DETECTION AND IDENTIFICATION METHODS AND NEW TESTS AS DEVELOPED AND USED IN THE FRAMEWORK OF COST 873 FOR BACTERIA PATHOGENIC TO STONE FRUITS AND NUTS

Pseudomonas avellanae

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SUMMARY

Pseudomonas avellanae, the causal agent of bacterial canker and decline of hazelnut (*Corylus avellana*) is currently reported in northern Greece and central Italy. Two lineages of the pathogen can effectively be differentiated by using BOX and ERIC-PCR. Three DNA-based techniques can be used to detect and putatively identify *P. avellanae* directly from infected plant samples. A conventional PCR targets the 16S rRNA gene of the pathogen and yields an amplicon of 762 bp. Another conventional PCR technique targets the *hrpW* gene sequences of the pathogen and yields an amplicon of about 350 bp. A Taq-Man real-time PCR was also developed to detect *P. avellanae* from hazelnut twigs. A reliable technique for artificial inoculation of hazelnut twigs is also available.

Key words: Hazelnut decline, *Corylus avellana*, BOX-PCR, ERIC-PCR, conventional PCR, TaqMan real-time PCR.

INTRODUCTION

Pseudomonas avellanae is the causal agent of bacterial canker and decline of hazelnut (*Corylus avellana*). So far, it has been reported only from northern Greece (Psallidas, 1993; M. Holeva, personal communication) and central Italy (Scortichini, 2002) where it has caused the death of many thousands of trees. Main symptoms are the rapid wilting of twigs and branches during spring to autumn and the occurrence of longitudinal cankers along the main trunk and branches. Currently, the identification procedures include the isolation on common bacterial media, the assessment of colony morphology and biochemical tests, conventional PCR and real-time PCR.

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HOST RANGE

Cultivated and wild *Corylus avellana* trees are the only species infected by *P. avellanae.* Apparently, two lineages of the bacterium have currently been discriminated using BOX-PCR and ERIC-PCR

DETECTION AND IDENTIFICATION

Isolation, media, and description colony morphology. Isolation should be carried out preferably from twigs and young branches. The epidermis should be carefully removed to reach the discoloured tissues beneath. Small fragments of tissue (1-3 mm) are crushed in a sterile mortars in the presence of sterile saline (0.85% NaCl in distilled water). Ten-fold serial dilutions are then made to obtain single colonies. Nutrient agar with 5% sucrose added (NSA) can effectively be used. After two-three days incubation at 25±2°C on NSA, *P. avellanae* colonies appear whitish, circular, domed, and 1-3 mm in diameter (Janse *et al.*, 1996).

Biochemical tests. The biochemical tests listed below are useful for discriminating *P. avellanae* from other *P. syringae* pathovars belonging to genomospecies 8 *sensu* Gardan *et al.* (1999), i.e. *Pseudomonas syringae* pv. *theae* and *P. syringae* pv. *actinidiae* (Scortichini *et al.*, 2002).

Fluorescence on King's B medium (KB)	+[a]
Fluorescence on CSGA medium	+[b]
Growth on Nutrient agar	-
Gelatin liquefaction	-
Casein hydrolysis	-
Arbutin hydrolysis	-
Esculin hydrolysis	-
Tyrosinase	-
Tween 80 lypolisis	-
Utilization of:	
Arabinose	+
DL-tartaric acid	+
D-xylose	+
L-arginine	-
L-tyrosine	-
Trigonelline	-

[a] Fluorescence disappears after several subcultures, especially with strains from central Italy.
[b] Strains from central Italy are negative.

For the methodology of these tests see: Lelliott and Stead (1987).

Reference strain: type-strain of the species: *P. avellanae* BPIC 631^T; CFBP 10963^T; DSM 11809^T; ICMP 9746^T; LMG 21662^T; NCPPB 3487^T; PD 2378^T

DNA-based techniques. Three DNA-based techniques can be used to detect and putatively identify *P. avellanae* directly from infected plant samples. A conventional PCR targets the 16S rRNA gene of the pathogen yielding an amplicon of 762 bp (Scortichini and Marchesi, 2001). The forward primer, PAV1 has the following sequence: 5'-GGCGACGATCCGTAACTG-GTCTGAGA-3', the reverse primer PAV22 has the following sequence: 5'-TTCCCGAAGGCACTCCTC-TATCTCTAAAG-3'. The PCR reaction mixture (50 µl) contains: 1X reaction buffer (10 mM Tris-HCl, pH 9,0; 50 mM KCl; 0.1% Triton X-100); 1.5 mM $MgCl_2$; 100 µm of each dNTP; 12 pmol of each primer; 0.5 U *Taq* DNA polymerase, and 6 µl of the bacterial DNA rapid extract solution. The following PCR conditions should be used: initial predenaturation at 95°C for 7 min; 30 cycles of 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min; final extension at 72°C for 3 min.

Another conventional PCR technique targets the *hrpW* gene sequences of the pathogen (Loreti and Gallelli, 2002) and yields an amplicon of about 350 bp. The forward primer WA has the following sequence: 5'-TC-CACAGGACGCCAGCAAGA-3', the reverse primer WC has the following sequence: 5'-TCGCGTGT-TACGCCACCATC-3'. The PCR reaction mixture (50 µl) contains: 0.2 mM dNTPs, 0.04 µg/µl BSA (bovine serum albumin), 2.0 mM $MgCl_2$, 0.5 mM of each primer, 0.05 U/µl Taq DNA polymerase, 1X PCR buffer, and 2 µl of the bacterial rapid DNA extract (20 ng). The following PCR conditions should be used: initial denaturation 94°C for 3 min followed by 30 cycles 94°C for 1 min, 62°C for 1 min, 72°C for1.5 min and a final elongation step of 5 min at 75°C.

A Taq-Man real-time PCR was also developed to detect *P. avellanae* from hazelnut twigs (Gervasi and Scortichini, 2009). The forward primer is: 5'-GCACTT-TAAGTTGGGAGGAA-3'; the reverse primer is: 5'-CAGAGTTAGCCGGTGCTTA-3'; the probe is: AAT-ACGTATCTGTTTTG-MGB. The amplification reaction mixture (25 µl) contains; 2 µl of DNA template; 1X PCR buffer II (Applied Biosystems, USA), 4 mM $MgCl_2$, 200 µm of each dNTPs, 300 nM of each primer, 200 nM TaqMan MGB probe, 1.25 U AmpliTaq Gold DNA polymerase. The cycling conditions are as follows: initial activation at 95°C for 10 min, followed by 45 two-step cycles comprising 10 sec at 95°C and 45 sec at 60°C.

Pathogenicity tests. A reliable technique for artificial inoculation of hazelnut twigs is currently available (Scortichini and Lazzari, 1996). The inoculation should be performed in early autumn (early October) on one-year-old twigs. The petiole of the leaf is removed and, immediately after, a 10 µl drop of bacterial suspension ($1\text{-}2\times10^5\text{-}10^6$ CFU ml^{-1}) is placed onto the leaf scar. The symptoms (twig dieback) will be appear in early spring.

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