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SHORT COMMUNICATION

Occurrence and characterization of *Pectobacterium brasiliense* causing soft rot on *Zamioculcas zamiifolia* plants in Greece

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Abstract

The soft rot inducing bacterium *Pectobacterium brasiliense* is considered one of the most virulent species among the Pectobacteriaceae. *P. brasiliense* affects a wide range of economically important crops and causes serious damages. In 2019 a bacterial disease was confirmed on *Zamioculcas zamiifolia* plants derived from commercial ornamental plant nurseries on the island of Crete in Greece. *Pectobacterium brasiliense* was isolated and subsequently identified morphologically, biochemically, physiologically and molecularly based on PCR with the specific primers BR1f/L1R and multilocus sequence analysis (MLSA). The pathogenicity of the isolates was confirmed using artificial inoculations on ZZ plants with subsequent re-isolation and re-identification. To our knowledge this is the first record of *Pectobacterium brasiliense* causing soft rot on *Zamioculcas zamiifolia* plants in Greece and worldwide.

Keywords *Pectobacterium brasiliense* · *Zamioculcas zamiifolia* · MLSA · Soft rot · Greece

Pectobacterium brasiliense (Pbr) is a bacterial plant pathogen causing potato blackleg and soft rot on in many parts of the world. It is considered as one of the most virulent species among Pectobacteriaceae family, affecting a wide range of economically important crops (Oulghazi et al. 2021; Tsror et al. 2021). The bacterium was first described in 2004 as *Erwinia carotovora* subsp. *brasiliensis* causing blackleg on potato in Brazil (Duarte et al. 2004). Pbr infects a wide range of host plants including both monocotyledonous and dicotyledonous, causing significant losses in 19 different plant species (particularly on potato) belonging to 10 families (Oulghazi et al. 2021), threatening the health of very important plants, leading to substantial economic losses (Szulta et al. 2023). Pbr cause the degradation of the cell wall of several plants and provoking soft rot diseases. Pbr is differentiated from other *Pectobacterium* species, due to its phenotypic and genetic characteristics as well as its higher virulence and aggressiveness (Duarte et al. 2004; van der Wolf et al. 2017). The pathogenicity of Pbr is based on its ability to produce a great range of enzymes (pectate lyases, polygalacturonases, cellulases and proteases) that degrade structural components of the cell wall and infect the host (Mejía-Sánchez et al. 2019). Strains of Pbr are virulent in both cool and warm temperatures, and high disease incidence occurs when cool and warm temperatures alternate (van der Merwe et al. 2010). *Zamioculcas zamiifolia* is an ornamental monocotyledonous plant belonging in the Araceae family and is known by several common names (aroid palm, ZZ plant or ZZ in short *etc*.). It has become a popular indoor potted plant worldwide mainly due to the glossy appearance of its foliage, the easy care and the efficiency to tolerate low light environment (Thongkham and Phavaphutanon 2018).

In February 2019, soft rot symptoms were observed on *Zamioculcas zamiifolia* (ZZ) plants which were growing at commercial ornamental plant nurseries on the island of Crete in Greece. The incidence of affected plants was estimated at approximately 30%, either by destroying completely most of them, or by significantly reducing their marketability. The infection initially was noticed by the chlorotic appearance of the plants (Fig. 1A) and the water-soaked lesions observed on petioles and lamina of the leaves (Fig. 1C, D). Frequently, the infection began as a watery spot in the contact area of the petioles with the soil substrate. Also, affected plants showed brown discolouration of internal tissues and progressively they rotted, wilted and finally collapsed. Often

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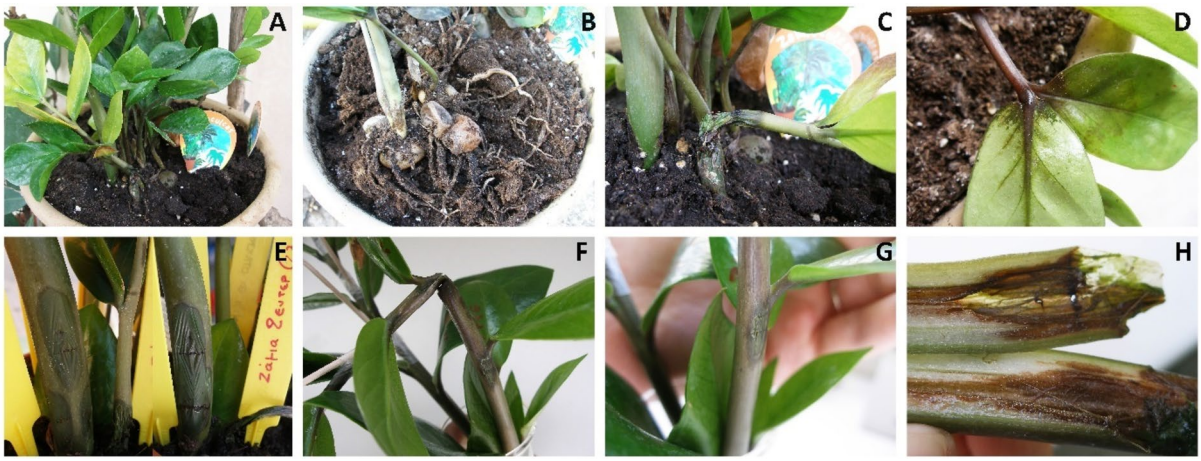

Fig. 1 Symptoms on affected *Zamioculcas zamiifolia* plants: chlorotic plant (**A**), decomposed mummified rhizomes (**B**) soft rot and water-soaked lesions on leaves petiole (**C**) and lamina (**D**). Symptoms after artificial inoculations: watery, soft rotted, wilted tissues (**E**, **F**, **G**) and brown discolouration of internal tissues (**H**)

the whole plant rapidly declined to an amorphous mass of foul-smelling tissues. Infection of the rhizomes was manifested by an extensive rotting, accompanied by unpleasant smell. Affected rhizome tissues were internally decomposed/mummified and only the bark remained intact (Fig. 1B). Microscopic observation from the borders of affected tissues revealed constantly the presence of bacteria oozing out of the cut section.

Tissues from affected leaves petiole, lamina and rhizomes were removed, surface sterilized with 10% ethanol and rinsed twice with sterile water. Small pieces from the margins of the infected tissues were cut aseptically and macerated, in sterile phosphate buffer (PBS) for 15 min. Loopfuls of the homogenates were streaked onto Nutrient Agar medium supplemented with 1% glucose (NAG) and incubated at 26 °C for 48 h. Whitish, circular, creamy, and slightly raised bacterial colonies with the characteristic "fried egg" morphology, were consistently isolated on NAG (Supplementary Fig. 1). Single bacterial colonies with the above phenotype were subcultured, purified and stored in 15% glycerol at -80 °C and as slant cultures on NAG for further characterisation. Five (5) representative isolates (HMU3737 = LMG32976, HMU3740, HMU3764, HMU3765, HMU3766) were identified initially based on their morphological, biochemical and physiological profile. The disease symptoms were not associated with the presence of phytopathogenic fungi since no fungal growth was observed on Potato Dextrose Agar (PDA) medium.

The phenotypic characteristics of five (5) isolates (Table 1) were evaluated according to Schaad et al. (2001) and Malathrakis and Goumas (1987). Strains of *Pectobacterium brasiliense* IPO3650 and IPO3671 (Leite et al. 2014; van der Wolf et al. 2017), *Dickeya solani* (Ds) IPO2222 (van der Wolf et al. 2014), as well as strains HMU3048 of *Pectobacterium carotovorum* (Pc) and HMU3211 of *Pectobacterium atrosepticum* (Pa) obtained from the collection of Plant Bacteriology Laboratory, of the Hellenic Mediterranean University (HMU, unpublished strains), were used as reference strains. In preliminary tests, all bacterial isolates from ZZ plants, were characterized Gram-negative with KOH 3% test, non-fluorescent on King' s B medium, facultative anaerobic and they caused pitting on crystal violet pectate (CVP) medium. Additionally, they were positive for catalase activity, indole production from tryptophan, and they reduced substances from sucrose. They were negative for oxidase production, phosphatase activity, hydrolysis of lecithin, sensitivity to erythromycin and did not produced acid from a-methyl-glucoside. Furthermore, they were efficient to grow at 37 °C in Nutrient Agar (NA), at 28 °C in Nutrient Broth (NB), in 2 and 5% sodium chloride (NaCl), but they did not develop at 39 °C. Carbon source utilization were evaluated using sterile microtiter plates according to Palacio-Bielsa et al. (2006) with the variations proposed by Trantas et al. (2022). All bacterial isolates from ZZ plants tested, did not utilize maltose, palatinose, sorbitol, D(-) tartrate, malonate, L-glutamate, dulcitol, and D-arabitol. On the contrary, they utilized lactose, trehalose, raffinose, a-D(+)melibiose, D-(+)-cellobiose, and D-mannitol. The phenotypic characteristics of all studied isolates, were identical with those of the reference strains IPO3650 and IPO3671 of Pbr, but they differed from the other *Pectobacterium* and *Dickeya* species used as reference strains (Table 1). Additionally, isolate HMU3737 was evaluated comparatively with the reference strains using the API 20 E and API 50 CH micro tests (bioMérieux, France). The tests were conducted according to the methods provided by the supplier and each test was repeated twice showing consistent results. Isolate HMU3737 (= LMG32976) showed 96.6% similarity

Table 1 Phenotypic characteristics of 5 isolates from *Zamioculcas zamiifolia* (HMU3737, HMU3740, HMU3764, HMU3765, HMU3766) in Greece, compared to reference strains of *Pectobacterium brasiliense* (IPO3650, IPO3671), *Pectobacterium carotovorum* (HMU3048), *Pectobacterium atrosepticum* (HMU3211) and *Dickeya solani* IPO2222

Phenotypic characteristics	*Zamioculcas zamiifolia* (5 isolates)	*Pectobacterium brasiliense* IPO3650 IPO3671	*Pectobacterium carotovorum* HMU3048	*Pectobacterium atrosepticum* HMU3211	*Dickeya solani* IPO2222
HR	+	+	+	+	+
Hugh and Leifson`s O/F test	+	+	+	+	+
Pectolytic activity (CVP)	+	+	+	+	+
Catalase	+	+	+	+	+
Oxidase	-	-	-	-	-
Phosphatase	-	-	-	-	+
Sensitivity to erythromycine	-	-	-	-	+
Hydrolysis of lecithin	-	-	-	-	+
Indole production	+	+	-	-	+
Reducing substances from sucrose	+	+	-	+	-
a-methyl glucoside	-	-	-	+	-
Growth at 37 °C in NA	+	+	+	-	+
Growth (NB at 28 °C)	+	+	+	+	+
Growth (NB at 39 °C)	-	-	-	-	-
Growth (NB in 2% NaCl)	+	+	+	+	+
Growth (NB in 5% NaCl)	+	+	+	+	-
Lactose	+	+	+	+	+
Trehalose	+	+	+	+	-
Maltose	-	-	-	-	-
Palatinose	-	-	-	+	-
Raffinose	+	+	+	+	+
Sorbitol	-	-	-	-	-
a-D(+)Melibiose	+	+	+	+	+
D(+)Cellobiose	+	+	+	+	+
D(-)Tartrate	-	-	-	-	-
Malonate	-	-	-	-	+
L-Glutamate	-	-	+	+	-
D-Manitol	+	+	+	+	+
Dulcitol	-	-	-	-	-
D-Arabitol	-	-	-	-	-

with Pbr reference strain IPO3650. Also, HMU3737 showed 96.6% similarity with Pc strain HMU3048, 91.6% with Pa strain HMU3211 and 86.6% with Ds strain IPO2222. Detailed results for the API tests are shown in Supplementary Table 1. The above results indicate that we cannot have a reliable identification in species level using conventional and API microbiological tests.

The 5 phenotypically characterized isolates (Table 1) were further studied using molecular methods. The bacterial isolates were grown in Luria Broth medium (LB) at 28 °C overnight with constant shaking. DNA was extracted and purified using the DNeasy Blood and Tissue kit, for Gram-negative bacteria (Qiagen, Germany). The genomic DNA was amplified with PCR using the specific primers BR1f (5'-GCGTGCCGGGTTTATGACCT-3') and L1R (5'-CARGGCATCCACCCGT-3') for *Pectobacterium brasiliense* which yield an 322 bp amplicon (Duarte et al. 2004). PCR reactions contained 50 ng DNA template, 0,2 µM of each primer, dNTPmix (0.2 mM each, Kapa Biosystems, South Africa), 1.5 mM $MgCl_2$ and 0.5 U KAPA Taq DNA polymerase (KK1016, Kapa Biosystems, South Africa). PCR amplification was carried out with the following thermal cycling program: initial denaturation at 95 °C for 5 min, followed by 30 amplification cycles of 94 °C for 30 s, annealing at 62 °C for 45 s and extension 72 °C for 90 s, ending with final extension at 72 °C for 7 min. The PCR products were analysed in 1.5% agarose gel, stained with Midori Green Advance (Nippon Genetics Europe,

Germany). Additionally, specific primers for Pc (Kang et al. 2003), Pa (De Boer and Ward 1995), and *Dickeya* sp. (Nassar et al. 1996) were used for the identification of the isolates. The five isolates from ZZ as well as the reference Pbr strains IPO3650 and IPO3671 used as positive control, showed the expected amplification (322 bp product) only with the specific primer pair for Pbr (BR1f/L1R) while negative controls (Pc HMU3048, Pa HMU3211, Ds IPO2222) showed no amplification. Regarding the other pairs of specific primers, each reference strain gave amplification only with the specific primer pair corresponding to each species.

Multilocus sequence analysis (MLSA) was performed using five housekeeping genes *acnA*, *gapA*, *mdh*, *pgi* and *proA* genes, which are present in most enterobacteria (Ma et al. 2007). The genes of the isolate HMU3737 were partially amplified using the primers shown in Supplementary Table 2 and were sequenced with Sanger method by Macrogen Europe, Amsterdam, the Netherlands. PCR reactions contained 50 ng DNA template, 0.4 μM of each primer, dNTPmix (0.2 mM each, Kapa Biosystems, South Africa), 1.5 mM $MgCl_2$, and 0.5 U KAPA Taq DNA polymerase (KK1016, Kapa Biosystems, South Africa). The PCR amplification consisted of an initial denaturation at 95 °C for 3 min, followed by 30 amplification cycles of: 94 °C for 30 s, annealing at 52 °C for 30 s, extension at 72 °C for 1 min and final extension at 72 °C for 6 min. The raw sequencing data were processed with software SnapGene (available at snapgene.com) and were edited manually based on the quality of chromatogram peaks (GenBank Accession Nos OL870305, OL870306, OL870307, OL870308, OL870309). The consensus sequences were aligned based on codons using the algorithm MUSCLE (Edgar 2004) and the partial sequences of five housekeeping genes were concatenated using MEGA version X (Kumar et al. 2018). A Maximum Likelihood (ML) tree was constructed based on the concatenated sequence of the five genes [*acnA* (332 bp), *mdh* (505 bp), *pgi* (543 bp), *gapA* (490 bp), and *proA* (701 bp)] using the program RAxML (Randomized Axelerated Maximum Likelihood) through raxmlGUI 2.0.0-beta.14 (Stamatakis 2014; Edler et al. 2019). GTRGAMMA nucleotide substitution model was used with 1000 bootstrap replicates and each codon position was analyzed individually. The ML tree (Fig. 2). was visualized using MEGA version X. Strains of *Pectobacterium brasiliense*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum* and *Pectobacterium betavasculorum* from Ma et al. (2007) study and GenBank were used for the phylogenetic analysis of the isolate HMU3737 (Supplementary Table 3). Two strains of *Dickeya dadantii* were selected for outgroup taxon of ML tree. The isolate HMU3737 was grouped with the strains

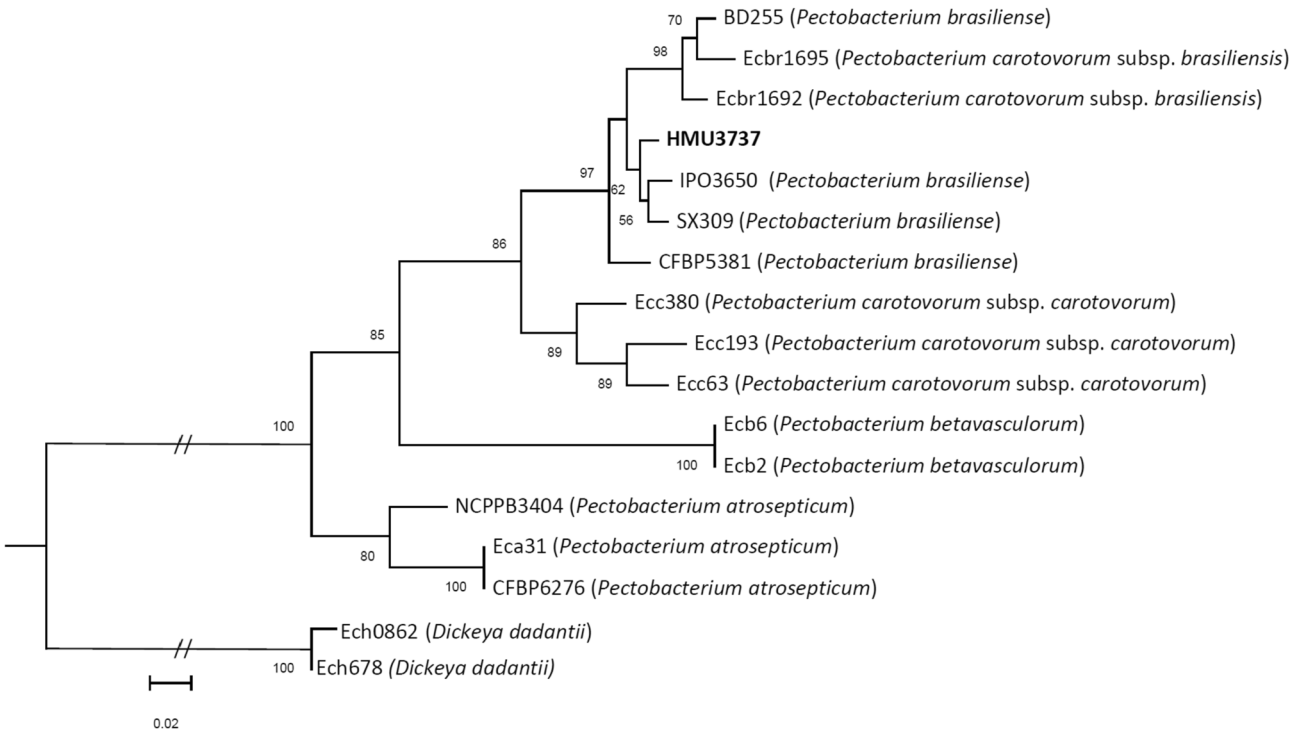

Fig. 2 Maximum likelihood tree (using the program RAxML, through raxmlGUI 2.0.0-beta.14), of *Zamioculcas zamiifolia* strain (HMU3737) isolated from Greece and strains of *Pectobacterium brasiliense*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium atrosepticum* and *Pectobacterium betavasculorum*, from Ma et al. (2007) study and GenBank, using the software MEGA version X. Two *Dickeya dadantii* strains were used for outgroup taxon

IPO3650 and SX309 in the Pbr clade indicating that it belongs to the species *Pectobacterium brasiliense*.

All bacterial isolates elicit hypersensitive response (HR) on tobacco (cv. Xanthi) plants within 24 h and also were pectinolytic in potato slices. The pathogenicity of the five studied isolates was confirmed by performing artificial inoculations on ZZ plants, by injecting with a syringe 20 µl bacterial suspension of 10^7 cfu/ml, into the leaves petiole. The inoculum was prepared from bacterial culture grown on NAG medium for 48 h, which was suspended in sterile PBS buffer. Three plant replicates were used for each of the five isolates tested and three control plants were treated with sterile distilled water. In each plant, three petioles were inoculated. The inoculated plants were maintained in a chamber at 26 °C and 16:8 photoperiod for 7 days. Typical disease symptoms resembled those of natural infections were observed within 2–7 days post inoculation (dpi). Initially, in ZZ plants were observed watery spots, which rapidly developed into soft rot, extending beyond the site of the initial inoculation point. Internally, the infection was accompanied by brown discoloration of the internal tissues. Soft rot usually caused falling of the plants petioles within 2–3 days (Fig. 1, E-H). Re-isolated bacteria were re-identified with the specific primers BR1f/L1 as *Pectobacterium brasiliense*, fulfilling Koch's postulates.

The results of this study suggest that the causal agent of *Zamioculcas zamiifolia* bacterial soft rot disease in Greece is *Pectobacterium brasiliense* based on morphological, physiological, biochemical genetical and phytopathological characteristics. Our findings based on phenotypic characteristics demonstrate that the isolates from ZZ plants showed the same profile with reference Pbr strains IPO3650 and IPO3671, in the majority of the tests. However, the differences compared to the rest of *Pectobacterium* species are not significant enough to allow a reliable identification at species level. Discrimination among *Pectobacterium* species using the traditional microbiological methods is possibly challenged by the diversity of the strains within each species (Nabhan et al. 2012). Thus, complementary to phenotypic assays, molecular methodologies must be used to identify and detect pectinolytic bacterial isolates, as also suggested by Czajkowski et al. (2015). The molecular analysis with specific primers for Pbr (BR1f/L1R, Duarte et al. 2004) indicated that all isolates from ZZ belong to Pbr, since they amplified the 322 bp product along with the reference Pbr strains IPO3650 and IPO3671. Our results coincide with those of other studies (Duarte et al. 2004; Naas et al. 2018; Tsror et al. 2021), confirming the identification of the studied isolates at species level. Importantly, according to the MLSA analysis based on concatenated partial sequences of five housekeeping genes (*acnA*, *gapA*, *mdh*, *pgi*, *proA*, Ma et al. 2007), the isolate HMU3737 from Greece was clearly grouped in the same cluster with Pbr strains and differentiated from other *Pectobacteriun* and *Dickeya* species, as already other researchers reported (Ma et al. 2007; Li-ping et al. 2020).

In conclusion, the results of our study confirm for the first time *Pectobacterium brasiliense* as the causal agent of soft rot in *Zamioculcas zamiifolia* plants, introducing this new host to the already long list of Pbr plant hosts. To our knowledge, there are no previous reports of the bacterium infecting ZZ plants in Greece and worldwide. Our results reinforce the knowledge of Pbr host distribution and potentially can be considered useful information to develop control practices for preventing economic losses.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s42161-023-01544-5.

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Data availability Data presented in this study has been submitted in the NCBI GenBank.

Declarations

Conflict of interest The authors declare no conflict of interest.

Consent for publication All authors have approved the manuscript and agree with its submission to Journal of Plant Pathology.

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