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Spread of levan-positive populations of *Pseudomonas savastanoi* pv. *savastanoi*, the causal agent of olive knot, in central Italy

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Abstract

Pseudomonas savastanoi pv. *savastanoi*, the causal agent of olive knot disease, has for a long time been included in subgroup 1b of phytopathogenic, fluorescent *Pseudomonas* species by the LOPAT determinative tests (production of levan, oxidase, pectinolytic and arginine dihydrolase activity, tobacco hypersensitivity). *Pseudomonas savastanoi* pv. *savastanoi* differs from the Pseudomonads in subgroup 1a only in being levan-negative. However, in 1990, during a survey on the spread of olive knot in Tuscany, levan-positive isolates of *P. savastanoi* pv. *savastanoi* were isolated from knots on two olive trees in an orchard in the province of Florence (Bagno a Ripoli). Some years later, to assess the further spread of levan production in populations of *P. savastanoi* pv. *savastanoi*, the survey was extended to 39 other orchards randomly scattered across Tuscany, and levan-positive bacteria were found in approximately 38% of these orchards. Phenotypic, genotypic and pathogenic characterisation allowed these levan-positive isolates to be assigned to *P. savastanoi* pv. *savastanoi*. The data suggest that in Tuscan olive orchards, levan-positive and levan-negative subpopulations of this phytopathogenic bacterium can coexist on the same plant. On the basis of the results obtained we suggest that subgroups 1a and 1b of the LOPAT determinative scheme should be combined, and that *P. savastanoi* should be considered a bacterial species that can be either levan-negative or levan-positive.

Introduction

Olive knot is a bacterial disease that occurs in almost all parts of the world where olive is grown. The first reported observations on this disease go back to the late 1870s (Savastano, 1878) and to the beginning of the twentieth century (Smith and Rorer, 1904). The bacterium also infects some other hosts, in particular oleander (*Nerium oleander*), ash (*Fraxinus excelsior*), and privet (*Ligustrum japonicum*), on which it causes diseases similar to olive knot. Isolates from these different host plants have been classified sometimes together and sometimes separately, as different species or pathovars (Young et al., 1978, 1991; Skerman et al., 1980; Janse, 1981; Gardan et al., 1992). Finally, in the Approved List of Names of Plant Pathogenic Bacteria 1864–1995 (Young et al., 1996) the olive knot pathogen was raised to the rank of species, *Pseudomonas savastanoi*, with a number of pathovars: *savastanoi, phaseolicola*, *glycinea*, *fraxini* comb. nov. and *nerii* comb. nov. In all studies on *P. savastanoi*, from Savastano and Smith until the latest published reports, isolates of the bacterium associated with olive were always levan-negative. Only Janse (1981) reported some levan-positive strains, which were, however, obtained from ash. Therefore, until the classification by Gardan and his colleagues, *Pseudomonas savastanoi* was included as the only member in subgroup 1b of the LOPAT determinative scheme for phytopathogenic, oxidase-negative and fluorescent bacteria (Lelliott et al., 1966): *P. savastanoi* is levan-negative,

whereas the *P. syringae* pathovars of subgroup 1a are all levan-positive. *Pseudomonas savastanoi* and the *P. syringae* pathovars were the same in the other characteristics tested by LOPAT (production of oxidase, pectinolytic and arginine dihydrolase activity, tobacco hypersensitivity).

Pseudomonas savastanoi has thus always been considered a typical levan-negative bacterium, having the general characteristics of *P. syringae* but with the following additions: levan is not produced; gelatin and harbutin not hydrolysed; D-gluconate, *meso*-inositol and mannitol are used as the sole carbon sources, but not erythritol, DL-homoserine, DL-lactate, quinate, D(-)tartrate or D-xylose. Isolates vary in the extent to which they use anthranilate, D-sorbitol, L(+)tartrate and trigonelline (Bradbury, 1986).

In 1990, during a survey carried out in Tuscany to trace the incidence and severity of olive knot disease (Tommasini, 1992) some levan-positive bacteria were isolated from the knots of two olive plants at Bagno a Ripoli, in the province of Florence. Levan-positive isolates were again isolated from knots on an olive plant in Latium, bordering on Tuscany in the south, and in 1992 from an olive plant in the province of La Spezia (Liguria), on the northern border with Tuscany. The isolates PFi1, PFi2, PFi3, PFi5, PFi6, and PFi8 isolated at Bagno a Ripoli were examined in the laboratory and assigned to *P. savastanoi* on the basis of some biochemical and pathogenic determinative tests (Iacobellis et al., 1993). In spite of these findings, the inability to produce levan on hypersucrose media is still thought to be one of the major determinative and discriminative phenotypic characteristics of *P. savastanoi* (Schaad et al., 2000). To elucidate the classification of this bacterium and ascertain the effective spread of levan-positive populations of *P. savastanoi*, and also to investigate the physiological, biochemical and pathological characteristics of those populations, it seemed of interest to follow up the investigations some ten years after the findings were first made.

One purpose of this work was thus to determine whether the levan-positive isolates of *P. savastanoi* found in some Italian olive orchards had been merely a chance occurrence or whether such isolates are well established in Tuscany. A second objective was to investigate more thoroughly the biochemical and pathological characteristics of the levan-positive isolates as compared with levan-negative ones. Bacteria were characterised both from a taxonomic point of view, using a polyphasic approach (biochemical tests, analysis of fatty acid profiles, sequencing of 16S rDNA), and from a pathological point of view, testing the pathogenicity/virulence of isolates directly by inoculation on the host species, and indirectly by amplifying portions of the genes *iaa*M, *iaa*H and *ipt* coding for some key enzymes of the biosynthetic pathways of IAA (*iaa*M and *iaa*H) and cytokinin (*ptz*), known virulence factors of this bacterial species (Surico et al., 1975, 1985; Comai and Kosuge, 1982). Levan-positive isolates were also checked for the gene *lsc*C (Hettwer et al., 1998), which codes for levan biosynthesis. This paper is an extension of the work by Iacobellis et al. (1993) and by Surico and Marchi (2003).

Materials and methods

Sampling procedures

Seventy-eight olive trees from 39 sites in Tuscany were sampled (two trees per site). Sites were mainly located in the area around Bagno a Ripoli, where levan-positive isolates were first detected in 1990. The location of the 39 sites sampled was recorded on a map (Figures 1 and 2). Sampling was carried out in the spring and early summer of 2001 and 2002 by cutting off from each tree at least two twigs with one or more knots. Twigs were placed in sterile plastic bags and kept in an ice chest, transported to the laboratory, and processed immediately. *Pseudomonas savastanoi* was isolated from at least three knots per tree (six knots per site).

Isolation from knots

To isolate *P. savastanoi*, knots were surface-disinfected with a paper towel moistened with 95% ethanol. Internal tissue of hydropic appearance was excised with a sterile scalpel, then ground in 200 μl of sterile distilled water (SDW). After 15 min, a loopful of the resulting suspension was streaked on plates of surface-dried 5% sucrose nutrient agar (SNA). SNA dishes were incubated at 26 °C for 3 to 5 days, after which single colonies were collected, checked twice for purity on SNA,

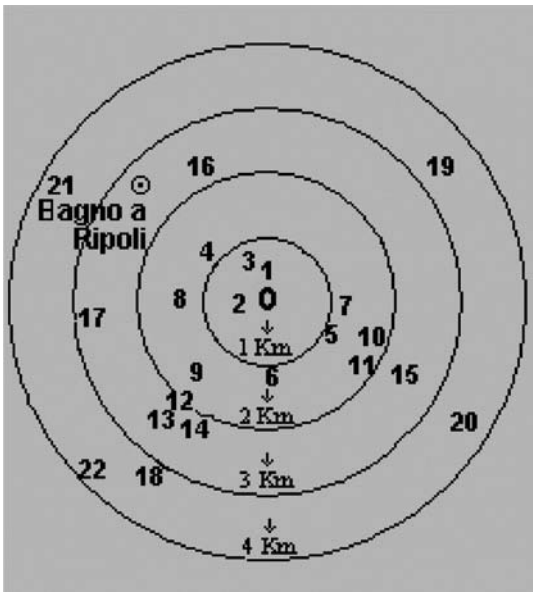

Figure 1. Detail of the area around Bagno a Ripoli (FI) from which 22 orchards were randomly chosen for isolation of *Pseudomonas savastanoi* from olive knots and leaves in 2001 and 2002. Nos. 2, 8, 13, and 16 represent orchards from which only levan-positive isolates were obtained; nos. 1, 4, 7, 9, 10, 12, 14, 15, 17, 19, 20, 21 and 22 orchards from which only levan-negative isolates were obtained, and nos. 3, 5, 6, 11 and 18 orchards from which both levan-positive and levan-negative isolates were obtained. No. 0 marks the orchard where levan-positive isolates were first found in 1990.

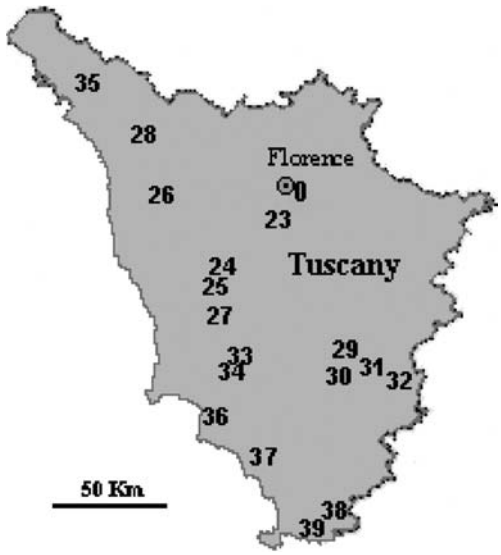

Figure 2. The location of 17 orchards in Tuscany randomly chosen for isolation of *Pseudomonas savastanoi* from olive knots and leaves in 2001 and 2002. Nos. 24, 25, 26 33, 34 and 35 represent orchards from which levan-positive isolates were obtained, and nos. 23, 27, 28, 29, 30, 31, 32, 36, 37, 38 and 39 orchards from which levan-negative isolates were obtained. No. 0 marks the orchard where levan-positive isolates were first found in 1990.

and stored on nutrient glycerol (1% v/v) agar (NGA) slants at 4 °C until use.

Isolation from the phylloplane

Fifty leaves per tree were randomly collected from ten diseased trees in five orchards (two trees per orchard) at Bagno a Ripoli (nos. 1, 2 and 8 in Figure 1, and nos. 23 and 29 in Figure 2), taken immediately to the laboratory, placed in 100 ml Erlenmayer flasks containing 40 ml of 0.05 M phosphate buffer (pH 7) and shaken for 2 h at room temperature. The resulting suspensions were serially diluted and 100 μl aliquots of each suspension were spread on SNA containing 66 mg l^{-1} cycloheximide and incubated at 26 °C. Single colonies were treated as above.

Characterisation of isolates

Twenty-two levan-positive isolates, twenty from knots and two from the phylloplane (Table 1) were tested to determine their phenotypical and phytopathological characteristics, their fatty acid profile, and the homology of their 16S-rDNA nucleotide sequence. In the characterisation tests, the isolates were compared with type strain NCPPB 639 (National Collection of Plant Pathogenic Bacteria, Sand Hutton, UK) and three reference strains of *P. savastanoi*: PvBa 229 (collection of the Department of Plant Pathology, University of Bari, Italy); ITM 105 and ITM 317 (collection of the ex Istituto Tossine e Micotossine da Parassiti Vegetali, CNR, Bari, Italy).

Phenotypic characterisation

The biochemical characteristics of the bacteria were determined by microbiological techniques described by Schaad (1988): Gram stain, formation of yellow colonies on YDC agar, LOPAT profile (Lelliott et al., 1966) and by some tests that according to Lelliott and Stead (1987) differentiate atypical *P. syringae* subsp. *savastanoi* strains from *P. syringae* subsp. *syringae*: gelatin liquefaction, and utilisation of quinate, erythritol and L-lactate

Table 1. Origin and peculiar characteristics of the *Pseudomonas savastanoi* isolates used in this study

Isolate	Sample origin					
	Orchard locality[a]	Plant part	Year collected	Fluorescence on KB	LOPAT group	Pathogenicity on olive
Levan-positive isolate						
PFi5	0	Knot	1990	–[b]	1a[c]	+
RM1L1	11	Knot	2001	–	1a	+
RM1R1	11	Knot	2002	–	1a	+
RM2R2	11	Knot	2002	–	1a	+
RM2L1	11	Knot	2001	–	1a	+
RMR3B1	11	Knot	2001	–	1a	+
RM4R1	11	Knot	2002	–	1a	+
CARA11	8	Leaf	2001	–	1a	+
CARA12	8	Knot	2001	–	1a	+
VER1B	3	Knot	2001	–	1a	+
MONB1	13	Knot	2001	–	1a	+
LEV4+	2	Leaf	2001	–	1a	+
PIC11	18	Knot	2001	–	1a	+
T3LAZ2	6	Knot	2001	–	1a	+
MLLI1	5	Knot	2001	–	1a	+
MLLI2	5	Knot	2001	–	1a	+
MLLI4	5	Knot	2001	–	1a	+
VOLT2	25	Knot	2002	–	1a	+
MMAR1	33	Knot	2002	–	1a	+
MMAR2	34	Knot	2002	–	1a	+
CAL1	26	Knot	2002	–	1a	+
C1	35	Knot	2002	–	1a	+
Levan-negative strain (type and reference strain)						
NCPPB639	Ex-Jugoslavia	Knot	1959	+	1b[d]	+
ITM 105	Amendolara, I	Knot	1982	+	1b	+
ITM317	Scanzano, I	Knot	1982	+	1b	+
PvBa 229	Bari	Knot	1968	+	1b	+

[a] see Figure 1 and Figure 2.
[b] –, negative reaction; +, positive reaction.
[c] 1a: positive for levan production and tobacco hypersensitivity, negative for oxidase production and pectinolytic and arginine dihydrolase activity.
[d] 1b: positive for tobacco hypersensitivity, negative for levan and oxidase production and for pectinolytic and arginine dihydrolase activity.

as the sole carbon source. All isolates were plated on King's B medium (KB) (King et al., 1954) and examined under UV light (254 and 365 nm) for fluorescent pigment production.

Pathogenicity tests

Wounds about 1 cm long were made in the bark of one-year-old olive stems (cv. Frantoio) with a scalpel dipped in a bacterial suspension containing about 10^9 cfu ml^{-1}. Each isolate was inoculated at six points places on two trees. Apical and subapical internodes were not inoculated. The wounds were protected with Parafilm M (American National Can, Menasha, WI) for three days. The trees were maintained in a greenhouse at 22–26 °C and 75–85% RH. Trees were inspected weekly symptom development over for 60 days. Control trees were treated in the same way with *P. savastanoi* strains ITM 317, ITM 105, NCPPB 639 and with SDW.

Fatty acid analysis

Analysis of the cellular fatty acid methyl ester (FAME) composition was performed at the Central Science Laboratory, Sand Hutton, York, UK, on five levan-positive isolates: three (MLLI1, MLLI2, MLLI4) from orchard 5, one (PFi5) from orchard 0 and one (T3LAZ2) from orchard 6. MLLI1, MLLI2, MLLI4 were from the same orchard and slightly differed from each other and from T3LAZ2

in the morphology of their colonies on SNA. The isolates tested were representative of all the isolates collected in regard to colony morphology. PFi5 was used for comparison. The fatty acid profiles were compared with those in two libraries, the commercially available TSBA40, and NCPPB3, a library containing almost all known plant-pathogenic bacteria and their close relatives common on plant surfaces, using Sherlock Microbial Identification System (MIS) software (MIDI, Newark, DE).

Bacterial DNA extraction and PCR amplification of 16S ribosomal DNA (16S rDNA)

Bacterial isolates were grown for 48 h at 26 °C on SNA. Total bacterial DNA was extracted with an Instagene Matrix Kit (Bio-Rad Laboratories, Hercules, CA) following manufacturer's instructions. PCR amplification of 16S rDNA was carried out in a Perkin Elmer 9600 thermocycler (Applied Biosystems, Foster City, CA). The reaction was performed in a final volume of 50 μl using the following amplification mixture: 1 X buffer [67 mM Tris–HCl; 16 mM $(NH_4)_2SO_4$; 0.01% Tween 20; pH 8.8]; 2 mM $MgCl_2$; 6% glycerol; 20 μM of each dNTP; 0.1 μM of primers fD1 and rD1; 2.5 U Polytaq Taq (Polymed, Firenze, Italy); approx. 20 ng of template DNA. The PCR consisted of 35 cycles of denaturation at 94 °C for 30 sec, annealing at 54 °C for 30 sec, extension at 72 °C for 1 min, preceded by 3 min at 95 °C for initial denaturation, and followed by 10 min at 72 °C for final extension. All reactions included both negative (DNA free) and positive controls. The successful amplification of the expected fragment (approx 1500 bp) was checked by electrophoresis in 1.5% (w/v) agarose gel at 4 V cm^{-1} for 1 h in TEB buffer (89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8) stained with 0.4 mg l^{-1} ethidium bromide and observed under UV light. The products of the amplification were purified with a Quiaquick PCR purification kit (Quiagen, Milan, Italy) following manufacturer's instructions and used directly for sequencing analysis.

16S rDNA sequencing and phylogenetic analysis

PCR amplicons were directly sequenced with their respective oligonucleotide primers. For each isolate and for the four reference strains used in this study, a portion of approximately 600 bp was sequenced using primers 27f and 685r1 (Lane, 1991). The nearly complete 16S rDNA gene sequences of isolates MLLI2 and T3LAZ2, and of the *P. savastanoi* reference strain ITM 105, was obtained by determining, both in forward and in reverse, contiguous overlapping sequences of PCR-DNA using primers fD1, rD1 (Weisburg et al., 1991), 530f, 926f, 685r1, 907r and 1392r (Lane, 1991). Sequencing was performed by the CIBIACI laboratories (Florence, Italy). 16S rDNA nucleotide sequences were compared with sequences of 23 validly described *Pseudomonas* species *sensu stricto* (Anzai et al., 2000) from the NCBI database GenBank. These species and their accession numbers are: *P. savastanoi* pv. *savastanoi*, AB021402; *P. aeruginosa*, Z76651; *P. agarici*, Z76652; *P. alcaligenes*, Z76653; *P. amygdali*, Z76654; *P. asplenii*, Z76655; *P. cichorii*, Z76658; *P. citronellolis*, Z76659; *P. ficuserectae*, Z76661; *P. fluorescens* biotype A, Z76662; *P. marginalis* pv. *marginalis*, Z76663; *P. mendocina*, Z76664; *P. oleovorans*, Z76665; *P. pseudoalcaligenes*, Z76666; *P. putida* biotype A, Z76667; *P. resinovorans*, Z76668; *P. syringae* pv. *syringae*, Z76669; *P. tolaasii*, Z76670; *P. viridiflava*, Z76671; *P. chlororaphis*, Z76673; *P. flavescens*, U01916; *P. stutzeri*, U26262; *P. corrugata*, D84012. Sequences were aligned using Clustal X (Version 1.81) and dissimilarities were converted to evolutionary distances according to Jukes and Cantor (1969). The construction of neighbouring joining trees and bootstrap analysis of 1000 resamplings were performed using the software package TREECON for Windows version 1.3b (Van de Peer and De Watcher, 1997), including *Escherichia coli* V00348 16S rDNA as the single-sequence (forced) outgroup.

Detection of iaa*M,* iaa*H,* ipt *and* Isc*C genes using PCR*

All isolates were tested for genes coding for *P. savastanoi* tryptophan mono-oxygenase (*iaa*M), indolacetamide hydrolase (*iaa*H), trans zeatin (*ptz*), and for the *P. syringae* pv. *glycinea* levansucrase C (*lsc*C), by PCR using the specific primers indicated below. Gene sequences were retrieved from the NCBI GenBank database, and the following four pairs of primers were designed using the Primer3 programme (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi): iaaMf, targeting

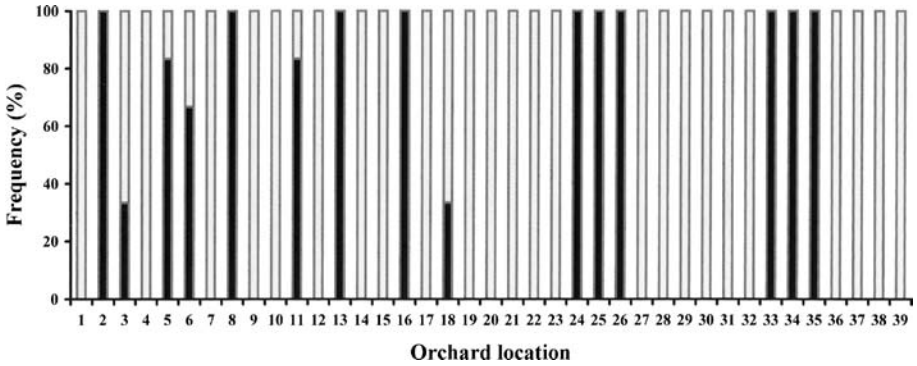

Figure 3. Frequency of isolation of levan-positive (black bars) and levan-negative (white bars) isolates of *Pseudomonas savastanoi* pv. *savastanoi* from each orchard surveyed. Levan-positive and levan-negative bacteria never occurred in the same knot but in orchards 3, 5, 6, 11 and 18 both types of isolates were present in different knots from the same plant (3 knots/plant; 2 plants/orchard).

position from 742 to 759 of *P. savastanoi iaa*M gene (accession no. M11035), 5′-ATGCGCTTTCCT CCSAGY-3′ and iaaMr, targeting position from 1032 to 1048 of the same gene, 5′-TGAGCCATM YCTGCCAT-3′; iaaHf, targeting position from 2681 to 2698 of *P. savastanoi iaa*H gene (accession no. M11035), 5′-TAAGAATACCCGGCGGC MT-3′ and iaaHr, targeting position from 3358 to 3374 of the same gene, 5′-GCATTRCTKGC CAGRTC-3′; ptzf, targeting position 618 to 637 of *P. savastanoi ptz* gene (accession no. X03679), 5′-GATTAGGAGGTGCGGATGAA-3′ and ptzr, targeting position 1079 to 1098 of the same gene, 5′-ACTCGTCTAACAACCCCGTG-3′; lscCf, targeting position from 3462 to 3478 of *P. syringae* pv. *glycinea lsc*C gene (accession no. AF 346402), 5′-ATCAGGGCGTGGAGTTGA-3′ and lscCr, targeting position from 3994 to 4011 of the same gene, 5′-GTCTGGAAAGGCTGCTCG-3′. PCR amplifications were done as described above for 16S rDNA amplification, with the exception of the annealing temperature with the lscCf and lscCr primers, which was 62 °C.

Results

Spread of levan-positive Pseudomonas savastanoi *populations in Tuscany*

Olive knots from 15 of the 39 sites investigated gave rise to bacterial cultures that formed typical levan-positive colonies on SNA and that were non-fluorescent on KB. Nine of these sites (nos. 2, 3, 5, 6, 8, 11, 13, 16 and 18 in Figure 1) were located within 4 km of the orchard from which these atypical isolates had been isolated for the first time in 1990 (no. 0 in Figure 1), and 6 were within about 108 km of that orchard (nos. 24, 25, 26, 33, 34 and 35 in Figure 2). Levan-negative fluorescent colonies of *P. savastanoi* were never found together with levan-positive ones in the same isolation plates (i.e. the two types of isolates never co-occurred in the same knot), but in 5 of the 15 orchards (nos. 3, 5, 6, 11 and 18) with levan-positive isolates, different knots from the same tree yielded both levan-negative and levan-positive isolates (Figure 3). Olive knots from the remaining 24 sites yielded bacterial isolates that formed levan-negative colonies on SNA and that were fluorescent on KB.

Morphology of levan-positive colonies

After five days on SNA, levan-positive colonies were grey-white to white, generally semi-translucent or translucent but sometimes opaque, mucoid, almost always radiated in varying degrees, generally circular, with entire, sometimes undulated margins, shiny or dull white, convex to domed, and about 3.5–4.5 mm diam. By contrast, colonies of levan-negative strains were gray to pale yellow after 5 days on SNA, with a matt centre, circular or irregularly round with entire or wavy margins, flat or slightly raised with a diameter of 3–5 mm. Levan-positive colonies with a similar morphology were also obtained when the leaves of olive trees whose knots had yielded levan-positive

Table 2. Species identification and similarity index (SI) values revealed by searching two databases using FAME profiles of five *Pseudomonas savastanoi* levan-positive isolates

Levan positive isolate	Identification	Closest match	SI (TSBA40 database)	Identification	Closest match	SI (NCPPB3 database)
T3LAZ2	*Pseudomonas*		0.908[a]	*Pseudomonas*		0.919
		P. cichorii	0.908		*P. syringae*	0.919
		P. viridiflava	0.900		*P. ficuserectae*	0.884
		P. savastanoi	0.856		*P. cichorii*	0.688
PFi5	*Pseudomonas*		0.917	*Pseudomonas*		0.866
		P. viridiflava	0.917		*P. syringae*	0.866
		P. savastanoi	0.863		*P. ficuserectae*	0.837
		P. cichorii	0.861		*P. caricapapayae*	0.701
MLLI1	*Pseudomonas*		0.530	*Pseudomonas*		0.508
		P. corrugata	0.530		*P. savastanoi*	0.508
		P. syringae	0.522		*P. corrugata*	0.349
		P. savastanoi	0.445			
MLLI2	*Pseudomonas*		0.500	*Pseudomonas*		0.484
		P. syringae	0.500		*P. savastanoi*	0.484
		P. corrugata	0.475		*P. corrugata*	0.275
		P. viridiflava	0.423			
MLLI4	*Pseudomonas*		0.606	*Pseudomonas*		
		P. syringae	0.606		*P. savastanoi*	0.550
		P. savastanoi	0.574		*P. corrugata*	0.398
		P. corrugata	0.562		*P. syringae*	0.283

[a]Strains with an SI of 0.6 or more and whose distance to the nearest SI is at least 0.100 are good library matches (Sasser, 1990). If SI is between 0.400 and 0.600 but the first SI is well separated from the second (> 0.100), the identification may be correct but the strain will be atypical. SI values lower than 0.400 suggest that the species to which the isolate belongs does not occur in the database.

isolates (orchards 2 and 8) were washed and the bacterial suspensions from those leaves were plated on SNA and KB.

Phenotypic characterisation and pathogenicity tests

All the 22 levan-positive isolates examined in this study were Gram-negative rods, occurring singly or in pairs, measuring 0.7 μm $\times$ 1.7–2.4 μm and with 1 to 3 polar flagella. All isolates were oxidase- and arginine dihydrolase-negative, did not macerate potato tuber slices, and induced a hypersensitivity response in tobacco when approx. 10^8 cfu ml^{-1} was injected into tobacco leaves (Table 1). No isolates liquefied gelatin or were able to use quinate, erythritol or L-lactate as the sole carbon source. When inoculated on the host, all these isolates produced the knots typical of the disease, although the size of the knots varied between isolates at different inspection dates. From newly formed gall tissue, bacteria with the same cultural characteristics were consistently reisolated.

Fatty acid analysis

The fatty acid profiles revealed that more than 74% of the total peak area of all cellular fatty acids of the five isolates examined (PFi5, MLLI1, MLLI2, MLLI4 and T3LAZ2) was accounted for by three major components: 16:0, 16:1 w7c and 18:1 w7c. The hydroxy-substituted acids 10:0-3OH, 12:0-2OH and 12:0-3OH contributed on average a further 11.31%. The most important minor components, occurring in all the isolates, were 12:0, 17:0 cyclo, 18:0, and 11methyl 18:1 w7c. Sixteen other fatty acids were also minor components (0.2%), or were found in amounts that varied strongly (from 0 to 2.46% of peak area) between isolates. However, while the fatty acid profiles (types, amounts and ratios) of PFi5 and T3LAZ-2 (Table 2) were fully consistent with some of the profiles of the '*Pseudomonas syringae* group' species (Anzai et al., 2000) included in both the TSBA40 and the NCPPB3 libraries, even if they could not be positively assigned to a given species (distance between the SI values less than 0.100), the fatty acid profiles of the MLLI group,

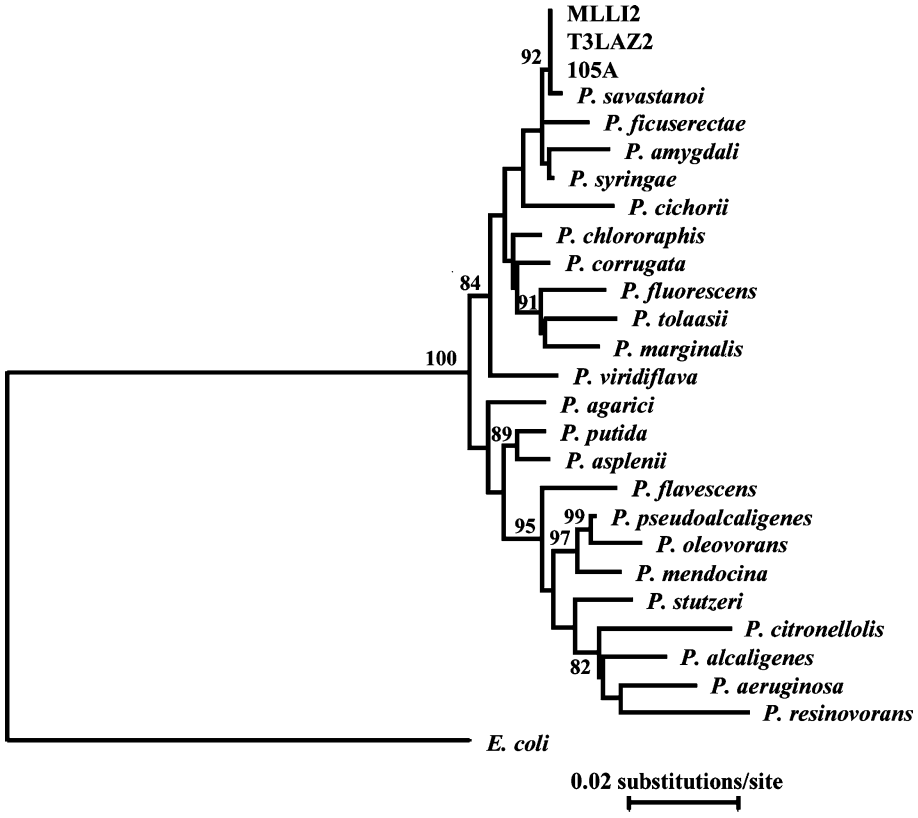

Figure 4. Phylogenetic tree derived from the similarities of 16S rDNA sequences of the levan-positive isolates T3LAZ2, MLLI2, the reference strain *Pseudomonas savastanoi* ITM 105, and 23 *Pseudomonas* species *sensu stricto*. Bootstrap proportions of confidence (1000 bootstrap repetitions) of 80% or more are indicated at the branch points. *Escherichia coli* (V00348) is used as the root organism.

although characteristic of the genus *Pseudomonas* (Table 2) and almost identical with each other, were different from the typical profiles of the *Pseudomonas* spp. strains in the TSBA40 library ($0.423 < SI < 0.606$). However, these data were not confirmed by the NCPPB3 library, which here revealed an acceptable similarity between the fatty acid profiles of isolates MLLI 1, 2 and 4 and the average profile of *P. savastanoi*. Nevertheless, the SI values were 0.484 for MLLI2, and 0.550 for MLLI4, suggesting that these isolates were atypical for *P. savastanoi* pv. *savastanoi*.

Genotypic identification of Pseudomonas *isolates*

The homology of the 16S rDNA sequences of all bacteria isolated in this study, downstream of primer 27f and over 600 bp, was more than 99.9% between the isolates themselves and between them and the *P. savastanoi* strains NCPPB 639, ITM 105, ITM 317 and PvBa 229 (data not shown). The inferred phylogenetic relationships derived from the almost complete 16S rDNA gene nucleotide sequences and the neighbour-joining analysis of pairwise comparisons (Figure 4) showed that isolates MLLI2, T3LAZ2 and ITM 105 clustered with *P. savastanoi* (more than 99.8% homology over 1500 bp).

Detection of iaa*M,* iaa*H,* ipt *and* Isc*C genes in bacterial isolates by the PCR test*

All the 22 levan-positive isolates and the *P. savastonoi* levan-negative strains used as controls, when tested by PCR amplification with the primer pairs iaaMf/iaaMr, iaaHf/iaaHr and ptzf/ptzr, yielded products of the expected size (approximately 307, 694 and 481, bp respectively). The primer pair lscCf/lscCr produced an amplicon of about 550bp (Figure 5) in all levan-positive isolates tested, but

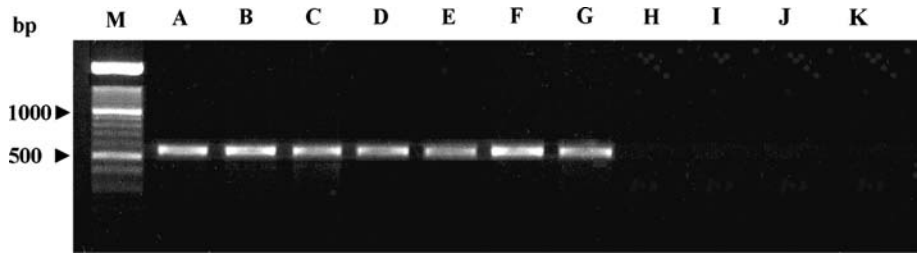

Figure 5. PCR reactions carried out for the detection of gene *lsc*C in *Pseudomonas savastanoi* isolates forming typical levan-positive colonies on NSA. Four *P. savastanoi* strains were used as controls. Lane M, molecular size standards; lane A, isolate RM1R1; lane B, CARA11; lane C, PIC11; lane D, LEV4+; lane E, PFi5; lane F, T3LAZ2; lane G, MLLI2; lane H, *P. savastanoi* NCPPB 639; lane I, *P. savastanoi* ITM 105; lane J, *P. savastanoi* PvBa 229; lane K, *P. savastanoi* ITM 317.

not in any of the levan-negative controls, indicating that the *lsc*C gene did not occur in the reference and type strains used in this study.

Discussion

All the 22 levan-positive isolates characterised in the study were positive for genes *iaa*M, *iaa*H and *ptz* of *P. savastanoi* in the PCR test, and caused knots when they were inoculated on the host tree. In addition, all isolates were negative for gelatin liquefaction, and were not able to use quinate, erythritol or L-lactate as their sole carbon source for growth. The approx 600 bp portion of 16S rDNA that was sequenced in all the isolates characterised in the study had a homology of more than 99.9% with the strains of *P. savastanoi* used as reference. A similar sequence homology (>99.8%) was observed between the nearly complete 16S rDNA sequence of the type strain of *P. savastanoi*, the sequence of reference strain *P. savastanoi* ITM105, and the sequences of the levan-positive isolates MLLI2 and T3LAZ2 examined in this study. It is our judgement that this result, on the whole, justifies assigning the Tuscan isolates to the pathovar *savastanoi*.

Other tests, however indicated that the bacterial isolates found in Tuscany had some peculiar characteristics that made them atypical for *P. savastanoi* pv. *savastanoi*, and were also not always the same for all these isolates. These characteristics included: the formation of domed colonies on hypersucrose media, some variations in the fatty acid profile, and a failure to produce fluorescent pigments on KB. The ecological significance of this last variant feature of isolates typical of *P. savastanoi* is unclear, but it is not all that surprising since it also occasionally occurs in other fluorescent Pseudomonads (Schaad, 1988; Bultreys et al., 2001), and in *P. savastanoi* itself (Ercolani, 1983). Of greater importance, however, are other differences found in this study. For example, fatty acid analysis found considerable differences between the profiles of the five levan-positive isolates examined. Bacteria isolated from trees growing less than 1 km apart had fatty acid profiles that were sometimes typical of species that belong to the *Pseudomonas syringae* group (isolates PFi5 and T3LAZ2) including *P. savastanoi*, but at other times (MLLI1, 2 and 4) they were atypical for species of the *P. syringae* group, so that the fatty acid profiles enabled these isolates to be classified only tentatively, and only to genus level (*Pseudomonas*). Interestingly, both libraries found that there was a degree of similarity between the profile of MILLI isolates and that of species of *P. corrugata*. Fatty acid profiling cannot however always be relied upon to provide a definitive identification, and this particular proposed match can be easily rejected since all our isolates were oxidase-negative, while *P. corrugata* is known to be oxidase-positive (Schaad, 1988). In any case isolates MLLI1 and 2, from different knots on the same tree (orchard 5), and MLLI4, from the second tree in orchard 5, had the same fatty acid profile.

There is a last peculiar characteristic of the isolates characterised, which is the production of levan. The capacity of these isolates to produce this hexopolysaccharide was first shown when dome-shaped colonies formed on a hypersucrose medium, and was confirmed by the PCR test for the *lsc*C gene, coding for the levansucrase enzyme

C of *P. syringae* pv. *glycinea* (Hettwer et al., 1998), which was positive for all the tested isolates but negative for all the strains of *P. savastanoi* used as controls in the trials. The production of levan is normally thought of as not being a property of *P. savastanoi*, so much so that this characteristic, together with some other properties (utilisation of trigonelline, ß-hydroxybutyrate, malonate, quinate, mannitol, inositol, sorbitol, DL-glycerate, L-lactate, production of indolacteic acid), has been used to argue that the pvs. *glycinea* and *phaseolicola* of *P. syringae* (both levan-positive) should not be assigned to *P. savastanoi* (Schaad et al., 2000).

The regional-scale survey showed that levan-negative and levan-positive populations can co-occur in the same orchard or even on the same plant (but not in the same knot). When a random sampling was carried out in a circular area of some 50 km^2 with at its centre the olive orchard in which the levan-positive isolates were first found in 1990 (arbitrarily assumed to be the original centre from which the levan-positive isolates had spread out), levan-positive isolates were found in almost 41% of all orchards surveyed. When the sampling area was extended to include a further 17 sites lying outside the borders of the comune of Bagno a Ripoli, the percentage of orchards in which levan-positive sub-populations were found was not significantly different (ca. 38%). It seems therefore that the levan-positive bacteria are gradually spreading throughout Tuscan territory. It should be added that levan-positive populations of *P. savastanoi* have also recently been isolated by other researchers (Scortichini et al., 2004) from olive trees grown in the provinces of Rimini (in Emilia and Romagna, a region bordering on Tuscany) and Grosseto (in Tuscany).

At present the data warrant no conclusions regarding the evolutionary origin of the levan-positive isolates. It seems certain however that these isolates in Tuscany, where olive cultivation is of long standing, are not a result of recent immigration from other regions, connected with the import of plantlets to establish new orchards, since the knots from which they had been isolated came from at least 20–30 year-old plants. A factor to be considered here is that a capacity to produce hexopolysaccharides confers a selective advantage on those bacteria, whether plant pathogenic or not, that have it. During the epiphytic growth of *P. savastanoi* (Ercolani, 1985), for example, hexopolysaccharides may help to form a biofilm matrix that holds the bacterial cells together. In this way a favourable micro-environment is created that: (i) enables the bacteria to attach themselves more strongly to the host; (ii) protects the cells from dehydration and UV radiation; and (iii) favours cell multiplication on account of the nutrients contained in the biofilm (Sutherland, 1985; Lindow and Brandl, 2003). On the other hand, during the parasitic growth of the bacterium, the hexopolysaccharides could become virulence factors, as occurs with other bacteria (El-Banoby and Rudolph, 1979; Kasapis et al., 1994; Jing et al., 1999). Taking all these considerations together, plus the fact that all *P. savastanoi* strains from olive characterised until 1990 were always levan-negative, a capacity to produce levan may have been recently acquired by the bacterium and may render it more competitive in nature. A similar idea was recently put forward by Gonzales et al. (2003), who isolated from various plants atypical populations of *P. viridiflava* (a pathogenic bacterium in the *Pseudomonas syringae* group) that differed from the type *P. viridiflava* strain in that they also produced levan in sucrose media. These authors also considered that the levan-positive strains of *P. viridiflava* they had isolated could recently have gained the ability to produce hexopolysaccharides in a hypersucrose medium, which may contribute to the epiphytic fitness of the strains, or even function as a virulence factor.

On the basis of the results obtained we suggest: that (i) subgroup 1b of the phytopathogenic, fluorescent *Pseudomonas* species based on the LOPAT determinative test should be eliminated; (ii) levan production should be considered a variable characteristic of *Pseudomonas savastanoi*.

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