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Occurrence of *Pseudomonas avellanae* (Psallidas) Janse et al. and related pseudomonads on wild *Corylus avellana* trees and genetic relationships with strains isolated from cultivated hazelnuts

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Abstract

Surveys in submediterranean forests of central Italy were carried out during 1996–98 to verify the possible presence of bacterial canker caused by *Pseudomonas avellanae* in wild hazelnut trees (*Corylus avellana* L.). Wilted twigs were noticed several times especially in summer. In other cases, wild *C. avellana* trees growing near to hazelnut orchards appeared completely wilted. Isolates that were pathogenic to *C. avellana*, showing a different degree of virulence, were obtained in both situations. Biochemical, physiological and nutritional tests as well as the comparison of whole-cell protein profiles, revealed the presence of 16 isolates identical to *P. avellanae* reference strains that had previously been isolated in the same area and five deviating isolates. Repetitive-PCR genomic fingerprinting performed by using BOX (Box elements), ERIC (Enterobacterial Repetitive Interkingdom Consensus) and REP (Repetitive Extragenic Palindromic) primer sets and analysed by means of UPGMA, revealed the existence of two main groups of pseudomonads pathogenic to *C. avellana*. Group A includes *P. avellanae* strains isolated in northern Greece and central Italy as well as the isolates obtained from the wild *C. avellana* trees grown near the cultivated hazelnut orchards. Group B includes strains previously isolated in northern, southern and other areas of central Italy as well as the isolates obtained from *C. avellana* wild trees showing twig dieback. Control measures should be taken to avoid the spread of bacterial canker of hazelnut in the forests of central Italy.

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Zusammenfassung

Auftreten von *Pseudomonas avellanae* (Psallidas) Janse et al. und verwandten Pseudomonaden an wilden *Corylus-avellana*-Bäumen und genetische Verwandtschaft mit Stämmen, die von Kultur-Haseln isoliert wurden

In den Jahren 1996–98 wurde in submediterranen Wäldern Mittelitaliens untersucht, ob *Pseudomonas-avellanae*-Canker bei wilden Haselbäumen (*Corylus avellana* L.) auftreten. Besonders im Sommer wurden mehrfach welke Zweige beobachtet, zudem waren wilde *C.-avellana*-Bäume neben Haselanlagen vollständig verwelkt. In beiden Fällen wurden für *C. avellana* pathogene Isolate gewonnen, die unterschiedliche Virulenzgrade zeigten. Biochemische und physiologische Untersuchungen sowie Nährstoff-Tests und Vergleiche der Proteinprofile der ganzen Zellen unterschieden 16 Isolate, die identisch mit zuvor im gleichen Gebiet isolierten Referenzstämmen von *P. avellanae* waren, sowie fünf abweichende Isolate. Durch repetitive PCR (rep-PCR) gewonnene genomische Fingerabdrücke, für die ERIC-, BOX-und REP-Primersätze verwendet wurden und die Analyse durch UPGMA erfolgte, zeigten die Existenz zweier Hauptgruppen von Pseudomonaden, die für *C. avellana* pathogen sind. Zu Gruppe A gehören *P.-avellanae*-Stämme aus Nordgriechenland und Mittelitalien ebenso wie die Isolate von den wilden *C.-avellana*-Bäumen neben den Haselanlagen. Gruppe B umfaßt Stämme, die zuvor im Norden, im Süden und in anderen Gebieten Mittelitaliens isoliert worden waren, ebenso die von wilden *C.-avellana*-Bäumen mit Zweigsterben stammenden Isolate. Um die Ausbreitung dieser Bakterienkrankheit der Hasel in den Wäldern Mittelitaliens zu verhindern, sollten Bekämpfungsmaßnahmen eingeleitet werden.

Introduction

Corylus avellana L. is a spontaneous tree distributed throughout the whole Europe from Portugal to the southern part of the Urals and from Norway (Lat. 68° N) to Spain, Italy and Greece. It is also spread in eastern and central Asia from Turkey and Syria to Iran as well as in northern Africa (i.e. Algeria) (Kasapligil, 1964). As a fruit tree species, it is known as hazelnut or European filbert and it is mainly cultivated in Turkey, Italy, Spain and in the USA (i.e. Oregon) (Lagerstedt, 1975).

During last 20 years, severe epidemics of hazelnut bacterial canker caused by *Pseudomonas avellanae* (Psallidas) Janse et al. have been reported in northern Greece (Psallidas and Panagopoulos, 1979; Psallidas, 1987) and in central Italy) (Scortichini and Tropiano, 1994; Scortichini and Angelucci, 1999). In both areas the bacterium has destroyed hundreds of hectares devoted to hazelnut cultivation by killing thousands of trees. The main symptoms are the sudden wilting of foliage, twigs, branches and of the whole tree during spring and summer. The pathogen enters the tree in autumn through the leaf scars and it is capable of systemically migrating along the woody tissue of the plant reaching also the roots (Scortichini and Lazzari, 1996). Most of the *C. avellana* germplasm was found to be very susceptible when artificially inoculated through leaf scars with some strains of the pathogen (Scortichini, 1998).

During surveys in submediterranean forests of central Italy, and on some wild *C. avellana* trees at one site of Denmark, symptoms (i.e. twig dieback) resembling those incited by *P. avellanae* towards the cultivated hazelnut were noticed (Fig. 1). In addition, some wild *C. avellana* trees close to cultivated hazelnut orchards in the Viterbo province, where the pathogen is particularly dangerous, appeared completely wilted. Since preliminary isolations yielded bacterial colonies morphologically similar to those of *P. avellanae*, a more detailed study was undertaken in order to identify and to determine the pathogenicity of these isolates. Moreover differentiation by means of repetitive-polymerase chain reaction (PCR) by using BOX (Box elements), ERIC (Enterobacterial Repetitive Interkingdom Consensus) and REP (Repetitive Extragenic Palindromic) primers (Louws et al., 1994) should provide more information on the genomic fingerprinting of the isolates obtained from the wild *C. avellana* trees with type and reference strains of *P. avellanae* populations previously isolated from cultivated hazelnut orchards of different geographic areas.

Materials and Methods

Surveys

Surveys to ascertain the possible presence of symptoms in wild *C. avellana* trees, resembling those incited by *P. avellanae* on cultivated hazelnut trees were undertaken in submediterranean forests of central Italy during spring, summer and autumn of the years 1996–98. Wilted twigs were noticed several times especially in summer (Fig. 1). In most cases, only one to three wilted twigs were present in the whole canopy of the tree. Other symptoms such as the complete wilting of branches or of the whole tree were

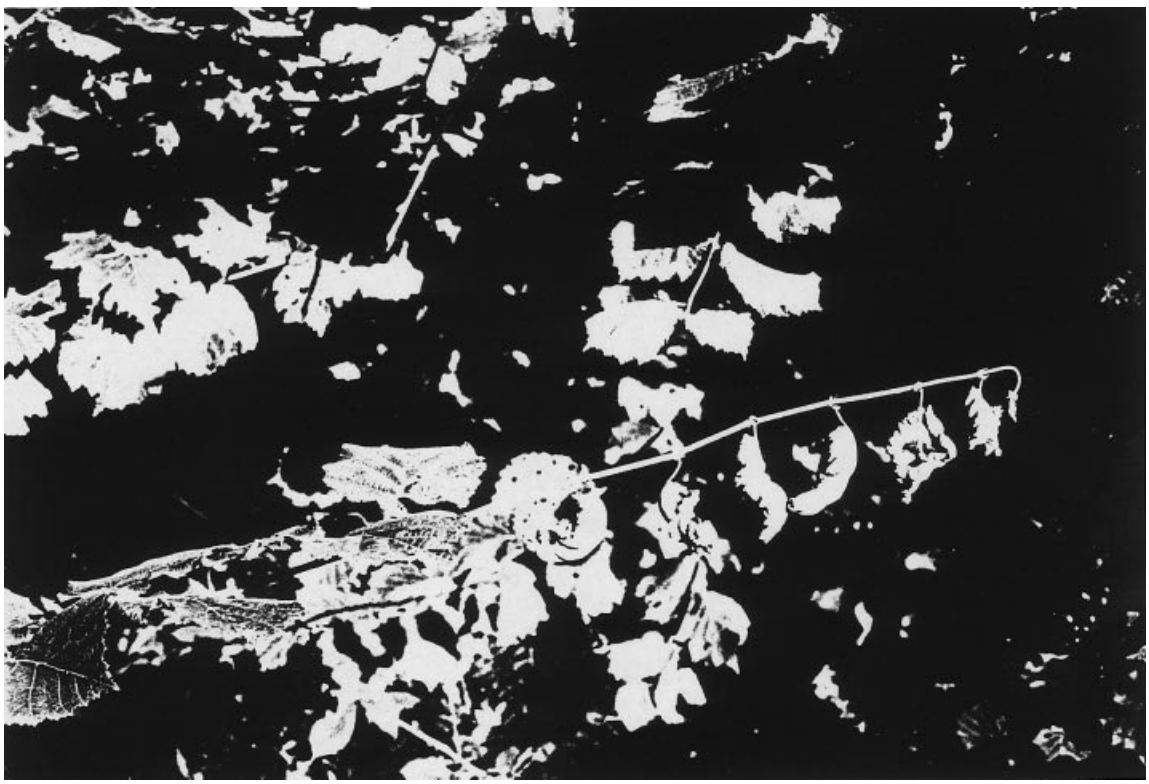

Fig. 1 Twig dieback incited by a phytopathogenic pseudomonad on a wild tree of *Corylus avellana* grown in a submediterreanean forest of central Italy

never observed. The wilted twigs were removed from the tree, put into a plastic bag and taken to the laboratory. Only the specimens without any sign of insect activity were processed for the isolation within 24 h of removal from the tree. In parallel, surveys were also performed in woods located near to cultivated hazelnut orchards. In some cases, completely wilted wild *C. avellana* trees were noticed. Diseased specimens were also collected and processed in these cases, following the procedures described above. Isolations were also carried out from specimen collected in the cultivated hazelnut orchards nearby.

Isolation

Isolations were carried out according to techniques described elsewhere (Scortichini and Tropiano, 1994; Scortichini and Lazzari, 1996). In all cases, nutrient agar with 5% (w/v) of sucrose added (NSA) was the substrate utilized. The plates were incubated at 25–27°C for 4 days. Then, pearl-white, levan-positive, oxidase-negative colonies were selected and streaked in purity on NSA for performing the identification tests. Twenty-six isolates were selected for identification and genetic characterization (Table 1).

Identification and characterization of the isolates

For the identification of the isolates obtained from the wild *C. avellana* trees, the techniques described by Psallidas and Panagopoulos (1979), Psallidas (1993), Scortichini and Tropiano (1994) and Janse et al. (1996) were followed. Biochemical and physiological tests were performed according to the techniques described by Lelliott and Stead (1987) and Schaad (1988). To characterize the isolates, their capability to assimilate 32 different compounds was assessed following the technique reported by Psallidas (1993). The compounds tested are listed in Table 2.

Pathogenicity tests

Pathogenicity tests were carried out with each of the 26 isolates suspected to belong to *P. avellanae*. To test the pathogenicity towards hazelnut, the techniques described by Scortichini and Lazzari (1996) and already adopted to assess the susceptibility of *C. avellana* germplasm towards the pathogen (Scortichini, 1998), were used. Briefly, in early autumn, adult *C. avellana* trees were inoculated through leaf scars located at the middle of 30–40 cm long, 1-year-old twigs, by using a micropipette to put 10 μl of a bacterial suspension in sterile physiological saline (0,85% of NaCl in distilled water) (SPS) containing between 1 and 2×10^5 colony-forming units (CFU)/ml (i.e. approximately 1000 bacterial cells per leaf scar). Six twigs were inoculated for each isolate. *Pseudomonas avellanae* type-strain BPIC 631 = NCPPB 3847 = ICMP 9746, isolated in northern Greece and *P. avellanae* ISPaVe 037 and 2059 isolated in central Italy were inoculated in the same way to compare the virulence of the isolates. A drop of SPS was placed on other leaf scars and served as control. Symptom appearance were checked during next spring (April–May). Re-isolations on NSA were performed after the appearance of symptoms to confirm the presence of the pathogen inside the twigs. To verify the specific pathogenicity of the isolates, pathogenicity tests were carried out with the following plant species: lemon (*Citrus limon* Osbeck), orange (*Citrus sinensis* Osbeck); lilac (*Syringa vulgaris* L.), pear (*Pyrus communis* L.), Japanese plum (*Prunus salicina* L.), peach (*Prunus persica* Stokes), apricot (*Prunus armeniaca* L.). Ripe lemon and orange fruits as well as pear fruitlets were surface-disinfected with 96% alchohol and then washed with sterile distilled water. For each isolate, a suspension in SPS containing between 1 and 3×10^8 CFU/ml was prepared from 24-h-old colonies grown on NSA. Some drops of the suspension were placed on the surface of the fruit that was then pricked using a sterile needle. *Pseudomonas syringae* pv. *syringae* NCPPB 3869, isolated from laurel (*Laurus nobilis* L) and previously shown to produce phytotoxin when inoculated in the same way (Scaloni et al., 1997), served as positive control. The fruits were subsequently put in a humid chamber at room temperature and checked for the appearance of symptoms for 7 to 9 days. Moreover, apricot, lilac, Japanese plum, peach and pear leaves of adult trees were prick-inoculated on the lower surface with the same suspension. Three leaves per species and per isolate were inoculated in eight different leaf areas. The trees were growing in open-air conditions and the inoculation were carried out at the beginning of spring when the relative humidity of the air is high (i.e. 80–90% during night and early morning). Again, *P.s.* pv. *syringae* NCPPB 3869 served as positive control.

Whole-cell protein comparison

In order to confirm the identity of the 26 isolates, the soluble extracts of whole-cell protein were collected according to Janse et al. (1996) and Scortichini and Angelucci (1999). After sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (PAGE), a visual comparison with *P. avellanae* type and reference strains that were representative of different areas of hazelnut cultivation was carried out.

Repetitive-PCR genomic fingerprinting

In order to establish a genetic relationship between the isolates obtained from wild *C. avellana* trees and *P. avellanae* strains previously isolated from cultivated hazelnut orchards, the repetitive-PCR (rep-PCR) technique which is capable of differentiate the genomic structure of phytopathogenic bacteria even at strain level (Louws et al., 1994; Louws et al., 1995) and had already been applied for differentiating the *P. avellanae* strains isolated in Italy from those obtained from Greece (Scortichini et al., 1998), was used. For this purpose, 25 *P. avellanae* strains that were judged to be representative of the different populations of the pathogen (Table 1) were compared with the isolates of the present study. For the preparation of DNA, the technique described by Smith et al. (1995) was followed. From each strain, a loop of a colony grown for 24 h on NSA at 25–27°C was suspended in SPS and centrifuged at 11000 r.p.m. (8.900 g) for 2 min. After discarding the supernatant, the pellet was suspended in SPS

Table 1 *Pseudomonas avellanae* type (T) and reference strains (R), field isolates from wild trees of *Corylus avellana* and from cultivated hazelnut utilized for rep-PCR genomic fingerprinting analysis. Isolates from wild trees are marked with an asterisk

Strain	Country	Province	Year of isolation
BPIC 631 (T)	Greece	Drama	1976
BPIC Fl 13 (R)	Greece	Kilkis	1976
BPIC 640 (R)	Greece	Kilkis	1976
ISPaVe-690 (R)	Italy	Viterbo	1996
ISPaVe-691(R)	Italy	Viterbo	1996
ISPaVe-2059 (R)	Italy	Viterbo	1994
ISPaVe-037 (R)	Italy	Rome	1993
ISPaVe-369 (R)	Italy	Rome	1995
ISPaVe-436 (R)	Italy	Rome	1995
ISPaVe-592	Italy	Cuneo	1995
ISPaVe-593	Italy	Cuneo	1995
ISPaVe-595	Italy	Cuneo	1995
ISPaVe-596	Italy	Cuneo	1995
ISPaVe-598	Italy	Cuneo	1995
ISPaVe-599	Italy	Cuneo	1995
ISF-Lan 1	Italy	Cuneo	1997
ISF-Lan 2	Italy	Cuneo	1997
ISF-Lan 3	Italy	Cuneo	1997
ISF-Lan 4	Italy	Cuneo	1997
ISF-C2	Italy	Avellino	1997
ISF-C3	Italy	Avellino	1997
ISF-C4	Italy	Avellino	1997
ISF-Lab 2	Italy	Rome	1997
ISF-Lab 3	Italy	Rome	1997
ISF-Lab 4	Italy	Rome	1997
DAN*	Denmark	Copenhagen	1998
N 1*	Italy	Rieti	1998
N 2*	Italy	Rieti	1998
S 1*	Italy	Rome	1997
SV 1*	Italy	Rome	1998
SV 2*	Italy	Rome	1998
SV 3*	Italy	Rome	1998
SV 4*	Italy	Rome	1998
SV 5*	Italy	Rome	1998
SV 6*	Italy	Rome	1998
SCR 1*	Italy	Viterbo	1998
SCR 2*	Italy	Viterbo	1998
SCR 3*	Italy	Viterbo	1998
SCR 4*	Italy	Viterbo	1998
SVT 1*	Italy	Viterbo	1998
SVT 2*	Italy	Viterbo	1998
SVT 3*	Italy	Viterbo	1998
SVT 4*	Italy	Viterbo	1998
SVT 5*	Italy	Viterbo	1998
SVT 6*	Italy	Viterbo	1998
SVT 7*	Italy	Viterbo	1998
SVT 8*	Italy	Viterbo	1998
SVT 9*	Italy	Viterbo	1998
SVT 10*	Italy	Viterbo	1998
SVT 11*	Italy	Viterbo	1998
SVT 12*	Italy	Viterbo	1998

BPIC, Culture Collection of Benaki Phytopathological Institute, Kiphissia-Athens, Greece; ISPaVe, Culture Collection of Istituto Sperimentale per la Patologia Vegetale, Roma, Italy; ISF, Culture Collection of Istituto Sperimentale per la Frutticoltura, Roma, Italy; T: P*seudomonas. avellanae* type strain = NCPPB 3487 = PD 2378.

up to an optical density corresponding to between 1 and 2×10^8 CFU/ml. Then, the suspension was put in boiling water for 10 min and, subsequently, the cell sample DNAs were stored at –20°C. ERIC, BOX and REP primer sets were synthesized by Eurogentec (Belgium). Amplification was performed on an MJ Research PTC 100 programmable thermal controller (MJ Research, Watertown, MS, USA) in 25 μl reaction volumes containing 200 μM deoxynucleoside triphosphates, 2 μM $MgCl_2$, primers at 60 pmol, Taq polymerase (Pharmacia Biotech, Sweden) 1.0 U and 4 μl of DNA cell sample. The PCR mixtures were overlaid with 25 μl of mineral oil. Thermal cycling was carried out as described by Louws et al. (1994): an initial denaturation cycle at 95°C for 7 min; 30 cycles of denaturation at 94°C, annealing at 44, 52 or 53°C for 1 min with REP, ERIC and BOX primers, respectively, extension at 65°C for 8 min, a single final extension cycle at 65°C and final soak at 4°C. Products of PCR amplifications were separated by gel electrophoresis on 2.0% agarose (Seakem LE, Rockland,

Table 2 Biochemical and nutritional characteristics of isolates obtained from wild *Corylus avellana* trees, cultivated hazelnut and reference (R) and type-strains (T) of *Pseudomonas avellanae*

	Wild *Corylus avellana* trees							Cultivated hazelnut					*P. avellanae*	
	SCR	SVT	Dam	S1	N1	N2	SV	595	592	Lan1	C2	Lab2	T631	R037
Levan	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Oxidase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Potato soft rot	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tobacco hypersensitivity	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Glucose metabolism	o	o	o	o	o	o	o	o	o	o	o	o	o	o
Fluorescence on KB	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Nitrate reduction	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Urease	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tyrosinase	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Aesculin hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Starch hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Casein hydrolysis	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Tolerance to 4% NaCl	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Tolerance to 0.05% of TTC (*)	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Utilization of:														
adonitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
amygdalin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
arbutin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
cellobiose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
dulcitol	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D(–)fructose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
glycerol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
meso-inositol	+	+	+	+	+	+	+	+	+	+	+	+	+	+
melezitose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
rutin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
salicin	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L(-)sorbose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
sucrose	–	–	–	–	–	–	–	–	–	–	–	–	–	–
D-xylose	+	+	+	+	+	+	+	+	+	+	+	+	+	+
glutamic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+
nicotinic acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L(+)tartaric acid	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-alanine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-arginine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-aspartic acid	+	+	+	+	+	+	+	+	+	+	+	+	+	+
L-glutamine	+	+	+	+	+	+	+	+	+	+	+	+	+	+
guanine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-histidine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DL-homoserine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
ipoxanthine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
isoleucine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-lysine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
L-methionine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
DL-ornithine	–	–	–	–	–	–	–	–	–	–	–	–	–	–
riboflavine	–	–	–	+	–	–	–	–	–	–	–	–	–	–
DL-serine	–	–	+	–	+	+	+	–	–	–	–	–	–	–
L-tryptophan	–	–	–	–	–	–	–	–	–	–	–	–	–	–

o, metabolism of glucose: oxydative; (*) TTC, Tetraphenyl tetrazolium chloride.

ME, USA) in 1 × TAE buffer, at 5 V/cm over 5 h, stained with ethidium bromide, visualized under a UV transilluminator Spectroline (Spectronic Corporation, Westburg, NY, USA) and photographed with a Polaroid type 55. The PCR amplifications were performed in triplicate with fresh sample preparations from the same extraction and master mix. For gel analysis the method followed by Smith et al. (1995) was adopted. Visual readings of the gels were taken and bands common to each triplicate amplifications were scored and 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 Dice's coefficients (Dice, 1945) and clustered by unweighted pair-group using arithmetic averages (UPGMA) by means of NTSYS software, version 1.80 (Exeter Software, New York, USA).

Results

Isolation and identification

From the diseased specimens of wild trees of *C. avellana*, NSA allowed the recovery of pearl-white, levan-positive, oxidase-negative bacterial colonies resembling *P. avel-*

lanae. Biochemical, physiological and nutritional tests were carried out with 26 isolates to compare them to *P. avellanae* type and reference strains. All isolates were catalase-positive, with a respiratory metabolism and they produce a fluorescent pigment on King's medium B (KB; King et al., 1954). After several re-streakings on NSA, the isolates obtained from the province of Viterbo, as the corresponding *P. avellanae* reference strains (Scortichini and Angelucci, 1999), showed no fluorescence on KB. In addition the isolates were negative to the following tests: nitrate reduction, potato soft rotting, presence of urease, tyrosinase and arginine dihydrolase and they did not hydrolyse aesculin, starch and casein. They tolerated up to 4% of NaCl and up to 0.05% tetraphenyl tetrazolium chloride. Nutritional tests revealed that riboflavine and L-serine pointed out differences among the isolates obtained from the wild trees grown in the forest. In fact, riboflavin was assimilated only by S1, whereas L-serine was assimilated by DAN, N1 and N2. and isolates named SV. *Pseudomonas avellanae* type and reference strains as well as the field isolates obtained from cultivated hazelnut (i.e. type ISF C, ISF Lab, ISF Lan, ISPaVe 592–599) and from wild *C. avellana* trees grown nearby the cultivated hazelnuts did not assimilate such compounds.

Whole-cell protein comparison

Profiles in SDS-PAGE of the whole-cell protein extracts revealed the identity of the isolates obtained from wild *C. avellana* trees close to the cultivated hazelnut orchards (i.e. isolates SCR and SVT) with *P. avellanae* reference strains of central Italy. The isolates obtained from the cultivated hazelnut orchards nearby also showed the identical profile. On the basis of biochemical, nutritional and pathogenicity tests as well as from the comparison of their whole-cell protein profiles it was assumed that the isolates from wild trees of hazelnut of type SCR and SVT were *P. avellanae*. Other isolates from wild trees (i.e. N1, N2, S1. DAN, SV) (Table 1) as well as some strains previously isolated from the cultivated hazelnut orchards of northern, central and southern Italy, showed slight quantitative and qualitative differences with the reference and type strains of *P. avellanae*.

Pathogenicity tests

All isolates from diseased twigs of wild *C. avellana* trees incited twig dieback when artificially inoculated in early autumn in hazelnut through leaf scars. The isolates of wild trees growing near the cultivated orchards (i.e. SVT and SCR) were more virulent. In fact, they caused a dieback in all six of the inoculated twigs. Moreover, some isolates, similarly to *P. avellanae* type ISPaVe 2059, reached the branches of the tree 7 months after the inoculation. The other isolates (i.e. N1, N2, S1, DAN, SV) incited dieback in two to four of the six inoculated twigs. Control twigs did not show any kind of symptom. Re-isolations on NSA, performed in early spring, yielded pearl-white colonies that were oxidase-negative, levan-positive with a respiratory metabolism. Whole-cell protein profiles comparison of the re-isolates confirmed their identity with the isolates used. All the isolates obtained from the wild *C. avellana* trees, apparently, did not incite any sign of infection on lemon and orange fruits and pear fruitlets nor on the foliage of the other plant species inoculated, whereas *P.s.* pv. *syringae* NCPPB 3869 incited lesions on fruits and fruitlets as well as necrotic lesions on the leaves of the tested host plants.

Rep-PCR genomic fingerprinting

Gel electrophoresis resulted in 36 reproducible clearly resolved bands used for UPGMA analysis: 19 for ERIC, 11 for BOX and six for REP primers. Representative genomic fingerprintings are shown in Figs 2, 3 and 4. The UPGMA analysis, performed with the 36 bands, by using Dice's coefficients identified two main groups of strains, namely A and B (Fig. 5). Group A included *P. avellanae* strains obtained from the province of Viterbo that showed a similarity of 100% with isolates collected from wild *C. avellana* trees close to the cultivated hazelnut orchards (i.e. isolates SVT and SCR). The isolates from wild trees were remarkably homogeneous. This group also included the strains isolated from northern Greece showing a similarity of around 90% with the previous subgroup. Group B included strains from northern, central and southern Italy as well as the other isolates obtained from the wild trees. This group showed around 25% of similarity with the other group. Within this cluster, isolates obtained from sites very distant to each other, such as Denmark and central Italy, showed a similarity of 80%. In addition, strains from northern Italy (i.e. ISF Lan 1, 2 and 4) also showed a similarity of 90% with an isolate obtained from a wild *C. avellana* tree grown in central Italy (i.e. S1). Another subgroup of isolates (i.e. SV), obtained from wild trees of central Italy, were shown to be homogenous.

Discussion

In this study two different groups (i.e. A and B) of phytopathogenic pseudomonads were capable of infecting wild *C. avellana* trees. In fact, isolates identical to *P. avellanae* reference strains (type SVT and SCR) could cause the complete wilting of wild trees located a few metres away from cultivated hazelnut orchards in central Italy. Their biochemical, physiological and nutritional reaction as well as their whole-cell protein and rep-PCR genomic fingerprinting revealed a perfect identity with the *P. avellanae* reference strains previously isolated in the same area. In addition, they did not induce any kind of symptoms when inoculated in other plant species. Such isolates are related at around 90% of similarity value with *P. avellanae* type and reference strains from northern Greece. At present, in central Italy, severe epidemics of bacterial canker are occurring and the strains isolated from such an area (i.e. province Viterbo) were repeatedly proved to be very aggressive towards germplasm of *C. avellana* (i.e. cultivars Tonda Gentile Romana and Nocchione) (Scortichini and Lazzari, 1996; Scortichini, 1998; present study). *Pseudomonas avellanae* was isolated from wild trees that might have been colonized by cells of the pathogen carried by rain and wind. Actually, the surveys performed on the cultivated orchards nearby allowed observation of the presence of bacterial canker symptoms

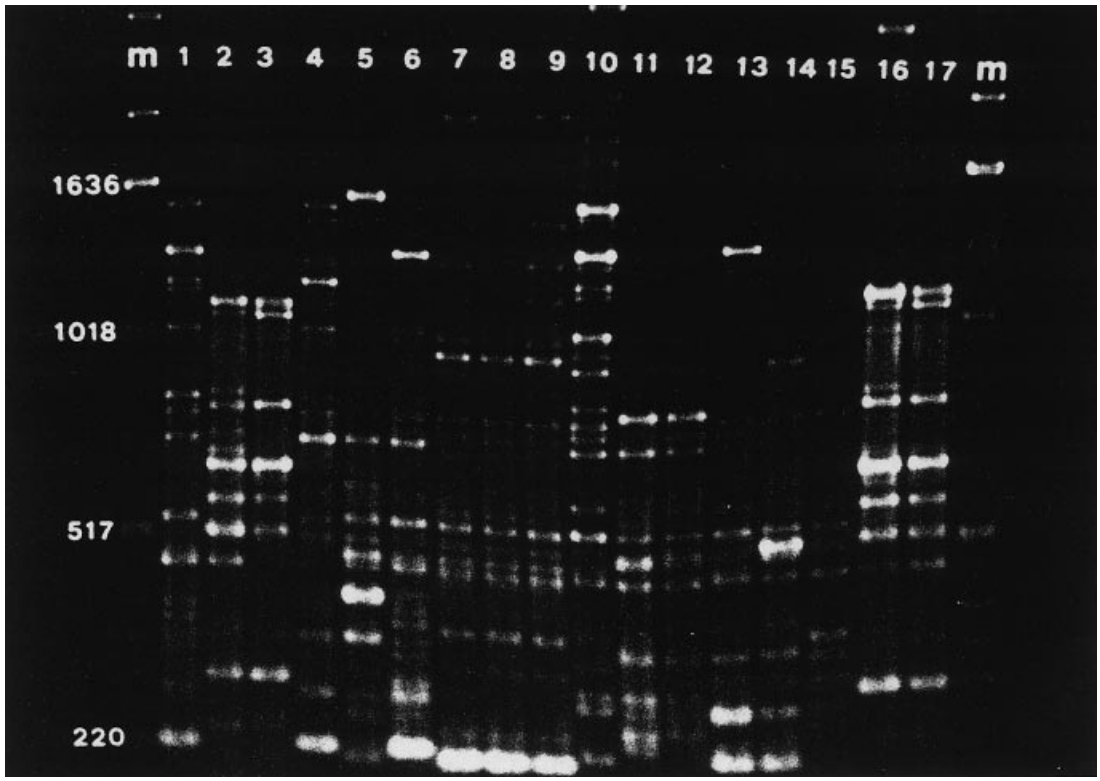

Fig. 2 Rep-PCR fingerprinting patterns from genomic DNA of *Pseudomonas avellanae* reference strains and field isolates recovered from wild trees of *Corylus avellana* obtained by using ERIC primer sets. m: DNA molecular size marker (1 kb ladder; Gibco-BRL, Life Technologies, Italia, S.r.l.); the sizes are indicated in base pairs. Lane 1: ISF Lan 1; lane 2: ISPaVe 2059; lane 3: ISPaVe 439; lane 4: ISPaVe 592: lane 5: ISPaVe 599; lane 6: ISF Lan 2; lane 7: ISF Lan 3; lane 8: ISF Lab 2; lane 9: ISF C4; lane 10: S1; lane 11: SV 1; lane 12: SV 2; lane 13: DAN; lane 14: N 1; lane 15: *P.syringae* pv. *syringae* NCPPB 3869; lane 16: SVT 1; lane 17: SCR 3

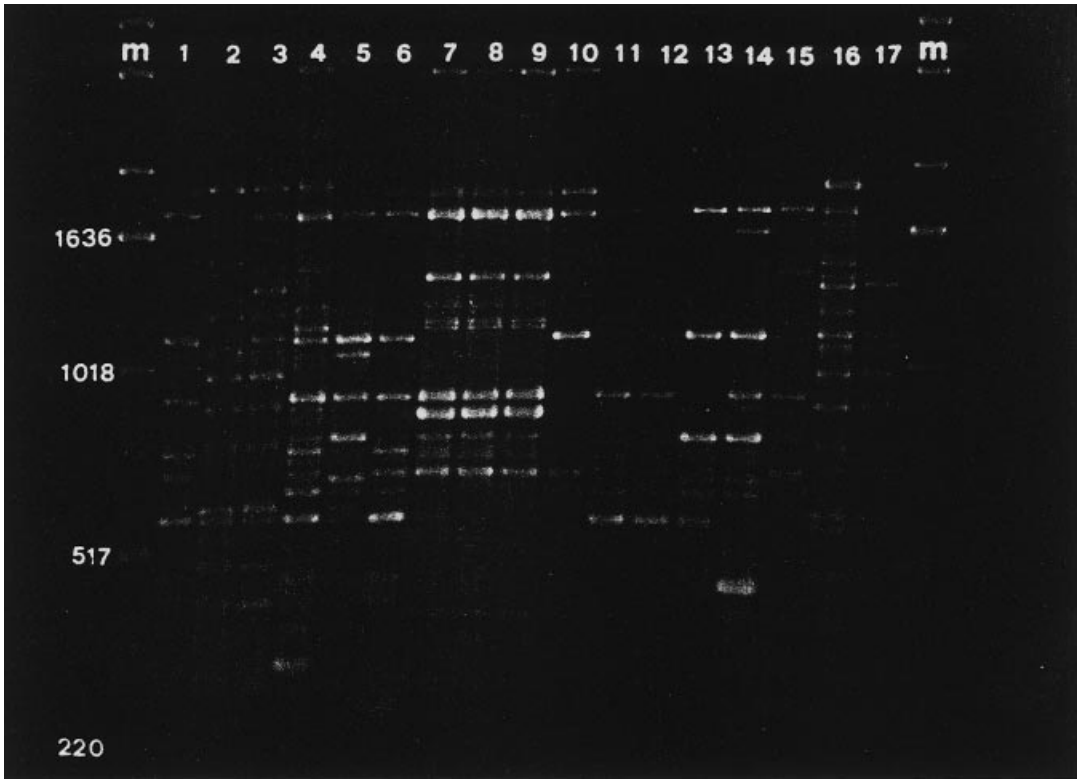

Fig. 3 Rep-PCR fingerprinting patterns from genomic DNA of *Pseudomonas avellanae* reference strains and field isolates recovered from wild trees of *Corylus avellana* obtained by using REP primer sets. M: DNA molecular size marker (1 kb ladder; Gibco-BRL, Life Technologies, Italia, S.r.l.); the sizes are indicated in base pairs. Lane 1: ISF Lan 1; lane 2: ISPaVe 2059; lane 3: ISPaVe 439; lane 4: ISPaVe 592: lane 5: ISPaVe 599; lane 6: ISF Lan 2; lane 7: ISF Lan 3; lane 8: ISF Lab 2; lane 9: ISF C4; lane 10: S1; lane 11: SV 1; lane 12: SV 2; lane 13: DAN; lane 14: N 1; lane 15: *P.syringae* pv. *syringae* NCPPB 3869; lane 16: SVT 1; lane 17: SCR 3

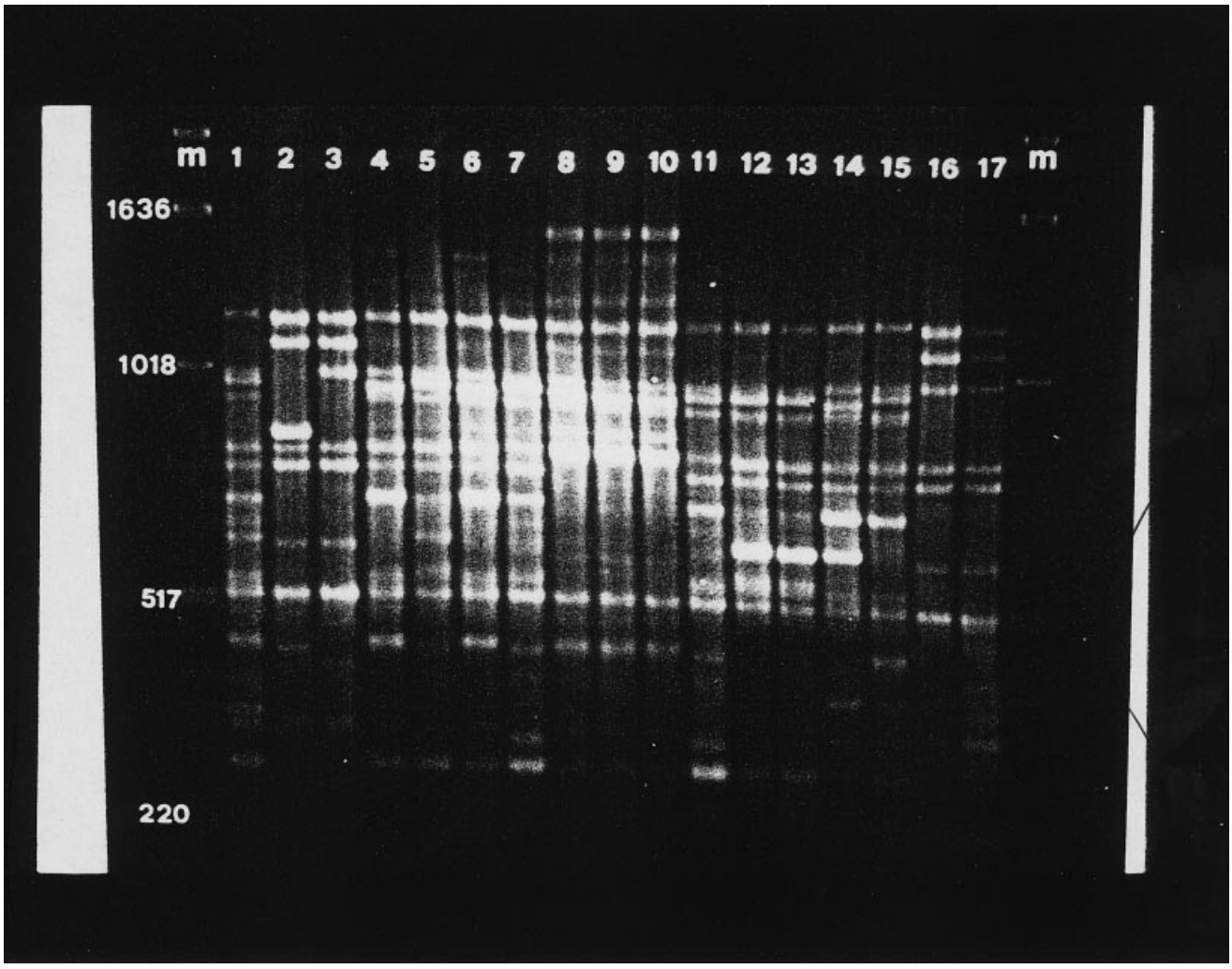

Fig. 4 Rep-PCR fingerprinting patterns from genomic DNA of *Pseudomonas avellanae* reference strains and field isolates recovered from wild trees of *Corylus avellana* obtained by using BOX primer set. M: DNA molecular size marker (1 kb ladder; Gibco-BRL, Life Technologies, S.r.l.); the sizes are indicated in base pairs. Lane 1: ISPaVe 593; lane 2: BPIC 631; lane 3: ISPaVe 2059; lane 4: ISPaVe 598; lane 5: ISPaVe 592; lane 6: ISPaVe 599; lane 7: ISF Lan 2; lane 8 ISF Lan 3; lane 9: ISF Lab 1; lane 10: ISF C 4; lane 11: S 1; lane 12: SV 1; lane 13: SV 2; lane 14: DAN; lane 15: N 1; lane 16: SVT 1; lane 17: SCR SCR 3

and *P. avellanae* was isolated from twigs and branches. In this case, it would seem that the infection course of the pathogen on the wild trees was quite rapid since the foliage appeared completely wilted in early summer at the time of the survey. These findings underline the risk of disease also for the wild *C. avellana* trees and control measures have to be immediately taken to avoid the spread of the disease in the forest close to the hazelnut orchards.

A second group of pseudomonads incited twig dieback towards wild *C. avellana* trees. Rep-PCR analysis performed with ERIC, BOX and REP primer sets, identified a similarity of around 25% with the other group. Within this second group, none of the strains isolated from wild trees showed a similarity of 100% with the strains previously obtained from the cultivated orchards. However, all of them incited twig dieback when they were artificially inoculated in hazelnut twigs. Their virulence, however, was lower when compared with that shown by the type and reference strains of *P. avellanae* isolated from northern Greece and central Italy. At the same time, they did not cause any sign of infection to the other plant species tested. Interestingly, the virulence of this group of strains proved to be lower than that of the first group and the strains isolated from the cultivated orchards were obtained from areas where the pathogen is not in an epidemic phase. Actually, surveys carried out in the submediterranean forests far from Viterbo area, never found severe wilting of the trees. It has also been noteworthy to isolate a phytopathogenic pseudomonad from a wild *C. avellana* tree grown in Denmark and to find it pathogenicity towards hazelnut cultivars typical of Italy. Such findings deserve further investigation as, in natural conditions, phytopathogenic pseudomonads might be co-adapted to *C. avellana* without causing severe damage. By contrast, very virulent populations of *P. avellanae* might be evolved in monocultural conditions such as that currently occurring in central Italy where there is around 20 000 ha of land on which hazelnut are cultivated as a sole crop. Rep-PCR genomic analysis performed with three different

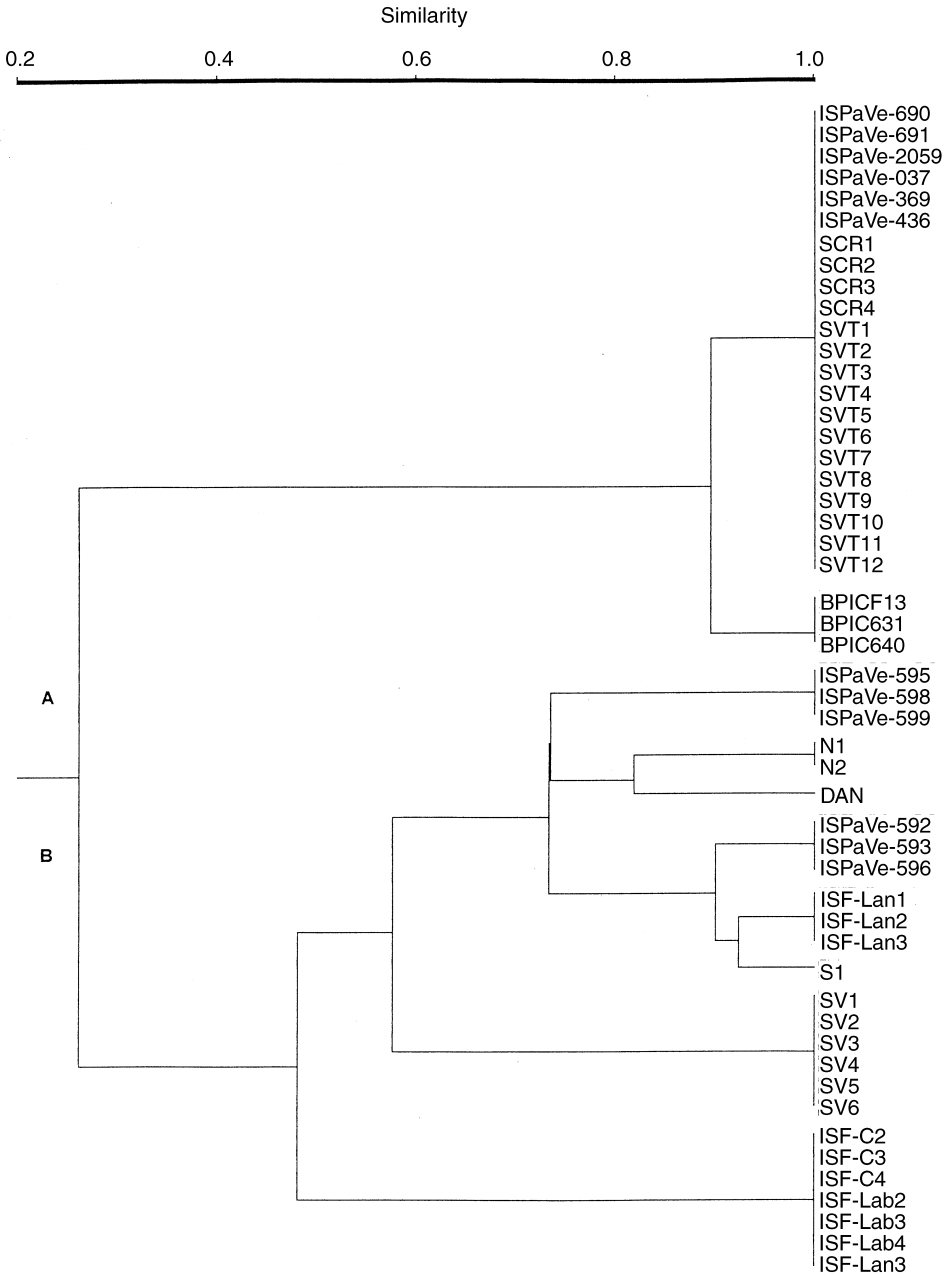

Fig. 5 Dendrogram showing relationships between *Pseudomonas avellanae* type and reference strains and field isolates obtained from wild trees of *Corylus avellana* (see also Table 1) based on rep-PCR with ERIC, BOX and REP primer sets. Similarities were calculated by using Dice's coefficients, and clustering was achieved by UPGMA

primers, clearly distinguished two groups of pseudomonads pathogenic to *C. avellana*. Such findings are interesting also from taxonomic point of view. By means of such a technique, Louws et al. (1995) were able to differentiate two distinct groups of strains within *Xanthomonas vesicatoria* (Doidge) Vauterin et al. whereas Vera Cruz et al. (1996) differentiated distinct genomic lineages in natural populations of *X. oryzae* pv. *oryzae* (Ishiyama) Swings et al. In addition, four groups of strains were identified within *Clavibacter michiganensis* ssp. *michiganensis* (Smith) Davis et al. (Louws et al., 1998). The present study distinguished at least two major groups of strains and within them other subgroups are present. Such genomic differences confirmed the results of the nutritional tests. In fact, the isolates of the first group yielded a nutritional profile identical to that of the *P. avellanae* type and reference strains, whereas the isolates of the group B showed variability in their utilization of the compounds tested. The two groups of strains seem genetically distant enough to hypothesize the existence of subspecies within *P. avellanae* or even a different species pathogenic to *C. avellana*. Investigations by using other techniques are currently being undertaken to further assess

the genomic structure of *P. avellanae* and the genetic relationships with the other pseudomonads pathogenic to *C. avellana*.

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