Short Communication

MIXED INFECTION OF *PECTOBACTERIUM CAROTOVORUM* subsp. *CAROTOVORUM* AND *P. CAROTOVORUM* subsp. *BRASILIENSIS* IN TOMATO STEM ROT IN ITALY

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SUMMARY

Grafted tomato plants grown in a soilless culture system in heated greenhouses in Sicily were affected by stem rot disease. Symptoms consisted of dark brown longitudinal discoloration of the basal part of the stem and petioles. Longitudinal sections of the stem revealed brown water-soaked or soft-rotted pith tissue and internal vascular discoloration. Bacterial isolates with two different colony morphologies were obtained from symptomatic tissues. Isolates were identified as *Pectobacterium* spp. on the basis of biochemical and molecular analyses. The analysis of the 16S rRNA gene sequence and of the concatenated sequences of the housekeeping genes *rpoS* and *pgi* revealed that the isolates of the two morphotypes belong to *P. carotovorum* subsp. *carotovorum* and *P. c.* subsp. *brasiliensis*, respectively. Isolates from both taxa reproduced disease symptoms on artificially inoculated tomato plants. This is the first report of *P. carotovorum* subsp. *brasiliensis* in Italy.

Keywords: *Pectobacterium*, tomato, diagnosis, gene sequencing, PCR.

In winter 2014-2015 grafted tomato plants grown in a soilless culture in a coconut fiber substrate in heated greenhouses in eastern Sicily (southern Italy) were affected by stem rot disease. Symptoms appeared in December during the ripening of the first fruit truss with a 30% disease incidence. Plants showed soft rot dark brown longitudinal discoloration of the stem mainly localised near the plant base, but also along the parts above. Epidermis slip off and decay of the petioles at the insertions with the stem were also observed. Symptoms were localised in the stem parts that were in contact with the plastic strings of the training system or near side shoot pruning scars (Fig. 1 a, b). Longitudinal sections of the stem revealed from brown water-soaked to rotted pith tissues also beyond the area affected externally, both of the scion and the rootstock, along with xylem discoloration.

A number of bacterial species have been reported in the Mediterranean region as being responsible for syndromes in tomato involving the browning, necrosis and/or rot of the internal part of the stem, mainly belonging to the genus *Pseudomonas* and *Pectobacterium* (Aysan *et al.*, 2005; Fiori *et al.*, 2005; Catara, 2007; Hibar *et al.*, 2007; Bella and Catara, 2010; Trantas *et al.*, 2013). To clarify the etiology of the disease, samples of symptomatic plants were subjected to phytopathological analysis.

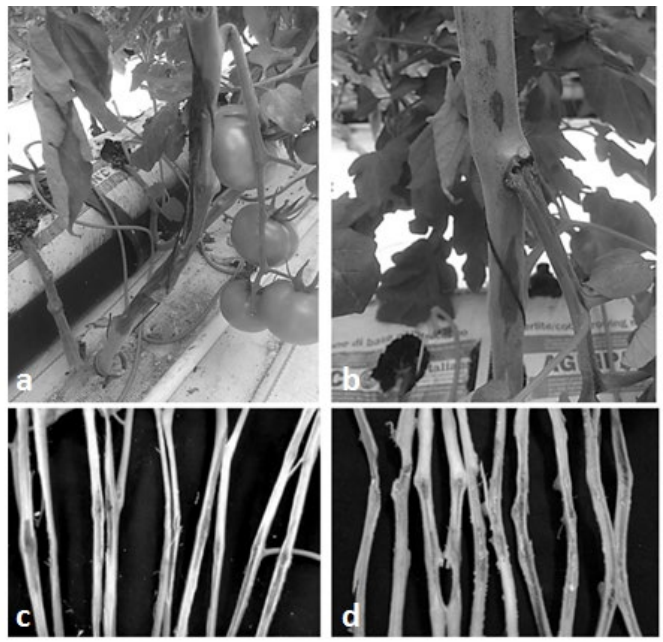

Fig. 1. Field symptoms (a, b): discoloration and rot of tomato stems induced by the two subspecies of *Pectobaterium carotovorum* in relation to stem wounds caused by plastic strings or pruning scars. Pathogenicity tests (c, d): browning and hollowing of the pith induced on tomato plantlets by *P. carotovorum* subsp. *brasiliensis* (c) and *P. carotovorum* subsp. *carotovorum* (d) inoculated by injection of 50 µl of bacterial suspension at the axil of the second true leaf.

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Table 1. List of *Pectobacterium carotovorum* strains isolated in this study, *Pectobacterium* strains used in the phylogenetic analysis and their GenBank accession numbers.

Species	Strain[1]	Origin	Host	GenBank Accession Nos.		
				pgi	*rpoS*	16srRNA
P c. subsp. *brasiliensis*	PVCT 203.1.1	Italy	*Solanum lycopersicum*	KX066083	KX066084	KX066076
	PVCT 203.1.4	Italy	*S. lycopersicum*	KX066080	KX066085	KX066077
	213 (ATCC BAA-418)	Brazil	*S. tuberosum*	JF926821	JF926841	AY207084.1
	371 (ATCC BAA-419)	Brazil	*S. tuberosum*	JF926820	JF926840	JF926726.1
	212 T (CFBP 6617) (ATCC BAA-417)	Brazil	*S. tuberosum*	JF926819	JF926839	NR_118228.1
	8 (ATCC BAA-416)	Brazil	*S. tuberosum*	JF926818	JF926838	JF926723.1
	1001	Canada	*S. tuberosum*	JF926817	JF926837	JF926759.1
	1009	Canada	*S. tuberosum*	JF926816	JF926836	
	C317.1	Syria	*S. tuberosum*	HM157084	HM157219	JF926725.1
	SCRI1073	Peru	*S. tuberosum*	HM157090	HM157201	JF926717.1
	JKI4.3.22	Germany	*S. tuberosum*	HM157094	HM157206	/
	SCRI 132	Japan	*S. tuberosum*	HM157100	HM157209	/
P. c. subsp. c*arotovorum*	PVCT 203.2.1	Italy	*S. lycopersicum*	KX066081	KX066086	KX066078
	PVCT 203.2.4	Italy	*S. lycopersicum*	KX066082	KX066087	KX066079
	CFBP 2046 T	Denmark	*S. tuberosum*	JF926812	JF926832	NR_118227.1
P. c. subsp. *odoriferum*	CFBP 1878 T	France	*Cichorium intybus*	JF926813	JF926833	NR_118225.1
P. atrosepticum	CFBP 1526 T	Scotland	*S. tuberosum*	JN600347	JN600353	NR_118295.1
P. wasabiae	CFBP 3304 T	Japan	*Eutrema wasabi*	JN600349	JN600355	NR_118294.1
P. betavasculorum	CFBP 2122 T	United States	*Beta vulgaris*	JN600348	JN600354	NR_118292.1
P. aroidearum	SCRI 109T	South Africa	*Zantedeschia aethiopica*	HM157096	HM157208	JN600323.1
Dickeya chrysanthemi	NCPPB 402 T	United States	*Chrysanthemum morifolium*	HM157124	HM157246	NR_118856.1

[1] The strains isolated in this study are underlined

Portions of tomato stems were surface disinfected and cut longitudinally. Small sections of affected vascular transition zones from diseased to healthy tissue were removed, ground in a small volume of sterile distilled water and plated on Nutrient Agar (Oxoid) supplemented with 1% D-Glucose (NDA). After incubation at 26°C for 48 h, numerous bacterial colonies with two different morphologies were observed. Single colonies from eight different isolation plates representative of the two morphologies were purified by re-streaking twice on NDA. All strains were routinely maintained on NDA at 4°C for short periods and preserved in 15% glycerol at −80°C for long-term storage. The strains were Gram-negative, oxidase-negative, facultative anaerobic and pectolytic, and did not fluoresce on King's B agar (Schaad *et al*., 2001).

The strains were also analyzed by polymerase chain reaction (PCR) with three primer sets: i) Y1/Y2, specific primers for *Pectobacterium* genus with a 434 bp PCR product (Darasse *et al*., 1994); ii) Y45/Y46, specific primers for *P. atrosepticum* (*Pa*) with a 439 bp PCR product (Fréchon *et al*., 1998); iii) BR1f/L1r, specific primers for *P. carotovorum* subsp. *brasiliensis* (*Pcbr*) with a 690 bp PCR product (Duarte *et al*., 2004). Two-µl aliquots of boiled lysis-prepared DNA from bacterial suspensions of approximately 10^7 cells ml^{-1} in molecular grade sterile water were used in PCR reactions. Reference strains for the different taxa used in this study were as follows: *P. carotovorum* subsp. *carotovorum* (*Pcc*) strain CFBP 2046^T; *P. atrosepticum* (*Pa*) strain NCPPB 549^T; *P. wasabiae* (*Pw*) strain NCPPB 3701^T; *P. carotovorum* subsp. *odoriferum* (*Pco*) strain NCPPB 3839; *P. carotovorum* subsp. *brasiliensis* (*Pcbr*) strain LMG 21370; *Dickeya chrysanthemi* (*Dc*) strain CFBP 2048^T (other collection designations: ATCC 11663^T, NCPPB 402^T).

The amplification of the 434 bp PCR amplicon with Y1/Y2 primers from the eight tomato strains revealed that they belonged to the *Pectobacterium* genus, hence *Dc* strain CFBP 2048^T was the only reference strain from which an amplicon was not obtained. PCR with Y45/Y46 primers did not amplify any PCR product except for the Pa reference strain NCPPB 549^T. Four of the eight strains isolated from tomato, namely strains PVCT 203.1.1, PVCT 203.1.2, PVCT 203.1.3 and PVCT 203.1.4, showed the expected amplification product of 690 bp with specific *Pcbr* primers Br1f/L1r, as well as the *Pcbr* reference strain LMG 21370.

Four bacterial strains (PVCT 203.1.4, PVCT 203.1.1, PVCT 203.2.4, PVCT 203.2.1) were further characterized by sequencing 16S rRNA, *rpoS* and *pgi* genes. PCR of 16S rRNA gene was conducted using the universal primers 27F/1492R (Lane, 1991), as previously described (Bella *et al*., 2012). Subgenic fragments from *rpoS* (RNA polymerase subunit sigma factor 38) and *pgi* (glucose-6-phosphate isomerase) genes were amplified by PCR with rpos1/rpos2 (Waleron *et al*., 2008) and pgi815F/ pgi1396R (Ma *et al*.,

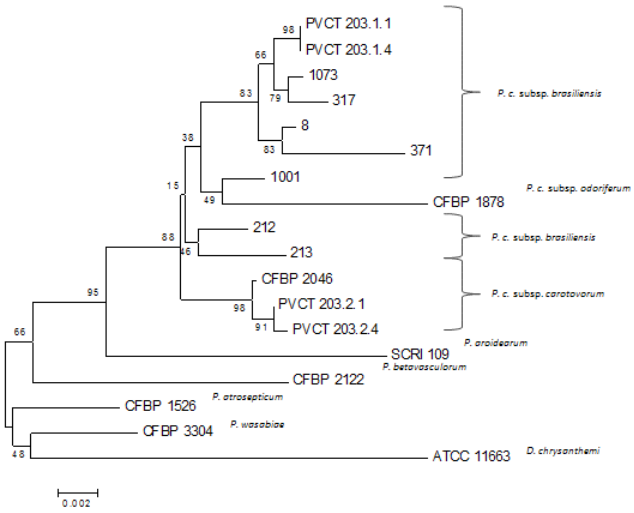

Fig. 2. Phylogenetic tree based on partial sequence of 16S rRNA gene (1437 positions) of *Pectobacterium* strains isolated in this study and type strains of *Pectobacterium* spp. and *Pectobacterium carotovorum* subspecies. The branching pattern was generated by the Neighbor-Joining method and the evolutionary distances were computed using the Jukes-Cantor model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. *Dickeya chrysanthemi* ATCC 11663^T (CFBP 2048^T, NCPPB 402^T) was included as an outgroup.

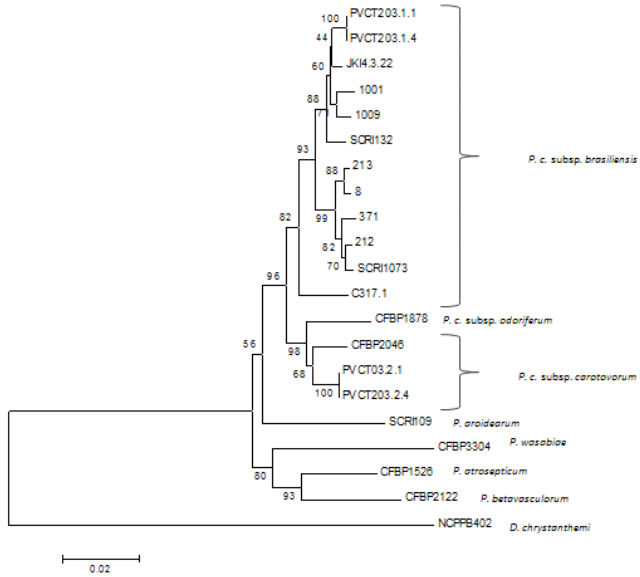

Fig. 3. Phylogenetic tree showing evolutionary relationships among strains isolated in this study and type strains of *Pectobacterium* spp. and *Pectobacterium carotovorum* subspecies based on concatenate partial sequences (1241 positions) of the two housekeeping *rpoS* and *pgi* genes by the Neighbor-Joining method. The evolutionary distances were computed using the Jukes-Cantor model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown. *Dickeya chrysanthemi* NCPPB 402^T (CFBP 2048^T, ATCC 11663^T) was included as an outgroup.

2007) primers, respectively. Sequences were determined on both DNA strands at the Bio Molecular Research Center (BMR), University of Padua, Italy. These sequences were aligned in MEGA version 6 (Tamura *et al*., 2013) with reference gene sequences obtained from the NCBI GenBank including type strains of *Pectobacterium* species and subspecies and *Dc* (Table 1).

The evolutionary history was inferred using the neighbor-joining method (Saitou and Nei, 1987). The evolutionary distances were computed using the Jukes-Cantor method (Jukes and Cantor, 1969). 16S rRNA gene sequences (1437 bp) from each of the four strains isolated in this study were aligned with fourteen 16S rRNA gene sequences obtained from the NCBI GenBank, including the type strains of *Pectobacterium* species and subspecies (Table 1). The NJ tree obtained showed that strains PVCT 203.2.4 and PVCT 203.2.1 clustered with the *Pcc* type strain, whereas strains PVCT 203.1.4 and PVCT 203.1.1 clustered with *Pcbr* strains (Fig. 2). Sequences were deposited in the GenBank under accession numbers KX066076-KX066087 (Table 1).

Since a low 16S rRNA gene sequence diversity was detected between the two subspecies (Nabhan *et al*., 2012a) we also analysed the two conserved genes *pgi* and *rpoS*. The phylogenetic tree inferred from concatenated DNA sequences of *pgi* and *rpoS* genes (1241 positions), showed that strains PVCT 203.2.4 and PVCT 203.2.1 were located in the same phylogenetic branch as the corresponding species type strain *P. carotovorum* subsp. *carotovorum* (*Pcc*) CFBP 2046^T, whereas strains PVCT 203.1.4 and PVCT 203.1.1 clustered with a different phylogenetic branch corresponding to *P. c.* subsp. *brasiliensis* (*Pcbr*) (Fig. 3).

Two inoculation methods were used to assess the pathogenicity of bacterial isolates on eight-week old tomato seedlings (cv. Ventero). Three plants per strain were either prick inoculated into the stem with a 24-h old bacterial colony or inoculated by injecting 50 µl of a bacterial suspension at the axil of the second true leaf. In the latter method the inoculum was prepared from 24 h cultures by suspending the bacterial cells in sterile distilled water up to a concentration of 10^6 cfu ml^{-1}. Negative and positive control plants were inoculated with sterile distilled water and *Pcc* strain CFBP 2046^T, respectively.

The plants were placed in a growth chamber with a 16/8 h day/night photoperiod and temperatures of 25 and 18°C, respectively. Pathogenicity tests on tomato plantlets highlighted that the strains had different degrees of virulence. Three days after prick inoculation of the stems of tomato plantlets, strains PVCT 203.2.1 and PVCT 203.2.4 identified as *Pcc* caused a severe collapse of the stems at the inoculation site, whereas those inoculated with *Pcbr* strains (PVCT 203.1.1 and PVCT 203.1.4) showed an internal brown discoloration and hollowness of 1-3 cm (data not shown). These differences were also observed in the second assay, with lower inoculum doses, where plantlets inoculated with *Pcc* strains showed browning, soft rotting and hollowing of almost all the stem pith. The lesions in

the plants inoculated with the *Pcbr* strain developed to a lesser extent (Fig. 1 c, d). In both cases external lesions were observed resembling those observed in greenhouse cultivated plants. No symptoms were observed in control plants. The pathogens were re-isolated from the diseased plants and re-identified by PCR.

Stem rot and pith necrosis caused by *Pcc* has been described in tomato and pepper in Italy (Fiori and Schiaffino, 2004; Fiori *et al*., 2005) as well as in other Mediterranean countries. Stem rot on solanaceous species caused by mixed infection of *Pectobacterium* spp. have already been reported in tomato, namely caused by *Pcc* and *Dc* (Aysan *et al*., 2005), and in eggplant by *Pcc* and *Pca* (Catara *et al*., 2001).

P. carotovorum subsp. *brasiliensis* was first described as causing blackleg disease in potatoes (*Solanum tuberosum* L.) in Brazil (described as *Erwinia carotovora* subsp. *brasiliense*) and has since been described as also causing soft rot in other species, i.e. *Capsicum annum* L., *Ornithogalum* spp., and *Dacus carota* subsp. *sativus*, sugar beet, cabbage and zucchini (Duarte *et al*., 2004; Ma *et al*., 2007; Nabhan *et al*., 2012b). *P. carotovorum* subsp. *brasiliensis* was recently reported in Florida on heirloom tomatoes affected by a syndrome similar to the one we observed (Rosskopf and Hong, 2016). Although this subspecies has not yet been validated in IJSEM, it is widely accepted that it represents a subspecific clade within *P. carotovorum* (Nabhan *et al*., 2012a). Strains of this taxon have been isolated in the USA, Canada, South Africa, Peru, Germany, Japan, Israel and Syria (Duarte *et al*., 2004; Ma *et al*., 2007; Nabhan *et al*., 2012b) and recently in New Zealand (Panda *et al*., 2012), the Netherlands (Nunes Leite *et al*., 2014), Korea (Dong-Hwan *et al*., 2014), and Poland (Waleron *et al*., 2015). Culture collection strains have also been reported to belong to this clade and about 20% of the *P. carotovorum* strains from potato collected in Syria have been identified as *P. carotovorum* subsp. *brasiliensis* (Nabhan *et al*., 2012b) as has one strain of tomato isolated in 1997 in Korea (Dong-Hwan *et al*., 2014). To our knowledge this is the first time that *P. carotovorum* subsp. *brasiliensis* has been reported in Italy. A careful revision of bacterial collections in Italy however would further clarify the exact distribution of the *Pectobacterium* subspecies.

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