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Occurrence of *Pseudomonas syringae* pv. *coryli* on Hazelnut Orchards in Sicily, Italy and Characterization by Fluorescent Amplified Fragment Length Polymorphism

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Abstract

Field surveys for ascertaining the sanitary status of hazelnut (*Corylus avellana* L.) orchards located in the provinces of Catania and Messina, Sicily, were carried out. Twig and branch diebacks were frequently observed on the local hazelnut cultivars. Identification of the bacterial pathogen associated with these symptoms was performed by means of biochemical, pathogenicity and molecular tests as well as by using fluorescent amplified fragment length polymorphic (fAFLP) analysis. The study revealed, for the first time, the presence of *Pseudomonas syringae* pv. *coryli* in the Sicilian hazelnut orchards. All the strains obtained did not show the presence of *syr*B gene. The fAFLP analysis pointed out the presence of three different lineages within the strains of this pathogen.

Introduction

In Italy, hazelnut (*Corylus avellana* L.) cultivation is mainly present in Campania, Latium, Sicily and Piedmont and a total of approximately 70 000 ha are cultivated. Many surveys recently carried out in the above regions have ascertained the occurrence of three distinct phytopathogenic pseudomonads: *Pseudomonas avellanae*, the causal agent of hazelnut bacterial canker and decline, which is present only in Latium (Scortichini et al., 2002); *P. syringae* pv. *coryli*, causal agent of bacterial twig dieback, found until now only in Piedmont and Sardinia (Fiori et al., 2003; Scortichini et al., 2005) and *P. syringae* pv. *syringae*, which is widespread in all areas of hazelnut cultivation causing partial wilting of the twigs (Scortichini et al., 2002, 2005). Moreover, in Sicily *Xanthomonas arboricola* pv. *corylina* has also recently been found to be dangerous for hazelnut cultivation (Cirvilleri et al., 2006a).

The cultivation of hazelnut in Sicily dates back to millennia, and currently, it spans over approximately 12 000 ha mainly located in the provinces of Messina, Catania and Palermo. During the surveys aimed to ascertain the sanitary status of the crop, twig diebacks and longitudinal cankers along the main branches and trunk were often noticed in some orchards located in the Messina and Catania provinces (Figs 1 and 2). A complete and widespread decline of the trees was never observed. Consequently, isolations were performed to ascertain the causal agent of such symptoms.

This study reports on for the first time the occurrence of *Pseudomonas s*. pv. *coryli* on hazelnut orchards cultivated in Sicily. Fluorescent amplified fragment length polymorphism (fAFLP) was used for identification and characterization purposes. This technique is accurate, discriminatory, reproducible and capable of standardization. fAFLP presents as a highly sensitive rapid technique used for human and plant pathogens typing (Ticknor et al., 2001; Kassama et al., 2002; Ahmed et al., 2003; Roumagnac et al., 2004; Cirvilleri et al., 2006b).

Materials and Methods

Isolation

Field surveys were carried out in the main hazelnut-growing areas of Sicily (Madonie and Etna areas). Samples from diseased twigs and branches were collected in spring and autumn 2005 from trees of the local hazelnut cultivar, namely 'Siciliana', at sites where twig dieback and partial wilting of trees were noticed. The samples were stored at 4–5°C and they were processed for isolation within 48 h after removal from the tree. All isolations were made by following the techniques described elsewhere (Psallidas and Panagopoulos, 1979; Scortichini and Tropiano, 1994). Samples from twigs and branches were surface-sterilized with 0.5%

Fig. 1 Longitudinal canker and twig diebacks on hazelnut (*Corylus avellana* L.) cv. 'Siciliana' trunk caused by *Pseudomonas syringae* pv. *coryli* in Sicily (southern Italy)

Fig. 2 Extensive necrosis of woody tissue along the trunk of hazelnut (*Corylus avellana* L.) cv. 'Siciliana'. *Pseudomonas syringae* pv. *coryli* was isolated from margin lesions

sodium hypochloride, rinsed with sterile distilled water (SDW) and the epidermis of a twig or branch was aseptically removed. Small pieces of tissue taken from the margin of the necrotic lesions were crushed in SDW and aliquots of 0.1 ml were plated on KB medium (King et al., 1954) and nutrient sucrose agar (NSA; Lelliott and Stead, 1987). The plates were incubated at 25–27°C for 2–4 days. Single colonies were further purified on nutrient agar (NA). All isolates were maintained at −20°C and subcultured on KB or NA as needed. Some representative strains of phytopathogenic pseudomonads used for comparison purposes are also reported in Table 1.

Biochemical and pathogenicity tests

Fluorescent colonies were purified on NA prior to identification, and the following biochemical tests were performed according to the procedures described by Lelliott and Stead (1987): levan production, presence of oxidase, soft rot activity on potato slices, presence of arginine dehydrolase, hypersensitivity reaction in tobacco leaves (LOPAT tests), metabolism of glucose, presence of tyrosinase, hydrolysis of aesculin and arbutin, gelatine liquefaction. In addition, the isolates belonging to LOPAT test group Ia, were evaluated for pathogenicity on lemon fruits and hazelnut, pear and lilac plants. With 24-h-old cultures grown on NA, suspensions in sterile saline (0.85% of NaCl in distilled water), photometrically adjusted to $1–2 \times 10^7$ cfu/ml, were prepared. Lemon (*Citrus lemon* L.) fruits were first surface-sterilized with sodium hypochlorite solution, then after rinsing with SDW, they were inoculated by puncturing the surface with a sterile needle and placing 10 μl of the bacterial suspension onto the wound and evaluated for symptom appearance 7 days after inoculation. For each isolate, 10 sites of two fruits were inoculated. Lemon fruits inoculated with sterile saline in the same way were used as control. In addition, pot-grown plants of pear (*Pyrus communis* L.) and lilac (*Syringa vulgaris* L.) were inoculated with a 20 μl drop of the bacterial suspension that was deposited on a fresh wound made on the midrib of the leaf. For each isolate, 10 different leaves were inoculated. Hazelnut cultivar 'Tonda Gentile Romana' pot-cultivated plants were inoculated in autumn (early October). Inoculations were performed by placing 10 μl of bacterial suspension ($1–2 \times 10^7$ cfu/ml) in the leaf scar immediately after the leaf removal. Three leaf scars per each isolate were inoculated. Control plants were wounded in the same way and treated with sterile saline. Symptom appearance (i.e. twig dieback) were checked in the following spring. Re-isolations were performed after symptoms appearance by using the techniques previously described.

Extraction of genomic DNA

Genomic DNAs of putative pathogenic fluorescent pseudomonads were extracted and purified using the DNA Purification Kit (Puregene, Gentra, Minneapolis, MN, USA), following the manufacturer's instructions. Each isolate was grown overnight in 5 ml of nutrient broth (NB; Oxoid CM1, Basingstoke, UK) at 27°C in a rotating incubator at 170 rpm. Cells were collected by centrifugation at $2500 \times \boldsymbol{g}$, and washed twice with SDW. The extracted DNA was quantified by electrophoresis on 1% agarose gel in the presence of an appropriate marker (High DNA mass ladder; Invitro-

Table 1
Strains of *Pseudomonas* spp. used in this study

Species/pathovar	Strains	Host	Country of origin	Area of origin	Year of isolation
Pseudomonas syringae pv. *coryli*	ISPaVe 592 = NCPPB 4273^T	*Corylus avellana*	Italy	Piedmont	1995
Pseudomonas syringae pv. *coryli*	ISPaVe 593	*Corylus avellana*	Italy	Piedmont	1995
Pseudomonas syringae pv. *coryli*	DPP 48	*Corylus avellana*	Italy	Sardinia	1999
Pseudomonas syringae pv. *coryli*	PVCT Sa27	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT Sa28	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT Sa30	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT Sa51	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT Sa52	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT Sa53	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT 7NOC	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT 38NOC	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas syringae pv. *coryli*	PVCT 44NOC	*Corylus avellana*	Italy	Sicily	2005
Pseudomonas avellanae	ISPaVe 011	*Corylus avellana*	Italy	Latium	1991
Pseudomonas syringae pv. *syringae*	PVCT 335.2	*Citrus sinensis*	Italy	Sicily	2000
Pseudomonas syringae pv. *syringae*	ISF 243	*Citrus reticulata*	Italy	Latium	1996
Pseudomonas syringae pv. *syringae*	ISF 293 = B359	*Setaria italica*	Australia	NK	
Pseudomonas syringae pv. *syringae*	ISPaVe 015 = NCPPB 3869	*Laurus nobilis*	Italy	Umbria	1992
Pseudomonas syringae pv. *syringae*	ISF 282	*Castanea sativa*	Italy	Umbria	1996
Pseudomonas syringae pv. *syringae*	ISF 355	*Hordeum vulgare*	Italy	Latium	1996
Pseudomonas syringae pv. *syringae*	HRI 1480A	*Pisum sativum*	UK	NK	
Pseudomonas syringae pv. *syringae*	ISF 357	*Hordeum*	Italy	Latium	1996
Pseudomonas syringae pv. *syringae*	ISF 300	*Triticum aestivum*	Italy	Latium	
Pseudomonas syringae pv. *syringae*	PVCT 23P	*Pyrus communis*	Italy	Sicily	1998
Pseudomonas syringae pv. *syringae*	PVCT 26P	*Pyrus communis*	Italy	Sicily	1998
Pseudomonas syringae pv. *syringae*	PVCT 1.3S	*Strelitzia reginae*	Italy	Sicily	2000
Pseudomonas syringae pv. *syringae*	ISF 288	*Pyrus communis*	Italy	Latium	1996
Pseudomonas syringae pv. *syringae*	ISF 281	*Pyrus communis*	Italy	Latium	1996
Pseudomonas syringae pv. *syringae*	PVCT 40	*Cynara scolymus*	Italy	Sicily	1992
Pseudomonas syringae pv. *syringae*	PVCT 169	*Cynara scolymus*	Italy	Sicily	1992
Pseudomonas syringae pv. *syringae*	AID 33	*Fragaria* × *ananassa*	Italy	Sicily	1988
Pseudomonas syringae pv. *syringae*	AID 76	*Fragaria* × *ananassa*	Italy	Sicily	1988

ISPaVe: CRA, Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; DPP, Plant Protection Department, University of Sassari, Italy; PVCT, Plant Pathology, University of Catania, Italy; ISF: CRA, Istituto Sperimentale per la Frutticoltura, Roma, Italy; HRI, Horticultural Research Institute, Warwick, UK; AID, Agricultural Industrial Development, Catania, Italy; ^{T}, type-strain; NK, not known.

gen-Life Technologies, Paisley, UK) and was used as a template for polymerase chain reaction (PCR) in both *syr*B and fAFLP analysis.

Presence of *syr*B gene

The presence of the *syr*B gene was assessed for all isolates by using the procedures described by Sorensen et al. (1998). Primers B1 (5′-CTTTCCGTGGTCTTGATGAGG-3′) and B2 (5′-TCGATTTTGCCGTGATGAGTC-3′) were synthesized by Eurogentec (Seraing, Belgium).

Fluorescent AFLP analysis

DNA template preparation using restriction enzymes *Eco*RI and *Mse*I (Invitrogen-Life Technologies) and adaptors ligation were carried out as reported by Vos et al. (1995). Briefly, bacterial genomic DNA (250 ng) from each isolate was digested with 10 U of both *Eco*RI and *Mse*I in 10X reaction buffer for 4 h at 37°C, in a final restriction volume of 25 μl. Restriction enzymes were heat-inactivated at 75°C for 15 min. In the same reaction mixture, 5 μl of *Eco*RI and *Mse*I adaptors (*Eco*RI forward: 5′-CTCGTAGACTGCGTACC-3′, *Eco*RI reverse: 5′-AATTGGTACGCAGTCTAC-3′, *Mse*I forward: 5′-GACGATGAGTCCTGAG-3′, *Mse*I reverse: 5′-TACTCAGGACTCAT-3′) were added at final concentrations of 2 and 20 μM, respectively, and ligated for 2 h at 20°C using 2 U of T4 DNA ligase in a 5X ligase buffer. The final ligation mixture volume was 50 μl. The PCR mixtures (50 μl) consisted of 5 μl of ligated DNA (dilution 1 : 20 with distilled water), 1 μl of 5 μM *Mse*I-primer (5′-GATGAGTCCTGAGTAAC-3′), 1 μl of 5 μM *Eco*RI-primer (5′-GACTGCGTACCAATTCA-3′) labelled at 5′-end with cy5 fluorophore, 1 μl of 10 mM each deoxynucleoside triphosphates (dNTPs), and 5 U of *Taq* polymerase. T4 DNA ligase, dNTPs and *Taq* polymerase were from Invitrogen-Life Technologies and all primers and oligos were from MWG Biotech, Inc. (High Point, NC, USA).

All PCRs were performed in a DNA thermal cycler (GeneAmp PCR system 9600; Perkin Elmer, Norwalk, CT, USA) following a previously described protocol (Kassama et al., 2002) with some modifications: 60 s at 94°C, 30 s at 65°C and 60 s at 72°C for one cycle; a 12 cycles touch down PCR with annealing temperature reduced from 65°C by 0.7°C at each cycle; and 25 cycles of 30 s of denaturation at 94°C, 60 s of annealing at

56°C, and 60 s of extension at 72°C. This 'touchdown' PCR protocol was used to minimize PCR artefacts.

Analysis of fragments

The AFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA sequencer (Beckman & Coulter, Fullerton, CA, USA). A mixture containing 2 μl of PCR products, diluted to 1 : 20 with Sample Loading Solution (SLS; Beckman & Coulter), 30 μl of SLS, 0.5 μl of CEQ DNA Size Standard Kit-600 (used to normalize the profile) and one drop of mineral oil were loaded on a capillary electrophoresis system CEQ 8000. The set-up of the CEQ 8000 was done according to the manufacturer's instructions.

The data, displayed as peaks in electropherogram files, were analysed by using the CEQ 8000 analysis software. The fragment sizes were determined by comparison with the internal DNA Size Standard Kit, limiting analysis to fragments between 60 and 700 bp. Reproducibility of peaks in electropherograms was checked by repeating fAFLP reactions two times on three strains examined in this study. Electropherograms of all fAFLP profiles were visually inspected for polymorphisms, with the presence (1) or absence (0) of fragments from 60 to 700 bp scored in a binary matrix and stored in Microsoft Excel 2000.

Cluster analysis

Cluster analysis was performed according to the unweighted pair-group method with average linkages (UPGMA) exporting the output files to PHYLIP 3.6 software package (Felsenstein, 2004). Similarity coefficients were determined by using the Dice's coefficient (Dice, 1945). After cluster analysis by UPGMA, a dendrogram was constructed and the robustness of the tree was assessed by bootstrap analysis (1000 repeated samplings; Felsenstein, 1985).

Results

Isolation, biochemical and pathogenicity tests

After 48 h of incubation, different colony types appeared. Fluorescent colonies grown on KB and levan-positive colonies on NSA, were purified by streaking on NA. On NA, after 48 h of growth, the colonies appeared creamy white and flat, with irregular margins, of about 2 mm in diameter. LOPAT tests were as follows: levan–positive, oxidase–negative, potato soft rot–negative, arginine dehydrolase–negative, tobacco hypersensitivity-positive (group Ia). In addition, the isolates possess an oxidative metabolism, hydrolysed arbutin and aesculin and did not liquefy gelatine.

All the isolates induced the wilting of hazelnut twigs, whereas no visible symptom was observed on lemon fruits and on lilac and pear leaves. No symptoms were observed in the control lemon fruits and plants. Re-isolations from symptomatic twigs yielded bacterial colonies on KB and NA that were identical to those used for the inoculations and belong to LOPAT group Ia.

Presence of *syrB* gene

PCR amplification with primers B1 and B2 gave rise to a 752 bp band in all *P. s.* pv. *syringae* strains analysed in this study, thus indicating that the *syr*B gene was present. In contrast, all *P. s.* pv. *coryli* strains failed to show amplification and hence they do not posses the *syr*B gene.

Fluorescent AFLP analysis

Fluorescent AFLP analysis with the selective primers *Mse*I + C and *Eco*RI + A showed the presence of different fingerprints with a number of bands ranging from 31 to 52. The sizes of the amplified fragments ranged from 60 to 700 bp and peak heights indicate the relative fluorescence of the detected fragments; this height did not vary between replicate runs with identical DNAs. The dendrogram of 31 *Pseudomonas* spp. strains analysed by fAFLP shows four main lineages (A, B, C and D) when using a 75% similarity index as cut-off point (Fig. 3). A few strains shared a very similar genomic profiles (PVCT 23P, PVCT 26P), while almost all the other strains did not. Lineages A and B, the largest lineages, consist of 12 strains each. Lineage A includes the *P. s.* pv. *coryli* strains isolated from hazelnut orchards in Sicily as well as those obtained in Piedmont and Sardinia. The strains of this lineage are clearly separated from both *P. avellanae* and *P. s.* pv. *syringae* strains used for comparison. The lineage B includes *P. s.* pv. *syringae* strains isolated from different herbaceous and woody plant species. Lineage C consists of a four-isolate lineage with two *P. s.* pv. *syringae* strains isolated from *Pyrus communis* and two others from *Cynara scolymu*, respectively. Lineage D comprises two *P. s.* pv. *syringae* strains isolated from *Fragaria* × *ananassa*. Finally, the unique *P. avellanae* strain (ISPaVe 011) clustered like an out-group. Bootstrap values depicted the robustness of lineages which is formed by fAFLP analysis. Lineages A and D had strong confidence at maximum of 93%, reflecting very close genetic background within constituents. Lineages B and C showed moderate consistency in clustering with 64% and 71%, respectively.

Discussion

Based on the results obtained from biochemical and pathogenicity tests as well as from the analysis performed by using fAFLP, we conclude that the twig and branch diebacks observed in the hazelnut orchards of Sicily surveyed in the present study are caused by *P. s.* pv. *coryli*. This is the first report of this pathogen in this area.

This pathogen, similar to the Langhe (Piedmont) and Gennargentu (Sardinia) districts, the other Italian areas where this bacterium was previously reported, apparently did not incite the total wilting of the trees. *Pseudomonas s.* pv. *coryli* has also been recently reported on hazelnut in Germany (Poschenrieder et al., 2006) where it causes, similar to Italy, twig dieback and canker along the trunk and branches of hazelnut cultivars different from those cultivated in Sicily. These

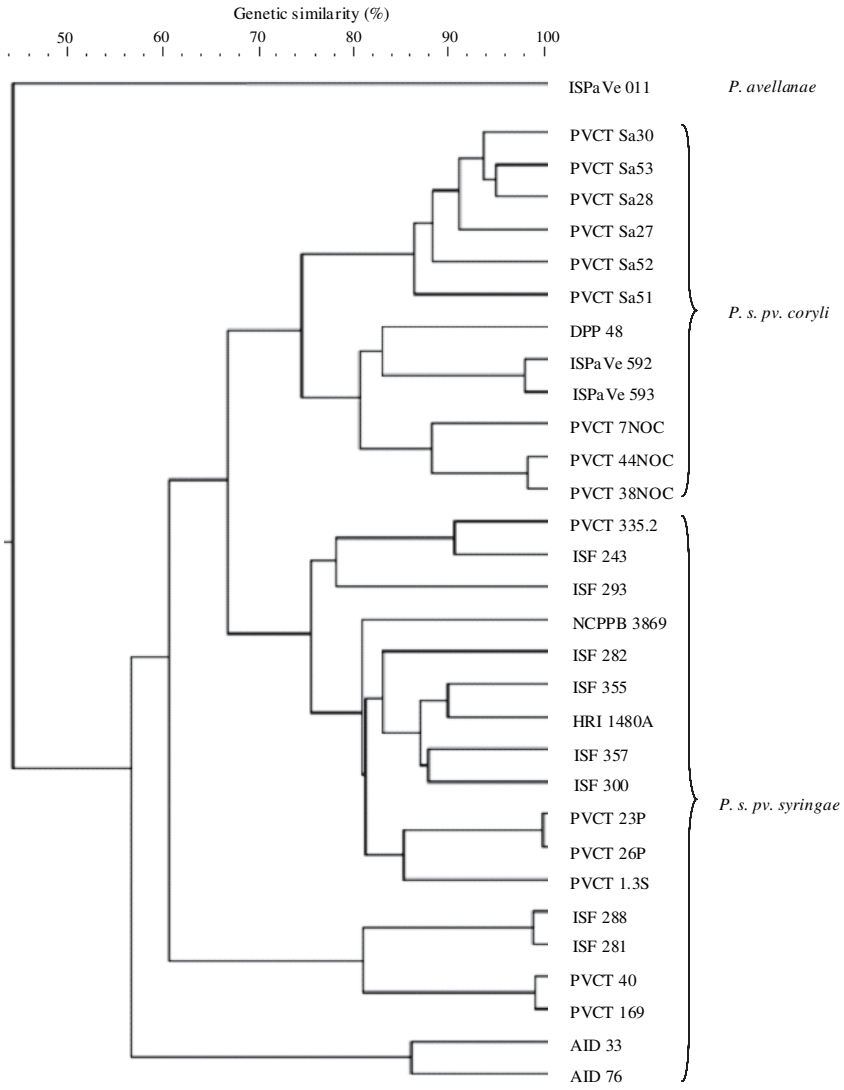

Fig. 3 Dendrogram of 31 *Pseudomonas* spp. strains analysed by fluorescent amplified fragment length polymorphic. Profiles in the range of 100–700 bp, were compared by numerical analysis using PHYLIP 3.6 software. Similarity between fingerprints was calculated with unweighted pair-group method with average linkages and the robustness of the tree was assessed by bootstrap analysis (1000 repeated samplings)

findings would suggest that this pathogen might be spread in many areas of hazelnut cultivation of the world and that it can also incite symptoms to Tonda Gentile delle Langhe, the sole hazelnut cultivar from which it was previously isolated in Italy (Fiori et al., 2003; Scortichini et al., 2005).

Pseudomonas s. pv. *syringae* has been found associated with hazelnut twig wilting in another area of hazelnut cultivation in Sicily, namely the province of Palermo (Scortichini et al., 2002). Further studies would seem worthwhile to ascertain if these two phytopathogenic pseudomonads can infect hazelnut trees in the same areas.

Fluorescent AFLP analysis confirmed its highly discriminative resolution. In fact, all the *P. s.* pv. *coryli* strains, including those isolated from Piedmont and Sardinia clustered separately from the other *Pseudomonas* spp. tested. Moreover, this technique revealed that, probably, there are different lineages of this pathogen. In fact, the *P. s.* pv. *coryli* strains isolated from the province of Catania (PVCT Sa), clustered separately from the ones obtained from the province of Messina (PVCT NOC). In addition, the *P. s.* pv. *coryli* representative strains previously isolated in Piedmont and Sardinia and used for comparative purposes, grouped in another lineage. Whether the different lineages represent a differentiated adaptation of the pathogen to diverse environments would deserve further studies.

Finally, like *P. s.* pv. *coryli*, strains of *P. s.* pv. *syringae* tested in the present study also appeared structured in different lineages. This would seem coherent with the occurrence of different populations subjected to varying environmental selection. fAFLP analysis made it possible to identify and characterize strains isolated from the same host, and to distinguish between groups of strains isolated from different hosts.

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