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## Short Notes

# First report of *Erwinia amylovora* in Tuscany, Italy

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**Summary.** 2-years-old plants of *Pyrus communis* showing symptoms of fire blight disease were sampled in an orchard in Tuscany (Italy) during Autumn 2020. Plants were obtained the previous spring from a commercial nursery located in a region where the disease is present since 1994. The collected material was processed in the lab in order to verify the presence of the bacterium *Erwinia amylovora*, the causal agent of fire blight. Pure isolates showing white mucoid colonies and levan producers on Lev-an medium were putatively assimilated to *E. amylovora*. DNA was extracted from the cultures and analysed with three molecular assays, including duplex PCR of the 29-Kb plasmid pEA29 and the *ams* chromosomal region, sequencing of the 16S rDNA and *recA* gene regions, two real-time PCR assays on symptomatic plant tissues. All tests confirmed the presence of *E. amylovora*. Symptomatic and surrounding plants were removed and immediately destroyed according to the regional phytosanitary protocol. This outcome poses a serious threat for fruit orchards in the area.

**Keywords.** Fire blight, AJ75/AJ76 and AMSbL/AMSbR primers, *recA* gene.

Fire blight symptoms were observed on 2-year-old pear trees (*Pyrus communis* ‘Williams’ and ‘Red Williams’) in October 2020 during an orchard phytopathological survey in south-eastern Tuscany (43.37966 N, 11.81162 E), an important fruit production area.

Plants were obtained in early spring 2020 from a fruit tree nursery located in a “protected area” within the Emilia Romagna region, where the pathogen causing this disease has been present since 1994 (EPPO, 114/1995). Plant material was standard, i.e. without phytosanitary certification proving disease-free status. At the time of the survey, the young pear trees showed a variety of symptoms, including shoot blight, stem canker or complete die-back, dried terminal shoots and shepherd’s crook (Figure 1). During autumn 2020, 21 infected plants were observed (19 *Pyrus communis*, two *Malus domestica*) out of more than 400 plants surveyed.



**Figure 1.** Symptoms of shoot blight and stem canker on symptomatic *Pyrus communis* 'Williams' and 'Red Williams' plants sampled during the survey.

**Table 1.** Isolates of *Erwinia* species used in this study. Sequence numbers in bold font were obtained in the present study.

Taxa	Strain	GenBank		Reference
		16S	<i>recA</i>	
<i>Erwinia amylovora</i>	1540	Z96088		Young and Park (2007)
	FB1-DZ		JN812979	Laala <i>et al.</i> (2012)
	ATCC15580	U80195		Arriel <i>et al.</i> , 2014
	IL6		AY217068	Geider <i>et al.</i> , (2006)
	IPSP_EA_001	MW786972	MW916100	This study
<i>E. chrysanthemi</i>	MAFF311151		AB713694	Suharjo <i>et al.</i> , (2014)
<i>E. mallotivora</i>	5705	Z96084	DQ859877	Young and Park (2007)
<i>E. persicina</i>	12532	Z96086	DQ859883	
	XHL2002230201	MT568607		Li <i>et al.</i> , 2021
<i>E. piriflorinigrans</i>	CFBP 5888 strain CECT 7348		GQ421460	López <i>et al.</i> , 2011
	CFBP 5882		GQ421461	
<i>E. psidii</i>	8426	Z96085	DQ859878	Young and Park (2007)
<i>E. pyrifoliae</i>	Ep1/96	AJ009930		Arriel <i>et al.</i> , 2014
	Ep16/96		AY217072	Geider <i>et al.</i> , (2006)
	14143	EF122435	DQ859885	Young and Park (2007)
<i>E. rhapontici</i>	ICMP 1582	Z96087	DQ859882	
	SUPP 355		LC406869	Tsuji <i>et al.</i> , 2020
<i>E. tasmaniensis</i>	Et4/99		AM292088	Geider <i>et al.</i> , (2006)
<i>E. tasmaniensis</i>	Et1/99		AM055718	
<i>E. tracheiphila</i>	5845	Y13250	DQ859879	Young and Park (2007)

Symptomatic shoot samples were collected, transferred to the laboratory on ice, and were processed for bacteria isolation and molecular analyses. Tissue samples were processed according to EPPO standard protocol (2013), as described below. Fragments of symptomatic tissues were surface sterilized in 1% NaClO solution, washed in sterilized distilled water and then ground in an antioxidant maceration buffer. Macerated tissues were enriched in liquid King's B medium (Sigma-Aldrich) and were incubated at 25°C for 48 h. To obtain single colonies, enriched suspensions were streaked onto Levan medium and nutrient glucose agar (NGA; 28 g L<sup>-1</sup> nutrient agar, 5 g L<sup>-1</sup> glucose: Oxoid), and incubated at 27°C for 48–72 h. Bacterial isolates were selected on the basis of colony morphology, and were purified and evaluated by KOH tests (Buch, 1982) to identify Gram negative bacteria. Isolates showing white mucoid colonies and levan production on Levan medium, were identified as putative *Erwinia amylovora*.

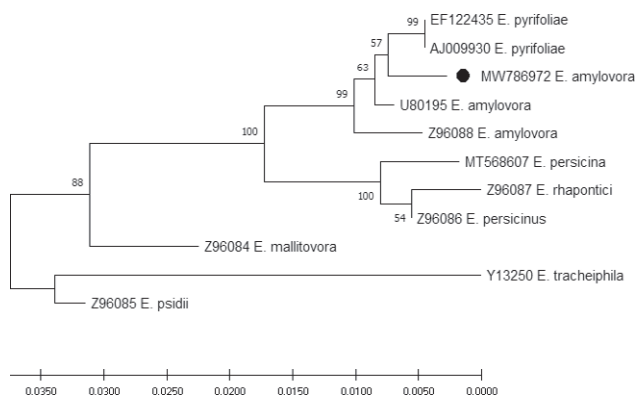
DNA extracted (Wizard® Genomic DNA Purification Kit: Promega) from the bacterial cultures was amplified by duplex PCR, as described by Hannou *et al.* (2013), using primers AJ75/AJ76 (844 bp fragment from the 29-Kb plasmid pEA29) and AMSbL/AMSbR (1.6-Kbp fragment from the *ams* chromosomal region). Both fragments, specific for *E. amylovora*, were successfully amplified.

The 16S rDNA and *recA* genes were amplified and sequenced using primers fD1 and rP1/rP2 (fD1: 5'-AGAGTTTGATCCTGGCTCAG-3'; rP1/rP2: 5'-GGYTACCTTGTTACGACTT-3'; Weisburg *et al.*, 1991), *recA1* and *recA2* (*recA1*: 5'-GGTAAAGGGTC-TATCATGCG-3'; *recA2*: 5'-CCTTCACCATACAT-AATTTGGA-3'; Waleron *et al.*, 2008).

Sequencing of 16S rDNA (GenBank accession no. MW786972; 1392 bp; Table 1) showed 99.61% similarity with *E. amylovora* (GenBank accession no. FN666575, isolate ATCC 49946). Sequencing of *recA* (GenBank accession no. MW916100; 711 bp; Table 1) showed 100% similarity with this reference *E. amylovora* isolate. Similarity with *E. pyrifoliae* (GenBank accession no. FP236842, strain Ep1/96) was of 99.21% for 16S and 96.18% for *recA*. Additional 16S and *recA* gene sequences from different strains of *E. amylovora*, and of different *Erwinia* species used for comparison, were obtained from NCBI (Table 1). Alignments were made using Geneious Prime (version 11.0.9) and phylogenetic analysis was performed with MEGA (version 10.2.2), using the Maximum Likelihood method and Tamura-Nei model. The trees obtained for both genes confirmed the species identification (Figures 2 and 3).

The presence of the pathogen was also confirmed by extraction of DNA with CTAB 2% (Li *et al.*, 2008) and





**Figure 2.** Phylogenetic analysis based on 16S rDNA gene sequences from diverse *Erwinia* spp. strains. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the greatest log likelihood (-2704.47) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Number of bootstrap replicates = 500. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 11 nucleotide sequences. There were a total of 1285 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). The sequence obtain in the present study is marked with a black dot.

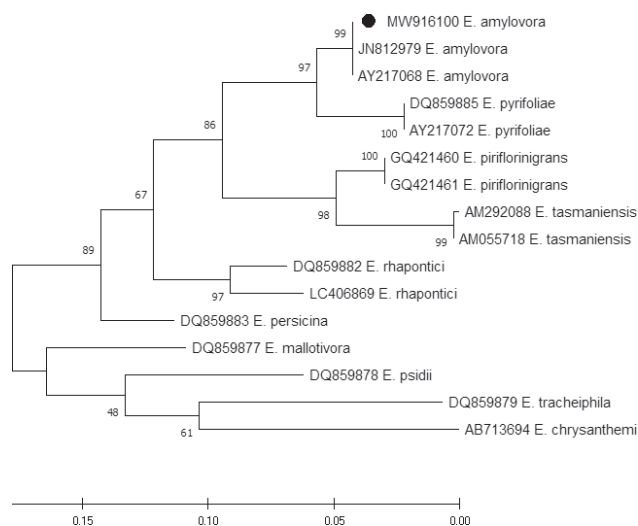
two real-time PCR protocols according to Gottsberger (2010) and Pirc *et al.* (2009) carried on the symptomatic plant tissue from which the pathogen had been isolated. All processed samples were identified as *E. amylovora*.

All symptomatic plants were removed and destroyed. In order to check for others possible outbreaks intensive monitoring started in the original orchard and the surrounding areas.

This is the first report of *E. amylovora* in Tuscany. This pathogen may pose a serious threat to apple and pear production in this area. Presence of this pathogen in Tuscany is also a clear example of the spread of a quarantine pathogen by the plants-for-planting pathway. This record supports the need to use certified plant material, especially when nurseries are located in protected areas.

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**Figure 3.** Phylogenetic analysis based on *recA* gene sequences from diverse *Erwinia* spp. strains. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model (Tamura and Nei, 1993). The tree with the greatest log likelihood (-1905.63) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Number of bootstrap replicates = 500. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Tamura-Nei model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. This analysis involved 16 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. There was a total of 399 positions in the final dataset. Evolutionary analyses were conducted in MEGA X (Kumar *et al.*, 2018). The sequence obtain in the present study is marked with a black dot.

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