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Identification of the causal agent of pistachio dieback in Australia

E. Facelli[1], C. Taylor[2], E. Scott[1], M. Fegan[3], G. Huys[4], R. D. Noble[5], J. Swings[4] and M. Sedgley[1]
[1]*School of Agriculture and Wine, University of Adelaide, Waite Campus, PMB 1, 5064 Glen Osmond, South Australia, Australia (Fax: +61-88-303-7109; E-mail: evelina.facelli@adelaide.edu.au);* [2]*Department of Primary Industries Victoria, P.O. Box 905, Mildura, Victoria, Australia;* [3]*CRC for Tropical Plant Protection, Department of Microbiology and Parasitology, The University of Queensland, 4072 St. Lucia, Queensland, Australia;* [4]*Laboratory of Microbiology, Ghent University, K.L. Ledeganckstraat 35, 9000, Gent, Belgium;* [5]*NSW Agriculture, Orange Agricultural Institute, Forest Road, Orange, 2800 New South Wales, Australia*

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Abstract

Symptoms associated with pistachio dieback in Australia include decline (little or no current season growth), xylem staining in shoots two or more years old, trunk μ and limb lesions (often covered by black, superficial fungal growth), excessive exudation of resin, dieback and death of the tree. Bacteria belonging to the genus *Xanthomonas* have been suggested as the causal agent. To confirm the constant association between these bacteria and the disease syndrome, the absence of other pathogens and the identity of the pathogen, we performed a series of isolations and pathogenicity tests. The only microorganism consistently isolated from diseased tissue was a bacterium that produced yellow, mucoid colonies and displayed morphological and cultural characteristics typical of the genus *Xanthomonas*. Database comparisons of the fatty acid and whole-cell protein profiles of five representative pistachio isolates indicated that they all belonged to *X. translucens*, but it was not possible to allocate the isolates to pathovar. Pathogenicity tests on cereals and grasses supported this identification. However, Koch's postulates have been only partially fulfilled because not all symptoms associated with pistachio dieback were reproduced on inoculated two-year-old pistachio trees. While discolouration was observed, dieback, excessive resinous exudate and trunk and limb lesions were not produced; expression of these symptoms may be delayed, and long-term monitoring of a small number of inoculated trees is in progress.

Introduction

Dieback of pistachios (*Pistacia vera* L.) in Australia was first reported in 1996 (Edwards et al., 1998b). Symptoms associated with the disease are: decline (little or no current season growth), xylem staining in shoots two or more years old, trunk and limb lesions (often covered by black, superficial fungal growth), excessive exudation of resin, dieback and, in some instances, death of the tree. There are no confirmed reports of symptoms on leaves. Symptoms have been observed on bearing scions (*P. vera* cvs Sirora and Kerman, and various male cultivars) but not on juvenile trees or any of the rootstocks used in Australian orchards (*P. terebinthus*, *P. atlantica* or *P. integerrima* cv. Pioneer Gold) (Facelli et al., 2002).

A *Xanthomonas* strain (Australian Collection of Plant Pathogenic Bacteria, DAR 69889) isolated from stained twigs from diseased trees at Kyalite, NSW, Australia, in 1994 was identified as the probable cause of the disease (Edwards et al.,

1998b). *Cladosporium*, *Alternaria*, *Fusarium* and *Phoma* spp. were also isolated (Edwards et al., 1998a), however, the occurrence of those fungi was very sporadic and none was considered likely to be a primary pathogen. Likewise, there was no consistent association of viruses, viroids or phytoplasmas with the disease (Edwards et al., 1998b). In 1998, one isolate from stained twigs from a diseased tree at Robinvale, Victoria and identified as *Xanthomonas* sp. (VPRI 21750a), was subject to 16S rRNA sequencing. VPRI 21750a shared 99.93% sequence similarity with *Xanthomonas translucens* pathovar (pv.) *graminis* (the only pathovar of *X. translucens* in the database at that time). Similar results were obtained by gas chromatography of fatty acid methyl esters (GC-FAME) of this and other strains, although similarity values were low and variable (0.125–0.660) (Taylor and Edwards, 2000; Facelli et al., 2002).

Mainly fungal diseases affect pistachio trees in cultivation in other parts of the world (Teviotdale et al., 2002). The three major diseases affecting Californian orchards are verticillium wilt (*Verticillium dahliae*), alternaria late blight (*Alternaria alternata*) and botryosphaeria panicle and shoot blight (*Botryosphaeria dothidea*). Fungi also cause sporadic diseases, such as botrytis blossom and shoot blight (*Botrytis cinerea*), armillaria root rot (*Armillaria mellea*) and phomopsis blight (*Phomopsis* sp.) (Michailides et al., 1995; Holtz, 2002). There are no reports of bacterial disease in pistachio orchards in California or elsewhere (Teviotdale et al., 2002).

We performed a series of isolations and pathogenicity tests to confirm the constant association between the bacteria and the disease syndrome, the absence of other pathogens and the identity of the pathogen. Brief details have been published previously (Facelli et al., 2002). We also assessed the pathogenicity of strains towards members of the *Poaceae* as *X. translucens* has been reported as a pathogen of cereals and grasses (Vauterin et al., 1995; Duveiller et al., 1997a).

Materials and methods

Isolation

Two-year-old shoots with xylem staining were collected from 10 trees in each of four orchards: Renmark, SA (34°10′ S, 140°45′ E); Kyalite, NSW (34°58′ S, 143°29′ E); Red Cliffs, Victoria (34°18′ S, 142°13′ E); Robinvale, Victoria (34°36′ S, 142°48′ E). Shoots of similar age without staining were collected from three trees in each of two experimental orchards apparently free from the disease: Department of Primary Industries Victoria (Mildura, Victoria 34°14′ S, 142°10′ E) and the University of Adelaide Waite Campus (Adelaide, SA 34°58′ S, 138°39′ E).

Shoot segments from each tree were dipped in 100% ethanol and flamed, the ends cut and bark removed with a sterilised scalpel. Four to six, 3–5-mm sections were incubated overnight in 9 ml of sterile distilled water (SDW) on a rotary shaker at 26 °C in the dark, and the suspension streaked on nutrient agar (NA, 13 g l^{-1} nutrient broth, Oxoid; 14 g l^{-1} agar, Oxoid technical #3) and sucrose peptone agar (SPA; Moffett and Croft, 1983) in duplicate. Plates were incubated at 28 °C in the dark. Four, 2-mm discs were placed on each of the media listed below (in triplicate, a total of 12 discs per tree per medium). Plates were incubated as above until bacterial colonies appeared (5–10 days) or for a maximum of four weeks. A loopful of the resultant bacterial growth was streaked for single colonies on NA and SPA. Fungi were sub-cultured and pure cultures established on potato dextrose agar (PDA, Difco) incubated in the dark at 25 °C. The general-purpose media were: NA; PDA; water agar (WA) and one-quarter strength PDA (¼ PDA). The specific media were: SPA, recommended for isolation of *Xanthomonas*; acidified PDA (APDA), recommended for isolation of *B. dothidea* (Ntahimpera et al., 2002; Teviotdale et al., 2002) and ethanol medium (EM), recommended for isolation of *V. dahliae* (Morgan et al., 1992; Teviotdale et al., 2002).

Identification

Slow growing bacteria which formed yellow colonies in 48–72 h were selected for further identification (Moffett and Croft, 1983). Each isolate, 8–10 isolates per orchard, was grown overnight at 26 °C in sucrose peptone broth. A 300 μl aliquot of the culture was vortexed with 700 μl of 100% sterile glycerol, snap-frozen in liquid nitrogen and stored at –80 °C. In a preliminary study, three isolates per orchard and isolate VPRI 21750a were sent to CSIRO Division of Land and Water,

Adelaide, for identification using GC-FAME. Subsequently, the variability among pistachio isolates was determined by rep-PCR using the BOXA1R primer as described by Louws et al. (1994). Five representative isolates (including the three tentatively identified at CSIRO) from each orchard and isolate VPRI 21750a were selected for this analysis. Representative isolates from each group determined by rep-PCR (one from each orchard and VPRI 21750a) were identified by GC-FAME analysis and their whole cell protein profiles were determined using sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). For the purpose of SDS-PAGE, isolates were grown on modified glucose–yeast extract–calcium carbonate (GYCA) medium for 48 h at 28 °C. Whole-cell proteins were solubilised by treatment with 20% SDS, separated by PAGE in a 12% (w/v) polyacrylamide slab gel, and stained with 0.25% Coomasie Blue (Vauterin et al., 1991). The resulting protein profiles were scanned with a LKB 2202 Ultroscan Laser Densitometer (LKB, Bromma, Sweden). Further processing and numerical analysis of the digitised SDS-PAGE data and comparison with the user-generated SDS-PAGE database of plant pathogenic xanthomonads (Vauterin et al., 1995) were performed using the GelCompar software package (version 4.1; Applied Maths, Belgium). Whole-cell FAME profiles of the representative isolates were determined using the Microbial Identification System (MIS, Microbial ID, (MIDI), Newark, DE). Bacteria were grown on trypticase soy broth (BBL) with agar (15 g l^{-1}) for one day at 28 °C. Fatty acids were extracted using the sample preparation procedures described in the Sherlock Microbial Identification Handbook. The resulting fatty acid methyl esters were analysed using a Hewlett Packard 6890 Gas Chromatograph and the peak areas were identified with the peak-naming component of the MIDI system (Sherlock version 4.5). The fatty acid profiles of the isolates were compared with composites of known reference strains in the database and given matches to known species in the form of a Similarity Index.

Pathogenicity tests on cereal and grasses

To confirm the similarity of pistachio strains to *X. translucens*, we assessed pathogenicity on (*Triticum aestivum* cvs Janz and Excalibur (wheat), and *Hordeum vulgare* cv. Franklin (barley)) and grasses that are common in pistachio orchards *Bromus diandrus* (bromegrass), *Avena fatua* (wild oats), *Lolium rigidum* (annual ryegrass), *Hordeum leporinum* (barleygrass) in a series of growth room and glasshouse experiments (Table 1). Due to limitations of space, experiments were conducted over time, with a variable number of hosts, strains and pots per treatment (Table 1). Overall, there were seven experiments. One pistachio strain representing each of the two groups determined by rep-PCR, namely VPRI 21750a (Robinvale) and DAR 75532 (Kyalite), and one strain of *X. translucens* from wheat, DAR 35705 [*X. campestris* syn. *translucens* pv. *translucens* (Jones, Johnson and Reddy 1917) Dye 1978 pv. (Pammel) Dowson 1939] were used in every experiment. *Xanthomonas axonopodis* syn. X. *campestris* pv. *malvacearum*, DAR 26904, was included in growth room experiment 2 (GR 2) as a negative control. In GR 3, other *Xanthomonas* isolates from pistachio were also included, namely DAR 77734 from Robinvale, DAR 75533 from Renmark and DAR 75535 from Red Cliffs. *T. aestivum* cv. Excalibur, a cultivar more susceptible to disease than cv. Janz (Wheeler and McMurray 2001) was used in GR 3 and glasshouse experiment 3 (GH 3). Strains were recovered from storage at −80 °C onto SPA. A slightly cloudy suspension (about 10^8 cells per ml in SDW) was prepared for each strain, the concentration estimated with a haemocytometer and confirmed by counting colony-forming units on SPA. This suspension was diluted to 10^6 cells per ml.

Each treatment (individual *Xanthomonas* strains or control) was sprayed onto plants in sets of three to ten pots, each containing three to five plants. SDW was applied to control plants. Pots were placed at random on the benches. Plants in every pot were observed for symptom expression. A pot was rated positive when at least one leaf had symptoms. One leaf per pot in a subset of five pots per treatment was assessed for the presence of bacteria in all experiments except for GH 3 (see below and Table 1). Seeds (three to five per pot) were sown in 0.9 l square pots filled with UC potting mix (Baker, 1957). Cereal plants were fertilised with Thrive® at the recommended rate seven days after sowing. In the growth room, day/night temperatures were 22/16 °C with a 14-h photoperiod. In the glasshouse, temperatures were

Table 1. Experiments to determine the pathogenicity of *Xanthomonas* strains from pistachio to selected cereals and grasses

Experiment[a]	Host	Number of pots per treatment used to assess symptoms[b]	Number of leaves per treatment used to assess presence of bacteria[c]	Number of treatments (strains and control)[d]
GR 1	*Triticum aestivum* cv. Janz	10	5	4
GR 2	*Triticum aestivum* cv. Janz	10	5	5
GR 3	*Triticum aestivum* cv. Excalibur	8	5	7
GR 4	*Bromus diandrus*; *Avena fatua*	8	5	4
GH 1	*Hordeum vulgare* cv. Franklin; *Lolium rigidum*	8	5	4
GH 2	*Lolium rigidum*	6	5	4
GH 3	*Triticum aestivum* cv. Excalibur; *Bromus diandrus*; *Hordeum leporinum*; *Avena fatua*	3	0	4

DAR 35705: *Xanthomonas campestris* pv. *translucens* from wheat; VPRI 21750a: pistachio strain from Robinvale; DAR 75532: pistachio strain from Kyalite; DAR 26904: *X. campestris* pv. *malvacearum*; DAR 77734: pistachio strain from Robinvale; DAR 75533: pistachio strain from Renmark; DAR 75535: pistachio strain from Red Cliffs (see text for details or locations).

[a]Experiments were conducted over time, in a growth room (GR) or glasshouse (GH).

[b]There were three to five plants per pot.

[c]One leaf per pot from a subset of five pots per treatment was used to assess the presence of bacteria in all experiments except GH 3.

[d]Strains DAR 35705, VPRI 21750a, DAR 75532 and SDW (control) were used in all experiments. In addition, strain DAR 26904 was used in GR 2 and strains DAR 77734, DAR 75533, DAR 75535 were used in GR 3.

between 18 and 24 °C, with natural daylight in winter (July–August). Plants with three to five expanded leaves were sprayed till run-off with the appropriate bacterial suspension or with SDW. After inoculation of the plants, pots were placed in SIGMA® sun bags that were kept closed for seven days and, in the growth room, the temperature was increased to 26/18 °C day/night. After bags were opened, plants were watered from below as required. Symptoms were assessed when bags were opened and 1 week later. GH 3 was set up with fewer pots to allow more frequent assessment (every two days) for a longer time (three weeks) and to observe the development of symptoms with a dissecting microscope. To assess the presence of bacteria, a piece of tissue (approx. 3×5 mm) from the margin of the lesions (or from the centre of the leaf blade in the control plants) was excised and finely chopped in a drop of SDW in a sterile plastic Petri dish (Duveiller et al., 1997b). The tissue was macerated for 15 min, covered to avoid desiccation, and the resulting suspension streaked on SPA amended with 150 mg l^{-1} Benlate® (BSPA). Identification of the bacteria as *Xanthomonas* sp. was based on colony morphology and characteristic slow growth as described above (Moffett and Croft, 1983). The Fisher's exact probability test (Siegel, 1956) was used to determine significant differences in comparison to controls when less than 100% of the pots were positive or when less than 100% of the assessed leaves yielded bacteria (Table 4).

Koch's postulates

Sixty two year-old potted pistachio trees (*P. vera* cv. Sirora scion on *P. terebinthus* rootstock) were used to test Koch's postulates. Trees were paired according to size and vigour. One tree of each pair was injected with 0.1 ml of SDW (control) and the other tree was injected with 0.1 ml of a suspension of VPRI 21750a (3×10^7 cfu per ml SDW). All injections were made in the trunk 5 cm above the graft union using a 27G (1/2) syringe. Inoculations were performed in March 1998 in a glasshouse cooled to around 23 °C, where the trees grew until the end of the experiment. Twelve weeks after inoculation, 15 pairs of trees were destructively sampled and assessed for symptoms and presence of bacteria. Three pairs were assessed 10 months after inoculation and a further two pairs were

sampled 15 months after inoculation. The remaining 10 pairs were planted in the field. Segments of trunk (approximately 6 cm) were dipped in 70% ethanol and flamed. The bark was removed and four to six, 2–3-mm-thick sections placed into SDW and incubated at room temperature (approximately 22 °C) for 24 h. The suspension was then streaked on SPA, and plates were incubated at room temperature for 4–7 days to determine the presence or absence of *X. translucens*.

Results

Isolation and identification

The only microorganism consistently isolated from diseased tissue was a bacterium, which produced yellow, mucoid colonies around and on top of the tissue. Bacterial growth was visible on wood discs, in general, after 48 h but in some samples, this took up to 12 days. Single colonies were small (<1 mm diam) and pale yellow when cultured on NA. On SPA, they were pale yellow, mucoid and domed. On both media, the colonies were visible after 48–72 h. The bacteria were motile, Gram-negative rods of about 0.4×1.5 μm. Overall, bacteria were recovered from 80 to 100% of the trees which had shoots with internal staining, but not from apparently healthy trees (Table 2). Fungal growth was observed only sporadically on samples from both healthy and diseased material, and there was no obvious difference in isolates obtained from these sources. The morphological and cultural characteristics of the bacterium matched those of the genus *Xanthomonas* (Moffett and Croft, 1983). Preliminary analysis of the fatty acid profiles was inconclusive. Similarity indices varied from 0.099 to 0.347, but the closest match was with *Xanthomonas* (Table 3). rep-PCR fingerprinting revealed the presence of two groups among the pistachio isolates: i.e. group 1, comprising isolates VPRI 21750a and DAR 77734 from Robinvale and group 2, comprising isolates DAR 75532, DAR 75533 and DAR 75535 from the other three orchards (Figure 1, Table 3). Identification of representative strains by GC-FAME analysis showed that pistachio isolates were members of the species *X. translucens*. The similarity indices of 0.347–0.484 were higher than those obtained in the preliminary assessment and indicated that the closest match was obtained with *X. translucens* pv. translucens (Table 3). This analysis also distinguished the pistachio isolates from the *X. translucens* pathovar from wheat (Figure 2). GC-FAME analysis also defined two groups among the *X. translucens* pistachio isolates, corresponding to the isolates from Robinvale (R4 and R) and those from other orchards, respectively (Figure 2). The same subgrouping was found by cluster analysis of digitised protein profiles (Figure 3). Following comparison with the SDS-PAGE database of pathogenic xanthomonads, the five selected pistachio isolates could be assigned to the species *X. translucens*. The isolates were most closely related to reference strains of pathovars *translucens, graminis* and *hordei* but it was not possible reliably to assign them to one pathovar.

Table 2. Isolation of *Xanthomonas* from diseased pistachio trees showing symptoms of dieback, using various culture media. Number of trees, of 10, from which *Xanthomonas* was recovered

Suspension[a]			Discoloured wood[b]					
Medium	NA	SPA	WA	¼ PDA	PDA	APDA	EM	NA
Orchard								
Red Cliffs	6	6	8 (55)	8 (78)	8 (68)	0	0	8 (55)
Kyalite	7	7	10 (52)	10 (107)	10 (84)	6 (14)	0	10 (80)
Robinvale	2	9	10 (95)	10 (94)	8 (40)	1 (1)	0	9 (66)
Renmark	4	6	8 (nr)	8 (54)	6 (28)	0	0	8 (35)

[a]Segments of 2-year-old shoots were incubated in sterile distilled water overnight and the resulting suspension streaked on nutrient agar (NA) and sucrose peptone agar (SPA).
[b]Discs cut from shoots were placed on different media (4×2 mm discs on three plates of each medium per tree). WA: water agar; PDA: potato dextrose agar; APDA: acidified PDA; ¼ PDA: one-quarter strength PDA; EM: ethanol medium. Numbers in brackets indicate the number of discs of 120 that yielded bacteria. nr: number of positive discs not recorded.

Table 3. Origin and identification (ID) of isolates from pistachio trees from four orchards used in identification. GC-FAME identification similarity indices and accession number of representative isolates

Isolate origin and ID in rep-PCR[a]	Accession[b]	Match on Sherlock® MIS library databases	Simil. index
Kyalite – K1	DAR 75532	*Xanthomonas translucens* pv. translucens	0.417
Red Cliffs – C1	DAR 75535	*Xanthomonas translucens* pv. translucens	0.484
Renmark – r3	DAR 75533	*Xanthomonas translucens* pv. translucens	0.374
Robinvale – R4	DAR 77734	*Xanthomonas translucens* pv. translucens	0.405
Robinvale – R	VPRI 21750a	*Xanthomonas translucens* pv. translucens	0.347
Kyalite – K6	Not lodged	*Xanthomonas* spp.[c]	Low
Kyalite – K8	Not lodged	*Xanthomonas* spp.[c]	Low
Red Cliffs – C3	Not lodged	*Xanthomonas* spp.[c]	Low
Red Cliffs – C5	Not lodged	*Xanthomonas* spp.[c]	Low
Renmark – r1	Not lodged	No match	
Renmark – r7	Not lodged	*Xanthomonas* spp.[c]	Low
Robinvale – R6	Not lodged	*Xanthomonas* spp.[c]	Low
Robinvale – R9	Not lodged	*Xanthomonas* spp.[c]	Low

[a]See text for details of the locations and Figure 1 for rep-PCR.
[b]DAR, HerbDAR, Plant Pathology Herbarium, NSW Agriculture. VPRI, Herbarium, Institute for Horticultural Development, Agriculture Victoria, Australia.
[c]Results from GC-FAME preliminary assessment.

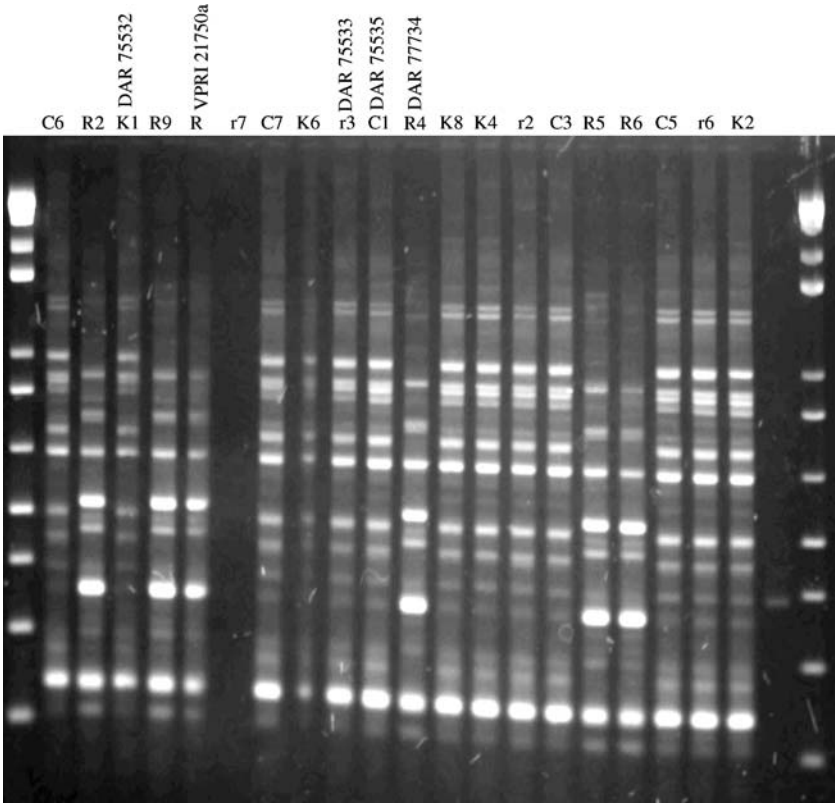

Figure 1. rep-PCR fingerprinting of pistachio isolates from four orchards. BOXA1R primer. 1 kb Plus DNA ladder from Invitrogen. C: isolates from Red Cliffs; R: isolates from Robinvale; r: isolates from Renmark; K: isolates from Kyalite. Two banding patterns are evident, *viz*. C6, K1, C7, K6, r3, C1, K8, K4, r2, C3, C5, r6, K2 and R9, R, R5, R6. See text and Table 3 for details.

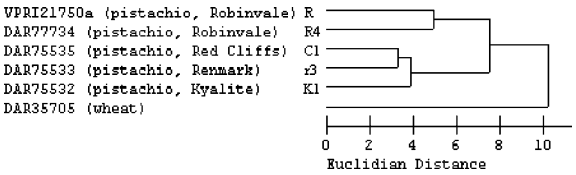

Figure 2. Dendrogram showing the relationships between representative pistachio isolates and a strain of *Xanthomonas translucens* from wheat (DAR 35705) based on fatty acid profiles determined by GC-FAME analysis. Closest match on Sherlock® MIS library databases was *X. translucens* pv. *translucens*.

The representative isolates were accessioned at NSW Agriculture's Plant Pathology Herbarium (Herb DAR) and accession numbers are given in Table 3.

Pathogenicity to cereals and grasses

The *X. translucens* pv. *translucens* strain DAR 35705, and the two pistachio isolates VPRI 21750a and DAR 75532 produced symptoms on wheat, barley, bromegrass, barleygrass and, to a lesser extent, on ryegrass. These symptoms ranged from water-soaked or yellow specks to extended water-soaked to yellow to necrotic streaks that were observed on two to six leaves in almost all inoculated plants. Ryegrass showed symptoms only on one to two leaves in two to three of the inoculated pots and these were evident only under a dissecting microscope. Only isolated light-greenish spots were observed on wild oats, also by microscopic examination. There were no symptoms on the control plants. In the first two experiments, *X. translucens* pv. *translucens* strain DAR 35705 and strain VPRI 21750a produced water-soaked streaks on *T. aestivum* cv. Janz, strain DAR 75532 produced abundant chlorotic specks and *X. campestris* pv. *malvacearum* strain DAR 26904 only isolated light-greenish spots. Similar results were observed on *T. aestivum* cv. Excalibur. On this cultivar, strains DAR 75533 and DAR 75535, from Renmark and Red Cliffs respectively, produced symptoms similar to those caused by strain DAR 75532 from Kyalite, while the Robinvale strain DAR 77734 caused symptoms similar to VPRI 21750a (Table 4). In GH 3, three weeks after inoculation, the three strains produced similar water-soaked lesions on wheat, bromegrass and barleygrass, and no symptoms on wild oats. More frequent observation over a longer period showed that the water-soaked or chlorotic specks joined and formed translucent streaks. Most of these lesions had superficial yellow droplets (Figure 4).

Koch's postulates

At 12 weeks after inoculation, no external disease symptoms were observed on either the control or the inoculated pistachio trees. Staining of the wood was observed in almost all the control trees (14 of 15) and in all the inoculated trees. Staining in the control trees was observed close to the injection point, spreading 1–5 mm above and below the injection point in 12 of 15 trees, whereas in two trees, the staining spread 10–25 mm above and below the injection point. Staining in the inoculated trees spread 1–205 mm above or below

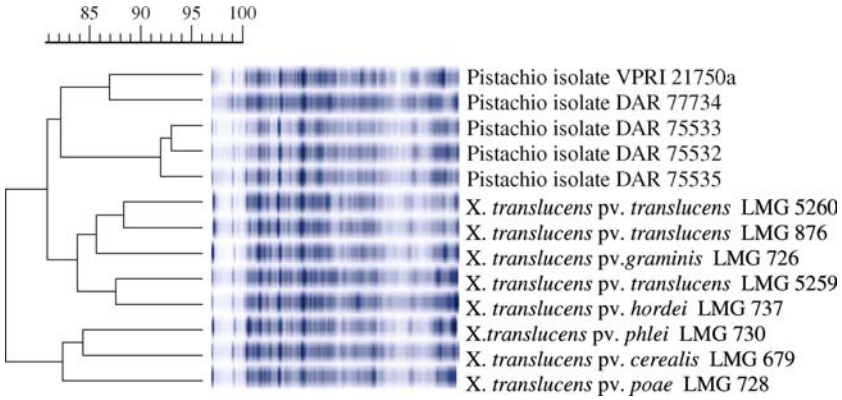

Figure 3. Cluster analysis of digitised protein profiles of selected pistachio isolates and reference strains of *X. translucens* pathovars. Dendrogram was constructed using the UPGMA method in which relatedness is expressed as percentages of the Pearson product–moment correlation coefficient. LMG, BCCM™/LMG Bacteria Collection, Ghent University, Gent, Belgium.

Table 4. Pathogenicity of selected *Xanthomonas* strains to cereals and grasses

Host	Experiment[a]	Disease parameters	*Xanthomonas* strains		
			From wheat	From pistachio	
			DAR 35705	VPRI 21750a	DAR 75532
Triticum aestivum cv. Janz	GR 1 & GR 2	Symptoms[b]	+	+	(+)
		Incidence %[c]	100	100	75**
		Recovery %[d]	100	100	100
Triticum aestivum cv. Excalibur	GR 3	Symptoms	+	+	(+)
		Incidence	100	100	75**
		Recovery	100	100	100
Triticum aestivum cv. Excalibur	GH 3	Symptoms	+	+	+
		Incidence	100	100	100
		Recovery	na	Na	na
Hordeum vulgare cv. Franklin	GR 2	Symptoms	+	+	+
		Incidence	100	100	100
		Recovery	100	100	100
Bromus diandrus	GH 3	Symptoms	+	+	+
		Incidence	100	100	100
		Recovery	na	Na	na
Avena fatua	GR 5	Symptoms	( )	( )	( )
		Incidence	25ns	38ns	25ns
		Recovery	40	60	40
Avena fatua	GH 3	Symptoms	–	–	–
		Incidence	0	0	0
		Recovery	na	Na	na
Hordeum leporinum	GH 3	Symptoms	+	+	+
		Incidence	100	100	100
		Recovery	na	Na	na
Lolium rigidum	GH 2	Symptoms	+	+	+
		Incidence	38ns	50*	38ns
		Recovery	80	80	80
Lolium rigidum	GH 3	Symptoms	+	+	+
		Incidence	50ns	33ns	33ns
		Recovery	60	40	0

[a]Data from GR 1 and GR 2 were combined because results were similar.
[b]Symptoms: +, water-soaked streaks; (+), bright yellow specks; ( ), light, isolated spots; –, no symptoms.
[c]Incidence: percentage of positive pots. Pots were rated as positive if at least one leaf had symptoms. Overall, symptoms were obvious on two to six leaves per plant and in most of the plants (three to five per pot). On *L. rigidum* and *A. fatua*, symptoms were observed using a dissecting microscope. ** and * indicate that the number of positive pots (from three to ten per treatment, depending on the experiment) was significantly greater than in the controls ($P < 0.01$ and $P < 0.05$ respectively); ns, not significant. Fisher's exact probability test was used to determine significant differences from the controls when fewer than 100% of the pots were positive.
[d]Recovery: percentage of leaves from which bacteria were recovered (from five leaves per treatment). na, not assessed. ** and * indicate that the number of leaves that yielded bacteria was significantly greater than in the controls ($P < 0.01$ and $P < 0.05$ respectively); ns, not significant. Fisher's exact probability test was used to determine significant differences from the controls when fewer than 100% of the leaves yielded bacteria.

the injection point. In nine of 15 trees it was observed more than 50 mm above and/or below the injection point. *Xanthomonas translucens* was isolated from 10 of the 15 inoculated trees, but not from the control trees. No external symptoms were observed on the three pairs of trees assessed 10 months after inoculation. Internal staining was found in both the control and inoculated trees, to a degree similar to that observed at 12 weeks. *Xanthomonas translucens* was isolated from one of the three inoculated trees, but not from the control trees. At 15 months after inoculation, internal staining was observed in both the control and inoculated trees, with the staining in the control trees extending 16 mm above and below the injection point. In the inoculated trees, staining extended more than 180 mm above and below the injection point. One of the inoculated trees had

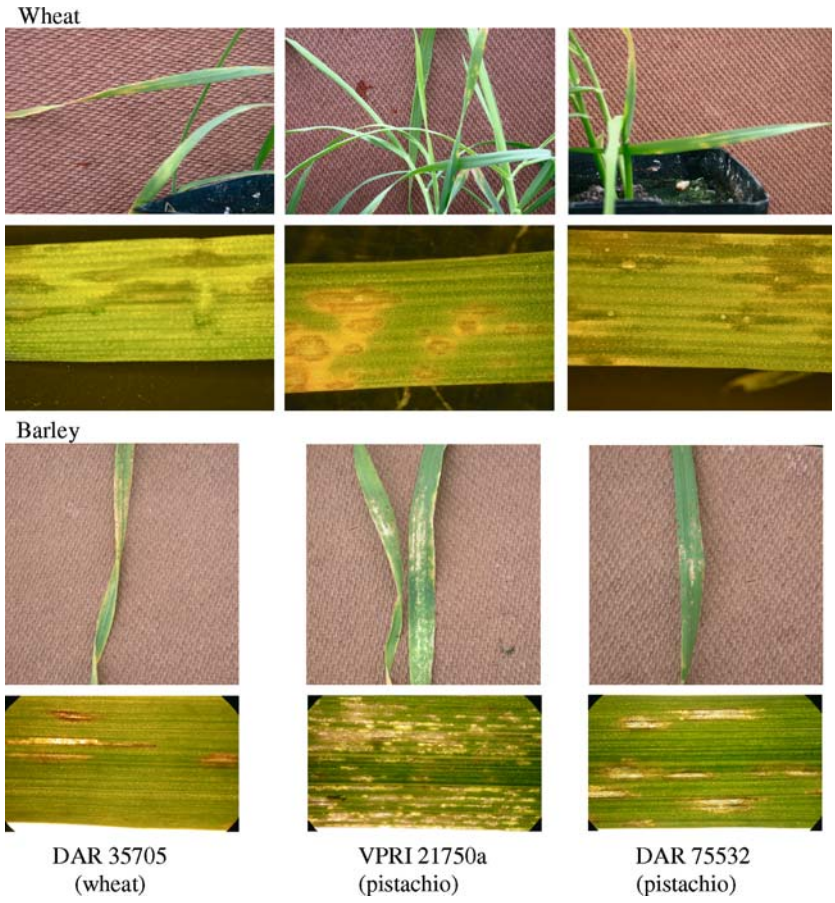

Figure 4. Symptoms observed on leaves of wheat and barley after inoculation with a *Xanthomonas translucens* strain from wheat (DAR 35705) and pistachio isolates (DAR 75532; VPRI 21750a).

developed a necrotic area around the injection point similar to that found under trunk and limb lesions on mature trees in the orchard. Staining was detected in the rootstock of both the inoculated trees. *Xanthomonas translucens* was isolated from the rootstock of one of those trees and from the scions of both trees, but not from the control trees. Trees planted in the field did not show external signs of disease.

Discussion

The results presented here confirm the association of xanthomonads with pistachio dieback (Edwards et al., 1998a; Facelli et al., 2002). Bacteria belonging to the genus *Xanthomonas* were the only microorganism consistently cultured from stained pistachio shoots. Although selective media were used in attempts to isolate the most common pathogens of pistachio, *V. dahliae* and *B. dothidea* (Teviotdale et al., 2002), neither of these fungi was detected. As not all symptoms associated with pistachio dieback were observed in young trees inoculated with strain VPRI 21750a, Koch's postulates have been only partially fulfilled. Dieback, excessive resinous exudate and trunk and limb lesions were not produced, however, these may develop as the disease progresses in the remaining trees, as they reach maturity. The remaining 10 pairs of trees that have been planted in the field will continue to be monitored for symptom development.

The relatively low similarity indices in the GC-FAME analysis (less than 0.5) indicate that the isolates from pistachio are atypical with respect to the GC-FAME patterns of strains of *X. translucens* in the databases (MIDI, 2003).

However, the fatty acid composition and profiles of the pistachio isolates (not shown) correspond with the average fatty acid profile of the pathovars of the '*translucens*' group (*X. translucens* pv. *cerealis, hordei, secalis, translucens* and *undulosa*) previously described (Vauterin et al., 1996). Although GC-FAME analysis has been used successfully for taxonomic analyses of the genus and to distinguish *X. translucens* from other xanthomonads (Yang et al., 1993; Vauterin et al., 1996), analysis of protein profiles by SDS-PAGE is a more reliable technique. This has been used widely in the identification of xanthomonads (Vauterin et al., 1991; 1992) and, in particular, in the taxonomy of xanthomonads associated with cereals and grasses (Vauterin et al., 1992; 1996). Based on SDS-PAGE analysis, the five selected pistachio isolates were clearly assigned to *X. translucens* but could not be clearly assigned to one of the known pathovars of this species.

The first pathogenicity tests on wheat suggested that some of the pistachio strains produced only a hypersensitive response (specks) on wheat. However, more frequent and close observation of the development of symptoms showed that the lesions produced were similar to those produced by *X. translucens* on cereal and grasses, that is, translucent stripes that initially were water-soaked (Fang et al., 1950; Duveiller et al., 1997b). The similarity in pathogenicity of the three strains tested was demonstrated by the production of the typical symptoms of bacterial leaf streak on barley and grasses. The absence of symptoms in oats has been associated with *X. translucens* pv. *cerealis* in some reviews (Alizadeh et al., 1995; 1997), but not in others. Bragard et al. (1997) considered the pathovar *undulosa* less aggressive than *cerealis* on oats. Similarly, the low incidence of symptoms produced by the three strains on ryegrass is associated with some strains of the pathovar *graminis* (Michel, 2001). However, the response of *Lolium* spp. to pathovars of *X. translucens* depends greatly on the ryegrass cultivar, age of the plants, the origin of the strains and the environmental conditions (Channon and Hissett, 1984; Leyns et al., 1987; Wang Hui and Sletten, 1995). Although the similarity of the effects produced by the three strains suggests that they are not only the same species but also the same pathovar, the delineation of two subgroups among the pistachio isolates by rep-PCR, GC-FAME and SDS-PAGE analyses was not reflected in the pathogenicity tests. Furthermore, GC-FAME indicated that the *X. translucens* isolate from wheat was different from the pistachio strains. Further taxonomic classification of the pistachio strains at the pathovar level was beyond the scope of this study. However, characterisation of pistachio isolates is in progress (Marefat et al., 2004).

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