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Rapid and specific detection of virulent *Pseudomonas avellanae* strains by PCR amplification

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Abstract

Specific oligonucleotides, based on *hrpW* (*hypersensitive response* and *pathogenicity*) gene sequences encoding harpin protein in phytopathogenic bacteria, were designed to detect and identify virulent strains of *Pseudomonas avellanae* by polymerase chain reaction (PCR). A population of virulent *P. avellanae* strains, isolated in central Italy (Viterbo region), was assessed with *hrpW*-derived primers, producing a specific band of about 350 base pairs in length. This target was successfully amplified from purified genomic DNA, from bacterial culture and from hazelnut bark tissue. No amplification was obtained when the PCR assay was performed on other plant-pathogenic species from the following genera *Agrobacterium*, *Erwinia*, *Brenneria*, *Pseudomonas*, *Ralstonia*, *Xanthomonas* or from hazelnut-associated bacteria, indicating the specificity of these primers. Moreover DNA from strain ISPaVe-MCB-596, isolated from north Italy (Piedmont region) and belonging to the less aggressive population of *P. avellanae*, did not amplify in PCR. The PCR assay with the primers described here provides a rapid, specific and sensitive diagnostic method for virulent *P. avellanae* strains and a useful tool to evaluate the progress of sanitation of the area.

Introduction

Bacterial canker of hazelnut (*Corylus avellana* L.), caused by *Pseudomonas avellanae*, is a major disease in hazelnut producing areas of central Italy (Scortichini and Tropiano, 1994; Scortichini and Angelucci, 1999) and in northern Greece (Psallidas and Panagopoulus, 1979; Psallidas, 1987). In these areas the bacterium has killed thousands of trees. The pathogen seems to pose a particular danger in the Latium region (Viterbo province) and the strains isolated proved to be very aggressive towards germplasm of *C. avellana* (Scortichini and Lazzari, 1996; Scortichini, 1998; Scortichini et al., 2000b). A second group of pseudomonads isolated from northern, southern and others areas of central Italy, characterized by a lower virulence, seems to be genetically distinct from the original strains (Scortichini et al., 2000b).

Current assays used to identify virulent strains of *P. avellanae*, by plating on culture media and the use of biochemical, physiological, nutritional and pathogenicity tests, are too labour-intensive for routine use. Whole cell protein analysis as well as repetitive-polymerase chain reaction (rep-PCR) fingerprinting have been used for the identification of this bacterium (Janse et al., 1996; Scortichini et al., 2000a). The development of a specific PCR assay for virulent *P. avellanae* would significantly improve the accuracy and efficiency of diagnosis. For this purpose, two primers from a harpin encoding gene were selected. The *hrp* cluster is required by bacterial plant pathogens to produce symptoms on a susceptible host and a hypersensitive response on resistant host or non-host plants (Willis et al., 1990). Specific primers based on *hrp* genes have been used in a sensitive and specific PCR method for the

detection of phytopathogenic bacteria (Leite et al., 1994).

Harpin protein seems to be characteristic of phytopathogenic bacteria. However, the encoding genes show some differences in the nucleotide sequences (He et al., 1993; Lindgren, 1997; Loreti et al., 2001). Recently, harpin-encoding genes (*hrpZ* and *hrpW*) were sequenced from an aggressive and a less aggressive *P. avellanae* strains, showing some sequence differences among the two strain (Loreti et al., 2001).

The objective of this study was to develop a PCR protocol based on specific primers that permitted identification of virulent *P. avellanae* populations, both from bacterial culture and from hazelnut bark tissue. Primers designed from the *hrpW* sequence were used in a rapid and specific PCR assay for the detection of the causal agent of the so-called 'moria' of hazelnut in central Italy, the *P. avellanae* bacterium.

Material and methods

Bacterial strains and media

P. avellanae strains were collected from *C. avellana* with symptoms of bacterial canker from the Viterbo province. All the isolates were compared with reference strains (ISPaVe-MCB-011, BPIC 631) by means of SDS–PAGE of whole cell protein and rep-PCR of genomic DNA. Strains were also characterized on the basis of LOPAT tests (levan production on 5% sucrose nutrient agar (NSA), Kovac's oxidase test, pectolytic activity on potato slice, arginine dihydrolase activity, tobacco hypersensitivity) and on the results of fluorescence on King's medium B, metabolism of glucose, aesculin and arbutin hydrolysis and catalase activity. All tests were conducted as described by Lelliot and Stead (1987) or Schaad (1988). A number of plant-pathogenic bacteria belonging to different species were also tested (Table 1). Hazelnut-associated bacteria were purified from symptomatic hazelnut samples in order to verify possible cross-reactions, in PCR assays, of non-pathogenic bacteria (saprophytic or opportunistic) present in infected hazelnut tissue (Table 1). These bacteria were characterized by their morphology and/or fluorescence on King's medium B, tobacco hypersensitivity, levan production on 5% sucrose nutrient agar (NSA), Kovac's oxidase test, aesculin hydrolysis, catalase activity, arginine dihydrolase activity and metabolism of glucose. Stock cultures were lyophilised and regenerated on NSA or nutrient agar (NA) media, at 26–27 °C for PCR assay.

Sample preparations

Bacterial genomic DNA was purified as described by Ausubel et al. (1996).

A rapid extraction, from bacterial cultures, was performed according to Smith et al. (1995), with minor modifications described by Scortichini et al. (1998). A loopful from a single colony grown overnight at 26–27 °C on NSA was suspended in sterile physiological saline buffer (SPS) (0.85% of NaCl in distilled water) and centrifuged at 10,000 rpm for 10 min. The pellet was suspended in SPS to a concentration of $1–2 \times 10^8$ colony forming units/ml (CFU/ml). After denaturation at 100 °C for 10 min, the sample (2 µl) was used in PCR assay or stored at −20 °C.

To verify the possible inhibition of PCR assays by plant substances, small pieces of hazelnut bark (0.04–1 g) were aseptically removed and crushed in sterile mortars containing SPS buffer (5 ml). Three tenfold dilutions (10^{-1}, 10^{-2} and 10^{-3}), were mixed with a bacterial suspension of ISPaVe-MCB-011 to obtain a concentration of 10^8 CFU/ml, and centrifuged for 10 min at 10,000 rpm. The pellets were suspended in 1 ml of SPS, denaturated at 100 °C for 10 min, and used in PCR assay (5 µl).

Detection of P. avellanae *from infected hazelnut bark tissue*

For direct detection of *P. avellanae* in infected hazelnut plants, artificially inoculated plants were used. Two to three-year-old hazelnut plants were inoculated by putting a drop (10 µl) of a bacterial suspension (1×10^5 CFU/ml) on a leaf scar (Scortichini et al., 1994). Strains ISPaVe-MCB-011, 1115, 1104 and BPIC-631 were used for inoculation. Control plants were inoculated with sterile distilled water. Transverse sections of bark tissue (0.04–1 g) were aseptically removed from the inoculated twigs. Three different DNA extraction methods were tested:

(1) method of Audy et al. (1994);
(2) method of hazelnut extraction described above; and
(3) method of DNA extraction from plant tissue (Dneasy Plant Mini Kit. Qiagen GmbH, Germany), with minor modification: bark tissue was crushed in 5 ml of SPS and the suspension centrifuged 10 min at 10,000 rpm; the pellet was used for the extraction.

Five microliters of each sample were used for PCR amplification. Viable bacterial counts from the samples were determined on NSA medium.

Table 1. Bacterial strains used in this study, their geographic origin and host of isolation

Species	Strain	Source*	Origin	Host
Pseudomonas avellanae	1104, 1105, 1106, 1107, 1108, 1109, 1110, 1111, 1112, 1113, 1114, 1115, 1116, 1117, 011, 012, 013, 037, 038, 596, 1208, 1209, 1210, 1211, 1212	ISPaVe-MCB	Italy	*Corylus avellana*
	631, F13	BPIC	Greece	*Corylus avellana*
Agrobacterium tumefaciens	At 20.N5	IPV-NA	Italy	*Prunus persica*
Brenneria nigrifluens	1061	ISPaVe-MCB	Italy	*Juglans regia*
Erwinia amylovora	2956	IPV-BO	Italy	*Pyrus communis*
Xanthomonas arboricola pv. *corylina*	1075, 232	ISPaVe-MCB	Italy	*Corylus avellana*
X. arboricola pv. *pruni*	1151	ISPaVe-MCB	Italy	*Prunus persica*
X. arboricola pv. *juglandis*	1013	ISPaVe-MCB	Italy	*Juglans regia*
X. axonopodis pv. *vesicatoria*	351	ISPaVe-MCB	Italy	*L. esculentum*
Ralstonia solanacearum	2892	IPV-BO	Italy	*Solanum tuberosum*
P. syringae pv. *tomato*	412	ISPaVe-MCB	Italy	*L. esculentum*
	2563	NCPPB	—	*L. esculentum*
P. syringae pv. *morsprunorum*	432	ISPaVe-MCB	Italy	*Prunus cerasus*
P. syringae pv. *actinidiae*	019, 020, 188, 189	ISPaVe-MCB	Italy	*Actinidia deliciosa*
P. savastanoi pv. *savastanoi*	1093, 1094	ISPaVe-MCB	Italy	*Olea europea*
	213	G. Surico	Italy	—
	317a	G. Surico	Italy	—
P. chicorii	1186	ISPaVe-MCB	Italy	*Cichorium intybus*
P. viridiflava	1181	ISPaVe-MCB	Italy	*Cichorium intybus*
P. corrugata	2445	NCPPB	—	—
P. syringae pv. *lachrimans*	3544	NCPPB	—	—
P. marginalis pv. *marginalis*	1184	ISPaVe-MCB	Italy	*Cichorium intybus*
P. savastanoi pv. *glycinea*	1155	ISPaVe-MCB	Italy	*Glicine max*
P. syringae pv. *syringae*	326	ISPaVe-MCB	Italy	*Magnolia purpurea*
	087	ISPaVe-MCB	Italy	*Prunus cerasus*
	1066	ISPaVe-MCB	Italy	*Pyrus communis*
	1070	IPV-NA	Italy	*Citrus limon*
	1071	IPV-NA	Italy	*Prunus armeniaca*
	1203, 1204	ISPaVe-MCB	Italy	*Prunus domestica*
	14a, 6a, 11a	ISPaVe-MCB	Italy	*Corylus avellana*
Hazelnut-associated bacteria	N5, P6-vt, P10, P11, P17, P20, P24, P30, P31, P34, P48, PM, PN, PD, PC	ISPaVe-MCB	Italy	*Corylus avellana*
Hazelnut-soil associated bacterium	TN	ISPaVe-MCB	Italy	Soil
P. putida	224	ISPaVe-MCB	Italy	Soil

*NCPPB: National Collection of Plant-Pathogenic Bacteria, York, UK; ISPaVe-MCB: Istituto Sperimentale per la Patologia Vegetale, Rome, Italy; IPV-NA, Istituto di Patologia Vegetale, Università degli Studi Federico II, Naples, Italy; IPV-BO: Istituto di Patologia Vegetale, Università degli Studi di Bologna, Italy; BPIC: Benaki Phytophathological Institute, Athen, Greece; G. Surico, DIBA Sez. Patologia Vegetale, Florence, Italy.

PCR assays

The PCR reactions were carried out in a 50 µl reaction mixture containing 0.2 mM dNTPs, 0.04 µg/µl BSA, 2 mM $MgCl_2$, 0.5 mM each primer, 0.05 U/µl Taq DNA polymerase (Promega Corp., Madison, USA; Pharmacia, Bjorkgatan, Sweden; M-Medical, Italy), 1X PCR buffer (Promega Corp., Madison, USA; Pharmacia, Bjorkgatan, Sweden; M-Medical, Italy). Genomic DNA (20 ng), or an aliquot (2 µl) of the bacterial rapid extract, or the hazelnut preparations (5 µl) were used as the template source. For detection of virulent *P. avellanae* strains, primer WA (forward primer: 5′-TCCACAGGACGCCAGCAAGA-3′)

was paired with primer WC (reverse primer: 5′-TGCGGTGTTACGCCACCATC-3′). Primers WA and WC were selected by comparing the nucleotidic *hrpW* sequences of *P. avellanae* ISPaVe-MCB-596, ISPaVe-MCB-011 (Loreti et al., 2001), *P. s.* pv. *tomato* DC3000 (Charkowski et al., 1998) and *P. s.* pv. *syringae* B728a (Charkowski et al., 1998). Oligonucleotide primers for PCR were purchased from Life Technologies (United Kingdom).

The thermal profile consisted of an initial denaturation step (94 °C, 3 min), followed by 30 cycles at 94 °C (1 min), 62 °C (1 min), 72 °C (1.5 min) and a final elongation step of 5 min at 72 °C. All the amplification products (amplicons) (14 µl) were analysed on 1% (w/v) agarose gel cast and run in TBE buffer (0.09 M Tris–borate, 0.002 M EDTA), stained with ethidium bromide and photographed under UV illumination. To detect the sensitivity of the PCR assay in the presence of large number of bacteria, cells of *P. avellanae* ISPaVe-MCB-011 were mixed with 20- and 200-fold excesses of *P. syringae* pv. *syringae* ISPaVe-MCB-14a. Each experiment was repeated a minimum of three times.

Results

Bacterial cultures

P. avellanae. All the bacterial colonies were pearl-white, levan-positive on NSA medium, Kovac's oxidase-negative, catalase-positive, hypersensitive reaction on tobacco-positive, metabolism of glucose-oxidative, produced a fluorescent pigment on King's medium B, arginine dihydrolase-negative, aesculin-negative, arbutine-negative, potato soft rotting-negative. Profiles in SDS–PAGE of whole cell protein extract and rep-PCR of genomic DNA of the isolates obtained from infected *C. avellana* were identical to the references strains.

Hazelnut associated bacterial cultures. On the basis of the main morphological characteristics, two types of cultures were selected by isolation from infected hazelnut tissue: the first group (P48, PD, PC, PM, PN), produced levan on NSA medium, and had the following characteristics: fluorescent pigment on King's medium B-positive, tobacco hypersensitivity-negative, Kovac's oxidase test-negative, aesculin hydrolysis-positive, catalase activity-positive, arginine dihydrolase-negative, metabolism of glucose-oxidative for PM and PN; -fermentative for P48, PD, PC. The second group (N5, P6-vt, P10, P11, P17, P20, P24, P30, P31, P34) produced yellow, translucent colonies on NA medium that differed from reference strains of *X. a.* pv. *corylina* (ISPaVe-MCB-1075 and ISPaVe-MCB-232) both in morphology on YDC medium and the profiles of SDS–PAGE of whole cell protein extracts.

Development of PCR conditions

By using the conditions described, amplicons of the expected size (about 350 bp) were obtained both from the bacterial rapid extraction preparation (Figure 1) and the genomic DNA purification (Figure 2). An analogous amplification product also was obtained from the diluted hazelnut preparations (from 10^{-1} to 10^{-3}) containing the bacterial suspension (Figure 3), but not from the non-diluted one. All *P. avellanae* strains, belonging to the virulent population, either from Greece or from Italy, produced an amplicon. No products were produced from the water controls, nor from other plant-pathogenic or hazelnut-associated bacteria. In particular, no amplicons were observed either from *P. s.* pv. *syringae* or from *P. s.* pv. *tomato* strains, in spite of the high identity of their HrpW amino acid sequences. Similarity between HrpW of *P. avellanae*

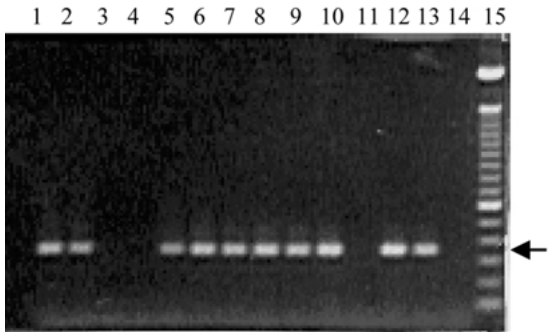

Figure 1. Electrophoretic analysis of PCR amplicons from the rapid extraction preparation of *P. avellanae* and other bacteria cultures, using specific *hrpW*-derived primer pair WA and WC. The arrow on the right shows the specific PCR product. Lanes 1 and 2, *P. avellanae* strains ISPaVe-MCB-011 and ISPaVe-MCB-012; lane 3, *P. avellanae* (from Piedmont) ISPaVe-MCB-596; lane 4, *P. s.* pv. *syringae* ISPaVe-MCB-14a; lanes 5–10, *P. avellanae* strains: BPIC-631, BPIC-F13, ISPaVe-MCB-038, ISPaVe-MCB-1106, ISPaVe-MCB-1110, ISPaVe-MCB-1115; lane 11, *P. s.* pv. *tomato* ISPaVe-MCB-412; lanes 12 and 13, *P. avellanae* strains ISPaVe-MCB-1113 and ISPaVe-MCB-1117; lane 14, water control; lane 15, 100 pb ladder (Gibco, Brl).

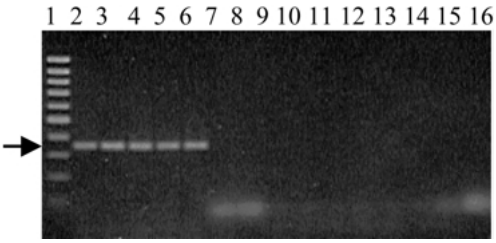

Figure 2. Electrophoretic analysis of PCR amplicons from the genomic DNA of *P. avellanae* strains and other bacteria, using specific *hrpW*-derived primer pair WA and WC. The arrow on the left shows the specific PCR product. Lane 1, 100 pb ladder (M-Medical, Genenco); lanes 2–6, virulent *P. avellanae* strains, ISPaVe-MCB-011, BPIC-631, ISPaVe-MCB-038, ISPaVe-MCB-1106, ISPaVe-MCB-1110, respectively; lane 7, *P. avellanae* ISPaVe-MCB-596 (from Piedmont); lane 8, *P. s.* pv. *syringae* ISPaVe-MCB-14a; lane 9, *P. s.* pv. *actinidiae* ISPaVe-MCB-019; lane 10, *P. putida* ISPaVe-MCB-224; lane 11, *P. s.* pv. *morsprunorum* ISPaVe-MCB-432; lane 12, *P. s.* pv. *tomato* ISPaVe-MCB-412; lane 13, *X. a.* pv. *corylina* ISPaVe-MCB-1075; lanes 14 and 15, hazelnut-associated bacteria TN (soil) and PN respectively; lane 16, water control.

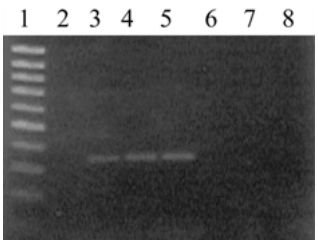

Figure 3. PCR amplicons obtained from hazelnut preparation (lane 2) and three decimal dilutions (lanes 3–5) containing a bacterial suspension (5×10^8 CFU/ml) of *P. avellanae* strain ISPaVe-MCB-011; lanes 6–8, healthy hazelnut preparation alone (dilutions from 10^{-1} to 10^{-3}); lane 1, 100 pb ladder (M-Medical, Genenco).

ISPaVe-MCB-011 was 70% for *P. s.* pv. *syringae* B728a and 95% for *P. s.* pv. *tomato* DC3000 (Loreti et al., 2001). No amplicons were produced from DNA of the less aggressive *P. avellanae* strain ISPaVe-MCB-596 or from extracts of healthy hazelnut.

Three different Taq polymerases (Promega Madison, USA; Pharmacia, Bjorkgatan, Sweden, M-Medical, Italy) were tested. All the enzymes were able to amplify the DNA from *P. avellanae* strains.

Sensitivity threshold

The level of detection by PCR was investigated both from the rapid bacterial extraction preparation and from genomic DNA. Bacterial extracts were serially diluted to 10^{-6} and 2 µl aliquots used in PCR. A band was visible on the gel corresponding to 2×10^3 CFU/PCR reaction with strain ISPaVe-MCB-011 (Figure 4a). This sensitivity threshold was maintained in the presence of 20- and 200-fold excess of *P. s.* pv. *syringae* strain ISPaVe-MCB-14a (Figure 5). For genomic DNA, aliquots (2 µl) of tenfold dilutions in the range of

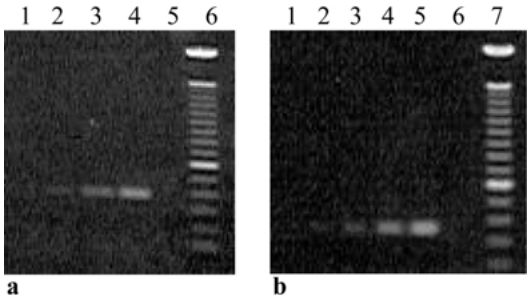

Figure 4. Sensitivity of detection from tenfold dilutions of the bacterial rapid extraction preparation (a) of *P. avellanae* strain ISPaVe-MCB-011. Lane 1, 10^{-3}; lane 2, 10^{-2}; lane 3, 10^{-1}; lane 4, non-diluted sample (2×10^5 CFU/PCR reaction); lane 5, water control; lane 6, 100 pb ladder (Gibco, Brl). Sensitivity of detection from genomic DNA (b) of *P. avellanae* strain ISPaVe-MCB-011. Lane 1, 2 pg; lane 2, 20 pg; lane 3, 0.2 ng; lane 4, 2 ng; lane 5, 20 ng; lane 6, water control; lane 7, 100 pb ladder (Gibco, Brl); for reference on the gel, the brighter band in the middle of the ladder is 600 pb long.

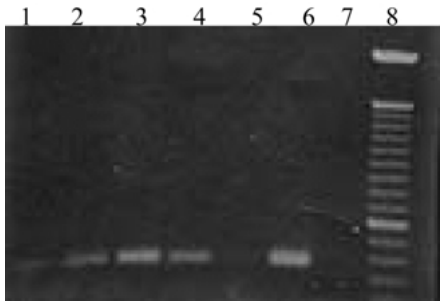

Figure 5. Sensitivity threshold of PCR assay in the presence of a large number of bacteria: cells of *P. avellanae* ISPaVe-MCB-011 were mixed with 200-fold excesses of *P. syringae* pv. *syringae* ISPaVe-MCB-14a. Lane 1, ISPaVe-MCB-011 (10^3 CFU/PCR reaction) mixed with ISPaVe-MCB-14a (1 : 200); lane 2, ISPaVe-MCB-011 (10^4 CFU/PCR reaction) mixed with ISPaVe-MCB-14a (1 : 200); lane 3, ISPaVe-MCB-011 (10^4 CFU/PCR reaction) mixed with ISPaVe-MCB-14a (1 : 20); lane 4, ISPaVe-MCB-011 (10^3 CFU/PCR reaction) mixed with ISPaVe-MCB-14a (1 : 20); lane 5, ISPaVe-MCB-011 (10^2 CFU/PCR reaction) mixed with ISPaVe-MCB-14a (1 : 20); lane 6, ISPaVe-MCB-011 DNA (0.2 ng); lane 7, water control; lane 8, 100 pb ladder (Gibco, Brl).

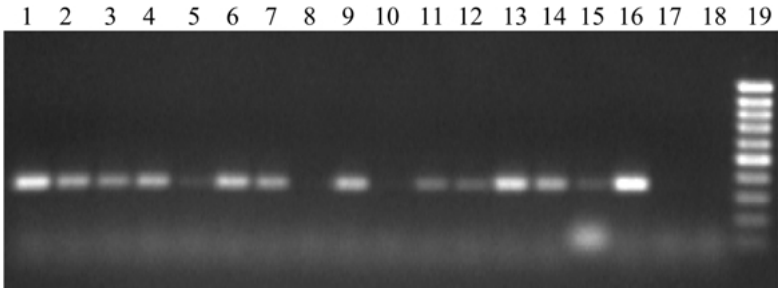

Figure 6. Detection of *P. avellanae* in infected hazelnut twigs. DNA from hazelnut tissue, previously inoculated with different *P. avellanae* strains, was extracted by Dneasy Plant Mini Kit (Qiagen GmbH, Germany) and amplified in PCR. Lane1, ISPaVe-MCB-011 positive control DNA (0.2 ng); lanes 2–5, 13,14, BPIC-631 inoculated twigs; lanes 6 and 7, ISPaVe-MCB-1115 inoculated twigs; lane 8, ISPaVe-MCB-596 inoculated twig; lanes 9, 11, 12, ISPaVe-MCB-011 inoculated twigs; lane 10, ISPaVe-MCB-14a inoculated twig; lanes 15 and 16, ISPaVe-MCB-1104 inoculated twigs; lane 17, uninfected hazelnut bark; lane 18, water control; lane 19, 100 bp (M-Medical, Genenco).

20 ng to 20 fg of strain ISPaVe-MCB-011 were used for amplification. The detection level was about 20 pg of DNA (Figure 4b).

PCR detection from infected hazelnut bark

Pseudomonas avellanae was detected directly from infected hazelnut bark extracted using the Dneasy Plant Mini Kit (Qiagen GmbH, Germany) (Figure 6), but not using the other hazelnut-DNA rapid extraction methods tested. From a total of 38 infected twigs 31 positive results were observed in PCR and isolation; three false negative PCR results (all positive in isolation) and four false negative isolation results (all positive in PCR).

No amplicons were obtained from DNA extracted directly from ISPaVe-MCB-596 and ISPaVe-MCB-14a inoculated twigs, and from water-control inoculated hazelnut twigs (Figure 6). Two hazelnut twigs produced levan-positive colonies on NSA, but was negative in the PCR assay. The colonies, purified on NSA medium, differed from *P. avellanae* strains in their morphology, appearing transparent, and gave a negative result in the PCR assays confirming the early result.

Discussion

A novel and rapid method for the identification and detection of *P. avellanae* virulent strains, responsible for the so-called 'moria' of hazelnut in central Italy, is reported. This bacterium is currently identified by traditional microbiological methods and pathogenicity assays, which are very time-consuming. In particular, the pathogenicity test needs almost six months for symptom development (Scortichini and Lazzari, 1996; Scortichini, 1998). Rep-PCR enabling distinction of specific populations of the pathogen according to its geographic origin, was useful for the identification of *P. avellanae* in central Italy (Scortichini et al., 2000a). Compared with strains originating from other areas of Italy, the *P. avellanae* population occurring in central Italy, is the most aggressive (Scortichini et al., 2000b). Accurate and specific identification of virulent *P. avellanae* strains is important, since a heterogeneous population of *Pseudomonas* species, including *P. s.* pv. *syringae*, were identified on infected hazelnut (Scortichini, 2000b; Loreti et al., 1999).

The use of specific *hrpW* primers for PCR amplification provides a means of distinguishing the most aggressive *P. avellanae* strains from hazelnut-associated pseudomonads and other saprophytic or opportunistic bacteria. Furthermore, PCR can be used in laboratories dealing with large numbers of samples. PCR detection of *P. avellanae* was efficiently performed either with purified DNA or with a bacterial extracts obtained with a rapid extraction method. To detect the bacterium by PCR from hazelnut preparations mixed with the bacterial suspensions, it was necessary to dilute the original preparation. However, PCR amplification was not successful with extracts from hazelnut bark using two rapid methods for DNA extraction. This could be explained by the inhibition of the PCR reaction due to the presence of inhibitory substances from hazelnut. Inhibitory substances were removed by the use of the Dneasy Plant Mini Kit, that provided, within one hour, a DNA preparation suitable for PCR amplification. By purifying the nucleic acid, *P. avellanae* was detected directly in the plant. This

method should be particularly useful for mass scale detection and indexing of plant propagation material.

In addition, about the same occurrence of false negative results were obtained in PCR as in isolation assays. To avoid the risk of false negative results, we suggest that PCR and isolation be carried out simultaneously. In this way, PCR false negative results could be avoided by monitoring the growth of cultures on NSA. By applying the bacterial rapid extraction method, levan-positive colonies can be quickly checked by PCR.

The limit of PCR sensitivity for *P. avellanae* was about 2×10^3 CFU/PCR reaction or about 20 pg of purified DNA. This degree of sensitivity was not reduced in the presence of excess *P. s.* pv. *syringae* strain ISPaVe-MCB-14a isolated from hazelnut. A similar detection limit was described for the diagnosis of *P. corrugata* (Catara et al., 2000) and for the identification of *P. s.* pv. *pisi* and *Xanthomonas fragariae* (Arnold et al., 1996; Pooler et al., 1996).

Finally, the use of specific primers based on *hrpW* sequence and PCR represent a useful tool for the diagnosis of *P. avellanae* strains responsible for the severe attack on hazelnut plants in central Italy. Furthermore, the use of this method can verify the efficacy of sanitation of an area. The destruction of wilted branches and diseased filberts are, until now, the only way to avoid the spread of the pathogen.

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