

# A pith necrosis caused by *Xanthomonas perforans* on tomato plants

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Accepted: 17 April 2013 / Published online: 30 April 2013  
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**Abstract** Pith necrosis is a common disease of tomato in Europe, mainly caused by *Pseudomonas corrugata* and other soil-borne species of *Pseudomonas*. During 2011–2012 a survey was conducted in soil-grown tomato crops in southeastern Sicily (Italy). Plants showed pith necrosis, brown discolouration of the vascular tissues, leaf chlorosis and sometimes wilting of leaves. Thirty bacterial isolates from symptomatic tissues, forming colonies on NA and KB, were identified by morphological, biochemical and physiological tests. Among them, seven isolates were analyzed for their 16S rDNA and 16S–23S spacer region sequence that resulted in 99 % identity to that of the *Xanthomonas perforans* type strain (GenBank accession number GQ46173 over 2.085 bp.). Additional sequences of *fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA* from one selected isolate were 100% identical to sequences of the *Xanthomonas perforans* type strain. *X. perforans* local

isolates showed similar genomic patterns with REP-PCR and fAFLP, and were clearly distinguished from other *Xanthomonas* spp. type strains. In stem-inoculation assays, bacteria isolated from symptomatic tomato plants identified as *P. fluorescens*, *P. putida*, *P. marginalis*, *P. citronellolis*, *P. straminea*, and *Pantoea agglomerans* induced discolouration of vascular tissues, while *Pectobacterium carotovorum* subsp. *atrosepticum* isolates induced soft rot. Conversely, the isolates here identified as *Xanthomonas perforans* were able to induce pith necrosis, vascular discolouration, longitudinal splits and external lesions on stems. This report of *X. perforans* causing pith necrosis on tomato represents a potentially serious problem that may limit the productivity of tomato crops.

**Keywords** Tomato bacterial diseases · Pith necrosis diagnosis · *Solanum lycopersicum* L

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## Introduction

In Italy, greenhouse fresh market tomato (*Solanum lycopersicum* L.) is a major vegetable crop reaching almost 7.280 ha of cultivated area with a total production of 630.451 t (ISTAT 2011). The areas of Sicily where the cultivation of tomatoes in greenhouses is most developed are located between the province of Syracuse and Ragusa. These areas are characterized by excellent agro-meteorological conditions that allow

the cultivation in unseasonal cycles at low cost as compared to other areas of Italy.

Due to the phase-out of methyl bromide, tomato cultivation in greenhouses has had remarkable phytopathological consequences over the last decade, with an incidence increase of soilborne diseases caused by fungal and bacterial pathogens. Several bacterial species can cause discoloration and necrosis of pith and vascular tissues, associated with leaf yellowing, wilting and premature dying of the whole tomato plants. The main species worldwide associated with pith necrosis is *Pseudomonas corrugata* (Scarlett et al. 1978; Clark and Watson 1986). *P. mediterranea* was later on identified as another pith necrosis pathogen (Catara et al. 2002). These pathogens cause an extensive pith necrosis that appears dry and disaggregated in the core. Other pathogens associated with tomato pith necrosis are *P. marginalis* (Bella and Catara 2009; Kudela et al. 2010), *P. viridiflava* (Alippi et al. 2003; Laysan and Uygun 2005) and *P. cichorii* (Wilkie and Dye 1974), but in such cases the main symptom was internal stem discoloration, with no rot of pith tissues.

Recently, vascular infections caused by bacteria have increased in importance in Sicily. In particular, infections caused by *Pseudomonas fluorescens* and *P. putida* resulted in the occurrence of chlorosis and vascular browning of tomato plants grown in greenhouses, open fields, and in soilless culture (Polizzi et al. 2007). The occurrence and severity of leaf chlorosis and necrosis were also reported to be related to abiotic stresses (e.g. high salinity or extreme temperature in the rhizosphere) which may occur more frequently in eastern Sicily (Castello et al. 2010; Dimartino et al. 2011).

In December 2011, pith necrosis symptoms were observed on soil-grown tomatoes in glasshouses located in Syracuse (Sicily, southern Italy). The plants showed symptoms of yellowing and wilting of lower leaves which progressed upwards, brown areas and longitudinal splits on stems, wilting of leaves and adventitious roots growth, brown discoloration of vascular tissues, and extensive pith necrosis. In a preliminary study (Aiello et al., unpublished), *P. corrugata* and *P. mediterranea* were found not to be associated with these symptoms. Considering the aggressiveness of the disease, the purpose of this study was to identify and characterize the species involved and to assess the pathogenicity of the isolates.

## Materials and methods

### Survey and pathogen isolation

During 2011–2012, a survey was conducted in two greenhouses (each approximately 1,000 m<sup>2</sup>) located in the province of Syracuse (Sicily, southern Italy) on tomato plants with typical symptoms of tomato pith necrosis. Twenty plants from each greenhouse were removed (canopy and root system) and were also evaluated for the root-knot (*Meloidogyne* spp.) and other root diseases. Symptomatic stem tissue samples were dipped for 1 min in 0.6 % of sodium hypochlorite, rinsed in sterile distilled water (SDW) and then blotted on sterile paper towels. Small pieces were placed into culture tubes containing SDW, vortexed and loopfuls of plant tissue suspension were streaked onto plates of nutrient agar (NA, Oxoid, Basingstoke UK) and King's medium B (KB) (King et al. 1954). The plates were incubated at 27 °C and examined after 48 h.

### Biochemical identification of isolates

Bacteria were always isolated from symptomatic tissues on NA and KB media. Thirty isolates were identified by morphological, biochemical and physiological tests. Initial identification of non fluorescent isolates was based on Gram reaction (KOH test), colony morphology, pigment production on yeast dextrose carbonate (YDC) medium, hypersensitive reaction, levan production on NA with 5 % sucrose, oxidase reaction, anaerobic growth, amylolytic and pectolytic activity (Schaad et al. 2001). Fluorescent isolates on KB medium were tested for levan production on NA with 5 % sucrose, oxidase, pectolytic and arginine dihydrolase activity, hypersensitivity reaction on tobacco leaves (LOPAT scheme) according to Lelliot and Stead (1987). The Biolog Identification MicroPlate™ System (Microlog TM System Release 4.2, Biolog Inc., Hayward, USA) was used for the identification of all bacterial isolates.

### Pathogenicity tests on tomato seedlings

The pathogenicity of 30 representative isolates was tested on 4 to 6 week-old tomato seedlings cv. Sir Elyan grown in 14-cm pots containing sterile peat (Table 1). Bacterial suspensions (10<sup>8</sup> CFU ml<sup>-1</sup>) in

**Table 1** Pathogenicity tests of the bacterial isolates on tomato plants cv. Sir Elyan recorded 15 and 90 days after stem inoculations

Strains	Cultivar from which were isolated	Internal stem symptoms <sup>a</sup>	External stem symptoms <sup>b</sup>	Mean length of lesions (cm) after 15 days	Mean length of lesions (cm) after 3 months	Number of wilted plants
<i>Xanthomonas perforans</i>						
1P4S1D	Dartagnan	PN	DL-LS	3.5±0.5	10.6±0.1	0
2P4S1D	Dartagnan	PN	DL-LS	4.3±0.5	11.5±0.1	0
1P4S1	Sir Elyan	PN	DL-LS	5.5±1.1	11.3±0.8	0
2P4S1	Sir Elyan	PN	DL-LS	4.9±0.7	9.6±0.4	2
1P6S1	Sir Elyan	PN	DL-LS	4.3±0.8	11.7±0.7	1
2P6S1	Sir Elyan	PN	DL-LS	6.8±0.5	11.3±0.8	1
4P1S2	Sir Elyan	PN	DL-LS	6.3±0.2	10.2±0.8	2
<i>Pantoea agglomerans</i>						
5P4S1D	Dartagnan	VD	–	1.8±0.5	1.9±0.4	0
5P1S2	Sir Elyan	VD	–	3.1±0.6	NT	0
6P1S2	Sir Elyan	VD	–	2.9±0.6	NT	0
7P1S2	Sir Elyan	VD	–	2.4±0.4	NT	0
8P1S2	Sir Elyan	VD	–	3.1±0.6	NT	0
<i>Pseudomonas straminea</i>						
4P4S1D	Dartagnan	VD	–	1.8±0.4	4.3±0.5	0
2P1S2	Sir Elyan	VD	–	3.0±0.6	4.1±0.5	0
<i>Pectobacterium carotovorum</i> subsp. <i>atrosepticum</i>						
3P2 S1D	Dartagnan	SR	–	–	NT	16
4P2 S1D	Dartagnan	SR	–	–	NT	16
5P2 S1D	Dartagnan	SR	–	–	NT	16
<i>Pseudomonas fluorescens</i>						
1P2S1	Sir Elyan	VD	–	1.5±0.5	NT	0
3P2S1	Sir Elyan	VD	–	2.7±0.5	3.1±0.2	0
4P2S1	Sir Elyan	VD	–	2.6±0.7	NT	0
1P4S2	Sir Elyan	VD	–	1.7±0.1	3.4±0.5	0
<i>Pseudomonas putida</i>						
1P1S2	Sir Elyan	VD	–	3.4±0.6	3.4±0.4	0
7P2S2	Sir Elyan	VD	–	3.4±0.8	3.8±0.8	0
<i>Pseudomonas marginalis</i>						
1P5S1	Sir Elyan	VD	–	1.0±0.3	NT	0
<i>Pseudomonas citroneolis</i>						
1P2S2	Sir Elyan	VD	–	3.7±0.6	NT	0
2P2S2	Sir Elyan	VD	–	3.7±0.5	NT	0
3P2S2	Sir Elyan	VD	–	2.4±0.4	NT	0
4P2S2	Sir Elyan	VD	–	1.7±0.2	1.8±0.2	0
5P2S2	Sir Elyan	VD	–	2.2±0.3	NT	0
6P2S2	Sir Elyan	VD	–	2.7±0.6	NT	0

Eight plants were used in each experiment. Experiments were repeated twice. ± standard deviation

<sup>a</sup> *PN* pith necrosis; *VD* vascular discolouration; *SR* soft rot

<sup>b</sup> *DL-LS* dark-brown lesions and longitudinal splits

*NT* not tested

SDW, prepared by growing the isolates for 24 h at 28 °C on NA, were stem-injected into the pith at the first true leaves using a sterile hypodermic syringe (two injections for each plant). Eight tomato plants were used for each isolate. SDW was used as a negative control. Plants were covered with plastic bags for 48 h and then maintained in a growth chamber at 27 °C and 70 % relative humidity with a photoperiod of 16 h. After 15 and 90 days, symptoms were evaluated and plants were sectioned to determine the presence and length of pith necrosis and vascular discoloration (Table 1). Bacteria re-isolated after short (15 days) and long (90 days) test periods were characterized on the basis of morphological, physiological and biochemical tests.

The yellow-pigmented *Xanthomonas* sp. isolate 1P4S1D found in the present survey was also tested for pathogenicity on tomato seedlings cv. Marmande, Missouri, Ciliegino Marasca, Datterino, and Big Rio Nano following the above described stem-inoculation procedure, and symptoms were evaluated 15 days after inoculation.

The yellow-pigmented *Xanthomonas* sp. isolate 2P4S1 and type strains *X. perforans* NCPPB4321, *X. euvesicatoria* NCPPB2968, *X. vesicatoria* LMG911, and *X. gardneri* NBPPB881 were tested for pathogenicity on tomato seedlings cv. Laetitia and Marinda, on *Capsicum annuum* L. seedlings cv. Airone, and on *Solanum melongena* L. seedlings cv. Birgah. Bacterial suspensions ( $10^8$  and  $10^5$  CFU ml<sup>-1</sup>) were stem-inoculated following the above described procedure, and were also sprayed-inoculated ( $10^8$  CFU ml<sup>-1</sup>) on young leaves previously wounded with carborundum powder. Eight seedlings were used for each isolate and for each cultivar and species. Plants were incubated as previously described and symptom development was observed periodically for 15 days. All experiments were made twice.

#### Molecular identification of *Xanthomonas* isolates by PCR assay

##### DNA extraction

Bacterial DNA was extracted using the DNA Purification Kit (Puregene-Gentra, Minneapolis, MN, USA) following the manufacturers' instructions. The extracted DNA was quantified spectrophotometrically (Eppendorf Biophotometer, Germany). All PCR

reactions were performed in a GeneAmp PCR system 9600 thermal cycler (Perkin Elmer, Norwalk, Conn., USA), using primers and PCR reagents from Invitrogen (Life Technologies, Pasley, UK).

##### PCR assays

Primers 10f (5' AGTTTGATCCTGGCTCAG) and 534r (5' ATTACCGCGGCTGCTGG) (Muyzer et al. 1993), which target the domain *Bacteria*, were used for the preliminary amplification of 16S rRNA gene. Thermal cycling consisted of an initial denaturation at 94 °C for 5 min, three cycles of 1 min at 94 °C, 2 min at 50 °C and 1 min at 72 °C, followed by 35 cycles of 20 s at 94 °C, 1 min at 50 °C and 1 min 72 °C, with a final extension of 10 min at 72 °C, and cooling to 10 °C.

Amplification of the 16S rRNA gene (*rrs*) and the internal transcribed spacer (ITS) located between *rrs* and the 23S rRNA gene (*rrl*) was performed with primers FGPS6-63 (5'-GGA GAG TTA GAT CTT GGC TCA G-3') and FGPL132'-38 (5'-CCG GGT TTC CCC ATT CGG-3') as previously described (Jones et al. 2000; Innes et al. 1995; Ponsonnet and Nesme 1994). To complete the sequencing of both strands of the amplified DNA fragment of about 2,100 bp, an internal sequencing primer (5'-ATA CGG CTA CCT TGT TAC GACT-3') was used. PCR amplifications consisted of an initial denaturation at 95 °C for 1.5 min, 22 cycles of 95 °C for 30 s, the annealing temperature for 30 s, and 72 °C for 4 min. The annealing temperatures were 57 °C for the first four cycles, 55 °C for the next four cycles, and 50 °C for the last 14 cycles. Finally, the mixtures were incubated at 72 °C for 10 min and then at 60 °C for 10 min.

Amplification of portions of the *fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA* genes from *Xanthomonas* was performed with previously designed degenerate primers (Almeida et al. 2010). PCR amplifications consisted of an initial denaturation at 94 °C for 3 min, five cycles at 94 °C for 30 s, 60 °C for 30 s, 68 °C for 45 s, followed by 25 cycles at 94 °C for 30 s, 58 °C for 30 s, 68 °C for 45 s, and final extension at 68 °C for 10 min.

All PCR products were sequenced in both directions (with the exception of part of the GPS6-63 – FGPL132'-38) with the appropriate primers using BigDye Terminator Ready Reaction Mix V 3.1 (ABI) with an ABI PRISM 3730 xl Genetic Analyzer

at the Sequencing Core Facility of BMR Genomics (CRIBI, Padova, Italy)

The resulting electrophorograms were analyzed with the Chromas computer software program (version 1.43; Techelysium Pty Ltd., Tewantin, Australia) and exported to FASTA format. Similarity searches of sequence data were carried out using the National Center for Biotechnology Information Blast Network Server (<http://www.ncbi.nlm.nih.gov/BLAST>) for comparison with known gene sequences in the GenBank (Altschul et al. 1990). Alignments and phylogenetic analysis of 16S rDNA sequences were carried out with Seaview (Gouy et al. 2010) using reference sequences downloaded from SILVA (<http://www.arb-silva.de/>).

#### *Repetitive sequence (REP) PCR*

REP-PCR analysis of isolates with ERIC and BOX primers (Louws et al. 1994) was performed as previously described (Cirvilleri et al. 2005) with some modifications. The ERIC and BOX programs comprised initial denaturation at 95 °C for 7 min, 35 cycles at 94 °C for 1 min, 50 °C for 1 min, 65 °C for 8 min, and a single final extension cycle at 65 °C for 8 min. Amplicons were separated by gel electrophoresis on 1.5 % agarose (BioRad) in 1X TAE buffer at 50 V for 6 h, stained with Gel-Red 1X for 2 h (Biotium, Corporate Place Hayward, Ca, USA), visualized and photographed under UV light with PowerShot G2 (Canon Inc., Lake Success, NY, USA). The 1-Kb DNA ladder (Invitrogen-Life Technologies) was used as a molecular size marker.

#### *fAFLP analysis*

fAFLP analysis was carried out as previously reported by Vos et al. (1995) and modified by Cirvilleri et al. (2007). DNA was digested with FastDigest EcoRI and MseI (Fermentas, Thermo Fisher Scientific Inc., Waltham, MA, USA) and EcoRI and MseI adaptors ligated C using two units of T4 DNA ligase. The PCR was performed using MseI-primer (5'-GATGAG TCCTGAGTAAC-3') and EcoRI-primer (5'-GACTGCGTACCAATTCA-3') labelled at the 5' end with cy5 fluorophore. All PCR reactions were performed in GeneAmp PCR system 9600 (Perkin Elmer) following a previously described protocol (Kassama et al. 2002). T4 DNA ligase, dNTPs and

Taq polymerase were purchased from Invitrogen-Life Technologies (Paisley, United Kingdom) and all primers and oligos were purchased from MWG Biotech Inc. (High Point, NC, USA).

The fAFLP products were separated with a CEQ 8000 Genetic Analysis System automated DNA sequencer (Beckman & Coulter, Fullerton, CA, USA) according to the manufacturer's instructions. The data were analysed using the CEQ 8000 analysis software. Reproducibility was checked repeating fAFLP reactions two times. The threshold for assigning a peak was set to 500 relative fluorescence units and any peak less than this value was not included in the analysis.

The presence and absence of fragments from 60 to 650 bp were scored as a binary matrix. Cluster analysis (UPGMA) was performed exporting the output files to PHYLIP 3.6 software package (Felsenstein 2004). Similarity coefficients were determined using the Dice's coefficient (Dice 1945) and the robustness of the dendrogram was assessed by bootstrap analysis (1,000 repeated samplings) (Felsenstein 1985).

## Results

### Survey and pathogen isolation

The disease symptoms were observed on tomato cv. Sir Elyan and Dartagnan and were documented in both of the greenhouse of the commercial crop surveyed (Fig. 1). These symptoms were detected on approximately 30 % of the plants. Transverse sections of stems revealed extensive pith necrosis and pink or brown discoloration of the vascular tissues (Fig. 1). Longitudinal splits of stems were often observed. Occasionally the plants showed symptoms of swelling at the crown, and internal decay of the lower stem (Fig. 1). Sometimes, internal stem browning along the entire length of the plants was observed. These symptoms usually appeared during the winter and the spring, mainly at ripening time, on mature tomatoes planted early or mid-autumn. On the root system severe symptoms of root-knot nematode (*Meloidogyne* spp.) and corky root infections caused by *Pyrenochaeta lycopersici* were detected. No disease symptoms resembling bacterial leaf spot of tomato were observed during the survey.

Bacteria were always isolated from the internal stem tissue of tomato plants on NA and KB media.



**Fig. 1** Bacterial symptoms observed at Syracuse, Sicily in 2011 on commercial cultivated tomato plants cv. Sir Elyan: yellowing (a); wilting (b); longitudinal split (c); vascular discolouration (d); pith necrosis (e–f)



All bacterial isolates were maintained in 15 % glycerol at  $-80^{\circ}\text{C}$  for long-term storage.

#### Biochemical identification of isolates

Among the 30 bacterial isolates, all were Gram negative, 13 were fluorescent on KB and showed the LOPAT characters of the Va group according to Lelliott and Stead (1987). Isolates were found to be negative for levan production, pectolytic activity and tobacco hypersensitivity, and positive for oxidase and arginine dihydrolase. Using Biolog analysis technique, the 13 fluorescent isolates were identified at species level on the basis of their metabolic fingerprints: four as *P. fluorescens*; two as *P. putida*; one as *P. marginalis*; and six as *P. citronnellis*.

Among the 17 non-fluorescent isolates, two were identified as *P. straminea*, three as *Pectobacterium carotovorum* subsp. *atrosepticum*, five yellow-pigmented isolates on YDC as *Pantoea agglomerans*, and seven yellow-pigmented isolates on YDC as *Xanthomonas* spp. *Xanthomonas* spp. isolates produced round, convex, mucoid, yellow colonies, were

aerobic, oxidase negative, positive for levan production, strongly amylolytic and pectolytic, induced HR on tobacco, and showed metabolic activity on dextrin, N-acetyl-D-glucosaminide, D-galactose, gentibiose, lactulose, acetic acid, cis-aconitic acid, malonic acid, propionic acid, D-alanine, and L-threonine. The results with Biolog GN Database confirmed that the isolates were similar to *Xanthomonas perforans* type strain NCPPB 4321 group A, as described by Jones et al. 2004.

#### Molecular identification of a representative *Xanthomonas perforans*

Isolate 4P1S2 was chosen to represent *X. perforans* obtained strain in the molecular identification study. The ribosomal sequence resulted identical over the overlapping 2064 nucleotides to accessions GQ461739.1 and GQ461740.1 (deposited as *Xanthomonas perforans* strain BC2642 and BC2923, respectively) and to accession AF123091.2 (deposited as *Xanthomonas axonopodis* strain XV938). In a ribosomal gene based phylogenetic analysis comprising type strains of all species of the genus

*Xanthomonas*, isolate 4P1S2 clustered with *X. perforans* and *X. euvesicatoria* (not shown). The sequence of the other five gene fragments (*fusA*, *gapA*, *gltA*, *gyrB*, *lacF*, and *lepA*) were used to interrogate PAMDB (the Plant-Associated Microbes DataBase, Almeida et al. 2010). All sequenced gene fragments were identical to those of the *X. perforans* NCPPB 4321 type strain, confirming the identification of isolate 4P1S2 as *X. perforans*.

#### Repetitive-sequence PCR

ERIC-PCR yielded 12 to 15 distinct and clearly resolved bands ranging in size from approximately 200 bp to over 3,000 bp (Fig. 2a), whereas PCR with BOX primers generated 14 to 16 distinct bands with molecular weights ranging from about 300 bp to over 3,000 bp (Fig. 2b). Both primers yielded highly similar pattern for the isolates tested.

All new isolates exhibited similar ERIC and BOX fingerprints whereas a high degree of genetic diversity was observed among the type strains of the four different *Xanthomonas* species associated with tomato

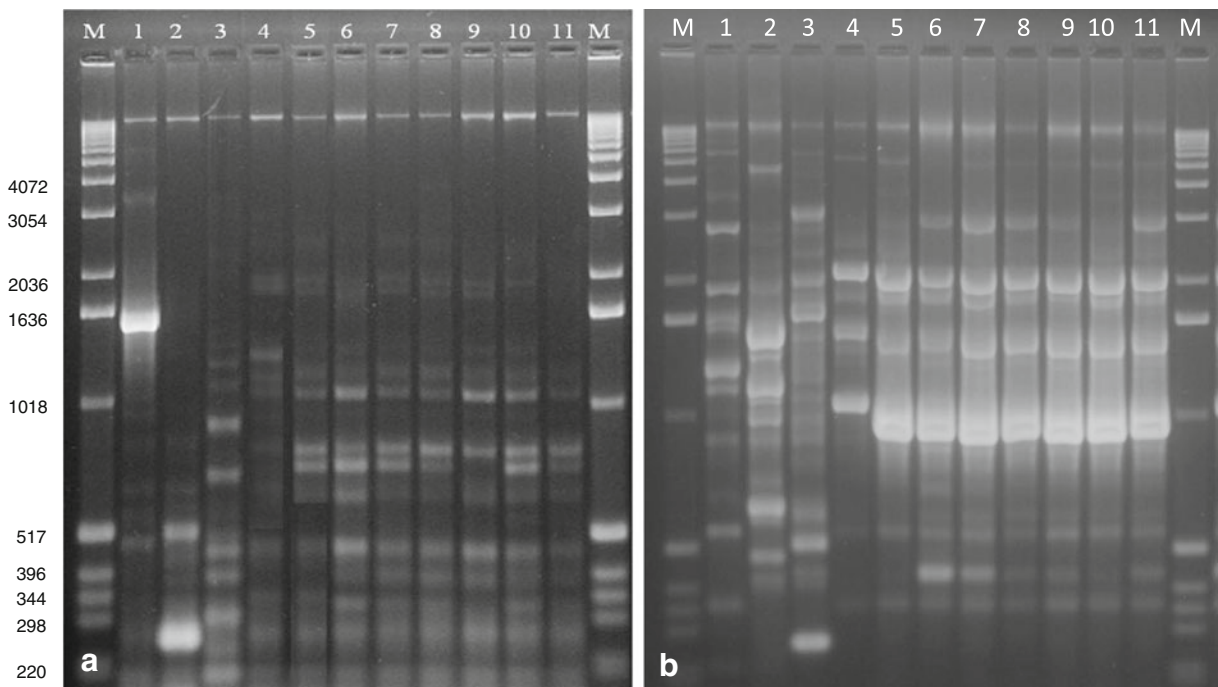
bacterial spot (Fig. 2a and b). They exhibited ERIC/BOX fingerprints that were most similar to *X. perforans* 4321 type strain (Fig. 2a and b). These local isolates showed just few differences in ERIC profiles: isolates 1P6S1, 2P6S1, 4P1S2 were found to lack a band at about 1,200 bp and isolate 2P4S1D presented an additional 1,000 bp PCR product.

#### Fluorescent AFLP analysis

Fluorescent AFLP analysis with the selective primers *MseI*+C and *EcoRI*+A generated a total of 236 fragments for the 11 tested isolates. The sizes of the amplified fragments ranged from 60 to 650 bp, with peak heights that did not vary between replicate runs with identical DNAs.

Dendrogram of *Xanthomonas* isolates analysed by fAFLP showed a main cluster including all *X. perforans* local isolates (Fig. 3), which could be further subdivided into two subclusters with 94 % genetic similarity.

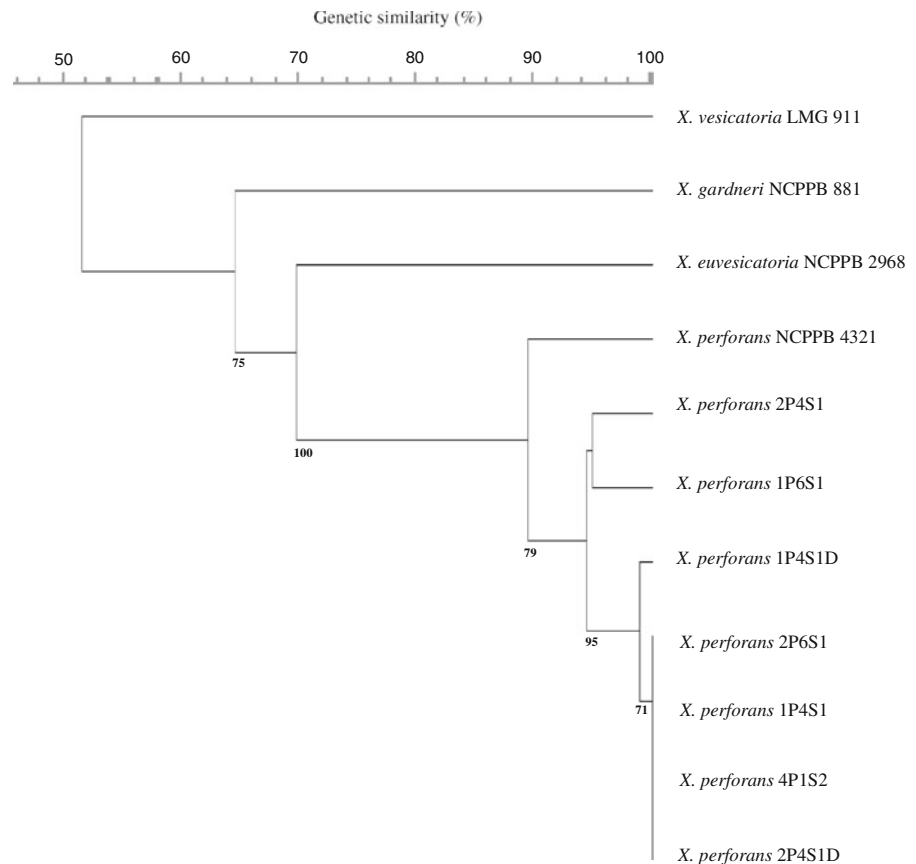
Local *Xanthomonas* isolates shared similar fAFLP genomic profile with *X. perforans* NCPPB 4321 type



**Fig. 2** PCR fingerprinting patterns from genomic DNA of the type strains of the tomato bacterial spot *Xanthomonas* species and the seven *Xanthomonas perforans* isolates associated with pith necrosis (this study) obtained by using ERIC (A) and BOX (B) primer sets. M: molecular size marker (1-kb ladder,

Invitrogen); the sizes are indicated in base pairs. Lane 1: *X. euvesicatoria* NCPPB 2968; lane 2: *X. gardneri* NCPPB 881; lane 3: *X. vesicatoria* LMG 911; lane 4: *X. perforans* NCPPB 4321; lane 5: 1P4S1D; lane 6: 2P4S1D; lane 7: 1P4S1; lane 8: 2P4S1; lane 9: 1P6S1; lane 10: 2P6S1; lane 11: 4P1S2

**Fig. 3** Dendrogram of *Xanthomonas perforans* strains associated with pith necrosis in this study and the type strains of tomato bacterial spot xanthomonads analysed by fluorescent amplified fragment length polymorphism. Profiles in the range of 100–650 bp, were compared by numerical analysis using Phylip 3.6 software. Similarity between fingerprints was calculated with unweighted pair-group method with average linkages and the robustness of the tree was assessed by bootstrap analysis (1,000 repeated samplings)



strain (90 % similarity index). Similarity indexes of local *Xanthomonas* isolates with *X. euvesicatoria* NCPPB 2968, *X. gardneri* NCPPB 881, *X. vesicatoria* LMG 911, were 70 %, 65 %, 52 %, respectively.

Bootstrap analysis supported the separation of the clade comprising *X. perforans* isolates from other species, but not the distinction of the local isolates from each other or from the *X. perforans* type strain (Fig. 3).

#### Pathogenicity tests on tomato seedlings

All 30 isolates tested produced symptoms on the tomato seedlings cv. Sir Elyan, with variable types of symptoms depending on the bacterial species (Table 1).

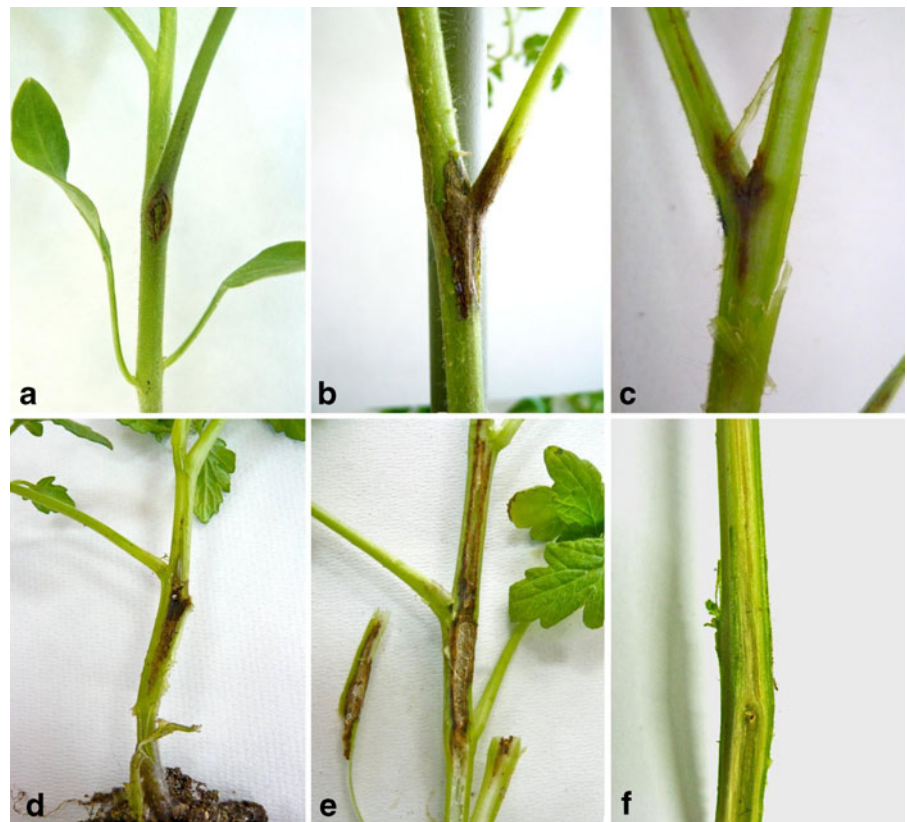
The isolates identified as *X. perforans* appeared to produce a more severe symptoms than those produced by the pseudomonads evaluated as they were able to produce external water-soaked spots and necrotic lesions on stems, besides pith necrosis when stem inoculated (Table 1, Fig. 4). Moreover, these isolates induced adventitious roots as early as 15 days after inoculation (Fig. 4). Ninety days after inoculation the

length of pith necrosis and vascular discolouration was extensive and, occasionally, chlorosis of basal leaves, wilting and death were observed. On the opposite, lesions length induced by *P. fluorescens*, *P. putida*, *P. marginalis*, *P. citronellolis*, *P. straminea*, and *P. agglomerans* increased only slightly (Table 1; Fig. 4f). Symptoms were observed around the inoculation sites, upwards to the apex and downwards to the roots. Longitudinal splits of stems corresponding to pith necrosis were about 3.5–7 cm long, and the length of the lesions varied according to the isolate (Table 1). The same symptoms were induced by *X. perforans* 1P4S1D in the tomato cultivars Marmande, Missouri, Ciliegino Marasca, Datterino, and Big Rio Nano with no relevant difference in lesion length among cvs.

*P. fluorescens*, *P. putida*, *P. marginalis*, *P. citronellolis*, *P. straminea* and *P. agglomerans* isolates, inoculated as control on the same cultivar, induced brown vascular discolouration and adventitious roots 15 days after inoculation, and *P. carotovorum* subsp. *atrosepticum* isolates induced soft rot 3–5 days post inoculation.



**Fig. 4** Symptoms induced on tomato plants cv. Sir Elyan by stem inoculations with *Xanthomonas perforans* 4P1S2 at  $10^8$  CFU ml<sup>-1</sup>: longitudinal split (a); external necrotic lesions (b); internal necrotic lesion (c); pith necrosis formed 15 days after inoculation (d); pith necrosis formed 90 days after inoculation (e) compared with vascular discolouration induced by *P. putida* isolate 7P2S2 (f)



No disease symptoms were observed on water-inoculated plants (negative control).

All *X. perforans* tested isolates and *X. perforans*, *X. euvesicatoria*, *X. vesicatoria* and *X. gardneri* type strains induced pith necrotic symptoms and brown-vascular discolouration when inoculated into stem of tomato cv. Laetitia and Marinda, pepper cv. Airone and eggplant cv. Birgah (Table 2). Symptoms were observed around the inoculation sites and up to the apex and downwards from the site of inoculation to the roots. The same symptoms were induced by *X. perforans* local isolates and *X. perforans*, *X. euvesicatoria*, *X. vesicatoria* and *X. gardneri* type strains when stem-inoculated at  $10^5$  CFU ml<sup>-1</sup>, even if the length of lesions were less extensive (Fig. 5) if compared with those caused by  $10^8$  CFU ml<sup>-1</sup>.

Moreover, all tested isolates induced leaf symptoms on tomato cv. Laetitia and Marinda when sprayed inoculated, with lesions first appearing as water-soaked spots enlarging slightly and becoming necrotic over time. No clear differences in the morphology of leaf lesions or the speed of lesion development after inoculation were noticed among *X. perforans* isolates.

On pepper cv. Airone *X. perforans* tested isolate, *X. perforans* 4321 type strain, *X. euvesicatoria* 911 T and *X. vesicatoria* 2968 type strain were not pathogenic on leaves, while *X. gardneri* 881 type strain produced visible necrotic reactions. On eggplant cv. Birgah all tested isolates were non-pathogenic on leaves.

All pathogenic bacteria were recovered from inoculated tomato plants. Re-isolated bacteria were recognized as identical to the original isolates by Biolog analysis.

## Discussion

In Italy greenhouse fresh market tomato is a major vegetable crop grown on almost 7,280 ha with a total production of 630,451 t (ISTAT 2001). Recently, following the elimination of methyl bromide from the Italian agrochemical market, some substantial changes occurred in cultural practices and these changes determined negative repercussions on phytopathological issues.

The occurrence and severity of some soil pests and diseases such as root-knot nematode (*Meloidogyne*

**Table 2** Symptoms induced by leaf and stem inoculations with *Xanthomonas perforans* associated with pith necrosis in greenhouse growing tomatoes and the tomato bacterial spot *Xanthomonas* after 15 days on Solanaceae hosts

<i>Xanthomonas</i> spp.	Inoculated host and cultivar							
	Tomato cv. Laetitia		Tomato cv. Marinda		Pepper cv. Airone		Eggplant cv. Birgah	
	Stem symptoms <sup>a</sup>	Leaf symptoms <sup>b</sup>	Stem symptoms <sup>a</sup>	Leaf symptoms <sup>b</sup>	Stem symptoms <sup>a</sup>	Leaf symptoms <sup>b</sup>	Stem symptoms <sup>a</sup>	Leaf symptoms <sup>b</sup>
<i>X. perforans</i> 4P1S2 <sup>c</sup>	+ (4.6±0.4)	+	+ (4.5±0.2)	+	+ (2.5±0.4)	–	+ (4.8±0.7)	–
<i>X. perforans</i> NCPPB4321 <sup>T</sup>	+ (4.8±0.6)	+	+ (4.4±0.5)	+	+ (6.2±0.5)	–	+ (7.9±1.3)	–
<i>X. euvesicatoria</i> NCPPB2968 <sup>T</sup>	+ (2.6±0.2)	+	+ (3.0±0.3)	+	+ (6.0±0.8)	–	+ (4.2±0.6)	–
<i>X. vesicatoria</i> LMG911 <sup>T</sup>	+ (5.1±0.6)	+	+ (5.2±0.3)	+	+ (4.4±0.2)	–	+ (3.3±1.1)	–
<i>X. gardneri</i> NCPPB881 <sup>T</sup>	+ (4.4±0.4)	+	+ (4.9±0.3)	+	+ (9.7±0.9)	+	+ (3.0±0.7)	–

Eight plants were used in each experiment. Experiments were repeated twice. ±standard deviation

<sup>a</sup> += development of lesions 5–15 days after inoculation; –= no development of lesions; <sup>b</sup> mean length of pith necrosis (cm)

<sup>c</sup> *Xanthomonas perforans* strains 1P4S1D, 2P4S1D, 1P4S1, 2P4S1, 1P6S1, 2P6S1 and 7P2S2 produced leaf and stem lesions similar to the lesions produced by *X. perforans* 4P1S2 (data not shown). <sup>T</sup> Type strain

spp.) and corky root infections (*Pyrenochaeta lycopersici*) have increased over the last years in Italy and elsewhere, mainly due to the phase-out of methyl bromide (Djian-Caporalino et al. 2011; Vitale et al. 2011; Klein et al. 2012). For this reason the tomato crops are under strict phytosanitary surveillance, with the aim of founding new control approaches and providing timing alerts on the occurrence of new disease.

During several surveys carried out in Sicily, severe symptoms of vascular discolouration and pith necrosis associated with leaf chlorosis were detected, and surprisingly *P. corrugata* and *P. mediterranea* were never found in symptomatic tissues. Conversely, we obtained several typical xanthomonads that were identified based on several methods as strains of *X. perforans*. This species, however, is known to cause the bacterial spot of tomato and pepper (BSTP). Symptoms of BSTP are different from those reported above, and include dark, circular-to-irregular water-soaked spots and necrotic lesions on leaves, sepals and fruits, reducing yield and fruit quality. *X. perforans* belongs to a group of bacteria whose taxonomy has changed in recent years. Traditionally contained in the taxon *X. campestris* pv. *vesicatoria*, this group of xanthomonads causing BSTP were split following the extensive reclassification of *Xanthomonas* based on DNA:DNA hybridization and phenotypic tests of Vauterin et al. (1995) into two taxa, *X. axonopodis* pv. *vesicatoria* (phenotypic group A) and *X. vesicatoria*

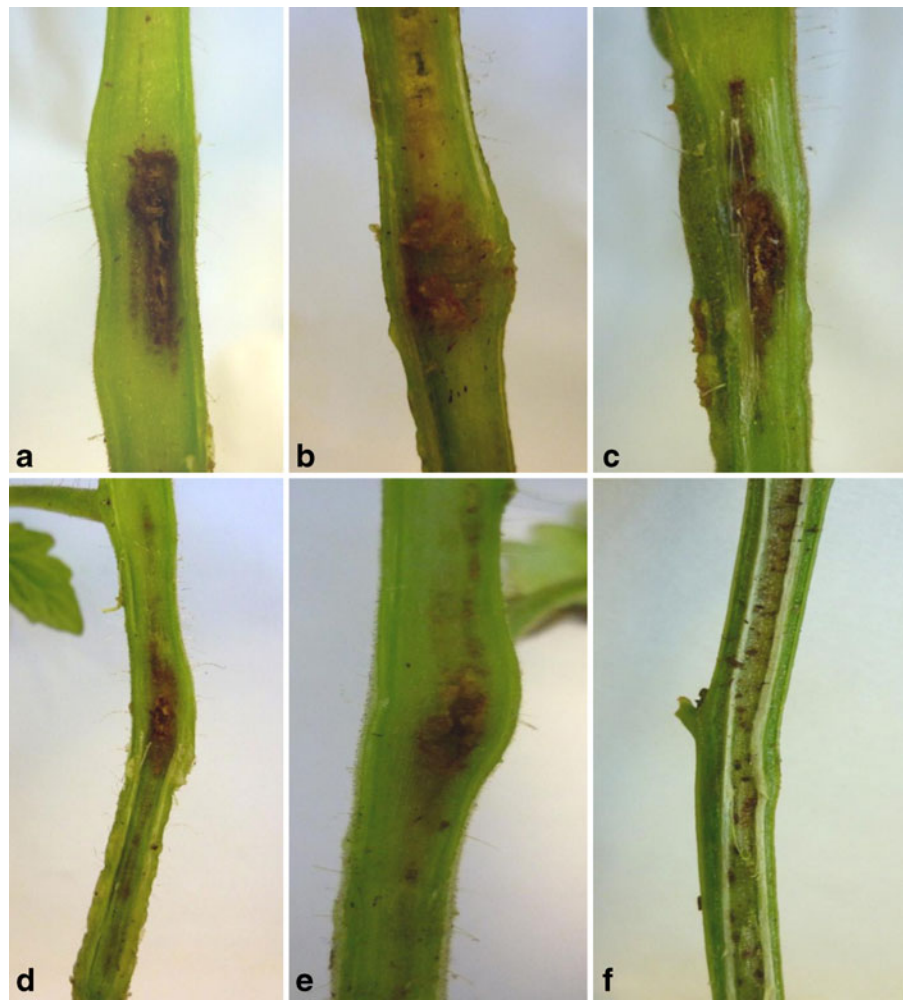
(phenotypic group B). In 2004, Jones et al. described two new additional species, *X. perforans* (phenotypic group C) and *X. gardneri* (phenotypic group D), and renamed *X. axonopodis* pv. *vesicatoria* as *X. euvesicatoria*. More recently this subdivision into four separate species was supported by a multilocus sequence-typing study (Almeida et al. 2010).

In contrast to the broad host range of the genus *Xanthomonas*, individual species and pathovar display a narrow host range and a marked tissue specificity (Vauterin et al. 1995), colonizing either the xylem or the intracellular spaces of nonvascular, mesophyll tissues (Bogdanove et al. 2011). Accordingly, *X. perforans* as well as other BSTP causing xanthomonads has never been reported as the cause of vascular diseases.

The association of *X. perforans* with a pith necrosis syndrome, supported by the fulfillment of Koch's postulates by reproducing the observed symptoms with artificial infections, is therefore in strict contrast with our knowledge of *X. perforans* and other BSTP associated xanthomonads as pathogens restricted to mesophyll tissues.

The reasons that promoted this unprecedented occurrence of association of *X. perforans* with a vascular syndrome are not known. It is possible that these isolates represent novel strains of *X. perforans* with expanded *in planta* behaviour following acquisition of unknown genetic elements. The recent work on the

**Fig. 5** Pith necrosis induced on tomato plants cv. Laetitia and pepper plant cv. Airone 15 days after stem inoculations with the bacterial spot *Xanthomonas* species at  $10^5$  CFU ml<sup>-1</sup>: *Xanthomonas perforans* 4P1S2 on tomato (this study) (a); *X. euvesicatoria* NCPPB 2968 on tomato (b); *X. perforans* NCPPB 4321 on tomato (c); *X. vesicatoria* LMG 911 on tomato (d); *X. gardneri* NCPPB 881 on tomato (e); *X. gardneri* NCPPB 881 on pepper (f)



genome sequence analysis of representative strains of BSTP causing xanthomonads (*X. vesicatoria* strain 1111, *X. perforans* strain 91–118 and *X. gardneri* strain 101) by Potnis et al. (2011) showed that those strains already possess some genetic determinants that are thought to be necessary for vascular colonization. They have orthologs of the type IV secretion system pilus protein PilQ, that was associated to twitching motility and migration to xylem vessel in *X. oryzae* pv. *oryzae* (Lim et al. 2008) and share genes for adhesins that are thought to be required for biofilm formation within vessels (Potnis et al. 2011). Since genes distinguishing mesophyll restricted from vascular xanthomonads have not been identified, Bogdanove et al. (2011), in their comparison of the complete genome sequence of *X. oryzae* pv. *oryzae* vs. *X. o.* pv. *oryzicola* and *X. c.* pv. *campestris* vs. *X. c.* pv. *raphani*, came to the conclusion that while host specificity involves a

complex set of adaptations at the level of gene content, the tissue specificity may be determined by subtle adaptation at the level of amino acid or non coding regulatory nucleotide sequence. Although it is not known to what extent these results can be extended to other *Xanthomonas* spp., they do not corroborate the hypothesis that a tissue specificity switch of a *X. perforans* strain could be accomplished with the gain of a limited number of genes through horizontal genetic transfer. Moreover, the fAFLP analysis of four isolates that is reported here revealed a degree of diversity among strains that is hardly compatible with the clonality expected in the first occurrence of a new pathogenic strain. In addition, type strains of other BSTP-causing xanthomonads were also capable of causing pith necrosis.

A more likely possibility is that the simultaneous occurrence of both root-knot nematode (*Meloidogyne*

spp.) and corky root infections which are responsible of severe injuries and wounds in the root system could have a role on promoting vascular bacterial infections. In addition, *X. perforans* might be associated with an endophyte or mild pathogen that provided a way for the infection of the pith. From the symptomatic material collected in our surveys, *X. perforans* has been frequently isolated in association with *P. fluorescens*, *P. putida*, *P. marginalis*, *P. citronellolis*, *P. straminea*, *P. agglomerans*, and *P. carotovorum* subsp. *atrosepticum*, which were able to induce vascular discoloration or soft rot. Among them, *P. fluorescens*, *P. putida*, and *P. marginalis* have been previously reported as responsible of tomato vascular discoloration (Lo Cantore and Iacobellis 2002; Molan and Ibrahim 2007; Polizzi et al. 2007; Bella and Catara 2009; Kudela et al. 2010; Dimartino et al. 2011). As shown above, BSTP xanthomonads are able to induce pith necrosis, if artificially stem-inoculated, suggesting that if they were able to reach the pith through the interaction with another organism, they would be able to incite symptoms of pith necrosis such as those observed during our surveys. Interactions with other microorganisms at time of infection are known to influence pathogen behavior (Llama-Palacios et al. 2002), although the extent of such interactions and their potential effect on the capability to invade host tissues that are not usually accessible have not been previously investigated. It is also possible that *X. perforans* isolates were associated in mixed infections with an unknown mild pathogen or endophyte. With the work reported here we demonstrated that the syndrome observed is completely reproduced by inoculation in the stem of *X. perforans*, but the way used by the pathogen to reach the pith in natural infections requires additional investigations.

This report provides further evidence for the risk posed by the emergence of plant pathogens associated with global trade, climatic change and modification of cultural practices. There have been other recent reports of bacterial plant pathogens (*P. syringae* pv. *actinidiae*, among many others; Scortichini et al. 2012) that became a serious threat for the cultivation of economically important crops following a change in their epidemiological traits and adaptation to local conditions. In this context it is of particularly concerning that a pathogen as widely spread as *X. perforans* may show a new pathogenic behavior

that may possibly provide it with novel epidemiological characteristics.

**Acknowledgments** The Authors thank C. Moretti of Dipartimento di Scienze Agrarie e Ambientali, Perugia (Italy) for supplying *Xanthomonas* type strains, and K. Geider, Institute for Plant Protection, Dossenheim, Germany, for helpful assistance at the beginning of the research.

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