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Genetic Diversity of *Xanthomonas arboricola* pv. *juglandis* (synonyms: *X. campestris* pv. *juglandis*; *X. juglandis* pv. *juglandis*) Strains from Different Geographical Areas shown by Repetitive Polymerase Chain Reaction Genomic Fingerprinting

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With 6 figures

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Abstract

A world-wide collection of 61 *Xanthomonas arboricola* pv. *juglandis* strains, isolated from Persian walnut (*Juglans regia* L.) or obtained from international culture collections and bacterial plant diseases laboratories, were studied by means of repetitive polymerase chain reaction (PCR) genomic fingerprinting using ERIC, BOX and REP primer sets and polyacrylamide gel electrophoresis. Cluster analyses were performed by UPGMA. Copper resistance, ability to hydrolize starch and quinate metabolism of the strains was also assessed. Pathogenicity was tested by inoculating leaves and nuts of Persian walnut seedlings. Polyacrylamide gel electrophoresis allowed very clear and reproducible differentiation of the PCR products. Cluster analysis showed the existence of three major groups of strains. The first two groups were 85% genetically similar, whereas the third clustered at 78% similarity with the other two. Each group could be divided into two subgroups which clustered according to the geographical origin of the isolates. In some cases, different genomic profiles were shown by strains from one country. This is possibly due to Persian walnut cultivation being mainly based on ecotypes and/or local seedlings that have become adapted to particular environments and so have allowed selection of different *X.a.* pv. *juglandis* populations. All strains were pathogenic and positive in starch hydrolysis and quinate metabolism tests. This is the first record of copper-resistant strains occurring outside California, USA.

Introduction

After preliminary studies, Pierce (1901) described the micro-organism inciting the 'bacteriosis of walnut' in California, USA and named it *Pseudomonas juglandis* Pierce. The name was soon after changed to *Bacterium juglandis* (Pierce) Smith by Smith (1905). It was later reclassified as *Phytomonas juglandis* (Pierce) Bergey et al. (Bergey et al., 1930), and as *Xanthomonas juglandis* (Pierce) Dowson by Dowson (1939). With the introduction of the pathovar concept to plant pathology, Dye (1978) proposed the name *Xanthomonas campestris* pv. *juglandis* (Pierce) Dye, a name officially accepted in the 'International standards for naming pathovars of phytopathogenic bacteria' (Dye et al., 1980). Recently, Vauterin et al. (1995) placed the pathogen within a new species, namely *Xanthomonas arboricola* as pathovar *juglandis* (Pierce) Vauterin et al. This last proposal, however, has still to be commonly accepted and the replacement of *X. arboricola* with *X. juglandis* (Pierce) Dowson has also been proposed (Schaad et al., 2000). Since studies on the genetic variation of many different strains of this pathogen from different geographical areas are lacking, it was useful to assess the genetic diversity of 61 *X.a.* pv. *juglandis* strains obtained either from international collections of phytopathogenic bacteria or directly from diseased specimens of *Juglans regia* L. The preliminary assessment of genetic variation of a micro-organism can facilitate investigation on its taxonomy, epidemiology and detection (Milgroom and Fry, 1997) as well as aiding breeding programs in the search for resistant germplasm (Norelli et al., 1984). The repetitive polymerase chain reaction (PCR) technique was chosen for this study because of its ability to differentiate bacterial taxa at the strain level (Louws et al., 1994, 1995), and to indicate intrapathovar diversity and identify pathovars with identical or similar profiles (Louws et al., 1994). The virulence of strains was also tested by pathogenicity tests and some phenotypic properties such as copper resistance, starch

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hydrolysis and quinate metabolism have been determined. The genetic variability found among strains of *X.a.* pv. *juglandis* obtained from different geographical areas is reported herein.

Materials and Methods

Bacterial cultures and isolation

The strains and isolates utilized in this study are shown in the Table 1. For isolation, the brilliant cresyl blue-starch (BS) semi-selective medium of Mulrean and Schroth (1981) was used, as it is based on the capability of *X.a.* pv. *juglandis* to hydrolize the starch contained in the medium and, consequently, to produce a pale blue zone surrounding the colonies. Isolations were made from visibly infected leaves; single leaflets were put into Erlenmeyer flasks each containing 200 ml of sterile physiological saline (SPS) (0.85% of NaCl in distilled water), and were shaken overnight at 150 r.p.m. Subsequently, 100 μl of the suspension were spread on to Petri dishes containing BS medium and incubated at 25–27°C for 5–7 days. Suspect colonies were streaked to purity on nutrient agar (NA) for use in additional tests to confirm the identity of the isolates. These tests were: observation of mucoid growth on glucose–yeast extract–calcium carbonate (GYCA) and yeast extract–dextrose–calcium carbonate (YDC) media, aesculin hydrolysis, protein digestion, growth at 35°C (Lelliott and Stead, 1987). Strains obtained from collections were revived on GYCA at 25–27°C.

Copper sensitivity, starch hydrolysis and quinate metabolism

Each strain was tested for copper sensitivity (Zevenhuizen et al., 1979), starch hydrolysis and quinate metabolism. For the former test, casitone–yeast extract–glycerol agar medium (CYEG) supplemented with 7, 20 or 60 μg/ml of cupric ions in the form of $CuSO_4.5H_2O$ was used. At a copper concentration of 7 μg/ml, all *X.a.* pv. *juglandis* strains tested by Lee et al. (1994) grew. At 10 μg/ml or more, the growth of strains started to be inhibited. Each strain was streaked to purity on CYGE plates and incubated at 25–27°C for 1 week. Positive growth was indicated by the subsequent appearance of colonies. The BS medium was used to test for starch hydrolysis; positive strains hydrolize the starch as described above. The ability to metabolize quinate is a stable phenotypic feature of *X.a.* pv. *juglandis* populations tested (Lee et al., 1992). For such tests the succinate–quinate medium (SQ) was used (Lee et al., 1992). Each strain was streaked on to the medium and incubated at 28°C for 6 days. The appearance of a deep-green-coloured area around the streak was considered as a positive reaction. All tests were performed twice.

Preparation of DNA and PCR analysis

Total genomic DNA was extracted by a modification of the technique described by Smith et al. (1995). Briefly, a loop (i.e. 3 mm diameter) full of a single colony of each strain grown in purity for 48 h on NA at 25–27°C was suspended in SPS and centrifuged at 8900 × ***g*** for 2 min. The pellet was suspended in SPS to an optical density

Table 1
List of *Xanthomonas arboricola* pv. *juglandis* strains studied. In brackets is reported, when known, the area of Persian walnut cultivation

Strain	Country	Year of Isolation
NCPPB 411 (T)	New Zealand	1957
NCPPB 412	New Zealand	1957
NCPPB 413	New Zealand	1957
NCPPB 414	New Zealand	1957
NCPPB 362	United Kingdom	1955
NCPPB 1659	United Kingdom	1964
NCPPB 1447	Romania	1963
NCPPB 2927	Iran	1977
NCPPB 3340	France	1984
PD 130	The Netherlands	1978
PD 157	The Netherlands	1987
PD 189	The Netherlands	1979
PD 2635	The Netherlands	1994
PD 2277	The Netherlands	1993
PD 2278	The Netherlands	1993
PD 2279	The Netherlands	1993
PD 1876	Hungary	1981
BPIC 279	Greece (Korinthos)	1970
BPIC 281	Greece (Korinthos)	1970
BPIC 282	Greece (Korinthos)	1970
BPIC 349	Greece (Arkadia)	1971
BPIC 733	Greece (Attiki)	1979
IVIA 1317.3	Spain	1993
IVIA 1325.2b	Spain	1993
IVIA 1321.1bb	Spain	1993
260 A2	Portugal (Alcobaça)	1994
261 C3	Portugal (Alcobaça)	1994
262 A1	Portugal (Alcobaça)	1994
257 A5	Portugal (Alcobaça)	1994
154 A	USA (California)	1999
154 B	USA (California)	1999
154 C	USA (California)	1999
154 D	USA (California)	1999
158 A	USA (California)	1999
158 C	USA (California)	1999
158 D	USA (California)	1999
ISF N1	Italy (Latium, Rome)	1991
ISF N2	Italy (Latium, Rome)	1995
ISF N3	Italy (Latium, Rome)	1999
ISF N4	Italy (Latium, Rome)	1999
ISF N5	Italy (Latium, Rome)	1999
ISF N6	Italy (Latium, Rome)	1999
ISF N7	Italy (Latium, Rome)	1999
ISF N8	Italy (Latium, Rome)	1999
ISF N9	Italy (Latium, Viterbo)	1999
ISF N10	Italy (Latium, Viterbo)	1999
ISF N11	Italy (Latium, Viterbo)	1999
ISF N12	Italy (Latium, Rieti)	1999
ISF N13	Italy (Latium, Rieti)	1999
ISF N14	Italy (Latium, Rieti)	1999
ISF N15	Italy (Latium, Rieti)	1999
ISF N16	Italy (Sicily, Palermo)	1999
ISF N17	Italy (Sicily, Palermo)	1999
ISF N18	Italy (Sicily, Palermo)	1999
ISF N19	Italy (Sicily, Palermo)	1999
ISF 964A	Italy (Piedmont, Turin)	1998
ISF 964B	Italy (Piedmont, Turin)	1998
ISF 1014C	Italy (Piedmont, Turin)	1998
ISF 1014D	Italy (Piedmont, Turin)	1998
ISF 1208	Italy (Piedmont, Turin)	1998
ISF 1209	Italy (Piedmont, Turin)	1998

T, pathotype strain; NCPPB, National Collection of Plant Pathogenic Bacteria, York, United Kingdom; PD, Plantenziekten-kundige Dienst, Wageningen, The Netherlands; BPIC, Benaki Phytopathological Institute Collection, Kiphissia-Athens, Greece; IVIA, Instituto Valencianio de Investigaciones Agrarias, Moncada-Valencia, Spain; ISF, Istituto Sperimentale per la Frutticoltura, Rome, Italy.

corresponding to 1 to 2×10^8 colony forming units (CFU)/ml. The suspension was placed in boiling water for 10 min and then stored at −20°C. The repetitive PCR (rep-PCR) method used was that of Louws et al. (1994). The Enterobacterial Repetitive Intergenic Consensus (ERIC), Repetitive Extragenic Palindromic (REP) and Box elements (BOX) primer sets were synthesized by Eurogentech (Seraing, Belgium). Amplification was performed on a MJ Research (Watertown, MS, USA) PTC 100 programmable thermal controller in 25 μl of reaction mixture containing 200 μM deoxynucleoside triphosphate, 2 mM $MgCl_2$, primers at 60 pmol, *Taq* polymerase 1.0 U and 4 μl of cell preparation sample, and overlaid with 25 μl of mineral oil. After thermal cycling (Louws et al., 1994), PCR products were separated by vertical gel electrophoresis on 6% acrylamide gels in 1 × TBE buffer, at 160 V, 4°C for 30 min, in Bio-Rad Mini Protean apparatus (Hercules, California, USA); the gels were stained with ethidium bromide and visualized under a UV transilluminator and photographed with a Polaroid film type 55. The PCR amplifications were performed in triplicate. Gel analyses were made as described by Smith et al. (1995). The gels were recorded and bands common to all three amplifications were recorded. For each primer and for each strain, bands were scored as present (1) or absent (0) and the readings were entered in a computer file as a binary matrix. Similarity coefficients for all pairwise combinations were determined by using Dice's coefficients (Dice, 1945) and clustered by unweighted paired group method using arithmetic averages (UPGMA) by means of NTSYS (Exeter Software, New York, USA), version 1.80. As outgroups the following xanthomonads were utilized: *Xanthomonas campestris* pv. *campestris* (Pammel) Dowson PD 1279, ISF C1, ISF C2; *X.c.* pv. *phaseoli* (Smith) Dye PD 514, *Xanthomonas vesicatoria* Vauterin et al. ISF PO 1.

Pathogenicity tests

Representative strains of the groups indicated by PCR analysis and the field isolates were tested for pathogenicity on leaves and fruit of *J. regia* seedlings under field conditions. Inocula were prepared from 48-hour-old cultures grown on NA, a loopful of the bacterial colony was suspended in SPS and photometrically adjusted to an optical density corresponding to 1 to 3×10^7 CFU/ml. Leaves and fruits were punctured at four locations with a sterile needle and, at the end of May, 10 μl of the bacterial suspension was placed on each wound. Leaves were inoculated either along the secondary veins or on the laminae. For each strain, two different leaves and fruits on the same plant were inoculated. Control plants were inoculated in the same way. Plants were checked for symptoms weekly, for 40 days after inoculation.

Results

Isolation

BS medium allowed the recovery of colonies capable of hydrolizing starch. Such colonies, obtained from diseased Italian walnut specimens were aesculin-positive, protein-digestion-positive, grew at 35°C and showed a mucoid growth on GYCA and YDC media. As all the colonies were pathogenic, they were all included in the present study (see Table 1).

Copper resistance, starch hydrolysis and quinate metabolism

Most of the strains grew on CYEG at a copper concentration of 7 μg/ml, but PD 157, IVIA 13.17.3, ISF N2, ISF N12, ISF N13, ISF N14, ISF N15 were inhibited. At a copper concentration of 20 μg/ml, only three of the four Portuguese strains (260 A2, 261 C3 and 262 A1) grew, and these also grew poorly at 60 μg/ml. All 61 *X.a.* pv. *juglandis* strains hydrolized starch contained in the BS medium and metabolized quinate.

Genomic fingerprinting

ERIC, BOX and REP primer sets gave reproducible genomic PCR profiles consisting of bands of approximately 100–1700 bp. The bands were very clearly differentiated by polyacrlamide electrophoresis. For UPGMA analysis, a total of 36 reproducible, clearly resolved bands were scored: 14 for primer ERIC, 13 for primer BOX and 9 for primer REP. ERIC and BOX primers were more discriminative than REP in differentiating the *X.a.* pv. *juglandis* strains. The construction of the similarity matrix showed that no strain from one country had the same profile as that of any strain from another area. Moreover, the overall genetic similarity of the strains from one country was always greater than that of strains from other countries. Representative genomic patterns are shown in Figs 1–5. The UPGMA analyses indicated genetic diversity among the strains isolated from different geographical areas. The 61 strains studied could be divided in three major groups, each of which could be divided into two subgroups according to the country of origin (Fig. 6). Group A1 included the strains from New Zealand, Romania and northern Italy. Group 2 included the strains isolated in The Netherlands and Hungary. Group B1 included the strains obtained from Portugal, Spain, France, California (USA), central Italy and the United Kingdom. Group B2 contained the strains from Greece. Group C1 included the strains from Sicily; group C2 was represented by the strain isolated in Iran. Groups A and B were related to approximately 85% similarity, whereas group C was related to the others to approximately 78%. The other xanthomonads utilized as outgroups showed the following similarities: *X.c.* pv *campestris* 43%, *X.c.* pv. *phaseoli* 40%, *X. vesicatoria* 39%. In some cases, *X.a.* pv. *juglandis* strains from one country or from different regions of a country did not show an unique profile. Strains collected in Italy, New Zealand, Greece and in The Netherlands showed more than one genomic fingerprint. The strains from northern, central and southern Italy differed genetically from each other and from strains from three different locations in Greece. In some other cases (i.e. California, Sicily), however, all the strains had a unique genomic profile. Four *X.a.* pv. *juglandis* strains taken from the same isolation plate showed two different profiles

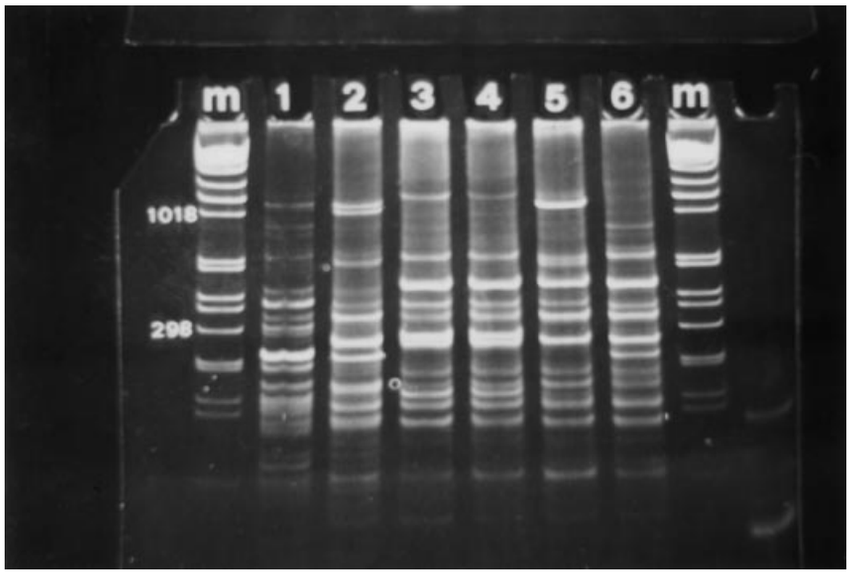

Fig. 1 PCR fingerprinting patterns from genomic DNA of *X.a.* pv. *juglandis* strains, obtained by using ERIC primer sets. m: molecular size marker (1-kb ladder, Gibco BRL, Life Technologies, Italy); the sizes are indicated in base pairs. Lane 1: PD 174; lane 2 ISF N15; lane 3: BPIC 279; lane 4: BPIC 282; lane 5: BPIC 349; lane 6: BPIC 733. Note the different profiles shown by the strains BPIC 349, isolated in Greece-Arkadia and BPIC 733, isolated in Greece-Attiki when compared with BPIC 279 and BPIC 282, isolated in Greece-Korinthos

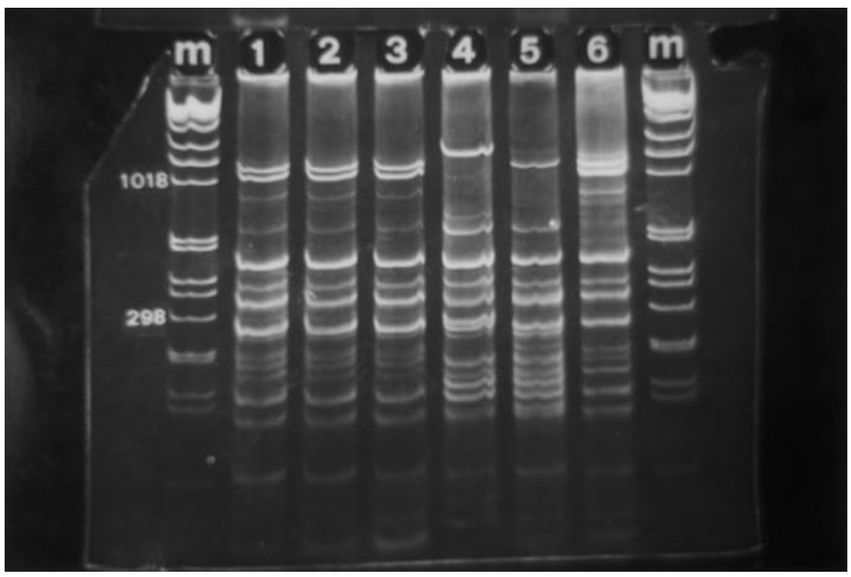

Fig. 2 PCR fingerprinting patterns from genomic DNA of *X.a.* pv. *juglandis* strains, obtained by using ERIC primer sets. m: molecular size marker (1-kb ladder, Gibco BRL, Life Technologies, Italy); the sizes are indicated in base pairs. Lane 1: 158 A; lane 2: 158 C; lane 3: 158 D; lane 4: NCPPB 412; lane 5: ISF 1209; lane 6: 260 A2. Note that the strains from California (i.e.158 A, C, D) each had a unique profile

(i.e. ISF N12 to ISF N15, from central Italy in the province of Rieti). Two strains (ISF N1 and ISF N2) isolated from the same walnut tree grown in the province of Rome, in 1991 and 1995, respectively, had the same profile.

Pathogenicity tests

At least one strain of each subgroup was tested for its pathogenicity. All the strains tested (i.e. NCPPB 411, group A1, PD 2635, group A2; 260 A2, 154 B, 158 A, group B1, BPIC 279, group B2, NCPPB 2927, group C2), as well as the Italian field isolates, induced symptoms typical of *X.a.* pv *juglandis* in Persian walnut. On fruit, necrosis developed around (i.e. 1–2 mm) the inoculation wound 10–15 days after inoculation and, sometimes, the necrotic spots tended to enlarge; some fruit fell prematurely. The inoculated leaf laminae and veins started to develop necrotic areas 7–11 days after inoculation. Some strains such as PD2635 were more aggressive in the leaf laminae than veins inducing large necrotic spots, whereas in inoculated veins it caused only small lesions. None of the controls developed necrosis.

Discussion

In this study it has been shown that genetic diversity exists among strains of *X.a.* pv. *juglandis* from different geographical areas of the world. This indicates that each area of Persian walnut cultivation has a different *X.a.* pv. *juglandis* population. Using ERIC, BOX or REP primer sets, no strain of a country had the same genomic fingerprint as a strain from another country. This also occurred with strains isolated from different regions of a single country. Thus, *X.a.* pv. *juglandis* strains from northern, central, and southern areas of Italy had different genomic fingerprints and so have been included in the three different groups, and the

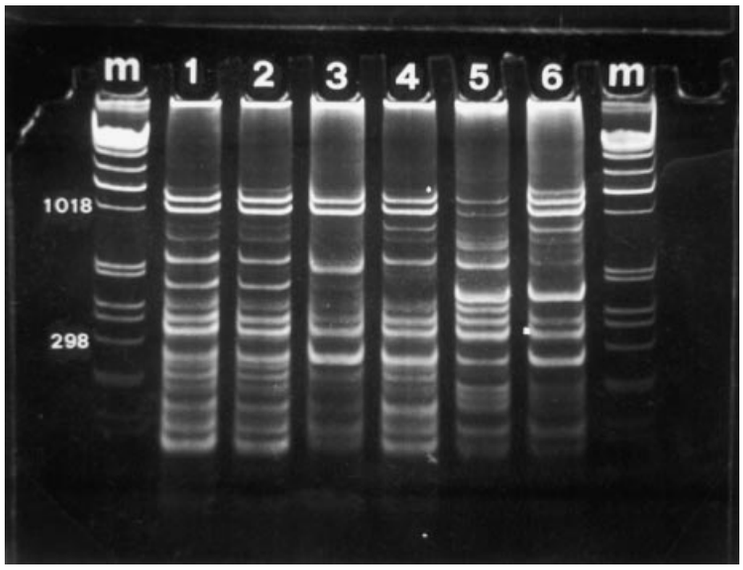

Fig. 3 PCR fingerprinting patterns from genomic DNA of *X.a.* pv. *juglandis* strains, obtained by using ERIC primer sets. m: molecular size marker (1-kb ladder, Gibco BRL, Life Technologies, Italy); the sizes are indicated in base pairs. Lane 1: ISF N12; lane 2: ISF N13; lane 3: ISF N14; lane 4: ISF N15; lane 5: NCPPB 2927; lane 6: 1317.3. Note that the strains ISF N12, ISF N13, ISF N14 and ISF N15 were obtained from the same isolation plate

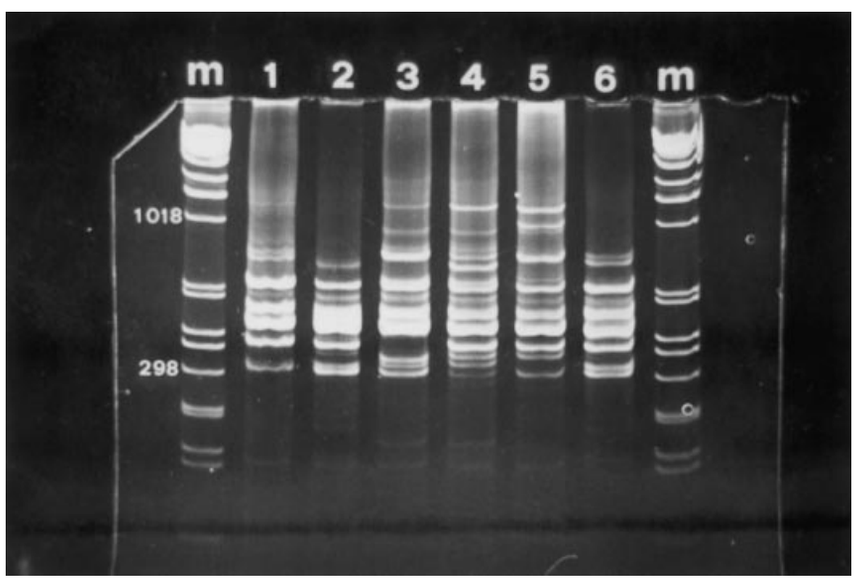

Fig. 4 PCR fingerprinting patterns from genomic DNA of *X.a.* pv. *juglandis* strains, obtained by using BOX primer sets. m: molecular size marker (1-kb ladder, Gibco BRL, Life Technologies, Italy); the sizes are indicated in base pairs. Lane 1: BPIC 279; lane 2: NCPPB 1447; lane 3: PD 1876; lane 4: PD 157; lane 5: 1325.2b; lane 6: ISF N6. Strains NCPPB 1447 and ISF N6 are included in group A1; strains PD 1876 and PD 157 in group A2; strain 1325.2b in group B1; strain BPIC 279 in group B2

Greek strains from Arkadia and Attiki also differed from each other and from the three strains from Korinthos. However, the overall genetic similarity of the *X.a.* pv. *juglandis* strains was high: groups A and B were related to each other to approximately 85% similarity, whereas group C was related to 78% with the other groups. Such diversity within *X.a.* pv. *juglandis* strains has already been shown by other techniques. Thus, using sodium dodecyl sulphate-polyacrylamide gel electrophoresis analysis of whole-cell proteins in an extensive study of *X. campestris* pathovars, Vauterin et al. (1991) placed nine strains, which were also used in the present study, in two distinct clusters: cluster 2, which includes PD 130, PD 157 and PD 189 from The Netherlands, were included in group A2 of the present study. Cluster 7, includes NCPPB 411, NCPPB 412, NCPPB 414 isolated in New Zealand, NCPPB 1447 isolated in Romania, NCPPB 362 and NCPPB 1659 isolated in the United Kingdom; in the present study, the first four strains belong to group A1 and the last two to B1. Moreover, by using contour-clamped homogenous electric field (CHEF) electrophoresis of 15 *X.a.* pv. *juglandis* strains isolated in South Africa, Du Plessis and Van der Westhuizen (1995) distinguished two clusters related at 54% similarity but with most of the strains showing a distinct profile.

Several molecular mechanisms promote genetic variations in micro-organisms that can account for the genetic diversity within bacterial populations; these include spontaneous mutations arising from DNA replication infidelity, the repair of DNA mismatches, transposition, site-specific recombination and horizontal DNA acquisition (Arber, 1997). Studies concerning these aspects on *X.a.* pv. *juglandis* are lacking, but the selective pressure of the host plant might play a relevant role in selecting different populations of the walnut

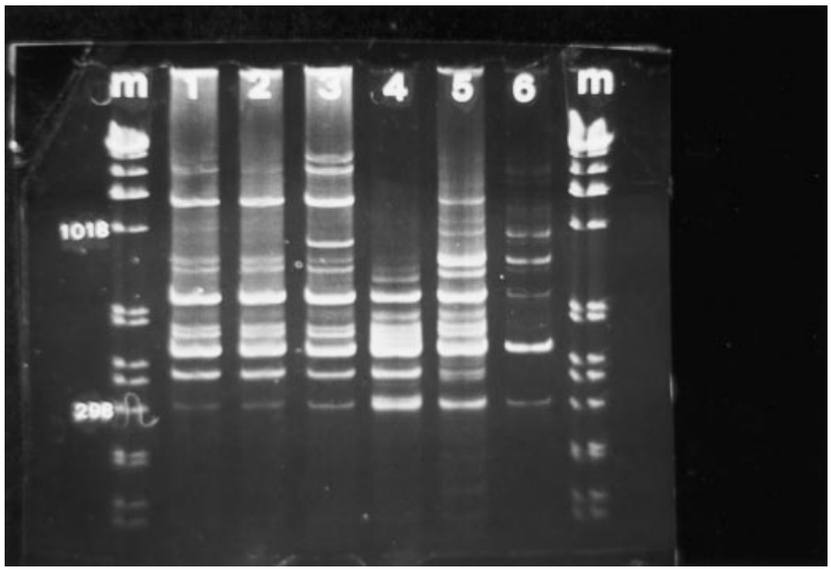

Fig. 5 PCR fingerprinting patterns from genomic DNA of *X.a.* pv. *juglandis* strains, obtained by using BOX primer sets. m: molecular size marker (1-kb ladder, Gibco BRL, Life Technologies, Italy); the sizes are indicated in base pairs. Lane 1: NCPPB 411; lane 2: NCPPB 412; lane 3: NCPPB 413; lane 4: NCPPB 1447; lane 5: NCPPB 1659; lane 6: NCPPB 2927. Strains NCPPB 411, 412 and 413 isolated from New Zealand. Note also the different profile of strain NCPPB 2927 (i.e. group C2), isolated in Iran

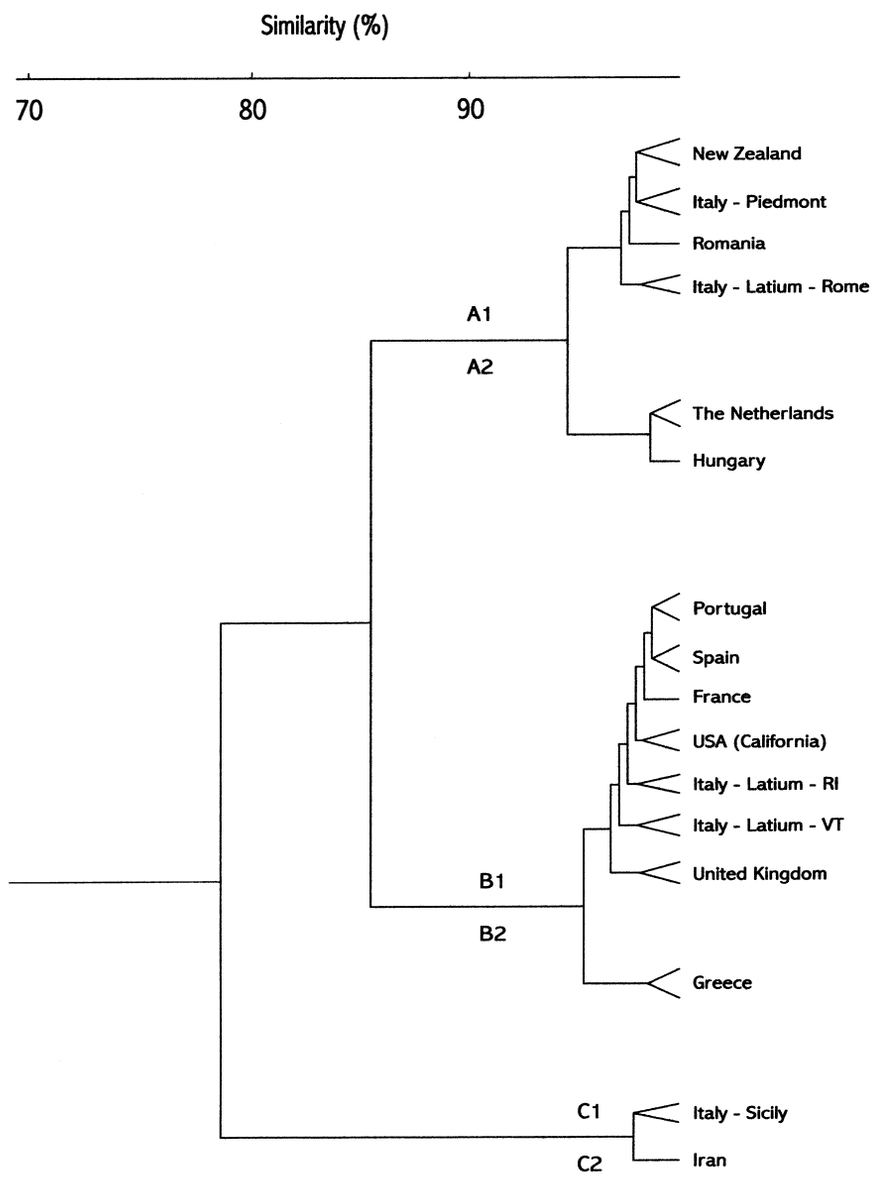

Fig. 6 Simplified dendrogram of *Xanthomonas arboricola* pv. *juglandis* strains obtained by UPGMA, on matrix calculated with Dice's coefficients. PCR patterns were obtained using ERIC, BOX and REP primer sets

pathogen. By studying the intra-population genetic variability of 27 naturalized populations of wild *J. regia* trees from different European and Asian areas by means of electrophoretic enzyme analysis, Malvolti et al. (1998) found that this species has genotypes adapted to local environmental conditions with alleles, in the different populations, that give evidence of distinctive gene pools. They found that walnut trees populations from Eastern Asia clearly differed from those grown in western, central and eastern Europe. It is also important to stress that, after the domestication of *J. regia*, ecotypes of the species were traditionally cultivated in each European and Asian country, and so-called cultivars are derived from different seedlings (Malvolti et al., 1993; Germain et al., 1997). Thus, different walnut ecotypes cultivated in different ecozones could have selected different *X.a.* pv. *juglandis* populations. Furthermore, in other pathosystems such as *Xanthomonas oryzae* pv. *oryzae* (Ishiyama) Swings et al./Rice, *Pseudomonas syringae* pv. *persicae* Prunier et al./Peach, *Erwinia amylovora* (Burrill) Winslow et al./Pear and *Pseudomonas avellanae* (Psallidas) Janse et al./Hazelnut, regional differencies in clusters of strains have been identified (Adhikari et al., 1995; Young et al., 1996; Zhang and Geider, 1997; Scortichini et al., 1998). The genetic diversity found in *X.a.* pv. *juglandis* strains might also be related to the relatively mild aggressiveness of the pathogen. Although this bacterium in some circumstances can cause economic losses approaching 60% (Olson et al., 1997), it very rarely kills trees. It causes damage to leaves, nut, flowers and twigs but the plant survives. This also might favour the selection of different genotypes of the bacterium co-infecting the same tree. In the present study genetic diversity was actually found among the strains isolated from the same leaf. A relevant genetic diversity was also found, for example, in fluorescent pseudomonads causing twig dieback on hazelnut (*Corylus avellana* L.) in a small area of cultivation (Scortichini et al., 2000); in this case also, the bacterium very rarely kills the trees. On the other hand, it was also found that within a population of a country, the strains isolated from different sites show the same genomic profile as well as strains isolated after 4 years from the same tree. Moreover, the strains isolated in California also showed a unique profile. This indicates that there is also the possibility that apparent clonal populations of *X.a.* pv. *juglandis* can establish in a particular environment.

In the present study it was also found for the first time that *X.a.* pv. *juglandis* strains showing resistance to copper occurred outside California. In fact, three of four strains isolated in Portugal showed this feature. Copper resistance of the walnut blight bacterium is considered a factor leading to failures in controlling the disease (Olson et al., 1997). However, the concentration of copper per hectare usually sprayed in the orchards (i.e. 4.5 kg/hectare per application, for a total of 10 or more applications per season) does not affect the growth of *X.a.* pv. *juglandis* strains which are resistant to 20–60 μg/ml of cupric ions in a bacterial culture medium, but this has still to be verified in orchard conditions. BS medium proved very effective in isolating *X.a.* pv. *juglandis* from leaves and twig lesions. Du Plessis and Van der Westhuizen (1995) found that some *X.a.* pv. *juglandis* strains isolated in South Africa did not hydrolize starch in this medium. This could be due to the absence of hydrolytic activity by such strains. The utilization of potato starch, however, is fundamental for indicating such a *X.a.* pv. *juglandis* feature (present authors, present study). In the isolation procedure, it was also found in the present study that the crushing of leaf lesions in SPS followed by spreading of the suspension on to BS medium, does not always permit the recovery of the pathogen. This could be due to the phenolic compounds released by the crushed leaf cells killing the bacterial cells. When the same sample was processed in parallel by simply placing the leaf overnight into Erlenmeyer flask containing SPS, followed by the plating of 100 μl of the suspension on BS medium, *X.a.* pv. *juglandis* isolates were readily obtained. Finally, this study confirms and stresses that breeding programmes to find Persian walnut germplasm that is tolerant to *X.a.* pv. *juglandis* should use more strains from different geographical areas (Woeste et al., 1992). Regional resistance breeding (Adhikari et al., 1995) might be an alternative strategy.

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Literature

Adhikari, T. B., C. M. Vera Cruz, Q. Zhang, R. J. Nelson, D. Z. Skinner, T. W. Mew, J. E. Leach (1995): Genetic diversity of *Xanthomonas oryzae* pv. *oryzae* in Asia. Appl. Env. Microbiol. **61,** 966–971.

Arber, W. (1997): Genetic bases for evolutionary development of microorganisms. In: Van der Zeijst, B. A. M., W. P. M. Hoekstra, S. D. A. Van Embden and A. S. W. Van Alfen (eds), Ecology of Pathogenic Bacteria, pp. 3–34. Royal Netherlands Academy of Art and Science, Amsterdam, The Netherlands.

Bergey's Manual of Determinative Bacteriology (1930): Phytomonas juglandis (Pierce) Bergey et al. 3rd edn. William and Wilkins, Baltimore, MD, USA.

Dice, L. R. (1945): Measurement of the amount of ecological association between species. Ecology **26,** 297–302.

Dowson, W. J. (1939): On the systematic position and generic names of the gram negative bacterial plant pathogens. Zentr. Bakter. Parasit. Infekt. II **100,** 177–193.

Du Plessis, H. J., T. J. Van der Westhuizen (1995): Identification of *Xanthomonas campestris* pv. *juglandis* from (Persian) English walnut nursery trees in South Africa. J. Phytopathol. **143,** 449–454.

Dye, D. W. (1978): Genus *Xanthomonas* Dowson 1939. N. Z. J. Agric. Res. **21,** 153–177.

Dye, D. W., J. F. Bradbury, M. Goto, A. C. Hayward, R. A. Lelliott, M. N. Schroth (1980): International standards for naming pathovars of phytopathogenic bacteria and a list of pathovar names and pathotypes. Rev. Plant Pathol. **59,** 153–168.

Germain, E., N. Aletà, A. Ninot, D. Rouskas, G. Zakinthinos, J. A. Gomes-Pereira, F. Monastra, F. Limongelli (1997): Prospections réalisées dans les populations de semis de noyer d'Espagne, de Grece, d'Italie et du Portugal: caracteristique des populations et description en collections d'études des préselections issues de ces prospections. Options Med. **16,** 9–40.

Lee, Y. A., D. C. Hildebrand, M. N. Schroth (1992): Use of quinate metabolism as a phenotypic property to identify members of *Xanthomonas campestris* DNA homology group 6. Phytopathology **82,** 971–973.

Lee, Y. A., M. Hendson, N. J. Panopoulos, M. N. Schroth (1994): Molecular cloning, chromosomal mapping, and sequence analysis of copper resistance genes from *Xanthomonas campestris* pv. *juglandis*: homology with small blue copper proteins and multicopper oxidase. J. Bacteriol. **176,** 173–188.

Lelliott, R. A., D. E. Stead (1987): Methods for the Diagnosis of Bacterial Diseases of Plants. Blackwell Scientific Publications for the British Society of Plant Pathology. Oxford, UK.

Louws, F. J., D. W. Fulbright, C. T. Stephens, F. J. De Bruijn (1994): Specific genomic fingerprinting of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequiences and PCR. Appl. Env. Microbiol. **60,** 2286–2295.

Louws, F. J., D. W. Fulbright, C. T. Stephens, F. J. De Bruijn (1995): Differentiation of genomic structure by rep-PCR fingerprinting to rapidly classify *Xanthomonas campestris* pv. *vesicatoria*. Phytopathology **85,** 528–536.

Malvolti, M. E., M. Paciucci, F. Cannata, S. Fineschi (1993): Genetic variation in Italian 'populations' of *Juglans regia* L. Acta Hort. **311,** 86–91.

Malvolti, M. E., N. Aletà, A. Ninot, M. Spada (1998): Walnut (*J. regia* L.) genetic resources in Europe. FAO Nucis Newsletter **7,** 20–23.

Milgroom, M. G., W. E. Fry (1997): Contribution of population genetics to plant disease epidemiology and management. Rev. Plant Pathol. **59,** 153–168.

Mulrean, E. N., M. N. Schroth (1981): A semiselective medium for the isolation of *Xanthomonas campestris* pv. *juglandis* from walnut buds and catkins. Phytopathology **71,** 336–339.

Norelli, J. L., H. S. Aldwinckle, S. V. Beer (1984): Differential host pathogen interactions among cultivars of apple and strains of *Erwinia amylovora*. Phytopathology **74,** 136–139.

Olson, W. H., R. P. Buchner, J. E. Adaskaveg, S. E. Lindow (1997): Walnut blight control in California. Acta Hort. **442,** 173–188.

Pierce, N. B. (1901): Walnut bacteriosis. Bot. Gaz. **31,** 272–273.

Schaad, N. W., A. K. Vidaver, G. H. Lacy, K. Rudolph, J. B. Jones (2000): Evaluation of proposed amended names of several pseudomonads and xanthomonads and recommandations. Phytopathology **90,** 208–213.

Scortichini, M., M. T. Dettori, U. Marchesi, M. A. Palombi, M. P. Rossi (1998): Differentiation of *Pseudomonas avellanae* strains from Greece and Italy by rep-PCR genomic fingerprinting. J. Phytopathol. **146,** 417–420.

Scortichini, M., U. Marchesi, M. T. Dettori, L. Angelucci, M. P. Rossi, C. Morone (2000): Genetic and pathogenic diversity of *Pseudomonas avellanae* strains isolated from *Corylus avellana* trees in north-west of Italy, and comparison with strains from other regions. Eur. J. Plant Pathol. **106,** 147–154.

Smith, E. F. (1905): Bacterium juglandis (Pierce) E. F. Smith. Bacteria in Relation to Plant Diseases. Carnegie Inst., Washington, DC, USA.

Smith, J. J., L. C. Offord, M. Holderness, G. S. Saddler (1995): Genetic diversity of *Burkholderia solanacearum* (synonym *Pseudomonas solanacearum*) race 3 in Kenya. Appl. Env. Microbiol. **61,** 4263–4268.

Vauterin, L., J. Swings, K. Kerster (1991): Grouping of *Xanthomonas campestris* pathovars by SDS-PAGE of proteins. J. Gen. Microbiol. **137,** 1677–1687.

Vauterin, L., B. Hoste, K. Kerster, J. Swings (1995): Reclassification of *Xanthomonas*. Int. J. Syst. Bact. **45,** 472–489.

Woeste, K. E., G. H. McGranaham, M. N. Schroth (1992): Variation among Persian walnut in response to inoculation with *Xanthomonas campestris* pv. *juglandis*. J. Am. Hort. Sci. **117,** 527–531.

Young, J. M., D. S. Jones, M. Gillings (1996): Relationships between populations of *Pseudomonas syringae* pv. *persicae* determined by restriction fragment analysis. Plant. Pathol. **45,** 350–357.

Zevenhuizen, L. P. T. M., J. Delfing, E. J. Eshuis, I. S. Scholten-Koerselman (1979): Inhibitory effects of copper on bacteria related to the free ion concentration. Microb. Ecol. **5,** 139–146.

Zhang, Y., K. Geider (1997): Differentiation of Erwinia amylovora strains by pulsed field gel electrophoresis. App. Env. Microbiol. **63,** 4421–4426.