Plant Pathology (2005) **54**, 789–798 Doi: 10.1111/j.1365-3059.2005.01247.x

Molecular characterization of *Erwinia amylovora* strains from different host plants through RFLP analysis and sequencing of *hrp*N and *dsp*A/E genes

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A total of 73 *Erwinia amylovora* strains obtained from 13 Maloideae host species and from *Rubus* spp., and isolated from different geographic areas, were assessed using RFLP and DNA sequencing analysis of the 3′*hrp*N gene and/or of a fragment of 1341 bp of the *dsp*A/E region. An *Erwinia pyrifoliae* strain, used as outgroup, was checked in the same way. For the three strains isolated from *Rubus* spp. and for one strain from *Amelanchier* sp., RFLP analysis of the *hrp*N gene using the *Rsa*I enzyme yielded a PCR product 60 bp smaller than that of all the other strains. Sequence analysis of the gene revealed this was due to the absence of a 60 bp fragment in the noncoding region downstream of the gene. The strain PD 2915, isolated from *Amelanchier* sp. grown in Canada, showed five same-sense substitutions and one missense substitution at position 868 of the *hrp*N gene, converting aspartic acid into asparagine. Also, restriction analysis of a fragment of 613 bp of the *dsp*A/E region with *Cfo*I revealed an RFLP pattern suitable for differentiating the *E. amylovora* strains isolated from *Rubus* spp. and *Amelanchier* sp. from all the others. In the *dsp*A/E coding region, the four strains showed 13–14 missense point mutations, in some cases yielding drastic amino acid substitutions. In addition, partial sequencing of the *dsp*A/E region of PD 2915 from *Amelanchier* sp. indicated a higher similarity to *E. amylovora* strains isolated from *Rubus* spp. than towards strains from other Maloideae hosts. The *E. pyrifoliae* strain showed 23 single nucleotide substitutions along the *hrp*N gene and 88% of nucleotide identity with *E. amylovora* strains in the portion of *dsp*A/E region. Artificial inoculations on immature pear fruits and young shoots of Maloideae and Ruboideae showed a restricted pathogenicity for the strains from *Rubus* and *Amelanchier*, with the latter inciting blight symptoms only on *Amelanchier*.

Keywords: *Amelanchier*, *Erwinia pyrifoliae*, fireblight, gene sequencing, *Rosaceae*, *Rubus*

Introduction

Erwinia amylovora, the causal agent of fireblight, is pathogenic on most species of the Maloideae including pear, apple, quince and various ornamentals. This bacterium has also been isolated from naturally infected Ruboideae species including *Rubus idaeus*, *Rubus fruticosus* and other brambles. Although strains obtained from both Maloideae and Ruboideae subfamilies are considered to belong to *E. amylovora* because of the high DNA–DNA homology value (Gardner & Kado, 1972), some pathogenic differences between them have been observed. In fact, *E. amylovora* strains from *Rubus* species have been reported not to be infective on apple seedlings and shoots (Ries & Otterbacker, 1977; Evans, 1996), whereas *E. amylovora* strains from apple and pear show an attenuated or suppressed pathogenicity towards tested blackberry (Starr *et al*., 1951) and raspberry (Evans, 1996) plants. Recently, a bacterium isolated in South Korea from the Asian pear, *Pyrus pyrifolia*, causing a shoot blight resembling symptoms of fireblight, was designated as a separate species, *Erwinia pyrifoliae* (Kim *et al*., 1999). Interestingly, the molecular characteristics of *E. pyrifoliae* resemble those of *E. amylovora* strains obtained from *Rubus* spp. more closely than those of strains isolated from Maloideae (McGhee *et al*., 2002).

When inoculated on nonhost plants such as tobacco, *E. amylovora* elicits a rapid and localized collapse of the leaf tissue, known as hypersensitivity reaction (HR) (Klement, 1982). Genetic studies have led to the identification of a gene cluster of 30 kb, named *hrp*, necessary for both pathogenicity on host plants and HR elicitation on nonhost plants (Bauer & Beer, 1991). Close to the *E. amylovora* *hrp* gene cluster, separated by only 4 kb, is the *dsp* region, coding for disease-specific function (Barny *et al*., 1990)

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Accepted 26 April 2005

and containing the *dsp*A/E and *dsp*B/F genes (Gaudriault *et al*., 1997; Bogdanove *et al*., 1998). The genes clustered in these regions encode three different types of protein, based on their function: regulatory, secretory and secreted. Regulatory proteins control expression of the other *hrp* genes; secretory proteins are structural components of a type III secretion system delivering proteins outside the bacterial cell. Four secreted proteins have been characterized: (i) the major HR elicitor HrpN (a glycine-rich harpin with a high degree of hydrophilicity), involved in pathogenicity (Wei *et al*., 1992); (ii) the DspA/E product, an essential pathogenicity determinant that shares 30% of protein homology with AvrE of *Pseudomonas syringae* pv. *tomato* (Bogdanove *et al*., 1998); (iii) the protein HrpW, which shares structural similarities with HrpN of *E. amylovora* and PopA of *Ralstonia solanacearum* (Kim & Beer, 1998); and (iv) HrpA pilus structural protein, which plays a key role in the secretion of Hrp proteins (Jin *et al*., 2001).

Among these factors, HrpN and DspA/E proteins, as well as the acidic exopolysaccharide amylovoran encoded in the 17 kb *ams* region of the chromosome (Bugert & Geider, 1995), play a fundamental role in *E. amylovora* pathogenicity (Kim & Beer, 2000; Bogdanove *et al*., 2000) and in elicitation of the oxidative burst (the massive production of reactive oxygen species) in pear microcuttings during the compatible interaction (Venisse *et al*., 2003).

Transposon-produced *hrp*N mutants cause weak fireblight symptoms (Barny, 1995), whereas *dsp*A/E mutants, obtained through insertional mutagenesis with the phage Mu::lacZ and through in-frame deletions, are not pathogenic (Barny *et al*., 1990; Bogdanove *et al*., 1998). As mutagenesis of *hrp*N revealed residual HR-inducing activity of Hrp-N fragments, HrpN might not be strictly required as an intact protein (Barny, 1995), and conservation of its sequence has not been strongly selected in mutational changes during evolution. It is possible that its DNA and amino acid sequences might be open to changes without affecting bacterial fitness and may be useful for strain differentiation (Jock & Geider, 2004).

Preliminary genomic DNA fingerprinting using repetitive sequence PCR indicated that three *E. amylovora* strains isolated from *Rubus* spp. and one strain obtained from *Amelanchier* sp. clustered separately from the other strains tested. To ascertain possible genetic variability in *hrp*N and *dsp*A/E genes of *E. amylovora*, RFLP analysis of both genes was carried out. In addition, for a subset of representative strains the 3′*hrp*N and 5′*dsp*A/E genes were studied through DNA sequence analysis to characterize the mutations found at nucleotide level. A host-range test was also carried out. An *E. pyrifoliae* strain, used as outgroup, was assessed in the same way.

Materials and methods

Bacterial strains and growth medium

Erwinia amylovora and *E. pyrifoliae* strains used in this study are listed in Table 1. Strains marked ISF were isolated for this study from diseased pear and *Cydonia oblonga* A (pear rootstock) specimens; all the others were obtained from international or national culture collections (Table 1). Pear twigs or fruits showing symptoms of fireblight were used for isolation. Fragments of tissue at the margins of lesions were crushed in sterile mortars containing 5 mL sterile saline solution (SS, 0·85% NaCl in distilled water). Tenfold serial dilutions in tubes were also performed. Subsequently, aliquots of 0·1 mL were spread on Petri dishes containing nutrient sucrose agar [NSA, 28 g nutrient agar (Oxoid) supplemented with 5·0 g L^{-1} sucrose]. Plates were incubated at 25–27°C for 3 days. Confirmatory tests (Lelliott & Stead, 1987) were carried out on the levaniform, whitish colonies suspected to belong to *E. amylovora*. These included the tobacco HR test; absence of fluorescent pigments on medium B of King *et al*. (1954); induction of necrosis and oozing in immature pear fruits; SDS–PAGE of whole-cell protein extracts; and comparison with *E. amylovora* type-strain NCPPB 683 and other representative strains previously isolated in Italy. The isolates reacting positively to these tests were considered to belong to *E. amylovora*. For the study, all strains were grown routinely on NSA at 25–27°C.

DNA preparation

To prepare total genomic DNA, a modification of the technique used by Smith *et al*. (1995) was used. For each strain, a loopful (diameter ≈3 mm) of a single colony that had been grown for 24 h on NSA at 25–27°C was suspended in SS and centrifuged at 12 000 ***g*** for 2 min. The supernatant was discarded and the pellet was suspended in bidistilled, filtered, sterilized water up to an optical density corresponding to $1–2 \times 10^8$ cfu mL^{-1}. The suspension was placed in boiling water for 10 min and then stored at –20°C.

Oligonucleotides for DNA amplification of *hrp*N gene and *dsp*A/E region and PCR assay conditions

Fragments of the *hrp*N gene and/or the *dsp*A/E region were amplified from 73 *E. amylovora* strains (Table 1) isolated from different host plants with primers 1–8 listed in Table 2. PCR was carried out in a total volume of 30 μL containing (final concentrations) 1 × PCR buffer (10 mM Tris–HCl, 50 mM KCl, 0·1% Triton X-100 pH 9); 50 pmol of each primer; 1 U *Taq* DNA polymerase (Promega); 0·2 mM each of dATP, dCTP, dGTP and dTTP (Promega); $MgCl_2$: 1·67 mM for *dsp*A/E1 amplification, 2·5 mM for pr-*dsp*A/E and *dsp*A/E2 amplifications, 2·8 mM for *hrp*N amplification; and 4 μL lysed cell suspension. All PCRs were performed in a Bio-Rad Gene Cycler version 1·5. Cycling parameters were 95°C for 7 min followed by 35 cycles of 94°C for 1 min, 54°C for 1 min and 72°C for 2 min, and a final incubation at 72°C for 5 min. Products were analysed by electrophoresis in 2% (wt/vol) agarose (Seakem) gel in 1 × Tris–borate–EDTA (TBE) buffer followed by ethidium bromide staining, and photographed under UV light.

Table 1 *Erwinia amylovora* and *Erwinia pyrifoliae* strains used in this study

Strain[a]	Host plant/cultivar	Origin	Year of isolation
NCPPB 683[T bc]	*Pyrus communis*	UK	1959
NCPPB 595[bc]	*P. communis*	UK	1958
NCPPB 2080[c]	*P. communis*	New Zealand	1955
BPIC 845[b]	*P. communis*	Greece	1984
BPIC 847[bc]	*P. communis*/P. Crassane	Greece	1984
BPIC 913[b]	*P. communis*/De Cayan	Greece	1985
BPIC 1093[b]	*P. communis*	Greece	1990
BPIC 1625[b]	*P. communis*	Greece	1991
PD 3891[bc]	*P. communis*/Wildeman	the Netherlands	2000
ICMP 13413[bc]	*P. communis*	Hungary	1996
IVIA 1892-1[bc]	*P. communis*	Spain	1998
UniBa-LE1[bc]	*P. communis*	Italy	1990
UniBa-LE2[bc]	*P. communis*	Italy	1990
UniBa-LE4[bc]	*P. communis*	Italy	1992
UniBa-LE8[bc]	*P. communis*	Italy	2001
UniBa-BR1[bc]	*P. communis*	Italy	1990
UniBa-BA5[bc]	*P. communis*	Italy	1994
UniBa-BA7[bc]	*P. communis*	Italy	1997
UniCt-Sic1[bc]	*P. communis*	Italy	1991
UniCt-Sic2[bc]	*P. communis*	Italy	1991
IPV-BO 2956[bc]	*P. communis*	Italy	1996
UniBa-Al 11[bc]	*P. communis*	Albany	1995
UniBa-Al 12[bc]	*P. communis*	Albany	1995
28CS[bc]	*P. communis*	Czech Republic	1990
85CS[c]	*P. communis*	Czech Republic	1990
90CS[c]	*P. communis*	Czech Republic	1990
ISF Joinee[bc]	*P. communis*	USA	unknown
ISF WC31[c]	*P. communis*	USA	unknown
ISF Ea 1/79 Rif[c]	*P. communis*	USA	unknown
ISF Ab 1[c]	*P. communis*/Abate	Italy	2001
ISF Conf 1[bc]	*P. communis*/Conference	Italy	2001
ISF Conf 2[bc]	*P. communis*/Conference	Italy	2001
ISF Will 1[bc]	*P. communis*/William	Italy	2001
ISF Will 2[bc]	*P. communis*/William	Italy	2001
ISF SM 1[bc]	*P. communis*/S. Maria	Italy	2001
ISF SM 2[bc]	*P. communis*/S. Maria	Italy	2001
ISF SM 2-1[bc]	*P. communis*/S. Maria	Italy	2002
ISF SM 2-5[bc]	*P. communis*/S. Maria	Italy	2002
ISF Ab 2-1[bc]	*P. communis*/Abate	Italy	2002
ISF Ab 2-5[b]	*P. communis*/Abate	Italy	2002
ISF SM-Fr 1[bcd]	*P. communis*/S. Maria	Italy	2002
ISF SM-Fr 2[bcd]	*P. communis*/S. Maria	Italy	2002
ISF SM-Fr 3[bcd]	*P. communis*/S. Maria	Italy	2002
ISF SM-Fr 5[bd]	*P. communis*/S. Maria	Italy	2002
ISF SM-Fr 6[bcd]	*P. communis*/S. Maria	Italy	2002
BPIC 918[b]	*Pyrus amygdalifoliae*	Greece	1986
ISF Cot 1[bc]	*Cydonia oblonga* A	Italy	2001
ISF Cot 2[bc]	*C. oblonga* A	Italy	2001
PD 2233[bc]	*C. oblonga*	Bulgaria	1993
PD 2913[bc]	*C. oblonga*	USA	unknown
H-902[bc]	*C. oblonga*	Hungary	1996
NCPPB 3159[bc]	*Malus domestica*	Italy	1981
PD 394[bc]	*M. domestica*/Mantet	the Netherlands	1983
PD 3368[bc]	*M. domestica*	the Netherlands	1998
ICMP 13415[bc]	*M. domestica*/Jonathan	Hungary	1996
H-895[bc]	*M. domestica*/G. Delicious	Hungary	1996
UniBaLE9[bc]	*M. domestica*	Italy	2001
IVIA 1767-3[bc]	*Malus* sp.	Spain	1997
NCPPB 2292[bc]	*Rubus idaeus*	USA	1949
NCPPB 2293[bc]	*R. idaeus*	USA	1949
PD 103[bc]	*Rubus* sp.	USA	1978

Table 1 *Continued*

Strain[a]	Host plant/cultivar	Origin	Year of isolation
UniBaBA6[bc]	*Cotoneaster* sp.	Italy	1995
UniBaLE10[bc]	*Cotoneaster* sp.	Italy	2001
IVIA 1525-1[bc]	*Cotoneaster* sp.	Spain	1996
PD 3678[bc]	*Crataegus monogyna*	Germany	1999
IVIA 198-11[bc]	*Crataegus* sp.	Spain	1996
PD 2912[bc]	*Eriobotrya japonica*	Israel	1997
PD 4071[c]	*Sorbus aucuparia*	Germany	1999
ICMP 13414[bc]	*Sorbus aria*	Hungary	1996
ICMP 13417[bc]	*Mespilus germanica*	Hungary	1996
PD 2914[bc]	*Dichomanthes* sp.	UK	1996
PD 2915[bc]	*Amelanchier* sp.	Canada	1996
IVIA 1951-2[bc]	*Pyracantha* sp.	Spain	1998
E. pyrifoliae CFBP 4172[bc]	*Pyrus pyrifolia*	South Korea	1996

[a]NCPPB: National Collection of Plant Pathogenic Bacteria, York, UK. PD: Culture Collection of Plant Protection Service, Wageningen, the Netherlands. BPIC: Benaki Phytopathological Institute Collection, Kiphissia-Athens, Greece. IVIA: Collection of Instituto Valenciano de Investigaciones Agraria, Moncada-Valencia, Spain. ISF: Culture Collection of the Istituto Sperimentale per la Frutticoltura, Roma, Italy. CFBP: Collection Francaise de bactéries Phytopathogènes, Angers, France. ICMP: International Collection of Micro-organisms from Plants, Auckland, New Zealand. IPV-BO: Culture Collection of the University of Bologna, Italy. UniBa: Culture Collection of the University of Bari, Italy. UniCt: Culture Collection of the University of Catania, Italy. H: Collection of the Plant Health and Soil Conservation Service, Pecs, Hungary. CS: Collection of the Institute of Plant Molecular Biology, Ceske Budejovice, Czech Republic.
[b]Strains assessed with RFLP of *hrp*N.
[c]Strains assessed with RFLP of *dsp*A/E.
[d]Strains isolated from diseased fruits.
[T]*Erwinia amylovora* type-strain.

Table 2 Primers used in this study for DNA amplification (annealing temperature 54°C) and sequencing of *dsp*A region and *hrp*N gene

Primer	Primer sequence
1 pr *dsp*A/E for	GCAGAATTTGCATTATGACC
2 pr *dsp*A/E rev	ATGATGCAGCGGCATTTT
3 *dsp*A/E for	GGGGGGCAGACTTTTTTTTAA
4 *dsp*A/E rev	CCAAAATTGTTCAGCTGATGC
5 *dsp*A/E2 for	ATAAGGTACCAACGCAGCAAA
6 *dsp*A/E2 rev	TTGATATCAAACAGGTGGCCT
7 *hrp*N for	GGTACCGGTATCGGTATGAA
8 *hrp*N rev	CTAATGCGCCCAGTAAATCC

RFLP analysis of *hrp*N and *dsp*A/E genes

For RFLP analysis of the 5′*dsp*A/E region and 3′*hrp*N gene, 15 μL aliquots of each PCR mixture containing ≈1·5 μg amplified DNA were digested with the following restriction endonucleases as recommended by the manufacturer (Promega): *Cfo*I, *Hae*III, *Mbo*I and *Msp*I for the *dsp*A/E region, and *Rsa*I for the *hrp*N gene. All digestions were carried out at 37°C for 6 h. The reaction products were analysed by agarose (Seakem) (3%, wt/vol) gel electrophoresis in 1 × TBE buffer containing 0·5 μg mL^{-1} ethidium bromide. Subsequently the gels were visualized under an UV transilluminator Spectroline (Spectronic) and photographed with a Polaroid type 55 film. The PCR amplifications were performed in duplicate.

Nucleotide sequence analysis

The 3′*hrp*N gene of *E. amylovora* PD 2915 and *E. pyrifoliae* CFBP 4172, as well as a portion (1341 bp) of the 5′*dsp*A/E region including the promoter region of *E. amylovora* NCPPB 2292, NCPPB 2293, PD 103, PD 2915 and *E. pyrifoliae* CFBP 4172, were amplified with the primers indicated in Table 2. The rDNA PCR was carried out in a total volume of 30 μL containing (final concentrations) 1 × PCR buffer, 50 pmol of each primer, 1 U *Taq* DNA polymerase (Promega), 0·2 mM each of dATP, dCTP, dGTP and dTTP (Promega), 1·6 mM $MgCl_2$, and 4 μL lysed cell suspension. The reaction mixtures were incubated in a Bio-Rad Gene Cycler version 1·5 at 95°C for 7 min followed by 35 cycles of 94°C for 1 min, 43°C for 1 min and 72°C for 2 min, and a final incubation at 72°C for 5 min. Before sequencing, the PCR products were separated from residual primers using the Qiaquick PCR Purification Kit (Qiagen) according to the manufacturer's instructions. All the sequences were determined at MWG-Biotech AG, Ebersberg, Germany directly from the purified fragments, using the corresponding amplification primers. The sequences were compared using the PC/Gene program CLUSTALW MULTIPLE SEQUENCE ALIGNMENT ver. 1·82 (Higgins *et al.*, 1994).

Pathogenicity tests

To test the host range of some representative *E. amylovora* strains (NCPPB 683 isolated from *Pyrus communis*; PD

103, NCPPB 2292, NCPPB 2293 isolated from *Rubus* spp.; and PD 2915 isolated from *Amelanchier* sp.), immature pear (*P. communis*) fruits and Maloideae and Ruboideae host plants were used. Artificial inoculations were carried out at the beginning of summer. Fruits were washed for 20 min in tap water and air-dried. For inoculation, strains were streaked for 24 h on NSA at 25–27°C. Then different doses of bacterial suspensions in SS were prepared. The immature fruits were stab-inoculated by means of a sterile needle with 10 μL of the following decreasing concentrations of bacterial suspension: 1–2 $\times 10^9$, 1–2 $\times 10^7$ and 1–2 $\times 10^5$ cfu mL^{-1}. *Erwinia pyrifoliae* CFBP 4172 was also inoculated in the same way. As negative controls, fruits were inoculated in the same way with: (i) only SS; and (ii) decreasing doses of *Pseudomonas avellanae* ISF 2059 suspensions (10 μL, 1–2 $\times 10^9$, 1–2 $\times 10^7$ and 1–2 $\times 10^5$ cfu mL^{-1}). For each strain, two different fruits and three sites on the same fruit were inoculated. Apple (*Malus domestica*) cv. Red Gala, pear cvs William (susceptible to fireblight) and Harrow Sweet (tolerant to fireblight), raspberry (*Rubus idaeus*) cv. Tulameen and serviceberry (*Amelanchier alnifolia*) pot-grown plants were inoculated with the same strains by two different methods, as follows. (i) Plants were inoculated by wounding the young shoot with a sterile needle and, immediately after applying 10 μL of bacterial suspension to the wound at decreasing concentrations (1–2 $\times 10^9$, 1–2 $\times 10^7$ and 1–2 $\times 10^5$ cfu mL^{-1}). (ii) Plants were inoculated by cutting two apical leaves of a young growing shoot with sterilized scissors previously plunged into an *E. amylovora* suspension of 1–2 $\times 10^7$ cfu mL^{-1}. *Erwinia pyrifoliae* CFBP 4172 was also inoculated in the same way. For each strain and technique, three and 10 shoots, respectively, were inoculated. Shoots inoculated with only SS served as negative control. The immature fruits and plants were kept in a room with 100% humidity. Symptoms were checked up to 15 days after inoculation. Finally, for each strain the HR was tested by infiltration of the bacterial suspensions (1–2 $\times 10^8$ cfu mL^{-1}) into the parenchyma of tobacco leaves.

Results

RFLP analysis and sequencing of *hrp*N

Restriction analysis of *hrp*N was performed using *Rsa*I. On amplification of the *hrp*N of 66 *E. amylovora* strains (Table 1), a difference in size in PCR products of the following strains was found: PD 2915 isolated from *Amelanchier* sp., and PD 103, NCPPB 2292 and NCPPB 2293 isolated from *Rubus* sp. Their PCR product was 60 bp smaller than that of the other strains (Fig. 1). After *Rsa*I restriction, however, the same two fragments of 51 and 112 bp were observed for 65 *E. amylovora* strains. In contrast, *E. amylovora* PD 103 yielded a fragment of 163 bp rather than the two fragments mentioned above. Sequence analysis of *hrp*N from PD 103, and comparison with *hrp*N of *E. amylovora* Ea321 (GenBank accession number M92994) isolated from *Crataegus* sp., revealed a point mutation at nt 834 of the coding region that eliminated

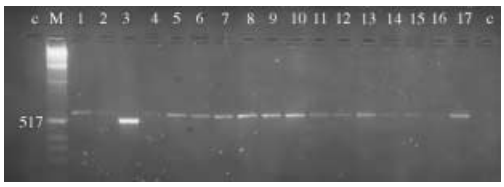

Figure 1 PCR amplification of *hrp*N gene with the primers shown in Table 2. *Erwinia amylovora* PD 2915, isolated from *Amelanchier* sp., grown in Canada (lane 3) yielded a PCR product 60 bp smaller that that of the other *E. amylovora* strains tested. c, negative control; M, molecular size marker (1 kb DNA ladder; Gibco-BRL). Lane 1, PD 2913; lane 2, PD 2914; lane 3, PD 2915; lane 4, PD 2233; lane 5, IVIA 1525-1; lane 6, IVIA 1951-2, lane 7, IVIA 1767-3, lane 8, IVIA 198-11; lane 9, ISF Cot 1; lane 10, BPIC 845; lane 11, BPIC 847, lane 12, BPIC 913, lane 13, BPIC 918, lane 14, BPIC 1093; lane 15, ISF Will 1; lane 16, ISF Conf 1; lane 17, ISF Will 2.

the *Rsa*I restriction site, but yielded no amino acid substitution at codon 278 coding for glycine. This identical substitution was also found in *E. amylovora* Ea246, isolated from *R. fruticosus* (GenBank accession number AF083620). *hrp*N sequence analysis of *E. amylovora* PD 2915 showed six single nucleotide substitutions (SNS) in the coding region that were not revealed by the *Rsa*I restriction analysis. Among these, five were identical nucleotide substitutions and one was a missense substitution at position 868 converting the codon AAT (for asparagine) into GAT (for aspartic acid), thereby changing a polar neutral amino acid into an acidic amino acid. 3′*hrp*N sequence analyses of PD 103 and PD 2915 also revealed that the PCR product size differences found with respect to the other strains were due to the absence of a 60 bp fragment in the noncoding region downstream of the *hrp*N gene. The 3′*hrp*N PCR amplification product of *E. pyrifoliae* CFBP 4172 showed no difference in size when compared with the *E. amylovora* strains. However, after *Rsa*I digestion two distinct fragments of 27 and 135 bp were observed. The *hrp*N sequence analysis of *E. pyrifoliae* CFBP 4172 showed 23 SNS along the 363 bp of the coding region. Only one was a missense substitution, revealing a change of G into A at position 1171 and converting the codon GCC (for alanine) into ACC (for threonine), thereby changing a nonpolar amino acid into a polar amino acid.

RFLP analysis of *dsp*A/E region

The 1341 bp *dsp*A/E region, which includes the promoter region and 1076 nt of the 5′*dsp*A/E coding sequence, was studied for 66 strains by amplifying three overlapping fragments of 427, 613 and 628 bp, followed by single digestion with the *Cfo*I restriction endonuclease. This enzyme was chosen after computer analysis revealed it to be a good candidate to distinguish *E. amylovora* strains by RFLP in this region. In addition, the portions were also double-digested. Restriction analysis of the 613 bp *dsp*A/E region with *Cfo*I yielded an RFLP pattern suitable for differentiating the *E. amylovora* strains isolated from *Rubus* sp. and *Amelanchier* sp. from all the other strains.

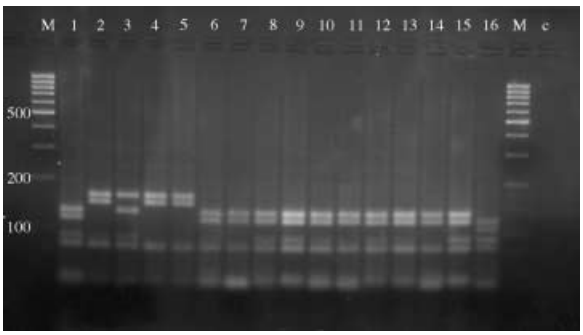

Figure 2 Restriction polymorphism of a fragment of 427 bp of the *dsp*A/E region from 15 *Erwinia amylovora* strains on digestion with *Cfo*I and *Msp*I endonucleases. *Erwinia pyrifoliae* is also included. M, molecular size marker (100 bp DNA ladder; Gibco-BRL). Lane 1, NCPPB 683; lane 2, PD 2915; lane 3, PD 103; lane 4, NCPPB 2292; lane 5, NCPPB 2293; lane 6, PD 2233; lane 7, PD 2912; lane 8, PD 2914; lane 9, PD 3368; lane 10, PD 3678; lane 11, PD 3891; lane 12, PD 4071; lane 13, NCPPB 3159; lane 14, IVIA 1951-2; lane 15, ISF SM Fr 1; lane 16, *E. pyrifoliae* CFBP 4172. C, negative control.

In fact, fragments of 152, 151, 131, 118, 36, 13 and 12 bp were obtained from 62 *E. amylovora* strains isolated from different Maloideae host plants, whereas fragments of 167, 152, 151, 131 and 12 bp (two *Cfo*I restriction sites have been removed by two mutational events) were obtained from the three strains isolated from *Rubus* spp. (NCPPB 2292 and 2293, PD 103) as well as with the strain isolated from *Amelanchier* sp. (PD 2915). The same grouping was also observed by double-digesting the 613 bp *dsp*A/E fragment with *Mbo*I and *Hae*III endonucleases.

The 427 bp *dsp*A/E fragment, amplified with primers 1 and 2 (Table 2), was double-digested with *Cfo*I and *Msp*I. Again, and even more clearly, it was possible to differentiate the strains isolated from Ruboideae and *Amelanchier* sp. from all the others (Fig. 2). The RFLP analysis of the 427 bp *dsp*A/E portion indicated for PD 2915 isolated from *Amelanchier* sp. a higher similarity with the strains obtained from *Rubus* spp. than with the other Maloideae strains. Remarkably, PD 103 isolated from *Rubus* sp. showed a unique restriction profile. Finally, RFLP analysis of the 628 bp *dsp*A/E fragment performed with *Cfo*I endonuclease allowed *E. amylovora* strains obtained from Ruboideae and *Amelanchier* sp. to be distinguished from the other strains.

The *dsp*A/E region of *E. pyrifoliae* CFBP 4172 was amplified with the same primer sets as were used for the *E. amylovora* strains, and subsequently digested with the same endonucleases. The RFLP patterns obtained allowed differentiation of *E. pyrifoliae* from all the *E. amylovora* strains (Fig. 2).

Partial sequencing of *dsp*A/E region

The *5′dsp*A/E region, including a short sequence of the upstream *hrp*W gene, of *E. amylovora* NCPPB 2292, 2293, PD 103 and PD 2915 (strains from *Rubus* sp. and *Amelanchier* sp.), was sequenced (1341 bp). Sequence analysis revealed various SNS in the *hrp*W and *dsp*A/E promoters and coding regions when compared with the corresponding sequence of *E. amylovora* CFBP 1430 (GenBank accession number Y13831) isolated from *Crataegus* sp. The DNA sequences from all four strains showed one point mutation in the *dsp*A/E promoter at the nucleotide position –63 from gene start, changing nucleotide G into T, and two point mutations in the *hrp*W promoter region at nucleotide positions 69 and 68 from gene start, converting AA into TG on the complementary strand of the *dsp*A/E gene. In the *5′dsp*A/E coding region, NCPPB 2292, 2293, PD 103 and PD 2915 showed 14, 13, 13 and 13 missense point mutations, respectively, in some cases yielding drastic amino acid substitutions that may influence the function of the protein. No SNS were found by sequencing the 613 *dsp*A/E fragment from seven strains (NCPPB 683, NCPPB 3159, PD 2233, PD 2912, PD 3678, PD 4071 and IVIA 1951–2) associated with different host plants from different geographical areas. The *5′dsp*A/E sequence analysis of *E. pyrifoliae* CFBP 4172, compared with *E. amylovora* CFBP 1430, showed 98 SNS in the *5′*-coding region, where the proportion of missense substitutions was 50%. The alignment and sequence comparison of *5′dsp*A/E genes of *E. amylovora* PD 2915, NCPPB 2292 and 2293, PD 103, CFBP 1430 (sequence information obtained from EMBL GenBank) and *E. pyrifoliae* CFBP 4172 (Fig. 3) revealed the following main features. (i) The *5′dsp*A/E regions from *E. amylovora* strains obtained from *Rubus* sp. and *Amelanchier* sp. were highly related (99% identity) and these sequences could be distinguished from that of *E. amylovora* CFBP 1430 by several mismatches (97% identity). (ii) The *dsp*A gene from *E. pyrifoliae* showed 88% nucleotide identity compared with *dsp*A/E from *E. amylovora* strains isolated from Maloideae or Ruboideae species. (iii) Comparison of the deduced amino acid sequences of *5′dsp*A/E genes under study showed 85% identity between *E. pyrifoliae* and *E. amylovora*, and 96% identity between *E. amylovora* CFBP 1430 isolated from *Crataegus* sp., and PD 2915 and NCPPB 2292, NCPPB 2293 and PD 103. The *dsp*A/E amino acid sequence alignment also revealed other peculiarities. In some cases *E. amylovora* PD 103 showed the same amino acid substitutions as PD 2915, NCPPB 2292 and 2293; it also maintained the amino acid residues of strains from Maloideae at amino acid positions 101 and 165; furthermore, it showed two amino acid substitutions seen in no other strain (change of aspartate into valine and of glycine into valine at amino acid positions 65 and 146, respectively). *Erwinia pyrifoliae* CFBP 4172, when compared with *E. amylovora* CFBP 1430, showed 49 amino acid differences, although it maintained the same amino acid residues in six positions that were different in *E. amylovora* strains isolated from *Rubus* spp. and *Amelanchier* sp.

Sequence accession numbers

The following nucleotide sequences were deposited in the EMBL nucleotide sequence database: *3′hrp*N sequences from *E. amylovora* PD 2915 and *E. pyrifoliae* CFBP 4172

```
PD 2915          MELKSLGTEHKAAVHTAAHNPVGHGVALQQGSSSSSPQNAAASLAAEGKNRGEMPRIHQP
NCPPB 2293       MELKSLGTEHKAAVHTAAHNPVGHGVALQQGSSSSSPQNAAASLAAEGKNRGEMPRIHQP
NCPPB 2292       MELKSLGTEHKAAVHTAAHNPVGHGVALQQGSSSSSPQNAAASLAAEGKNRGEMPRIHQP
PD 103           MELKSLGTEHKAAVHTAAHNPVGHGVALQQGSSSSSPQNAAASLAAEGKNRGEMPRIHQP
CFBP 1430        MELKSLGTEHKAAVHTAAHNPVGHGVALQQGSSSSSPQNAAASLAAEGKNRGKMPRIHQP
E.pyrifoliae     MVLKLQGTEHKTAVQIAAHNPVGQGVALQQGSSSSSPQNAAASLASEGKNRGKMPKVHQS
                 * **  *****:**: *******:**********************:******:**::**.

PD 2915          STAADGISAAHQQKKSFSLRGFLGTKKSSKSAPQAQPGTTLSKGATLHDLLARDDGETQH
NCPPB 2293       STAADGISAAHQQKKSFSLRGFLGTKKSSKSAPQAQPGTTLSKGATLHDLLARDDGETQH
NCPPB 2292       STAADGISAAHQQKKSFSLRGFLGTKKSSKSSPQAQPGTTLSKGATLHDLLARDDGETQH
PD 103           STAAVGISAAHQQKKSFSLRGFLGTKKSSKSAPQAQPGTTHSKGATLHDLLARDDGETQH
CFBP 1430        STAADGISAAHQQKKSFSLRGCLGTKKFSRSAPQGQPGTTHSKGATLRDLLARDDGETQH
E.pyrifoliae     STAADGVSAAHQQKKSFSLKGFLGLKKSSKSLPQTQPGATHSKGTTLRDLLAQDDGETQH
                 **** *:************:* ** ** *:* ** ***:* ***:**:****:*******

PD 2915          EAAAPDAARLTHSGGVKRRNMDDMAGRPMVKGGSGEDKVPTQQKGRQLNNFGQMRQTMLS
NCPPB 2293       EAAAPDAARLTHSGGVKRRNMDDMAGRPMVKGGSGEDKVPTQQKGRQLNNFGQMRQTMLS
NCPPB 2292       EAAAPDAARLTHSGGVKRRNMDDMAGRPMVKGGSGEDKVPTQQKGRQLNNFGQMRQTMLS
PD 103           EAAAPDAARLTHSGGVKRRNMDDMAVRPMVKGGSGEDKVPTQQKRRQLNNFGQMRQTMLS
CFBP 1430        EAAAPDAARLTRSGGVKRRNMDDMAGRPMVKGGSGEDKVPTQQKRHQLNNFGQMRQTMLS
E.pyrifoliae     EAAAPDAARS---GGVKRHNLDDMAGRP---------------KQHQLNNFSQMRQTMLS
                 *********    *****:*:**** **               * :*****.********

PD 2915          KMAHPASANAGDRLQHSPPHIPGSHHEIKEEPVGSTSKATTAHADRVEIAQEDDDSEFQQ
NCPPB 2293       KMAHPASANAGDRLQHSPPHIPGSHHEIKEEPVGSTSKATTAHADRVEIAQEDDDSEFQQ
NCPPB 2292       KMAHPASANAGDRLQHSPPHIPGSHHEIKEEPVGSTSKATTAHADRVEIAQEDDDSEFQQ
PD 103           KMAHPASANAGDRLQHSPPHIPGSHHEIKEEPVGSTSKATTAHADRVEIAQEDDDSEFQQ
CFBP 1430        KMAHPASANAGDRLQHSPPHIPGSHHEIKEEPVGSTSKATTAHADRVEIAQEDDDSEFQQ
E.pyrifoliae     KMTHPASADAGERLHHSPPRIPGSHHEIEELPVGSTSKAATAHTGKGEIAHEDDDSEFQQ
                 **:*****:**:**:****:********:* ********:***:.: ***:*********

PD 2915          LHQQRLARERENPPQPPKLGVATPISARFQPKLTAVAESVLEGTDTTQPPLKPQSMLKGS
NCPPB 2293       LHQQRLARERENPPQPPKLGVATPISARFQPKLTAVAESVLEGTDTTQPPLKPQSMLKGS
NCPPB 2292       LHQQRLARERENPPQPPKLGVATPISARFQPKLTAVAESVLEGTDTTQPPLKPQSMLKGS
PD 103           LHQQRLARERENPPQPPKLGVATPISARFQPKLTAVAESVLEGTDTTQPPLKPQSMLKGS
CFBP 1430        LHQQRLARERENPPQPPKLGVATPISARFQPKLTAVAESVLEGTDTTQSPLKPQSMLKGS
E.pyrifoliae     LHQQRLARERENPPQPPKLGVTTPTAARYQPKLTAIAESVLEGTDSTQSPLKPQSMLKGS
                 **********************:** :**:******:*********:**.***********

PD 2915          GAGVTPLALTLDKGKLQLAPDNPPALNTLLKQTLGKDTQHYLAHHASSDGSQHLLLDNT
NCPPB 2293       GAGVTPLALTLDKGKLQLAPDNPPALNTLLKQTLGKDTQHYLAHHASSDGSQHLLLDNT
NCPPB 2292       GAGVTPLALTLDKGKLQLAPDNPPALNTLLKQTLGKDTQHYLAHHASSDGSQHLLLDNT
PD 103           GAGVTPLALTLDKGKLQLAPDNPPALNTLLKQTLGKDTQHYLAHHASSDGSQHLLLDNT
CFBP 1430        GAGVTPLAVTLDKGKLQLAPDNPPALNTLLKQTLGKDTQHYLAHHASSDGSQHLLLDNK
E.pyrifoliae     GAGVTPLAVALDKGKLQLASTNPPALNTLLKQTLGKDAQHYLAHHASSDGSQHLLLDNK
                 ********::*********. ****************:********************.
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Figure 3 Similarity of deduced amino acid sequences of a fragment of the *dsp*A/E of *Erwinia amylovora* strains PD 2915, NCPPB 2292, NCPPB 2293, PD 103, CFBP 1430; and of *Erwinia pyrifoliae* CFBP 4172. Asterisks indicate identity; dot pairs, conservative substitutions; single dots, less conservative substitutions; absence of dot, nonconservative substitutions; dashes, absence of amino acid sequence.

Table 3 Pathogenicity tests and symptoms observed after 15 days on pear immature fruits and on wound-inoculated shoots using *Erwinia amylovora* and *Erwinia pyrifoliae* strains

		Pear plant cv.				
	Pear fruit	William	Harrow Sweet	Apple	Raspberry	Serviceberry
NCPPB 683	+	+	+[a]	+	–**	–**
PD 103	–	+/–***	–	+/–***	+	–
NCPPB 2292	–	+/–***	–	+/–***	+	–
NCPPB 2293	–	+/–***	–	+/–***	+	–
PD 2915	–	–	–	–	–	+
E. pyrifoliae CFBP 4172	+*	–	–	–	–	–

+, Spread of infection departing from site of inoculation and fireblight symptoms.

[a]Fireblight symptoms only at highest dose.

*Necrosis evident 15 days after inoculation using highest dose.

**Initial wilting evident 15 days after inoculation.

***Slight necrosis surrounding site of inoculation using medium and highest doses.

(accession numbers AJ631304 and AJ631305, respectively); *5′dsp*A/E sequences from *E. amylovora* NCPPB 2292 (AJ698834); *E. amylovora* NCPPB 2293 (AJ698835), *E. amylovora* PD 103 (AJ640077), *E. amylovora* PD 2915 (AJ698952) and *E. pyrifoliae* CFBP 4172 (AJ699305).

Pathogenicity tests

Results of the pathogenicity tests are shown in Tables 3 and 4. On immature pear fruits, only *E. amylovora* NCPPB 683 induced, at any dose tested, necrosis and oozing of

Table 4 Mean distance (mm) of twig necrosis induced by *Erwinia amylovora* and *Erwinia pyrifoliae* strains on growing shoots of some Maloideae and Ruboideae species recorded 15 days after inoculation performed by cutting two apical leaves off the shoot (minimum and maximum distance of necrosis in parentheses)

	Pear cv.				
	William	Harrow Sweet	Apple	Raspberry	Serviceberry
NCPPB 683	232 (13–385)	72 (44–160)	105 (40–128)	28 (0–44)	17 (0–31)
PD 103	21* (0–31)	8* (0–11)	9* (0–14)	65 (29–88)	2* (0–08)
NCPPB 2292	28* (0–36)	6* (0–12)	11* (0–19)	71 (41–91)	1* (0–88)
NCPPB 2293	19* (0–29)	2* (0–08)	16* (0–20)	69 (29–82)	6* (0–08)
PD 2915	14 (0–28)	4* (0–08)	12* (0–17)	13 (0–26)	78 (27–99)
E. pyrifoliae CFBP 4172	9* (0–19)	2* (0–08)	3* (0–09)	4* (0–09)	6* (0–11)

*Necrosis at tip of shoot observed 8 days after inoculation, not enlarging afterwards.

bacterial exudate. *Erwinia amylovora* PD 103 and NCPPB 2292 and 2293, as well as PD 2915, did not incite any apparent symptoms on immature pear fruit even at the highest dose. *Erwinia pyrifoliae* CFBP 4172 induced a slight necrosis with a slimy exudate only when inoculated at the highest dose; an enlargement of the necrosis was observed 15 days after the inoculation. No symptom was observed on fruits inoculated with SS and *P. avellanae*.

On wound-inoculated shoots of apple and pear cv. William, *E. amylovora* NCPPB 683 type-strain isolated from *P. communis* induced complete wilting 8 days after inoculation at any dose tested. Shoots inoculated with the highest dose also showed withering of the apical leaves. Pear cv. Harrow Sweet exhibited blight symptoms only when inoculated at the highest dose. In contrast, inoculation of raspberry and serviceberry with the highest dose produced only initial wilting (1·0–1·5 cm) of the shoot after 15 days. On apple and pear, *E. amylovora* PD 103, NCPPB 2292 and 2293 isolated from *Rubus* spp. incited, at the medium and highest doses, only slight necrosis surrounding the inoculation site and no apparent symptoms on serviceberry. On raspberry, complete wilting of the shoot was observed only at the highest dose 8 days after the inoculation, whereas 1 week later wilting was also noticed for the shoots inoculated with medium and low doses. Apple, raspberry and pear shoots after inoculation with *E. amylovora* PD 2915 isolated from *Amelanchier* sp. remained symptomless. On serviceberry, an initial wilting of the shoot was observed 8 days after inoculation, and shoots showed fireblight symptoms 1 week later. *Erwinia pyrifoliae* CFBP 4172 did not cause any symptoms, at any dose, to shoots of any of the plant species tested. The inoculation performed by cutting the apical leaves confirmed these results (Table 4) and the restricted pathogenicity of the strains isolated from *Rubus* spp. and *Amelanchier* sp. was again observed. These strains produced limited necrosis at the shoot tips of the apple and pear cultivars tested, but caused typical fireblight symptoms solely on host plants from which they were originally isolated. Finally, all strains incited an HR on tobacco leaves 24 h after inoculation by infiltration.

Discussion

Analysis of the 3′*hrp*N region of 66 *E. amylovora* strains, isolated from *Rubus* spp. (Ruboideae) and from Maloideae, showed a diversity between these two *E. amylovora* subgroups. In fact, PD 2915 isolated from *Amelanchier* sp., and NCPPB 2292, 2293 and PD 103 obtained from *Rubus* spp., showed a PCR product 60 bp smaller than that of all the other strains tested. Moreover, the subsequent restriction analysis performed with *Rsa*I enabled only PD 103 and *E. pyrifoliae* to be distinguished from all the other *E. amylovora* strains tested. The subsequent DNA sequence analysis of PD 103 revealed the presence of a same-sense point mutation abolishing the corresponding *Rsa*I restriction site, thus differentiating this strain from all the others. Significant differences in 3′*hrp*N DNA sequence, undetectable with *Rsa*I restriction digestion, were also found for PD 2915 isolated from *Amelanchier* sp. In fact, this strain showed six SNS in the coding region: five synonymous nucleotide substitutions and one missense substitution at nucleotide position 868 converting the codon ATT (for asparagine) into GAT (for aspartic acid). The contribution of this missense mutation to the specificity of pathogenicity of PD 2915 was not assessed; however, the present data support the utilization and usefulness of sequence information of *hrp*N for strain differentiation (Jock & Geider, 2004). Sequence analysis of *E. pyrifoliae* showed the presence of 23 SNS in the 3′*hrp*N coding region, only one of which was a missense point mutation, converting alanine into threonine.

Differentiation of strains from Ruboideae and from *Amelanchier* sp. was also achieved by restricting fragments of the *dsp*A/E region. The partial sequence of the *dsp*A/E region yielded even more information about the diversity of these strains. Interestingly, results with PD 2915 from *Amelanchier* sp. showed that it is more similar to strains from *Rubus* spp. than to the other Maloideae strains, as 13 missense point mutations were identified in the 5′*dsp*A/E coding region. In some cases these mutations produced drastic amino acid substitutions which might influence protein function.

Pathogenicity tests performed in this study confirmed the diversity of the strains from *Amelanchier* sp. and *Rubus* spp., and confirmed the disease-specific functions of the *dsp*A/E gene (Barny *et al*., 1990; Gaudriault *et al*., 1997; Bogdanove *et al*., 1998). In fact, such strains showed a restricted pathogenicity, inducing insignificant or no symptoms on apple and pear or on immature pear fruits, and causing typical fireblight symptoms only on the host plants from which they were originally isolated. In addition, the present study demonstrated that a strain from *Amelanchier* sp., a plant species of the Maloideae subfamily, showed reduced aggressiveness towards some other Maloideae species such as pear and apple.

The four deviating strains described here originated from North America. Recently, Jock & Geider (2004) found that *E. amylovora* strains from Europe showed identical *hrp*N sequences, whereas North American isolates from fruit tree species and raspberry showed more variability. For example, a North American raspberry strain could be clearly distinguished from the other *E. amylovora* strains from fruit trees, showing six amino acid insertion sequences in the centre of *hrp*N gene (Jock & Geider, 2004). The results obtained in the present study regarding strain PD 2915, isolated from *Amelanchier* sp. grown in Canada, supports the hypothesis that the genetic diversity of *E. amylovora* is higher in North America, its centre of origin (McManus & Jones, 1995; Jock & Geider, 2004), and that this has influenced the molecular evolution of *E. amylovora* genes, such as *hrp*N and *dsp*A/E, involved in oxidative burst in host plants (Venisse *et al*., 2003)

This study also showed the usefulness of RFLP analysis for rapid assessment of possible diversity within *E. amylovora* isolates. Digestion of a 613 bp fragment of the *dsp*A/E region with *Cfo*I endonuclease allowed strains from *Rubus* spp. and *Amelanchier* to be distinguished from all the other strains. Differences in *dsp*A/E region sequences were also ascertained for *E. pyrifoliae* CFBP 4172 which showed 88% nucleotide identity with *5′dsp*A/E from *E. amylovora* strains isolated from hosts belonging to the Maloideae and Ruboideae. The amino acid analysis of this DNA portion showed 49 amino acid differences when compared with *E. amylovora* CFBP 1430. However, *E. pyrifoliae* CFBP 4172 conserved the same amino acid residues in six positions. These amino acid residues resulted in modified *dsp*A/E from *Rubus* spp. and *Amelanchier* sp. The possibility that these amino acids might be important for the function of DspA/E protein involved in the induction of specific pathogenic symptoms in *Rosaceae* species deserves further study.

Acknowledgements

This work was financed by the Italian Ministry of Agriculture and Forestry in the framework of the project 'PRIA-Ricerche sul pero finalizzate alla riduzione dell'impatto ambientale e alla valorizzazione della qualità'; publication no. 6. The authors wish to thank the following colleagues for supplying *Erwinia amylovora* strains: C. Bazzi (University of Bologna, Bologna, Italy), C. Cariddi (University of Bari, Bari, Italy), V. Catara (University of Catania, Catania, Italy), J. D. Janse (Plant Protection Service, Wageningen, the Netherlands), V. Kudela (Institute of Plant Molecular Biology, Ceske Budejovice, Czech Republic), M. M. Lopez (Instituto Valenciano de Investigaciones Agrarias, Moncada-Valencia, Spain), C. Manceau (INRA-Pathologie Végétale, Beaucouzé, France), J. Németh (Plant Health and Soil Conservation Station, Pecs, Hungary), P. Psallidas (Benaki Phytopathological Institute, Kifissia-Athens, Greece) and D. E. Stead (Defra Central Science Laboratory, York, UK).

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