European Journal of Plant Pathology (2006) 114:435–440
DOI 10.1007/s10658-006-0003-7

Short communication

Characterization of *Erwinia amylovora* strains from Croatia

Edyta Halupecki[1,*], Carlo Bazzi[2], Susanne Jock[3], Klaus Geider[3,4], Damir Đermić[5] and Bogdan Cvjetković[1]
[1]*Department of Plant Pathology, Faculty of Agriculture, Svetošimunska 25, 10000, Zagreb, Croatia;* [2]*Department of Agroenvironmental Sciences and Technologies, University of Bologna, Viale Fanin 42, 40127, Bologna, Italy;* [3]*Max Planck Institute Ladenburg, Schwabenheimer Str. 101, D-69221, Dossenheim, Germany;* [4]*Federal Biological Research Centre for Agriculture and Forestry (BBA), Schwabenheimer Str. 101, D-69221, Dossenheim, Germany;* [5]*Department of Molecular Biology, Ruđer Bošković Institute, Bijenička 54, 10000, Zagreb, Croatia; *Author for Correspondence (Phone: +385-1-239-3731; Fax: +385-1-239-3730; E-mail: edermic@agr.hr)*

Accepted 11 January 2006

Key words: fire blight, pathogenicity, PFGE, SSR

Abstract

Erwinia amylovora is the causative agent of fire blight, a destructive disease of rosaceous plants. The European population can be divided into several subtypes according to differences in restriction fragment length polymorphism of the *Xba*I genomic DNA digest analysed with pulsed-field gel electrophoresis. This technique was also used to determine the genetic relatedness of six Croatian isolates to the *E. amylovora* types found in the countries surrounding Croatia. The isolates belong to the Pt2 pattern type that is characteristic of the East Mediterranean basin. All tested isolates gave essentially the same total cell protein pattern in SDS-polyacrylamide gel electrophoresis. The number of short-sequence DNA repeats in plasmid pEA29 of six isolates was determined by PCR assays and ranged from four to seven. The isolates examined showed high pathogenicity in immature pear fruits. Differences were also revealed in microbiological assays such as amylovoran synthesis, levan formation, siderophore production and colour on coliform medium.

Erwinia amylovora is the bacterium that causes fire blight, a destructive disease of apple and pear fruit trees as well as many other rosaceous plants. Severe losses, especially for apple and pear production, are due to partial destruction or death of infected plants. In Europe, the disease was first detected in 1955 in England and since then it has spread to most European countries (Bonn and van der Zwet, 2000). The symptoms of fire blight were first observed in Croatia in 1995, in the eastern part of the country near the city of Osijek (Cvjetković et al., 1996). Although many quarantine measures were undertaken, the disease spread to the adjacent counties in the West. The bacterium can be transmitted by many vectors such as insects, birds, wind and infected or contaminated plant material (van der Zwet and Beer, 1995). Commercial exchanges of plant material can play an important role in the dissemination of the bacterium. Characterization of the natural populations of *E. amylovora* can help to trace its spread and the ways the pathogen was dispersed. One of the methods used for differentiating bacterial strains is comparison of their total cell protein patterns obtained through polyacrylamide gel electrophoresis (Kersters and De Ley, 1975) and it was used earlier for the characterization of some *E. amylovora* strains (Vantomme et al., 1982). Recently, *E. amylovora* isolates have been characterized by assaying restriction fragment length polymorphism (RFLP) of their genomic DNA, using pulsed-field gel electrophoresis (PFGE)

(Zhang and Geider, 1997; Zhang et al., 1998; Jock et al., 2002; Jock and Geider, 2004). The PFGE patterns indicated sequential dissemination of the pathogen from different geographical origins in Europe and the Mediterranean region. The PFGE patterns were assigned to types Pt1 to Pt4 and to minor pattern types Pt5 and Pt6 (Jock et al., 2002). Fire blight, caused by *E. amylovora* strains of different PFGE pattern types, has been reported in several countries neighbouring Croatia (e.g. Hungary, Serbia and Montenegro, Italy, Slovenia), and also Austria, Greece and Albania. In central Europe (e.g. Austria) strains of Pt1 type prevail, and Pt2 in Hungary and in the Mediterranean basin. In north-eastern Italy, Pt3 pattern type strains dominated, which was connected to plant imports (Zhang et al., 1998). Since Croatia is one of the regions where zones with different PFGE pattern types may overlap, the aim of this study was to characterize *E. amylovora* isolates from Croatia and, by comparing them with the strains from other European regions, to reveal the possible origin(s) of fire blight in Croatia. Strains of *E. amylovora* can be distinguished by means of differences in size of the 1-kb PCR fragment obtained from plasmid pEA29 (Lecomte et al., 1997). Different numbers of a short-sequence DNA repeats (SSR) were shown to be responsible for the size variations in this DNA fragment, where the nucleotide sequence 'ATTACAGA' was repeated from 3 to 15 times (Kim and Geider, 1999). The change in the number of repeats of SSR motif can be a result of stress (e.g. environmental pressure), but is rare during normal propagation. The SSR-number can define individual strains after their isolation if they were propagated under mild laboratory conditions (Jock et al., 2003). The number of SSR repeats in *E. amylovora* strains from Croatia was checked, but also growth morphology on various semi-selective media to distinguish the isolated strains from each other.

During four years of the survey (1998–2001), we isolated 40 *E. amylovora* strains from different host plants with fire blight symptoms at various locations in the continental part of Croatia. Isolations were made from the margins of blighted tissue of different plant organs (e.g. fruits, leaves, shoots) on nutrient agar (NA) (Difco Laboratories, USA) and the isolates were identified as *E. amylovora* by using conventional laboratory methods (Schroth and Hildebrand, 1988). All isolates of *E. amylovora* had similar colony morphology and colour on plates containing NA; they did not produce fluorescent pigment on the KB medium (King et al., 1954) and their oxidase reaction was negative. All of them were also serologically identified as *E. amylovora* by ELISA-DASI enrichment method using monoclonal antibodies (Plant-Print Diagnòstics, Spain) according to Gorris et al. (1996). To confirm identification, the polymerase chain reaction (PCR) analysis was performed with a pair of primers P29A and P29B, specific for plasmid pEA29 according to Bereswill et al. (1992). The expected 1 kb DNA fragment was amplified from all 40 isolates indicating that all harboured pEA29, common for *E. amylovora*. The pathogenicity of the isolates EaCro listed in Table 1 was evaluated by the inoculation of immature pear fruits (Schroth and Hildebrand, 1988) and with a hypersensitive response (HR) assay on *Nicotiana tabacum* var. *xanthi*, according to Klement (1963). All isolates induced a HR on tobacco leaves and caused development of necrosis followed by the appearance of exudate droplets on infected tissue of immature pear fruits. The dynamics of necrosis development and amount or appearance of exudate was similar for all isolates and agreed with pathogenicity on immature pear fruits.

The *E. amylovora* isolates were further characterized by total cell protein electrophoretic profiles and by pulsed-field gel electrophoresis of their genomes. Total cell protein extracts from six *E. amylovora* isolates (EaCro; Table 1) were separated using discontinuous sodium dodecyl sulphate (SDS)-polyacrylamide gel electrophoresis (Laemmli, 1970; Kersters and De Ley, 1975; Jackman, 1985). The strains from Croatia matched strain Ea OMP-BO 1077.7/94 from Italy (data not shown) confirming a previous report (Vantomme et al., 1982) that *E. amylovora* is a homogenous species.

PFGE analysis of strains from Europe and the Mediterranean basin revealed several RFLPs of *Xba*I-digested chromosomal DNA (Zhang and Geider, 1997) and clustered into three groups corresponding to their geographic origin (Zhang et al., 1998). Their analysis revealed that three Croatian isolates analysed belonged to the Mediterranean group Pt2. They originated from the same location (town of Osijek) and they were isolated in 1996 from hawthorn and pear. Additionally, the isolate from pear had 8 SSR repeats,

Table 1. Properties of isolates from Croatia, compared to other *Erwinia amylovora* strains

E. amylovora strain (country, host, year of isolation):	Fe	LE	Cf	M2	SSR
1. Ea1/79 (Germany, Cotoneaster sp.)	6	+	+	+	6
2. Ea1/79Sm (Sm-resistant)	5	(+)	+	+	6
3. Ea1/79HR1 (from HR lesion)	8	++	++	+	6
4. Ea1/79SSR5 (with change in SSR)	7	++	++	++	5
5. Ea266 (Canada)	8	++	++	++	7
6. EaCro8DJ (Croatia, Đakovo, *Malus* sp., 1998)	8	+	++	+	6
7. EaCro16K (Croatia, Kutjevo, *Pyrus communis*, 1999)	4	+	–	+	7
8. EaCro29J (Croatia, Jarmina, *Malus* sp., 2000)	6	(+)	++	+*	6
9. EaCro54SP (Croatia, Sesvete, *Cydonia oblonga*, 2000)	3	+	+	+	5
10. EaCro58VE (Croatia, Vetovo, *Mespilus* sp., 2000)	3	+	++	+*	5
11. EaCro65VT (Croatia, Virovitica, *Malus* sp., 2000)	3	+	+	+*	4
12. Ea345.90 (Switzerland)	8	+	++	++	4
13. Ea508.94 (Switzerland)	6	+	+	+*	7

Fe: Siderophore formation on Fe-CH agar (after 1 d) [labelling: diameter of halo in mm]; LE: Levan formation on LBsucrose (after 2 d) [size of colony with levan; ++, large; +, intermediate; (+), small]; Cf: coliform agar (after 2 d) [colour of colony: ++, pink; +, slightly pink; – ,white]; M2: yellow colour and mucoidy of colonies on MM2Cu (after 3 d) [++, fluidal; +, colonies with intermediate or low (*) mucoidy]; SSR, repeat numbers revealed through polyacrylamide gel analysis [compared to migration of PCR fragments from *E. amylovora* strains with sequenced SSR regions].

while the other two isolates, derived from hawthorn, had 5 SSR repeats (Kim and Geider, 1999). The infected area has since significantly enlarged and fire blight now occupies a large part of continental Croatia. In this work, we analysed *E. amylovora* strains isolated from four different host plants and from six locations within the infected areas. Genomic DNA of *E. amylovora* isolates (EaCro; Table 1) was digested with *Xba*I restriction enzyme and the digests were assayed by pulsed-field gel electrophoresis (PFGE) as described earlier (Zhang and Geider, 1997; Zhang et al., 1998; Jock et al., 2002). Electrophoretic bands of these isolates were identical with reference strain Ea4/82, isolated in Egypt (Figure 1); therefore the strains belong to the East European/East Mediterranean PFGE pattern type Pt2. PFGE analysis confirmed that these isolates from Croatia were homogenous and highly related, although Croatia is situated in a region where other PFGE pattern types occur in neighbouring countries. Accordingly, fire blight had probably spread into Croatia from one or a few origins of the east of the Mediterranean region. Only isolate EaCro16K (Figure 1, lane 3) had a slightly different pattern in PFGE analysis with an additional low molecular weight DNA fragment of about 20 kb while all other bands were identical with those found in strains of Pt2 pattern type. Previously, one or two low molecular weight bands of 50 kb or less were found for pattern types Pt1, Pt3 and Pt4, and these deviations were named Pt1s, Pt3s

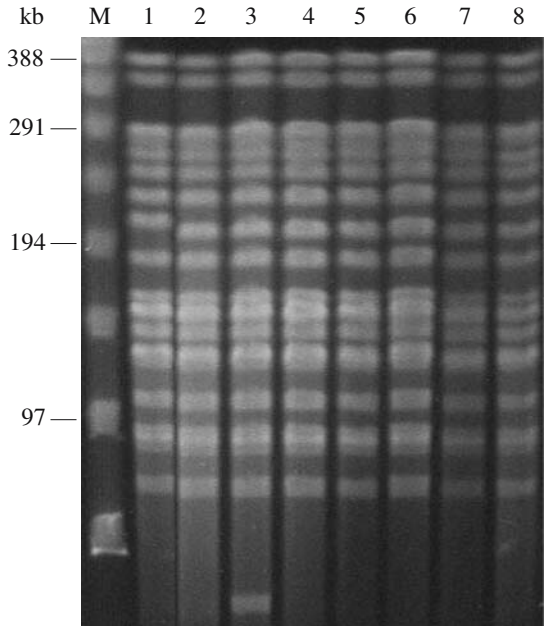

Figure 1. PFGE analysis of *Erwinia amylovora* natural isolates from Croatia. *Xba*I-digested genomic DNAs were analysed and their RFLP monitored. Lane 1, (reference strain Ea1/79 from Germany, Pt1); 2, (EaCro8DJ); 3, (EaCro16K); 4, (EaCro29J); 5, (EaCro54SP); 6, (EaCro58VE); 7, (EaCro65VT); 8, (reference strain Ea4/82 from Egypt, Pt2). M, λ DNA marker (sizes in kb are at the left margin). The origins of the EaCro isolates are described in Table 1.

and Pt4s (Jock et al., 2002). This change can be due to the presence of a plasmid or can be derived from base pair changes in the genome. The pattern type of isolate EaCro16K was named Pt2s and, to our knowledge, it is the first report of such a fragment in isolates of the pattern type Pt2. Further analysis is required to determine the nature of this RFLP band.

In order to differentiate six EaCro strains (Table 1), they were assayed on various media. The morphology of the colonies was observed on the semi-selective medium MM2Cu (Bereswill et al., 1998) for yellow colonies and amylovoran synthesis, as well as on CCT agar (Ishimaru and Klos, 1984) and on nutrient agar enriched with 5% sucrose (LBsuc) for levan production. After three days of growth on CCT medium the colonies were smooth, about 5 mm in diameter and light blue opalescent with regular margins as expected for *E. amylovora* colonies. On minimal medium MM2Cu *E. amylovora* colonies are yellow and most of them show mucoid appearance which is characteristic for *E. amylovora* strains (Bereswill et al., 1998). Six EaCro isolates plated on the semi-selective medium MM2Cu developed yellow colonies after 3 d of growth, but three strains (EaCro29J, EaCro58VE and EaCro65VT) were not significantly mucoid (Table 1). Prolonged incubation of the MM2Cu agar plates yielded colonies of high or intermediate mucoidy including the strains from Croatia with low amylovoran synthesis after 3 d of incubation (Figure 2). After two days of growth on LBsuc agar the colonies were dome-shaped, white and shiny, typical of levan producing *E. amylovora* except that of strain EaCro29J with low levan formation. The Fe-CAS-HDTMA agar was prepared according to Schwyn and Neilands (1987) with glucose as the carbon source and a low phosphate medium instead of MM9 salts. The rapid diagnosis (RD) medium (Kritzman et al., 2003) was the commercial preparation of the coliform agar (Merck, Germany). Only strain EaCro16K did not show the typical pink colony colour on Cf agar. All assays on different media were performed twice and the morphology of the colonies was unchanged in both experiments. With the growth morphology on different agar types, typical pattern morphology can be assigned to individual *E. amylovora* from Croatia. Three strains were barely mucoid on MM2Cu-agar. This feature is a stable characteristic and has also been found for other *E. amylovora* strains (Bereswill et al., 1998; Jock et al., 2000) and is associated with a strain. Hignett (1988) assumed a requirement for capsulated and uncapsulated cells for efficient interaction of *E. amylovora* with plant tissue. Although both phenotypes can be isolated from the same plant, low EPS producers are still virulent in contrast to *ams* mutants (Bugert and Geider, 1995), which are non-virulent on fire blight host plants. Levan synthesis can also vary between *E. amylovora* strains and low producers occur naturally (Bereswill et al., 1997). Levan synthesis is totally absent for the Asian pear pathogen *Erwinia pyrifoliae* (Rhim et al., 1999) and the related *Erwinia* strains from Japan (Kim et al., 2001).

In addition to divergent, but slowly changing PFGE patterns, a relatively frequent change of short sequence DNA repeats (SSRs) in the 1 kb *Pst*I fragment from plasmid pEA29 is a molecular tool for strain differentiation (Jock et al., 2003). In some strains the SSR numbers remained constant under laboratory conditions as well as under natural conditions over several years (Ruppitsch et al., 2004). Most frequent SSR repeat numbers for previously analysed *E. amylovora* strains from Europe are 5, 6 and 7 (Jock et al., 2003; Ruppitsch et al., 2004). Six isolates of *E. amylovora* (EaCro; Table 1) were analysed on polyacrylamide gel for their short sequence DNA repeats (SSR) numbers using the PCR primers RS1 and RS2c (Kim and

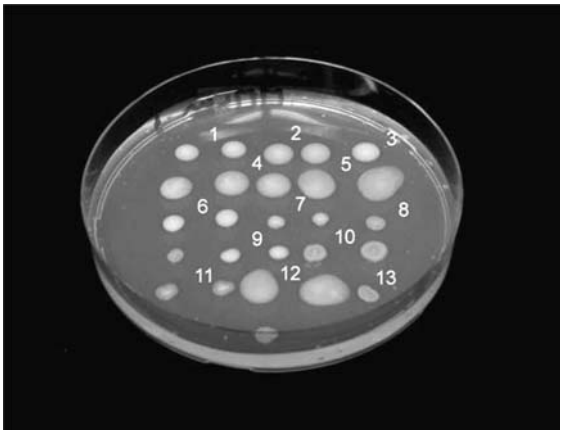

Figure 2. Growth morphology of *Erwinia amylovora* strains from Croatia and other countries on MM2Cu agar. The plates were incubated for a prolonged time (6 d) to allow extensive amylovoran synthesis of the colonies. The order of the strains (from upper left corner down to right corner) is identical with the listing in Table 1 (each strain was transferred in duplicate).

Geider, 1999) in order to distinguish them in more detail (Table 1). Within analysed strains from Croatia, those with 5 SSR repeats prevailed. Nevertheless, the occurrence of several SSR numbers indicates non-homogenous *E. amylovora* isolates in Croatia.

Under laboratory conditions, the microbiological properties of the strains seem to be remarkably stable. Field conditions could influence these features providing a tool for distinction of the isolates. It is impossible to trace the properties found on plates or for the SSR numbers back to a defined time before strain isolation. A conclusion about changes before introduction into Croatia is therefore speculative. Grouping the *E. amylovora* strains from Croatia into PFGE pattern type Pt2 is the strongest support for their East Mediterranean origin.

Acknowledgements

This work was supported by the Croatian Ministry of Science, Education and Sports (grant 0178042) and a scholarship from the Italian Ministry of Foreign Affairs (259EM929; 25521/99) to E.H. We thank M. Mohammadi for SSR assays.

References

Bereswill S, Jock S, Aldridge P, Janse JD and Geider K (1997) Molecular characterization of natural *Erwinia amylovora* strains deficient in levan synthesis. Physiological and Molecular Plant Pathology 51: 215–225.

Bereswill S, Jock S, Bellemann P and Geider K (1998) Identification of *Erwinia amylovora* by growth in the presence of copper sulfate and by capsule staining with lectin. Plant Disease 82: 158–164.

Bereswill S, Pahl A, Bellemann P, Zeller W and Geider K (1992) Sensitive and species-specific detection of *Erwinia amylovora* by polymerase chain reaction analysis. Applied and Environmental Microbiology 58: 3522–3526.

Bonn WG and van der Zwet T (2000) Distribution and economic importance of fire blight. In: Vanneste J (ed.) Fire Blight: The Disease and its Causative Agent *Erwinia amylovora* (pp. 37–53) CABI Publishing, New York, Wallingford, Oxon.

Bugert P and Geider K (1995) Molecular analysis of the *ams*-operon required for exopolysaccharide synthesis of *Erwinia amylovora*. Molecular Microbiology 15: 917–933.

Cvjetković B, Šubić M and Žlof V (1996) Prvi nalaz bakterijske paleži (*Erwinia amylovora*) u Hrvatskoj. Glasnik zaštite bilja 1: 13–14.

Gorris MT, Cambra M, Lecomte P, Llop P, Chartier R, Paulin JP and López MM (1996) A sensitive and specific detection of *Erwinia amylovora* based on the ELISA-DASI enrichment method with monoclonal antibodies. Acta Horticulturae 411: 41–46.

Hignett RC (1988) Effects of growth conditions on the surface structures and extracellular products of virulent and avirulent forms of *Erwinia amylovora*. Physiological and Molecular Plant Pathology 32: 387–394.

Ishimaru C and Klos EJ (1984) New medium for detecting *Erwinia amylovora* and its use in epidemiological studies. Phytopathology 74: 1342–1345.

Jackman PJH (1985) Bacterial taxonomy based on electrophoretic whole-cell protein patterns. In: Goodfellow M and Minnikin DE (eds.) Chemical Methods in Bacterial Systematics (pp. 115–128) Academic Press, London.

Jock S and Geider K (2004) Molecular distinction of American *Erwinia amylovora* strains and of two Asian pear pathogens by analysis of PFGE patterns and *hrpN* genes. Environmental Microbiology 6: 480–490.

Jock S, Donat V, López MM, Bazzi C and Geider K (2002) Following spread of fire blight in Western, Central and Southern Europe by molecular differentiation of *Erwinia amylovora* strains with PFGE analysis. Environmental Microbiology 4: 106–114.

Jock S, Jacob T, Kim W-S, Hildebrand M, Vosberg H-P and Geider K (2003) Instability of short-sequence DNA repeats of pear pathogenic *Erwinia* strains from Japan and *Erwinia amylovora* fruit tree and raspberry strains. Molecular and General Genomics 268: 739–749.

Jock S, Rodoni B, Gillings M, Kim W-S, Copes C, Merriman P and Geider K (2000) Screening of ornamental plants from the Botanic Gardens of Melbourne and Adelaide for the occurrence of *Erwinia amylovora*. Australasian Plant Pathology 29: 120–128.

Kersters K and De Ley J (1975) Identification and grouping of bacteria by numerical analysis of their electrophoretic protein patterns. Journal of General Microbiology 87: 333–342.

Kim W-S and Geider K (1999) Analysis of variable short-sequence DNA repeats on the 29 kb plasmid of *Erwinia amylovora* strains. European Journal of Plant Pathology 105: 703–713.

Kim W-S, Hildebrand M, Jock S and Geider K (2001) Molecular comparison of pathogenic bacteria from pear trees in Japan and the fire blight pathogen *Erwinia amylovora*. Microbiology 147: 2951–2959.

King EO, Ward MK and Raney DE (1954) Two simple media for the demonstration of pyocyanin and fluorescein. Journal of Laboratory and Clinical Medicine 44: 301–307.

Klement Z (1963) Rapid detection of the pathogenicity of phytopathogenic pseudomonads. Nature 199: 299–300.

Kritzman G, Shwartz H, Marcus R, Manulis S, Klietman F, Oppenheim D, Zilberstaine M and Shtienberg D (2003) Testing a rapid diagnostic medium for *Erwinia amylovora* and development of a procedure for sampling blossoms in pear orchards. Phytopathology 93: 931–940.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.

Lecomte P, Manceau C, Paulin JP and Keck M (1997) Identification by PCR analysis on plasmid pEA29 of isolates of *Erwinia amylovora* responsible of an outbreak in Central Europe. European Journal of Plant Pathology 103: 91–98.

Rhim S-L, Völksch B, Gardan L, Paulin J-P, Langlotz C, Kim W-S and Geider K (1999) *Erwinia pyrifoliae*, an *Erwinia* species different from *Erwinia amylovora*, causes a necrotic disease of Asian pear trees. Plant Pathology 48: 514–520.

Ruppitsch W, Stöger AR and Keck M (2004) Stability of short sequence repeats and their application for the characterization of *Erwinia amylovora* strains. FEMS Mirobiology Letters 234: 1–8.

Schroth MN and Hildebrand M (1988) *Erwinia*-''Amylovora'' or non-soft rot group. In: Schaad NW (ed) Laboratory Guide for Identification of Plant Pathogenic Bacteria (pp. 37–43), 2nd edn, APS Press, The American Phytopathological Society, St. Paul.

Schwyn B and Neilands JB (1987) Universal chemical-assay for the detection and determination of siderophores. Analytical Biochemistry 160: 47–56.

van der Zwet T and Beer SV (1995) Fire Blight – its nature, prevention and control. A practical guide to integrated disease management. USDA Agriculture Information Bulletin No. 631. USDA, Washington.

Vantomme R, Swings J, Goor M, Kersters K and De Ley J (1982) Phytopathological, serological, biochemical and protein electrophoretic characterization of *Erwinia amylovora* strains isolated in Belgium. Phytopathologische Zeitschrift 103: 349–360.

Zhang Y and Geider K (1997) Differentiation of *Erwinia amylovora* strains by pulsed-field gel electrophoresis. Applied and Environmental Microbiology 63: 4421–4426.

Zhang Y, Merighi M, Bazzi C and Geider K (1998) Genomic analysis by pulsed-field gel electrophoresis of *Erwinia amylovora* strains from the Mediterranean region including Italy. Journal of Plant Pathology 80: 225–232.