Tomato	USA	10
Pseudomonas viridiflava	PV 17	ATCC 43558	Tomato	USA	10
Pseudomonas viridiflava	PV 18	ATCC 43559	Tomato	USA	10
Pseudomonas viridiflava	PV 20	–	Onion	USA	10
Pseudomonas viridiflava	Pvalb7	–	Basil	Argentina	7
Pseudomonas viridiflava	Pvalb8	–	Basil	Argentina	7
Pseudomonas viridiflava	PV 5776	ICMP 5776	Bean	Iran	11
Pseudomonas viridiflava	PV 12363	ICMP 12363	Basil	Iran	11
Ralstonia solanacearum	RsUSA	G2	Tomato	USA	12
Ralstonia solanacearum	RsC	BV R596–10	Tomato	Argentina	13
Erwinia carotovora ssp. *carotovora*	Ecc	Ecc	Tomato	Argentina	7

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NDA, no data available.

for each strain was determined. MIC for sensitive strains ranged between 40 and 120 μg mL^{-1}; strains were considered as having low resistance when their MIC ranged between 160 and 240 μg mL^{-1}, and as having high resistance when their MIC were between 280 and 320 μg mL^{-1} or greater as described by Sundin *et al.* (1989).

Similarly, susceptibility to streptomycin sulphate and oxytetracycline hydrochloride was assessed on SPA medium supplemented with 100 μg mL^{-1} streptomycin sulphate and 50 μg mL^{-1} oxytetracycline hydrochloride, respectively (Ritchie & Dittapongpitch, 1991; Spotts & Cervantes, 1995).

Pathogenicity tests

Plants of tomato [*Lycopersicon esculentum* cvs Tommy, BHN 110, Presto, Iván, Ringo and Platense (línea 7 Gorina); pepper (*Capsicum annuum*, cv. Dominó) and chrysanthemum (*Chrysanthemun* × *morifolium* = *Dendrathema* × *grandiflorum*, cv. Dignity Alba] were grown in a plastic greenhouse at 35 ± 5°C. They were inoculated 1 month after transplanting to pots with sterile potting mix. Inoculations were made by injecting 0·5 mL of a bacterial suspension of about 10^7 cfu mL^{-1} into the stems. The injections were performed at two different points: 10 and 30 cm above the potting mix. Three replicates per cultivar × bacterial strain were performed. Control plants were inoculated with sterile distilled water. Plants were maintained in a humid chamber for 3 days after the inoculations (Lopez *et al.*, 1994). External (adventitious roots and necrotic areas) and internal (pith necrosis) symptoms were recorded 45 days after inoculation.

In addition, tobacco plants (*Nicotiana tabacum*, cv. Samsum) were inoculated with the same inoculum and in the same manner but at only one point of injection (10 cm from the substrate). Internal symptoms were evaluated 40 days after the inoculation. Controls plants were inoculated with water. For each plant species and cultivar tested, bacteria were isolated on KB agar.

Serological techniques

Antisera were prepared from strains Pc 1 and Pc 16. Female rabbits weighting ~2 kg were intramuscularly injected once a week for 4 weeks with 2 mL of a 1:1 emulsion of each bacterial suspension (10^9 cfu mL^{-1}, 1 h treatment at 100°C) and Freund's incomplete adjuvant (Siverio *et al.*, 1993). Afterwards, the rabbits received four intravenous injections of 1 mL of each bacterial suspension at 4-day intervals. The rabbits were bled 3 days after the last injection. The antiserum titre was determined by the Ouchterlony double diffusion test (Hampton *et al.*, 1990). In addition, four antisera to cells of *P. corrugata* were included in ELISA tests: IPV55-6FP provided by M. Fiori (University Di Sassari, Italy) and AS NCPPB2445T, AS 536T and AS 592P provided by M. M. López [Instituto Valenciano de Investigaciones Agrarias (IVIA), Valencia, Spain].

ELISA-I was used to determine differences in serological response among known strains of *P. corrugata* and the newly isolated strains of *Pseudomonas* spp. causing pith necrosis. Additional species of *Pseudomonas* and other bacteria were used as reference strains. The ELISA-I assays were done according to Siverio *et al.* (1993). Bacterial antigens were added directly to the plates in coating buffer at a concentration of 10^9 cfu mL^{-1}. *Pseudomonas corrugata* antisera were diluted in PBS as follows: IPV55-6FP, 1:5000; NCPPB 2445T, 1:6000; AS 536, 1:200; AS 592P, 1:1000; Pc 1, 1:250; Pc 16, 1:1000. For the detection studies, alkaline phosphatase-conjugated goat antirabbit immunoglobulin diluted 1:3000 in PBS, Tween 20 0·05% and 3% nonfat powdered milk was used. P-nitrophenyl phosphate substratum was freshly prepared at a concentration of 0·7% in Diethanolamina 10% and plates were read at OD 405 after 1 h. Values that were twice those of the negative control in each plate were considered positive.

Growth of cells and extraction of DNA

Strains of *P. corrugata* and other *Pseudomonas* species were grown on KB while *R. solanacearum* and *E. c. carotovora* were grown on SPA for 24 h at 28°C. For the preparation of bacterial DNA template, a rapid procedure using whole cells from plates was applied. Briefly, cells from about one to two single colonies picked up by using a 1-μL plastic disposable loop were suspended in 300 μL NaCl 1 M. Each sample was vortex-mixed and centrifuged at 16 000 ***g*** for 4 min, the supernatant was removed and the pellet resuspended in 300 μL double-distilled water, vortex-mixed and centrifuged at 16 000 ***g*** for 3 min. The supernatant was removed and the pellet was resuspended in 150 μL of an aqueous suspension of 6% resin. The mixture of cells and resin was incubated at 56°C for 20 min and vortex-mixed for 30 s. Finally, the mixture was incubated at 99°C for 8 min, and vortex-mixed for 1 min. Bacterial debris and resin were removed by centrifugation, and a subsample of the supernatant DNA was used as template for PCR amplifications.

Repetitive (rep)-PCR fingerprinting

The rep-PCR method with BOXA1R, REP (REP1R-I and REP2-I) and ERIC (ERIC1R and ERIC2) primers were used (Versalovic *et al.*, 1994). Polymerase chain reaction (PCR) was carried out in a final volume of 25 μL according to the protocols of Louws *et al.* (1994) using 10 μL of the supernatant of crude DNA preparation as template source. The reaction mix was overlaid with a drop of mineral oil and incubated in a thermal cycler according to the specifications of Louws *et al.* (1994) for each primer. Five microlitres of the PCR reactions were run on a 1·6% agarose gel, in TBE buffer 0·5×, and observed under UV light after staining with ethidium bromide (Ausubel *et al.*, 1992). Fingerprints generated were compared visually. In addition, photographs of PCR profiles were analysed with Gelcompar vs. 4·1 software (Applied Maths, Kortrijk, Belgium) using the UPGMA clustering algorithm and DICE coefficient for a combined gel.

Amplification of specific PCR products

Two pairs of primers, PC 1/1 and PC 1/2, and PC 5/1 and PC 5/2 (Catara *et al.*, 2000), were combined and used in a single PCR reaction for the amplification of two specific PCR products (either 1100 or 600 bp) according to the *P. corrugata* strain tested (Catara *et al.*, 2000). The PCR reactions were carried out in a final volume of 25 μL, containing PCR buffer, 1·5 mM $MgCl_2$, 2 mM of each dNTP, the four primers (10 pmol of each) and 1·25 U *Taq* polymerase (Catara *et al.*, 2000). Whole cells were used as template source and obtained by gently touching one single bacterial colony (picked up by using a sterile toothpick) and then touching the bottom of the PCR tube with the contaminated toothpick. The reaction mix was overlaid with a drop of mineral oil and incubated in a thermal cycler as described by Catara *et al.* (2000). PCR products were separated in a 1·6% agarose gel in TBE buffer 0·5×, and observed under UV light after staining with ethidium bromide. The strains were distinguished by the presence of either of these two possible bands: 1100 bp (band I) or 600 bp (band II).

RFLP analysis of PCR-amplified 16S rRNA

A fragment of 1·5 kb of 16S rRNA gene was amplified by PCR using primers rD1 and fD1 (Weisburg *et al.*, 1991) and whole cells prepared as described above as a template. Subsamples of 8 μL were incubated overnight at 37°C with restriction enzymes *Alu*I, *Cfo*I, *Msp*I, *Rsa*I and *Hae*III. RFLP analysis was performed by electrophoresis in a 2% agarose gel. Banding patterns were analysed using the UPGMA clustering algorithm and DICE coefficient, and a band tolerance of 3%.

Sequence analysis

A 260-bp DNA region of genes encoding 16S rRNA was amplified by using Y1 and Y2 primers (Young *et al.*, 1991). The Y1 and Y2 amplification product was purified and concentrated with isopropanol as described by van Berkum *et al.* (1996), and both strands were sequenced by using the dideoxy chain terminator procedure. Sequence data were analysed with software SEQUAID, Clustwal W, and the Pileup program of the University of Wisconsin Genetics Computer Group (Madison, WI, USA) package vs. 9·0 (GCG). The aligned partial 16S rRNA sequences were analysed together with the sequences of reference strains from the Gene Bank (http://www2.ncbi.nlm.nih.gov/cgi-bin/GenBank).

Numerical analysis

A total of 71 characteristics from 42 strains were used for the numerical taxonomy analysis. Biochemical and physiological characteristics, resistance to copper and antibiotics, pigment production on the media tested, results obtained by using the API 20 E galley, PCR amplification of the two specific bands, induction of HR in tobacco, and pathogenicity tests were subjected to analysis. Results were ranked as 1 (positive) or 0 (negative). The distance matrix was calculated by using the DICE coefficient and the cluster analysis was performed using the unweighted pair group method with averages (UPGMA) (Rohlf, 1994). The analysis was performed using SIMQUAL and SHAN programs (NTSYSpc version 2·0, software package, Exeter Software, Setauket, NY, USA).

Results

Biochemical and physiological tests

The bacterial strains from Argentina were either typical of the species *P. corrugata* or *P. viridiflava* except for two isolates, Ps 2 and Ps 17, which were unclassified as *Pseudomonas* spp. (Table 3).

Resistance to copper, oxytetracycline, and streptomycin

Three levels of copper sensitivity were revealed among the Argentinian *Pseudomonas* populations isolated from tomato and pepper plants with TPN. All known *P. corrugata* strains were resistant to copper with MCI values ranging between 200 and 320 μg mL^{-1}. Variable results were obtained with *P. viridiflava* strains; MIC values ranged from < 40 to > 320 μg mL^{-1}. *Pseudomonas* spp. (isolate Ps 2) was highly resistant whereas Ps 17 showed low resistance (Table 2).

Of *P. corrugata* strains, 44 and 50%, respectively, were resistant to oxytetracycline and streptomycin. In contrast, only 20 and 26%, respectively, of *P. viridiflava* strains were resistant to these antibiotics.

Pathogenicity tests

Strains identified as *P. corrugata* produced black pith cavities as well as adventitious roots and browning or discoloration of stem surfaces on all tomato and pepper plants tested. Although none of the *P. corrugata* strains from France (namely Pc 29, Pc 30, Pc 31, Pc 32, and Pc 33) (Table 1) produced any symptoms to chrysanthemum, all the other strains produced typical symptoms (Table 3). Results obtained with tobacco plants were variable depending upon the strain tested. All *Pseudomonas* spp. causing TPN were pathogenic in tomato, pepper and chrysanthemum, and produced identical symptoms on all the hosts tested. No symptoms were observed on control plants inoculated with water. Bacteria isolated from lesions and reinoculated resulted in the same symptoms and therefore fulfilled Koch's postulates.

Serology

The results of the ELISA-I-tests are shown in Table 4. Antisera prepared against Argentinean strains Pc 1 and Pc 16 reacted with five and 14 out of 18 *P. corrugata* strains tested, respectively. Antisera IPV55/6FP, NC2445T,

Table 2 Resistance of *Pseudomonas* isolates causing tomato and pepper pith necrosis to copper sulphate, expressed as minimal inhibitory concentrations (MIC) of cupric sulphate in casitone–yeast–glycerol agar

Strain	MIC copper sulphate in CYEG ($\mu g\ mL^{-1}$)	Copper sensitivity
Pseudomonas corrugata		
Pc 1	280	HR
Pc 3	200	LR
Pc 4	200	LR
Pc 5	200	LR
Pc 15	280	HR
Pc 16	280	HR
Pc 2445	320	HR
Pc 536·7	320	HR
Pc 592·4	240	LR
Pc 25	240	LR
Pc 26	200	LR
Pc 1394	280	HR
Pc 28	280	HR
Pc 10883	240	LR
Pc 10146	320	HR
Pc 10900	280	HR
Pc 10904	320	HR
Pc 10906	280	HR
P. viridiflava		
Pv 5776	240	LR
Pv 12363	180	LR
Pv 1	280	HR
Pv 2	280	HR
Pv 11	40	S
Pv 16	280	HR
Pv 17	40	S
Pv 18	80	S
Pv 20	40	S
Pvir 8	> 320	HR
Pvir 9	< 40	S
Pvir 11	< 40	S
Pvir 14	> 320	HR
P. viridiflava Pvalb7	40	S
P. viridiflava Pvalb8	40	S
Pseudomonas spp. Ps 2	320	HR
Pseudomonas spp. Ps 17	180	LR

S, sensitive; LR, low resistance; HR, high resistance.

536T, and 592P reacted with six (33%), seven (39%), eight (44%) and seven (39%) *P. corrugata* strains, respectively. *Pseudomonas viridiflava* strain 9, *P. fluorescens* and *P. s. syringae* reacted against all the sera tested. In contrast, *P. corrugata* 1394 and *P. cichorii* did not react against any sera. Strains PV 5776, PV 2 and Pvir 9 of *P. viridiflava* reacted against all sera, while strains Pvir8 reacted against two sera (33%), Pvir 11 against five sera (83%) and Pvir 14 reacted against three sera (50%).

rep-PCR fingerprinting

The analysis of 18 *P. corrugata* strains by using primers BOX, REP and ERIC in combination with PCR generated 16 genomic patterns, each consisting of a variable number of bands: between five and 12 bands with molecular sizes ranging from 150 bp to > 2 kb for BOX-PCR; six to 15 bands ranging from 150 bp to 1·3 kb for ERIC-PCR, and five to 10 bands ranging from < 100 bp to 2·5 kb for REP-PCR (Fig. 1). Bands common among different profiles were identified: *P. corrugata* strains Pc 10883 and Pc 10906 from France had identical BOX-, REP- and ERIC-PCR fingerprint profiles, as did Pc 1 and Pc 5 strains from Argentina (Fig. 1). Analysis of the combined BOX-, REP- and ERIC-PCR patterns revealed that all the strains clustered in three major groups (A, B and C) at 57, 63 and 70% level of similarity, respectively, and similarity between clusters A, B and C was about 55% (Fig. 1). Cluster A comprised strains Pc 22 (NCPPB2445 from England, type strain); Pc 27 (PD1394 from Turkey); Pc 23 (Pc 536·7) and Pc 24 (Pc 592·4) from Spain; Pc 30 (CFBP10146), Pc 29 (CFBP10883), Pc 32 (CFBP10904), Pc 33 (CFBP10906) and Pc 31 (CFBP10900) from France. Cluster B comprised Pc 15, Pc 16, Pc 25 and Pc 26, and cluster C strains Pc 1, Pc 3, Pc 4, Pc 5 and Pc 28, both from Argentina. All Argentinian strains were grouped within clusters B and C, while strains from other countries were grouped into cluster A.

Analysis of *P. viridiflava* strains resulted in 15 profiles; each *P. viridiflava* strain tested had a unique BOX-, REP- and ERIC-PCR fingerprint profile, although bands common to different profiles were observed (Fig. 2). The analysis of the combined patterns revealed that strains clustered into two groups, A and B. Cluster A comprised strains from Argentina (Pvir 8, Pvir 11, Pvalb7, Pvalb8, Pvir 14, Pvir 9) and strains Pv 5776 and Pv 12363 from Iran, which clustered at a similarity level of about 55%; while group B comprised strains from the USA (PV 1, Pv 11, Pv 2, Pv 16, Pv 17, Pv 18 and Pv 20) that clustered at a similarity level of about 60%. The similarity between clusters A and B was 55%.

Amplification of specific PCR products

All the strains of *P. corrugata* examined produced a single band following amplification by PCR with primers PC 1/1, PC 1/2, PC 5/1 and PC 5/2. Only two strains from Spain (536·7 and 592·4 isolated from tomato and pepper) and one from France (CFBP 10906 from tomato) showed the band II of 600 bp, while the rest of the strains (83%) showed the band I of 1100 bp. Representative PCR results are shown in Fig. 3. As expected, none of the *P. viridiflava* strains or other *Pseudomonas* species listed in Table 1 produced any bands following the amplification by PCR with these primers.

RFLP analysis of 16S rRNA

The use of endonucleases *Alu*I, *Hae*III and *Msp*I in the RFLP analysis of 16S rRNA gene allowed 18 *P. corrugata* strains to be grouped into 11 genotypes. The dendrogram cluster analysis is shown in Fig. 4. RFLP patterns of Pc 5, Pc 15, PC 16, Pc 22, Pc 27 and Pc 28 were identical. The

Table 3 Characters used to differentiate *Pseudomonas* spp. causing tomato and pepper pith necrosis

	Pseudomonas corrugata													*Pseudomonas viridiflava*			TPN	
	Pc 1	Pc 3	Pc 4	Pc 5	Pc 15	Pc 16	Pc 2445	Pc 536·7	Pc 592·4	Pc 25	Pc 26	Pc 1394	Pc 28	Pc 10883	Pc 10146	Pc 10900	Pc 10904	Pc 10906
Gram reaction	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PBH inclusions	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescence on KB	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Levan	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Potato soft rot	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	+	+	–	–	+	–	–	–	–	+	–	+	–	+	–	–
Tobacco HR	–	+	+	+	+	+	+	+	+	–	–	–	+	+	+	+	+	+
Growth at 37°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tween 80 hydrolysis	+	+	–	–	+	+	–	–	–	+	+	+	+	–	–	–	+	+
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tyrosine degradation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on TTZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pigmentation on GP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Fluorescence on GP	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth on T-5	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Litmus test (acid production)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin liquefaction	+	+	+	+	+	+	–	–	+	+	+	+	+	+	+	+	+	+
Nitrate reduction to nitrite	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Indol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Acetoine production (V-P)	–	+	+	–	–	–	+	+	–	–	–	–	–	–	+	–	–	+
D-arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-celobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Sorbitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D(+)-trehalose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Meso-tartrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pathogenicity on:																		
Tomato	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pepper	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chrysanthemum	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–	–	–	–
Tobacco	–	–	+	+	–	+	+	+	+	+	+	+	+	–	+	–	+	+

Table 3 *continued*

	Pseudomonas viridiflava															TPN	
	PV 5776	PV 12363	PV 1	PV 2	PV 11	PV 16	PV 17	PV 18	PV 20	Pvir 8	Pvir 9	Pvir 11	Pvir 14	PValb7	PValb8	Ps 2	Ps 17
Gram reaction	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Catalase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
PBH inclusions	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Fluorescence on KB	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Levan	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Potato soft rot	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Tobacco HR	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Growth at 37°C	–	+	–	–	–	–	–	+	–	+	+	+	+	–	–	+	+
Growth at 4°C	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Tween 80 hydrolysis	+	+	–	+	–	–	+	+	–	–	–	–	–	+	+	–	–
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tyrosine degradation	+	+	+	+	+	+	+	+	+	–	+	+	–	+	+	–	+
Growth on TTZ	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pigmentation on GP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Fluorescence on GP	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Growth on T-5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
Litmus test (acid production)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Gelatin liquefaction	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction to nitrite	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Indol	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Acetoine production (V-P)	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
D-arabinose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
L-arabinose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
D-celobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-galactose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
D-glucose	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	–	+	+	–	+	+	+	+	+	+	+	+	+	+	+	–	+
Maltose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Manitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–
L-rhamnose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	–
Sucrose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	–
D(+)-trehalose	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	+	+
Citrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-malate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Malonate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Propionate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pyruvate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Meso-tartrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Pathogenicity on:																	
Tomato	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Pepper	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Chrysanthemum	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–	+
Tobacco	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	–

Table 4 Serological reactions of *Pseudomonas corrugata* strains, other *Pseudomonas* spp. and *Ralstonia solanacearum* by ELISA-I. *Erwinia carotovora* ssp. *carotovora* (Ecc) was used as negative control

	Antisera					
Strain	1	16	IPV55-6FP	NC2445T	536T	592P
Pc 1	+	+	–	–	–	–
Pc 3	+	–	–	–	–	–
Pc 4	+	–	–	–	–	–
Pc 5	+	–	–	–	+	–
Pc 15	–	+	–	–	–	–
Pc 16	–	+	–	–	–	+
Pc 2445	+	+	–	+	–	–
Pc 536·7	–	+	+	+	+	+
Pc 592·4	–	+	+	+	+	+
Pc 25	–	+	–	–	–	–
Pc 26	–	+	–	–	–	–
Pc 1394	–	–	–	–	–	–
Pc 28	–	+	–	–	+	–
Pc 10883	–	+	+	+	+	+
Pc 10146	–	+	+	+	+	+
Pc 10900	–	+	+	+	+	+
Pc 10904	–	+	–	–	–	–
Pc 10906	–	+	+	+	+	+
Ps 2	–	+	–	–	–	+
Pvir 8	–	+	–	–	–	+
Pvir 9	+	+	+	+	+	+
Pvir 11	–	+	+	+	+	+
Pvir 14	–	+	–	+	–	+
Ps 17	–	+	–	–	–	–
R. solanacearum C	–	+	–	–	–	–
R. solanacearum USA	–	+	–	–	–	–
P. viridiflava PV 2	+	+	+	+	+	+
P. viridiflava PV 5776	+	+	+	+	+	+
P. fluorescens Fr 2	+	+	+	+	+	+
P. fluorescens NRRL	+	+	+	+	+	+
P. cichorii Pcich1	–	–	–	–	–	–
P. s. syringae ATCC	+	+	+	+	+	+
E. c. carotovora Ecc	–	–	–	–	–	–

two strains from Spain (Pc 23 and 24) also showed identical RFLP patterns between them, as did two strains from France (Pc 29 and Pc 30). The patterns of these two French strains were also very similar to patterns from Pc 31; these strains from Spain and France formed group B, clustering together at a level of similarity of more than 85%, while all nine Argentinean strains clustered together in group A at a similarity level of about 79%. Group A also contained Pc 22 (the type strain from the UK), Pc 27 (from Turkey) and Pc 32 (from France).

Similar analysis of 13 *P. viridiflava* strains resulted in three main clusters (A, B and C, Fig. 5) using *Alu*I, *Hae*III, *Cfo*I and *Rsa*I digestions. Cluster A contained Pvir 8, and Pvir 14 strains from pepper and tomato from Argentina; both strains clustered together with a similarity of 86%. Within cluster B, patterns generated by Pv 12363 from Iran, and Pv 1, Pv 16 and Pv 11 from the USA were identical and closely related to patterns generated by Pv 20 and Pv 18 from USA, and Pvir 9 and Pvir 11 from Argentina. Patterns generated by Pvalb 7 and Pvalb 8 from Argentina were identical. Strains in group B showed a similarity level amongst themselves of about 88%. Cluster C contained one strain, Pv 5776 from Iran. Groups A, B and C clustered together at a similarity level of 65%.

Sequence analysis

Homology of 100% occurred between strain Pvir 9 and the reference strain of *P. viridiflava* PVZ76671, whilst *P. corrugata* (Gene Bank No. D84012) showed a homology of 99·54% to strain Pc 23 and 100% to the strains Pc 22 and Pc 27.

Numerical analysis

A dendrogram displaying the similarity among the strains of *P. corrugata* (n = 18) and *P. viridiflava* (n = 15) in comparison with reference strains (n = 9) based on the numerical analysis of 71 traits is shown in Fig. 6.

All the *P. corrugata* strains clustered together at a similarity level of about 93%, while all the *P. viridiflava* strains clustered together at a level of 94%. The rest of the *Pseudomonas* species, including both TPN strains clustered at a level of about 65%; *R. solanacearum* and *P. fluorescens* strains tested clustered together at a level of 93 and 87%, respectively, while *E. c.* ssp. *carotovora* clustered with *P. corrugata* strains at 53%.

Within the cluster of *P. corrugata,* three distinct groups were recognized at levels of about 97, 96·5 and 95%, respectively (named C, B and A). Group A contained seven Argentinian strains and strain Pc 32 from France and Pc 27 from Turkey; group B contained two Argentinian strains, three strains from France, and the type culture Pc 22 from the UK. Group C contained two strains from Spain and one from France.

Within the cluster of *P. viridiflava* strains, three groups, D, E and F, were recognized at similarity levels of about 98, 98 and 97%, respectively. Group D contained two Argentinian strains from basil and six strains from USA, group E contained four Argentinian strains (two from pepper and two from tomato exhibiting symptoms of pith necrosis), and group F contained one strain from USA and two from Iran.

Pseudomonas spp. Ps 17 clustered separately at a level of about 80%, indicating that it belonged to another species, but closely related to the *P. corrugata* group, while *Pseudomonas* spp. Ps 2 clustered separately at a level of 65%.

Discussion

In order to investigate the diversity within native bacterial populations isolated from tomato and pepper exhibiting pith necrosis symptoms in Argentina, 15 isolates from tomato and pepper were collected and compared with reference strains of *P. corrugata*, *P. viridiflava*, *P. cichorii*, *P. fluorescens*, *P. syringae* pv. *syringae*, *R. solanacearum* and *E. carotovora* ssp. *carotovora*. The collection of isolates was characterized by traditional phenotypic characteristics, copper and antibiotic sensitivity, ELISA,

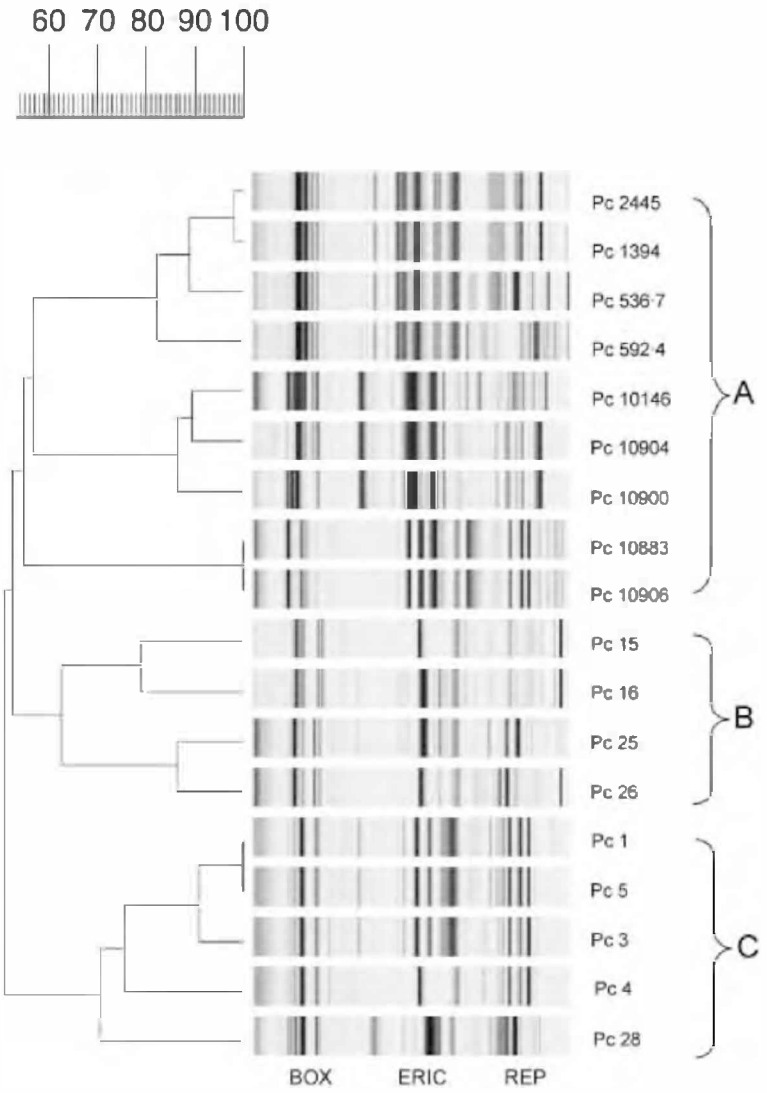

Figure 1 Similarity of 18 strains of *Pseudomonas corrugata* based on the presence and absence of bands generated using primers BOX, REP and ERIC. The clustering shown is based on the UPGMA clustering algorithm and DICE coefficient with a band tolerance of 3%.

pathogenicity, DNA fingerprinting and RFLP analysis of a fragment of 16S rRNA gene. The results confirmed that three pseudomonads, *P. corrugata*, *P. viridiflava* and *Pseudomonas* spp., cause pith necrosis on tomato and pepper in Argentina; in addition, considerable genetic diversity was revealed.

Using the numerical analysis data, all *P. corrugata* strains formed a single phenon that clustered at a level of about 93% comprising three subphenons A, B, and C, while all the *P. viridiflava* strains clustered into a separated phenon at a level of 94% that comprised three groups (D, E and F) (Fig. 6). The existence of *Pseudomonas* spp. closely related to *P. corrugata* has been reported by Sutra *et al.* (1997) and Catara *et al.* (1997), but the biochemical test results obtained here are not in agreement with the amended description for *Pseudomonas* according to Sutra *et al.* (1997). Results reported here showed that native Argentinian populations causing TPN symptoms exhibit genetic diversity but that they are nevertheless related, as observed by ELISA, results from which suggest that the antisera cross-reacted with *P. corrugata*, *P. viridiflava* and TPN *Pseudomonas* spp.

All the *P. corrugata* strains tested here were copper-resistant. This is most likely due to the heavy use of copper sprays by growers, as described in California (Cooksey & Azad, 1992). It has been reported that *P. syringae* pv. *tomato* accumulates copper as part of the resistance mechanism encoded by the *cop* operon located on pPT23D plasmid (Bender & Cooksey, 1987). The mechanism is suggested to be common to copper-resistant *Pseudomonas* species (Cooksey & Azad, 1992). This is the first report on copper resistance in *P. corrugata*. The *P. corrugata* strains in this study demonstrated different levels of resistance to copper. All the highly resistant strains, however, were from Argentina where copper sprays are commonly used in tomato and pepper crops for controlling fungal diseases. Also within the Argentinean collection of *P. corrugata*, a high proportion of strains were resistant to oxytetracycline and streptomycin. On the other hand, most of the *P. viridiflava* strains were susceptible to

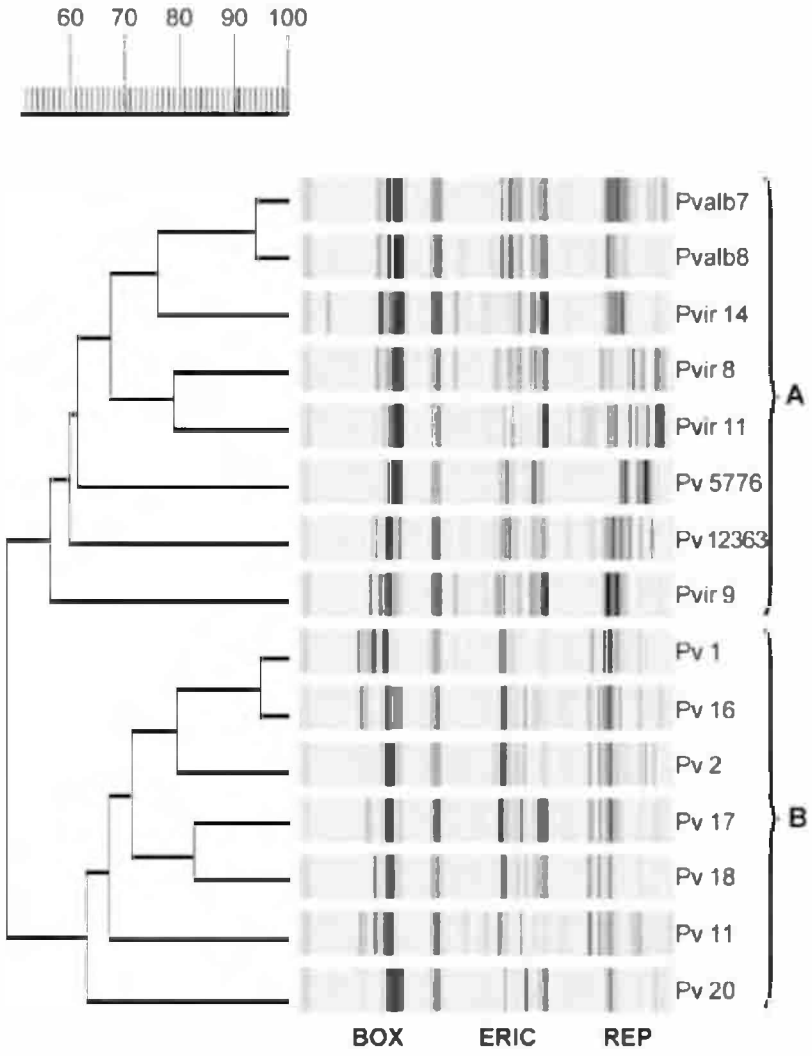

Figure 2 Similarity of strains of *Pseudomonas viridiflava* based on the presence and absence of bands generated using primers BOX, REP and ERIC, respectively. The clustering shown is based on the UPGMA clustering algorithm and DICE coefficient with a band tolerance of 3%.

copper, streptomycin and oxytetracycline, regardless of their geographical origin.

A high degree of heterogeneity in the serological reactions of *P. corrugata* was previously described by Siverio *et al.* (1993), Lopez *et al.* (1994) and Catara *et al.* (1997). They reported cross-reactions between *P. corrugata* and fluorescent *Pseudomonas* species causing TPN symptoms and indicated that these strains shared common antigen epitopes in spite of their phenotypic heterogeneity. These results may be due to a direct relationship between serological reactions and lipopolysacharide (LPS) profiles between strains of the same species or strains of closely related species of the genus *Pseudomonas*. Achouak *et al.* (2000) also reported cross-reactions between *P. corrugata*, *P. fluorescens* bv. I, II and III, *P. putida* biotype 1 and *P. brassicacearum*, by using an antisera prepared from *P. brassicacearum*. In the study reported here, using 18 strains of *P. corrugata*, only Pc 27 (PD1394) from Turkey failed to react against any of the six sera tested. While results of the rest of the strains were very variable, a similar phenomenon has been reported previously for *P. corrugata* strains isolated from several European countries (Lopez *et al.*, 1994; Catara *et al.*, 1997; Sutra *et al.*, 1997). Most of the *P. viridiflava* strains examined reacted against all or most of the sera (reactivity between anti-*P. corrugata* sera and *P. viridiflava* strains); these cross-reactions confirmed that *P. corrugata* and *P. viridiflava* also share common epitopes in spite of their heterogeneity.

By using primers corresponding to ERIC-sequences in combination with PCR, Achouak *et al.* (2000) found 55 genomic fingerprint patterns within a collection of 236 strains of *P. corrugata* isolated from two different soil types from the area of Grignon (France). Results from this study confirmed the high genetic diversity of the species. Using ERIC primers for the Argentinian strains, the number of bands and their molecular sizes are in agreement with data obtained by Achouak *et al.* (2000) for ERIC, including a common band of 517 bp found in most of the strains. No previous study in which BOX or REP primers were used has been reported. A close relationship was found between geographical origin of strains and combined fingerprint patterns: all the strains from France

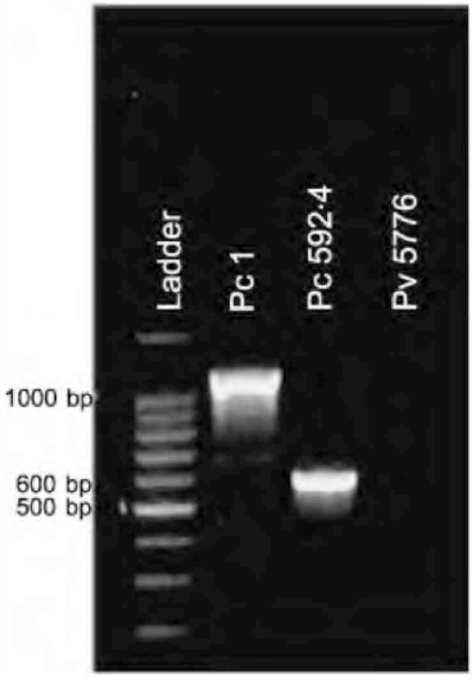

Figure 3 PCR amplification with a mixture of type I and type II primers (PC1/1, PC1/2, PC5/1, and PC5/2). Lanes: 1, ladder; 2, *Pseudomonas corrugata* Pc1 from Argentina, *P. corrugata* Pc 24 from Spain; 3, *P. viridiflava* PV 5776.

and Spain, and the strains from England and Turkey, typed into group A, while groups B and C contained strains from Argentina. No relationship was found between host and fingerprint patterns since strains from tomato and pepper belonged to the same group.

A low correlation was observed between fingerprint patterns obtained by rep-PCR and those obtained by 16S rRNA-RFLP analysis (Figs 1 and 2 vs. Figs 4 and 5). This is expected since 16S rRNA varies little at the species level. Achouak *et al.* (2000) found a unique restriction pattern by using *Alu*I within the *P. corrugata* population from France. The same pattern was observed in this study in strains from France (Pc 33, Pc 29, Pc 30 and Pc 31) and Spain (Pc 23 and Pc 24). With the exception of Pc 1 and Pc 4, the rest of the *P. corrugata* strains from Argentina showed the same *Alu*I pattern, different from the French strains; on the other hand, all the Argentinian strains, Pc 32 from France, Pc 22 from England (NCPPB type strain), and Pc 1394 from Turkey showed the same *Hae*III pattern, different from the pattern observed within the French and Spanish strains (Fig. 4). This result is attributed to a level of genetic diversity present in the 16S rRNA, as observed in other organisms (Prior *et al.*, 1998).

No relationship was found between fingerprint patterns obtained by rep-PCR and those obtained by 16S rRNA-RFLP analysis in *P. viridiflava* strains also. This result agrees with that of Catara *et al.* (2000) regarding the

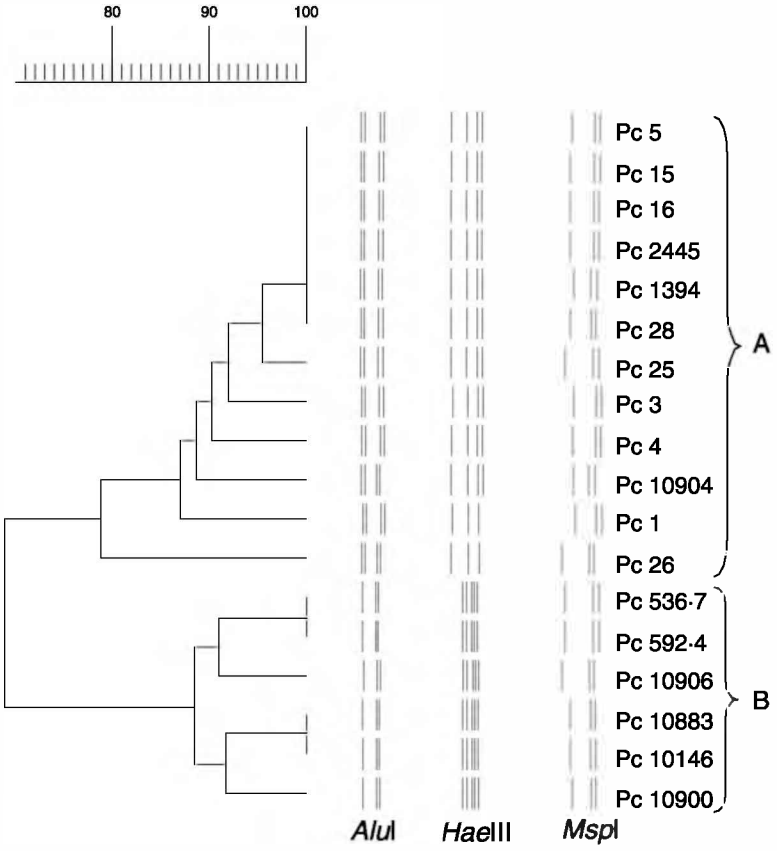

Figure 4 Similarity of *Pseudomonas corrugata* strains based on combined restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rRNA by using the restriction enzymes *Alu*I, *Hae*III and *Msp*I. The clustering shown is based on UPGMA and the DICE coefficient with a band tolerance of 3%.

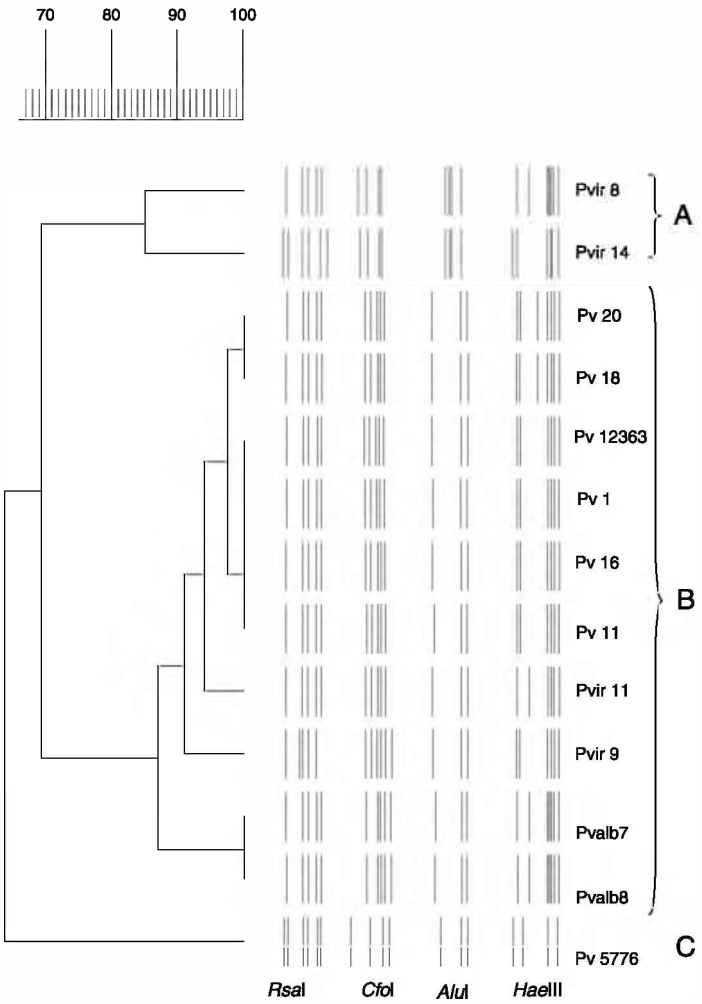

Figure 5 Similarity of *Pseudomonas viridiflava* strains based on combined restriction fragment length polymorphism (RFLP) patterns of PCR-amplified 16S rRNA by using the restriction enzymes *Rsa*I, *Cfo*I, *Alu*I and *Hae*III, respectively. The dendrogram shown is based on UPGMA and the DICE coefficient with a band tolerance of 3%.

existence of two distinct genomic groups within *P. corrugata*. The most frequent amplification product was a genotype I-specific band of 1100 bp found in 83% of *P. corrugata* strains tested. All Argentinian isolates showed the genotype I band. The genotype II-specific band of 600 bp was only found in three European strains (two from Spain and one from France). None of the *P. viridiflava* or other *Pseudomonas* spp. tested produced any PCR-specific product. The genotype II group involving the two strains from Spain and one from France coincides with group C of strains observed in the dendrogram constructed from data obtained by numerical analysis of biochemical, physiological and pathogenicity tests (Fig. 6). The strains belonging to groups A and B from the numerical analysis (Fig. 6) represent the genotype I (band of 1100 bp). Similar results occurred when observing the dendrogram obtained from data from rep-PCR and 16S rRNA-RFLP analysis; the strains of *P. corrugata* that represent genotype II belong to the same clusters (Figs 1 and 3).

There is a general tendency among the strains of *P. corrugata* and *P. viridiflava* to group according to geographical origin and not by host of isolation. This suggests that the populations are not host-specific. This is the first report of a pith necrosis being caused by *P. viridiflava* on greenhouse-grown tomato and pepper plants in Argentina and the first report of a pith necrosis in pepper plants being caused by *P. viridiflava*. The four strains identified as *P. viridiflava* isolated from tomato and pepper exhibiting pith necrosis symptoms were highly aggressive for both hosts. The 16S rRNA sequence of strain Pvir 9 was 100% homologous when compared with the sequence of the reference type strain deposited in GenBank (*P. viridiflava* accession no. PVZ76671).

This work clearly demonstrated that *P. corrugata* and *P. viridiflava* are the dominant species causing pith necrosis of tomato and pepper in Argentina.

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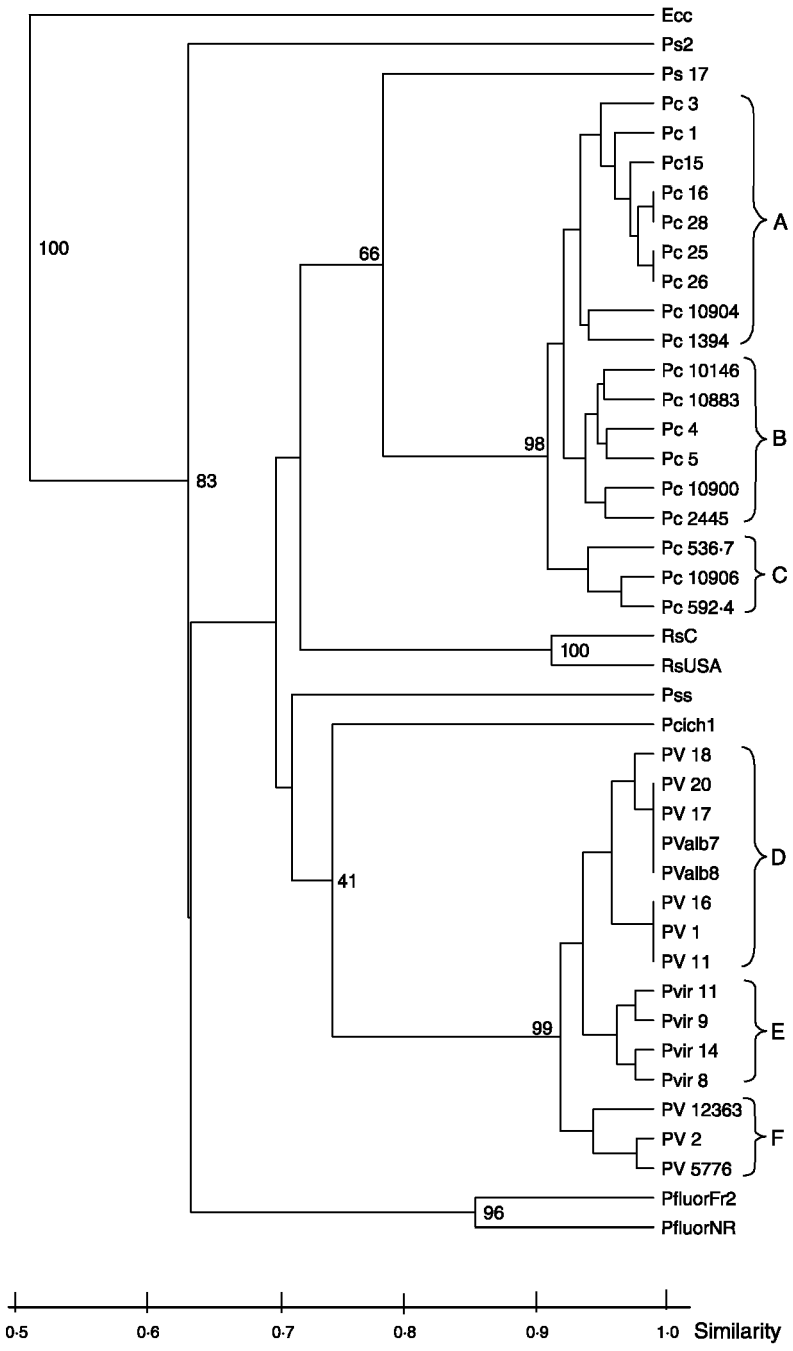

Figure 6 Dendrogram displaying the similarity among the strains of *Psuedomonas corrugata* (n = 18), *P. viridiflava* (n = 15) and TPN-*Pseudomonas* spp. (n = 2) in comparison with reference strains (n = 7) based on morphological, physiological and biochemical tests, antibiotic resistance and pathogenicity. The distance matrix was calculated using the DICE coefficient and UPGMA clustering algorithm. Bootstrap values are indicated at the branching points (500 resampled data sets).

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