European Journal of Plant Pathology (2006) 115: 419–430
DOI 10.1007/s10658-006-9033-4

Characterisation of *Pseudomonas syringae* strains associated with a leaf disease of leek in Australia

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Accepted 29 May 2006

Key words: Allium diseases, fatty acid analysis, pathovar, seed transmission

Abstract

A necrotic leaf disease of leek (*Allium ampeloprasum* Porrum Group) is reported in Australia for the first time. The fluorescent pseudomonad consistently associated with diseased tissue was identified as *Pseudomonas syringae* by LOPAT tests (+,–,–,–,+), carbon utilisation, bean and lemon inoculations and fatty acid methyl ester analysis. It was confirmed as *P. syringae* pv. *porri* by pathogenicity to leeks, bulb onions, spring onions, shallots and garlic, and by genetic analysis using 16S rDNA PCR, REP, ERIC and BOX PCR, and IS50 PCR. Comparison with reference strains of pv. *porri* from other countries showed similarity to known strains of pv. *porri*. The Australian leek strains were generally uniform in their biochemical reactions although three strains tested varied in their pathogenicity to other *Allium* spp. and varied from published data. All Australian strains shared the same genetic profile with strains from New Zealand, France and California. However, Japanese strains from leek and onion were distinct from the Australian strains and those from New Zealand, France and California. Data strongly support the hypothesis that the pathogen is seed-borne.

Introduction

Leeks (*Allium ampeloprasum* Porrum Group) have become an important component of vegetable production in Australia. Production has expanded over the past 20 years from increased domestic demand and establishment of more export markets in Japan, Hong Kong and Singapore. The leek industry was valued at $17.2 million (Australian) in 2002. The main leek production areas in Australia are the outer metropolitan areas of Melbourne, Adelaide and Perth with smaller plantings in Stanthorpe and Redlands in Queensland. Most seed is imported from Europe, and is grown in speedling (plug) trays in nurseries for up to 100 days before being planted in the field. Leeks are grown all year, except for 1 month in mid-winter, with larger growers planting up to 200,000 seedlings every week in adjacent plantings. Several varieties of leeks are used throughout the year and summer crops are generally irrigated with overhead sprinklers. Leeks are harvested from 4 months after planting in spring and summer to 7 months after planting in autumn and winter crops. Varying rotations are used, from leek on leek for several years, or with up to 5-year rotations with other vegetable crops and cereals.

Pseudomonas syringae was first recorded as a disease of *Allium* by Goto (1972), who described a leaf spot of onions in Japan, where eye-spot lesions had water-soaked margins and a yellow halo. Lelliot (1952) described a bacterial disease of leeks caused by a pseudomonad, later attributed to a strain of *P. syringae* by Hale (1975). Samson et al. (1981) reported similar symptoms in France and published a description of this pathogen (Samson et al., 1998), naming it *P. syringae* pv. *porri*. This disease has been confirmed as present in New Zealand (Hale, 1975), France (Samson, 1981) and USA (Koike et al., 1999). All reports have described similar symptoms on leek plants, with flower stems having sunken lesions with necrotic centres and olive-green water-soaked edges. Leaf lesions were water-soaked with a yellow halo and which often coalesced into long yellow to light brown streaks and leaf tips withered. Koike et al. (1999) suggested that the bacterium was seed-borne and spread through the crop by the treatment of transplants and by overhead irrigation.

A leaf disease with symptoms similar to those described above was observed in 2001 and 2002 in leek seedlings and mature plants in South Australia, Victoria and Western Australia. Symptoms appeared initially as small pale lesions with water-soaked margins which then developed into rusty-brown leaf spots, sometimes with water-soaked, slightly raised margins, occasionally with a yellow halo in some cultivars (Figure 1), or as yellow streaking down the centre or edge of leaves following leaf veination. Disease in seedlings reduced plant vigour and caused death of transplants after 3–4 weeks in the field. Damage to mature plants resulted in extensive death of leaf tissue and curling of leaves if a lesion extended only up one side of the leaf (Figures 2, 3). Lesions could extend from the bulb (Figure 4) to leaf tip. Bacterial ooze from sections of water-soaked tissue suggested a bacterial disease.

This paper reports on the first detection of bacterial blight of leeks in Australia, the characterisation of the causal bacterium, *Pseudomonas syringae* pv. *porri*, and comparison with other strains of the pathogen.

Figure 1. Early stage symptoms of bacterial leaf spot of leek.

Figure 2. Shepherd's crook symptoms on leek caused by elongated leaf lesions.

Figure 3. Shepherd's crook symptoms on leek caused by elongated leaf lesions.

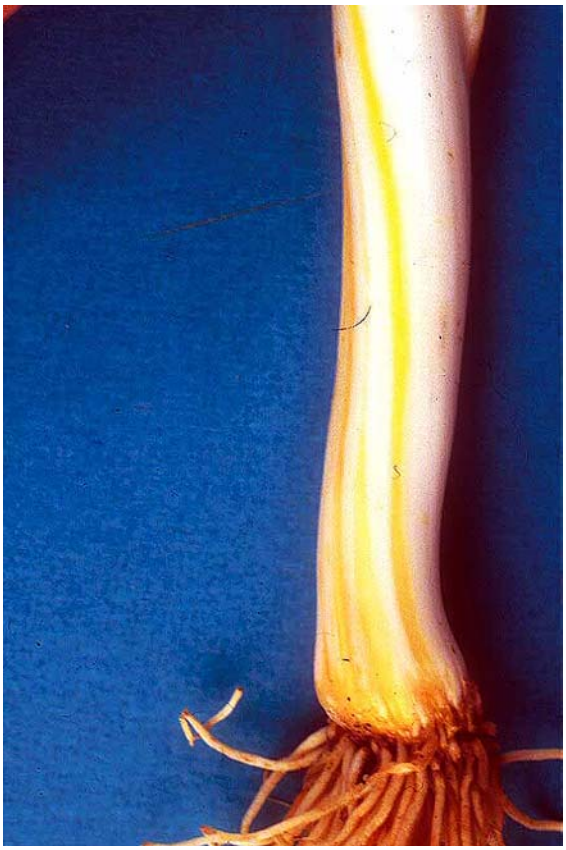

Figure 4. Lesions caused by *P. syringae* pv. *porri* extending through to the root cap.

Materials and methods

Isolation

Diseased tissues (Figure 1) were examined in 2001 and 2002 for bacterial streaming. Small pieces were dissected in sterile distilled water (SDW); where ooze was observed, small sections of adjacent tissue were macerated and placed in 2 ml SDW for 20 min, after which loopfuls were streaked onto a Kings medium B agar (KB), (King et al., 1954) and sucrose peptone agar (SPA), (Hayward, 1960) for isolation and identification. Plates were incubated at 27 °C and examined after 24–48 h. Cultures of interest were stored on beads using Microbank™ storage tubes (PL.160, Pro-Lab Diagnostics, Ontario, Canada) at −70 °C. Selected strains were deposited in the Australian Collection of Plant Pathogenic Bacteria (ACPPB in Herb. DAR, WFCC 365).

Comparative strains

Characterised strains of *P. syringae* pv. *porri* from leek and a strain from onion were imported through quarantine for comparative tests (Table 1). In addition, the following Australian strains from ACPPB were included: a strain (DAR 61456) from leek grown from seed which had been imported into Australia from Japan and three strains of *P. syringae* pv. *syringae* from cherry (DAR 30499, 33426) and lilac (DAR 35712). The pv. *syringae* strains were included to determine the relationship of the field strains to a known pathovar possessing similar pathogenic and phenotypic characteristics.

Physiological and biochemical tests

The LOPAT tests of Lelliott et al. (1966) for levan, oxidase, potato rot, arginine dihydrolase and

tobacco hypersensitivity were performed on all strains, including those re-isolated from pathogenicity tests. Carbon source utilisation tests were performed on Biolog GN Microplates™ (Biolog Inc., Hayward, CA, USA) to the manufacturer's instructions and scored after 24 and 48 h. Additional tests for hydrolysis of aesculin and ability to liquefy gelatine, and for the utilisation of adonitol, *p*-hydroxybenzoate, betaine, beta-hydroxybutyrate, erythritol, glutarate, homoserine, inositol, lactate, malonate, mannitol, quinate, sorbitol, L+-tartrate and trigonelline were carried out by the method of Young and Triggs (1994). Beans and immature lemons were inoculated by the method of Fahy and Hayward (1983). All tests were replicated twice and repeated at least twice.

The whole cell fatty acid methyl ester profiles were determined using the MIDI System (Microbial ID, Inc. (MIDI), Newark, DE, USA). Bacteria were grown on trypticase soy broth (BBL) with 1.5% agar (Bacto) (TSBA) for 1 day at 28 °C. Fatty acids were extracted following the sample preparation procedures described in the Microbial Identification System Handbook and analysed using a Hewlett-Packard 6890 gas chromatograph.

Pathogenicity tests

Pathogenicity studies to confirm Koch's postulates were undertaken initially on seedlings. Later studies examined whether the bacteria isolated from the leaf lesions could also cause the symptoms observed on inflorescence stalks and whether these stalk infections could result in infected seed in the developing seed heads. All pathogenicity tests were conducted at least twice and SDW was used as the negative control in all tests.

Table 1. Isolates from leek and other sources used for comparison in this study

Isolate DAR	Host	Location	Year	Source	Strain number	Other collection designation	Reference	Restriction digestion pattern of 16S rDNA fragments[a]
61456	Leek	Adelaide, SA, ex Japanese seed import	1988	ACPPB				Profile 3 2/1/1/2/3/1/2
75283	Leek	Nairne, SA	2001	This study				Profile 2 2/1/1/2/2/2/2
75284	Leek	Lenswood, SA	2001	This study				Profile 2 2/1/1/2/2/2/2
75287	Leek	Langhorne Creek, SA	2001	This study				Profile 2 2/1/1/2/2/2/2
75554	Leek	Clyde, Vic	2002	This study				Profile 5 2/1/1/2/2/4/2
75555	Leek	Murray Bridge, SA	2002	This study				Profile 5 2/1/1/2/2/4/2
75556	Leek	Langhorne Creek, SA	2002	This study				Profile 5 2/1/1/2/2/4/2
77311	Leek	Langhorne Creek, SA	2001	This study				Profile 5 2/1/1/2/2/4/2
77312	Leek	Langhorne Creek, SA	2001	This study				Profile 5 2/1/1/2/2/4/2
77313	Leek	Lenswood, SA	2001	This study				Profile 5 2/1/1/2/2/4/2
77314	Leek	Lenswood, SA	2001	This study				Profile 5 2/1/1/2/2/4/2
77315	Leek	Lenswood, SA	2001	This study				Profile 5 2/1/1/2/2/4/2
75520	Onion	Japan	1969	ICMP	3414	CFPB1787NCPPB 2737(Goto strain a)	Samson et al. (1988)	Profile 4 3/2/2/3/1/3/3
75521	Leek	New Zealand	1973	ICMP	3644	NCPPB 2693		Profile 5 2/1/1/2/2/4/2
75522	Leek	France	1978	ICMP	8961	CFBP1908 NCPPB 3364	Holopathotype strain	Profile 5 2/1/1/2/2/4/2
75523	Leek	California	1999	S. Koike	96-01		Koike et al. (1999)	Profile 5 2/1/1/2/2/4/2
75524	Leek	California	1999	S. Koike	96-02			Profile 5 2/1/1/2/2/4/2
30499	Cherry	Young, NSW	1978	ACPPB				Profile 6 2/3/2/4/1/1/4
33426	Cherry	Armidale, NSW	1979	ACPPB				Profile 1 1/1/1/1/1/1/1
35712	Lilac	Orange, NSW	1981	ACPPB				Profile 7 4/3/3/5/4/1/4

[a]Digestion enzymes: *Cfo* I/*Dde* I/*Hae* III/*Hin* f I/*Msp* I/*Nci* I/*Rsa* I.

Inoculum preparation
Isolates of bacteria were grown on KB agar. Bacterial cells from 24 h-old cultures of each strain were suspended in SDW to give the required concentration of $\sim 5 \times 10^6$ cfu ml^{-1} unless otherwise stated.

Seedlings
Preliminary pathogenicity tests were carried out using two strains from leek from different outbreaks and locations, (DAR 75283 and 75555) recovered from leaf tissue in South Australia. Leek seed (each of cvs Missile, Musselburgh, Harpoon, Nova and Admiral) was germinated in 100 mm pots containing sterile 1:1 perlite:vermiculite mix, watered and maintained in a growth room at 25 °C for 2–4 weeks. Seedlings were transferred into 5 cm pots and grown for a further 3–4 weeks before inoculation. Plants were placed in a humidity chamber (95%) at 17–25 °C for 24 h prior to inoculation. Plants were then removed and spray-inoculated to visible moisture and returned to the humidity chamber for a further 24 h, after which they were kept in a greenhouse for a further 7–10 days. Symptoms were described, and differences in susceptibility assessed for each treatment by recording the number of plants displaying symptoms of characteristic water-soaked spots on the leaves. Bacteria were isolated from the developing lesions and identified to confirm Koch's postulates.

Host range
Seeds of leek (cvs Missile, Harpoon, Musselburgh), onion (cvs Cream Gold, Hunter River White), shallot (Ambition F1) and spring (Welsh) onion (cv. Longwhite bunching) were sown in Speedling™ trays containing pasteurised potting mix. One seed was sown per cell so that each tray contained eight rows, each containing eight plants of each species. Trays were replicated six times and kept in a greenhouse at 25/15 °C day/night for 7 weeks prior to inoculation. Garlic (cv. unknown) was grown in 100 mm diam pots from individual cloves. Trays were placed in a humid chamber for 24 h prior to spray-inoculation as above with one of the strains listed in Table 3. Trays were returned to the humidity chamber for a further 24 h, after which they were maintained in the greenhouse and results noted for 21 days when isolations were undertaken from lesions developing on each species. Negative controls were spray-inoculated with SDW and positive controls with known pv. *porri*. In addition, a known pathogenic strain of *P. syringae* pv. *syringae*, DAR33426, was included for comparative purposes. Pathogenicity was scored by counting the number and percentage plants in each cultivar displaying characteristic lesions. White marks caused by hypersensitive reactions were scored separately.

Inflorescence stalks
Mature plants of leek (cv. Musselburgh) and spring onion that had developed inflorescence stalks ('pipes') were transplanted from a domestic garden into 20 cm diam pots and placed in the greenhouse. Stalks of two plants of each species were stab-inoculated at several locations about 3 cm apart with a 25G needle attached to a hypodermic syringe filled with a suspension of each bacterium, including positive and negative controls, as listed in Table 3. Slight pressure was applied to the needle to wound the stem and to the hypodermic plunger so that a drop of inoculum was placed at the wound site. Plants were placed in the humidity chamber for 24 h then returned to the greenhouse. Pathogenicity of the strains was scored according to the symptom severity and given a positive score for the appearance of symptoms. A negative result indicates the production of no symptoms, while + + + implies the most severe reaction.

Seed transmission
The stalks of seed heads on mature 7 month-old leek plants, cv. Missile, in a commercial planting were inoculated with a strain of *P. syringae* pv. *porri* (DAR 75555). A bacterial suspension (1 ml of approximately 10^8 cells ml^{-1}) was injected into the seed stalk approximately 20–30 cm below the seed head of 72 plants using a 12G hypodermic syringe. Sterile water was injected as a control treatment into a further 72 plants. Plastic bags sprayed inside with water to provide humidity were placed over the seed heads and sealed around the stem. These bags were removed 48 h later. Plants were assessed after 10 days for stem lesions on the seed stalk and 8 weeks later the seed heads were removed, dried and the seed recovered. Forty leek seeds collected from each of 16 inoculated plants were immersed in 70% ethanol for 3 min and macerated in 10 ml sterile water using a

mortar and pestle. Suspensions (100 μl) of aliquots ranging from undiluted to 10^{-4} dilution were spread onto KB and sucrose peptone agar plates, which were then dried for 5 min in a laminar airflow cabinet. After drying, plates were incubated at 28 °C for 48 h before being assessed for the presence of fluorescing and levan-positive colonies characteristic of *Pseudomonas* spp. In addition, 30 sterilised seeds were germinated and sown in pasteurised potting mix as described above and symptoms developing on seedlings were observed for 10 weeks after germination.

Molecular analysis

Bacterial cultures were grown on Nutrient Agar (Oxoid) and the DNA was extracted using the DNeasy© Tissue Kit (Qiagen,Vic., Australia). A loopful of bacterial cells was harvested from the plate, washed in 1 ml of 1× phosphate buffered saline, resuspended in the kit's extraction buffer and processed according to manufacturer's instructions.

16S rDNA PCR

One micro litres of DNA template was used undiluted in a final volume of 25 μl. Each reaction contained 200 μM of each deoxyribonucleotide triphosphate (dNTP, Promega, Annandale, NSW, Australia), 1 μM primer 2F and 1 μM primer 5R (Ochiai and Kaku, 1991), 2 mM $MgCl_2$ and 1.25 U of Taq Polymerase (Invitrogen, Mt Waverly, Vic., Australia) in buffer (50 mM Tris (pH 9), 20 mM NaCl, 1% Triton X-100, 0.1% gelatine). Amplification was an initial denaturation (94 °C, 3 min), followed by 30 cycles of denaturation (94 °C, 1 min), annealing (55 °C, 1 min) and extension (72 °C, 2 min), with a final extension of 72 °C for 5 min.

Six micro litre of the amplified 16S fragment was digested in the appropriate buffer (supplied) using 5 U of each restriction enzyme, in a final volume of 20 μl. Fragments were digested separately with each of the enzymes *Cfo* I, *Dde* I, *Hae* III, *Msp* I and *Rsa* I (all from Roche) and *Hinf* I (Promega), at 37 °C for approximately 1 h. Digestion products were electrophoresed on 1–2% agarose gels (Promega) in 1× TBE buffer; gels were stained with ethidium bromide and images captured by UV transillumination using a Gel Doc 2000 (Bio-Rad, Reagents Park, NSW, Australia). All fragments were co-electrophoresed with a 100 bp marker (New-England Biolabs, Ipswich, USA). Isolates producing the same restriction pattern as each other for any one enzyme were recorded as having the same number for that enzyme; each enzyme produced between 3 and 5 different patterns from the 20 strains used in this study.

REP, ERIC AND BOX PCR

Extracts were prepared using the Qiagen DNeasy Tissue kit and diluted 1:10 in TE buffer. PCR mix contained 200 μM dNTPs (Promega), 1 × PCR Buffer (20 μmM Tris–HCl (pH 8.4), 50 mM KCl; Invitrogen) 4 mM $MgCl_2$ (Invitrogen), 1% Bovine Serum Albumin (Promega), 1 μM of each primer, 1 μl of template DNA, 6 μl GeneReleaser (BioVentures, Murfreesboro, TN, USA) and 1.25 U of Taq polymerase (Invitrogen) in a final volume of 25 μl. Reactions were overlayed with 20 μl of paraffin oil (Sigma Pharmaceuticals, Victoria, Australia). BOX PCR used the primer BOX1AR primer (Koeuth et al., 1995). Amplification in Hybaid Touchdown Thermocycler (Integrated Sciences) included an initial denaturation step (94 °C, 3 min), followed by 40 cycles of denaturation (94 °C, 30 s), annealing (58 °C, 30 s) and extension (68 °C, 6 min). The final extension was 68 °C for 12 min. ERIC and REP PCR reactions included the primer pairs ERIC1R and ERIC21, and REP1R and REP21 (Versalovic et al., 1991). The cycling conditions of these PCR cycles were: initial denaturation (94 °C, 3 min), followed by 30 cycles of denaturation (94 °C, 30 s), annealing (54 °C, 30 s) and extension (68 °C, 6 min), with a final extension of 68 °C for 12 min.

IS50 PCR

DNA extracts were diluted 1:10 in 10 mM Tris–Cl pH 8.0 and 1 mM ethylenediamine tetracetic acid; 1 μl of the dilution was used as template in a final volume of 25 μl. Each reaction contained 200 μM dNTPs (Promega), 4 mM $MgCl_2$, 1% bovine serum albumin (Promega), 1 μM of IS50 primer (Ullrich et al., 1993), 1.25 μl of dimethyl sulphoxide (Sigma-Aldrich), 1.25 U of Taq polymerase (Invitrogen) and 1 × PCR Buffer supplied. Cycling was initial denaturation (95 °C, 3 min), followed by 35 cycles of denaturation (94 °C, 1 min), annealing (38 °C, 1 min) and extension (72 °C, 3.5 min), with a final extension of 72 °C for 10 min. Amplified products were analysed by

agarose gel electrophoresis as above. Fragments that varied only in intensity between strains were not classed as polymorphic. Only the presence or absence of fragments was scored, with fragments that amplified from one strain but not from another counted as polymorphic. Fragments that varied only in intensity between strains were not classed as polymorphic.

Results

Isolation and pathogenicity studies

Bacteria, weakly fluorescent blue-white on KB, were consistently isolated from leaf lesions of naturally infected plants and from lesions developing on plants inoculated in pathogenicity tests. In the LOPAT tests, strains were oxidase, potato rot and arginine dihydrolase negative, and levan and tobacco HR positive. All Australian leek strains produced a brown diffusible pigment on TSBA and BUG™ agar after 2 days, as did the known pv. *porri* strains, consistent with the observations of Samson et al. (1998). The strain 61456 from leek derived from seed from Japan, 75520 from onion from Japan, and the pv. *syringae* strains did not produce the pigment. The leek varieties Missile, Musselburgh, Harpoon, Nova and Admiral were all susceptible in the initial pathogenicity test (Figure 5). Sunken tan spots, similar to those observed in the field, appeared within 7 days. Yellowing of infected leaves developed within 13 days of inoculation and subsequently progressive necrosis developed. Eleven strains from leeks were deposited in Herb DAR.

Figure 5. Leaf lesions on 7 week-old leek seedlings, cv. Musselburgh, 14 days after inoculation with *P*. syringae pv. *porri* DAR 75283.

Physiological and biochemical tests

The leek strains from Australia were generally uniform in their biochemical reactions and are compared in Table 2 with the published data of Young and Triggs (1994) and Samson et al. (1998). The leek strains were distinctly different from pv. *syringae* and the nutritional profiles closely resembled those of pv. *porri*. The reaction of lemons and beans to inoculation confirmed the results. Only pv. *syringae* strains produced the dark, sunken water-soaked lesions, on at least one fruit, which are characteristic of pv. *syringae* infection. Eight of the eleven strains were identified by Biolog as pv. *porri* based on carbon source utilisation tests (Table 2).

The two strains with Japanese origins were identified by Biolog as pv. *tabaci* B, indicating that they had similar nutritional profiles. The carbon tests run according to the method of Young and Triggs (1994) showed slight differences between the strains (Table 2) but they were distinctly separated by fatty acid analysis, the Japanese onion strain grouping with pv. *syringae* (Figure 6). However, the nutritional profile of the onion strain differed markedly from pv. *syringae* in that it was negative for trigonelline, aesculin and gelatin hydrolysis. Both strains with Japanese origins utilised galacturonic acid, which was negative for all the reference strains and all the Australian strains from leek. In addition, these two strains and one from California did not utilise homoserine, in contrast to all the other leek strains. The results for inositol were variable.

Based on fatty acid methyl ester profiles, all Australian leek strains and the reference strains of pv. *porri* clustered at a Euclidian Distance (ED) of less than 3, indicating little variability between strains. (Sherlock Library Generation System V4.0) (Figure 6). The apparent clustering of the reference strains of pv. *porri* separately from the Australian leek strains with an ED of 2, is not significant. Reference strains of pv. *syringae* and the onion strain from Japan clustered quite separately, being linked to the leek strains with an ED

Table 2. Comparative results of carbon utilisation and biochemical tests for leek pathogens and characterised pathovars

	Adonitol	Benzoate	Betaine	Butyrate	Erythritol	Glutarate	Glyceate	Homoserine	Lactate	Malonate	Mannitol	Quinate	Sorbitol	L + tartrate	Trigonelline	Aesculin	Gelatine	Biolog	
																		Similarity	ID[b] pathovar
75283	–	+	–	–	–	w[a]	+	+	w	+	+	+	+	–	–	–	–	0.149	*syringae*
75284	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	–	0.511	*tomato*
75287	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	Delayed	0.662	*porri*
75554	–	+	–	–	–	–	+	+	–	+	+	+	+	–	–	–	Delayed	0.853	*porri*
75555	–	+	–	–	–	–	+	+	–	+	+	+	+	–	–	–	–	0.853	*porri*
75556	–	+	–	–	–	–	+	+	–	+	+	+	+	–	–	–	–	0.595	*porri*
77311	–	+	–	–	–	w	+	+	w	+	+	+	–	–	–	–	–	0.358	*pisi*
77312	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	–	0.634	*porri*
77313	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	–	0.463	*porri*
77314	–	+	–	–	–	w	w	+	w	+	+	+	+	–	–	–	–	0.649	*porri*
77315	–	+	–	–	–	w	w	+	w	+	+	+	+	–	–	–	Delayed	0.520	*porri*
Onion Japan	–	+	–	–	+	w	w	–	w	w	+	+	–	–	–	–	–	0.459	*tabaci* B
Leek Japanese seed	–	+	+	v[c]	v	–	+	–	w	+	+	+	w	–	–	–	–	0.474	*tabaci* B
Leek NZ	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	Delayed	0.555	*porri*
Leek France	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	–	0.661	*porri*
Leek CA 1	–	+	–	–	–	w	+	+	w	+	+	+	+	–	–	–	–	0.445	*porri*
Leek CA 2	–	+	–	–	–	w	w	–	w	+	+	+	w	–	–	–	–	0.549	*porri*
pv. *syringae*33426	–	+	+	+	+	+	+	–	+	+	+	+	+	–	+	+	+	0.724	*lachrymans*
Data of Young and Triggs (1994)	–	+	–	±	–	±	+	+	–	±	+	+	+	–	–	–	±		
Data of Samson et al. (1998)			–	–	–			+ delayed	–	+	+		+ delayed	–	–		+ Delayed		

[a]w indicates weakly positive.
[b]As pathovars of *Pseudomonas syringae*.
[c]Variable, indicates a different result when the test was repeated.

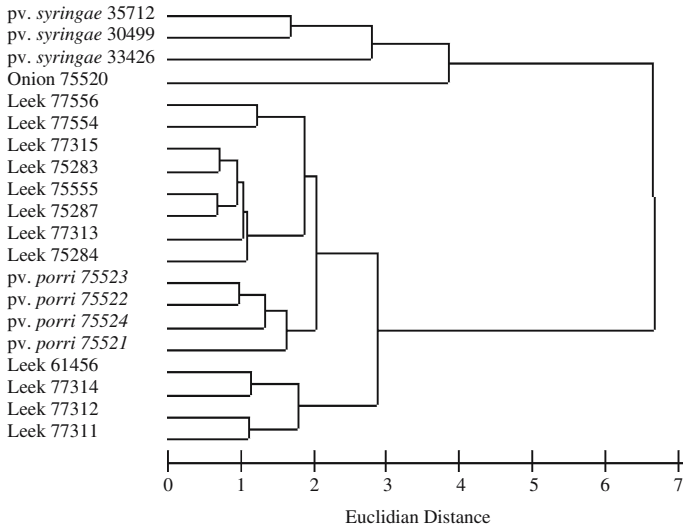

Figure 6. Dendrogram of fatty acid profiles of 11 strains from leek compared with recognised strains of *Pseudomonas syringae* pv. *porri*.

of 7, a distance considered sufficient to separate subspecies or biotypes.

The Japanese strain from onion, DAR 75520, had phenotypic characters (Table 2) similar to those of pv. *porri* but this strain clustered with pv. *syringae* in the fatty acid analysis. These results confirmed comments by Samson et al. (1998) that Japanese onion strains were distinct from leek strains.

Seed transmission

Seed from 10 of the 16 seed heads tested produced colonies on KB and SPA characteristic of *Pseudomonas*. Prolific fluorescent colonies were observed on KB with the concentrations of fluorescent colonies on KB and levan colonies on SPA ranging from 10 to 1000 cfu ml^{-1} in the suspension derived from 30 seeds. Biolog tests confirmed the fluorescent colonies as *P. syringae*, but colonies were not characterised to pathovar level. Typical disease symptoms appeared in 22 of the seedlings arising from seed collected from inoculated plants, with the first symptoms of twisted leaves observed 3 weeks after germination.

Host range

Although the *Allium* spp. showed some variation in reaction when inoculated with the Australian leek strains (Table 3), pathogenicity was established, the pathogen was re-isolated and identified, and Koch's postulates satisfied. Similarly, strong reactions were observed on inflorescence stalks of leek and spring onion when wound-inoculated. Tan, water-soaked lesions developed rapidly and, particularly in leek, coalesced to form extended lesions down the length of the stalks.

Molecular analysis

A 16S rDNA band estimated at 1050 bp was amplified from all strains. The amplified product from each strain was individually incubated with each of six restriction enzymes. The resulting patterns were assigned numbers for each enzyme (Table 1); strains with the same number had the same digestion pattern. A six-digit digestion profile was thereby generated for each strain, dividing the strains into seven different groups (Table 1). The strain from leek derived from seed from Japan (DAR61456), the Japanese onion strain (DAR75520) and the strains of pv. *syringae* (DAR30499, DAR35712, DAR33426) each had their own distinct profile. A single polymorphic band observed with one enzyme (*Nci* I) divided the strains from leek into two groups – one (assigned profile number 2 in Table 1) contained three strains from South Australia (DAR75283, DAR75284 and DAR75287) and the second (profile 5) the reference strains and remaining field

Table 3. Results of host range inoculations. Percentage of plants in Allium cultivars displaying lesions 21 days after inoculation with various Pseudomonas strains and reaction of inflorescence stems to wound inoculation

Isolate	Leek cvs			Onion cvs			*Allium* spp.		Stem inoculation; disease rating	
	Missile	Harpoon	M'burgh	Cream Gold	Hunter River White	Spring Onion	Shallot	Garlic	Leek	Spring Onion
75283 Leek SA	63	70	50	54	77	45	92	100	+ +	+ +
75555 Leek SA	31	11	25	12.5	0	0	67	100	+ +	+ + +
75554 Leek Vic	79	76	81	95	91	52	94	80	–	Weak +
75523 Cal 1	64	25	42	77	46	60	58	100	+ +	+ + +
75524 Cal 2	91	100	75	50	91	17	83	100	+ +	–
75521 NZ	46	71	43	52	73	46	58	100	+ + +	–
75522 Holopathotype Strain	46	50	59	42	46	30	93	80	+ +	Weak +
75520 Onion Japan	10	10	0	0	4	4	33	0	Weak +	Weak +
33426 *Pseudomonas syringae*	0	0	0	14	23	8	25	Hypersensitive reaction	–	Hypersensitive reaction
Water-only control	0	0	0	0	0	0	0	0	–	–

strains. This indicates little variation between the strains from leek in this region of the genome.

DNA fingerprinting was conducted to examine whether the strains in this study could be differentiated by analysis of a greater proportion of the genome. Amplification with the IS50 primer revealed some genomic diversity and a representative gel is shown in Figure 7. Leek isolates had

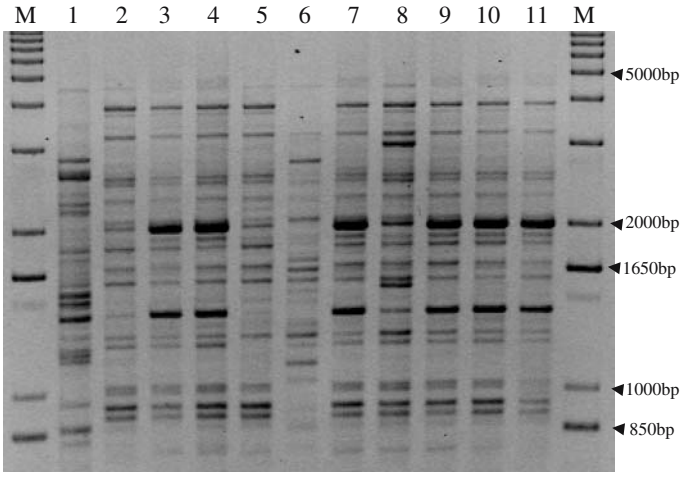

Figure 7. DNA fingerprinting using IS50 primer. M = 1 kb plus marker; Lane 1 = DAR33426 *Pseudomonas syringae* pv. *syringae*; Lanes 2–5 and 7–11 = strains of *Pseudomonas syringae* from leek (Lane 2 = DAR75283; 3 = DAR75284; 4 = DAR75287; 5 = DAR61456; 7 = DAR75521; 8 = DAR75522; 9 = DAR75523; 10 = DAR75524; 11 = DAR75554); Lane 6 = DAR75520 *Pseudomonas syringae* from onion.

DNA fingerprint profiles that were distinctly different from those of reference strains of *P. syringae* pv. *syringae* (compare Lanes 1 and 2, Figure 7) and from that of the Japanese onion strain (shown in Lane 6, Figure 7). All leek strains produced identical profiles, with the exception of DAR75522 (reference strain from France) and DAR61456 (leek strain from Japanese seed). In contrast, the use of repetitive element PCR (i.e. amplification individually with ERIC-, BOX- and REP-primers) did not consistently discriminate between any of the strains from leek (results not shown). Polymorphic bands were not consistently observed between leek strains. In all cases, however, the profiles amplified from the leek strains were clearly distinct from those amplified from strains from other hosts.

Discussion

We adopted a polyphasic approach to determining the pathovar designation of the bacterial strains associated with a new disease of leeks in Australia and included several strains from other countries as 'standards' for comparison. The pathogen isolated from Australian leeks has been identified to pathovar level by pathogenicity to various *Allium* spp. Although pathovars can only be distinguished by pathogenicity, Young and Triggs (1994) published minimum determinative tests for *syringae* pathovars and Schaad's guide (Schaad et al., 2001) places heavy reliance on nutritional separation of pathovars (although it does not include pv. *porri*). There was considerable uniformity across all leek strains used in this study in carbon source utilisation although, for inositol utilisation, Samson and Benjama (1984) recorded pv. *porri* as delayed positive and Young and Triggs (1994) recorded inositol utilisation by pv. *porri* as positive. We found that utilisation was inconsistent when the tests were repeated. Although inositol is listed by Young and Triggs (1994) in their minimum determinative tests for pathovars of *P. syringae*, the test is not discriminatory for pv. *porri*.

Analysis of fatty acid profiles of the Australian and other leek strains clustered all strains together and they were distinctly separated from pv. *syringae*. The fatty acid methyl ester profile of pv. *porri* is not included in the MIDI database and it is generally considered that fatty acid methyl esters are not discriminatory at the pathovar level. However, the Australian strains consistently had the closest matches in the database to pv. *papulans* and to pv. *coronafaciens*, confirming the results of Koike et al. (1999). An association between strains of *P. syringae* pathogenic to *Allium* sp. and *P. coronafaciens* has been shown in other studies. *Pseudomonas coronafaciens* and pv. *porri* were included in the same DNA hybridisation group (Gardan et al., 1999) and a study using multilocus sequencing typing and phylogenetic analysis of *P. syringae* pathovars (Sarkar et al., 2004) showed that one of the four major groups of strains contained only pathogens of monocots, including a strain from oats, *P. coronafaciens* and a strain of pv. *syringae* from spring onion. However, *P. syringae* pv. *porri* was not included in that study.

Our pathogenicity tests contrast with those of Samson et al. (1998) whose strains were not pathogenic to onion, spring onion, shallot or garlic. Koike et al. (1999) reported pathogenicity of his leek strains to onion and garlic but they did not use spring onion or shallot. Hale (1975) reported only weak pathogenicity of his bacterium to onion. These variable results may be due to the cultivars used, the age of the cultures since original isolation, or the inoculation/environment conditions employed by the different research groups, the latter point being noted by Koike et al. (1999).

The molecular methods used were internally consistent. The leek isolates were genetically very similar to each other, whether analysis was based on one target or fingerprinting, and they were different from isolates from other sources.

This is the first report of *P. syringae* pv. *porri* in Australia. The disease appears to be widespread and leeks with distinctive leaf lesions have been observed in all leek-growing areas. The spread of the disease since it was first observed suggested seed-borne transmission, as much of the leek seed used in Australia is from common sources in Europe. Koike et al. (1999) also suggested that the pathogen may be seed-borne. ACPPB has a culture of pv. *porri* isolated in 1988 from a diseased leek that had been grown in Quarantine from seed imported from Japan. These results are consistent with seed transmission. We have shown that infecting stems of developing seed heads could produce infected seed, which when germinated produced seedlings with typical symptoms of the disease. The widespread use of seed from Europe

in Australia and the distribution of the disease further support the assertion by Koike et al. (1999) that the pathogen may have originated in Europe. Isolates of *P. syringae* pv. *porri* are genetically distinct from the other tested pathovars within *P. syringae*, but little genetic variation was detected between strains of *P. syringae* pv. *porri* from different locations both within Australia and from New Zealand, France and USA, consistent with dispersal of a common strain through infected seed lots. Seed merchants need to be aware of the disease status of crops grown for export seed and should consider implementing a certification scheme to ensure growers are supplied with seed containing a minimal level of bacterial contamination.

Acknowledgements

A portion of this work was funded by the Vegetable Industry and the Australian Government through Horticulture Australia Limited. We thank the Australian growers who participated in the initial disease survey.

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