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Occurrence of *Pseudomonas fluorescens* and *P. putida* associated to tomato pith necrosis in Brazil

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ABSTRACT

In 2016, several reports emerged of fresh-market staked tomato plants with severe symptoms of pith necrosis and premature death in commercial cultivation areas in the states of Paraná and Minas Gerais, Brazil, which are similar to those caused by *Pseudomonas corrugata*. Four bacterial strains were isolated from infected tissue samples and characterized as Gram-negative, aerobic, and fluorescent on King's B Medium. LOPAT tests were performed and the isolate UFU H120 was classified into group Vb (+ + − + −) and the isolates UFU H3, UFU H6, and UFU H21 into group Va (− + − + −). The pathogenicity of the isolates was confirmed by artificial inoculation on tomato plants of Santa Cruz type, cv. Kada Gigante, and Koch's postulates were accomplished successfully. The isolates sequences of their 16S rRNA gene region were compared with those deposited in GenBank, the isolate UFU H120 aligning with *Pseudomonas fluorescens* (99,45% similarity) and the isolates UFU H3, UFU H6, and UFU H21 with *Pseudomonas putida* (99.2, 99.53 and 99.64% similarity, respectively). Notably, *P. fluorescens* and *P. putida* are most known as saprophytic bacteria normally present in the soil, although both of these species have already been reported to infect tomato plants in Italy. However, so far, there have not been any reports of such bacteria being phytopathogenic on tomato or any crop in Brazil. Thus, to our knowledge, this is the first report of pith necrosis of such occurrence in Brazil.

Keywords: *Solanum lycopersicum*, fluorescent *Pseudomonas*, pith necrosis.

RESUMO

Ocorrência de *Pseudomonas fluorescens* e *Pseudomonas putida* em tomateiro no Brazil

Em 2016 em tomateiro tutorado para consumo *in natura*, foram observadas plantas com sintomas graves de necrose da medula e morte prematura da planta, em áreas de cultivo comercial nos estados do Paraná e Minas Gerais, Brazil, similares aos causados por *Pseudomonas corrugata*. Quatro cepas bacterianas foram isoladas a partir de amostras de tecido infectado e caracterizadas como Gram negativas, crescimento aeróbico e fluorescimento em meio de cultura King B. Testes LOPAT foram realizados e o isolado UFUH120 foi classificado no grupo Vb (+ + - + -) e isolados UFU H3, UFU H6 e UFU H21 no grupo Va (- + - + -). A patogenicidade dos isolados foi confirmada pela inoculação artificial em tomate Santa Cruz cv. Kada Gigante e os postulados de Koch realizados com sucesso. O sequenciamento da região do gene 16S rRNA dos isolados foi realizado e as sequências obtidas comparadas com as depositadas no GenBank, o isolado UFU H120 foi alinhado com *Pseudomonas fluorescens* (99.45% de similaridade) e os isolados UFU H3, UFU H6 e UFU H21 com *Pseudomonas putida* (similaridades de 99,2, 99,53 e 99,64%, respectivamente). *Pseudomonas fluorescens* e *P. putida* são mais conhecidas como bactérias saprofíticas presentes no solo, embora as duas espécies tenham sido relatadas na Itália, causando doença no tomateiro. No entanto, até o momento não há relatos que essas bactérias sejam fitopatogênicas no tomate ou em qualquer cultura no Brasil. Sendo este, por parte do conhecimento dos autores, o primeiro relato da ocorrência dessas espécies causando necrose da medula em lavouras de tomateiro no país.

Palavras-chave: *Solanum lycopersicum*, *Pseudomonas* fluorescentes, necrose da medula.

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Pith necrosis is a tomato (*Solanum lycopersicum*) disease caused by different bacteria belonging to the genus *Pseudomonas*. The disease is important because results in yellowing of the leaves, and impaired development of the plant that may be related to lower productivity (Monteiro *et al*., 2019).

The species *Pseudomonas cichorii* (Wilkie & Dye, 1974; Trantas *et al*., 2013), *P. corrugata* (Scarlett *et al*., 1978; Clark & Watson, 1986), *P. viridiflava* (Alivizatos, 1986; Malathrakis & Goumas, 1987), *Pseudomonas fluorescens* (Malathrakis & Goumas, 1987; Lo Cantore & Iacobellis, 2002; Saygili *et al*., 2004; Polizzi *et al*., 2007), *Pseudomonas mediterranea* (Catara *et al*., 2002; Trantas *et al*., 2015), *Pseudomonas putida* (Polizzi *et al*., 2007; Dimartino *et al*., 2011), and *P. marginalis* (Bella & Catara, 2010; Kudela *et al*., 2010) have already been reported causing pith necrosis in tomato plant, and also *Xanthomonas perforans* in association with *P. fluorescens*, *P. marginalis*, *P. putida*, *P. citronellolis,* and *P. straminea* (Aiello *et al*., 2013).

In Brazil, the pith necrosis caused by *Pseudomonas corrugata* (Rodrigues Neto *et al*., 1989; Quezado-Duval *et al*., 2007), *P. mediterranea* (Rodrigues *et al*., 2010), and *P. viridiflava* (Monteiro

et al., 2019) have been reported. Other species of *Pseudomonas* are associated to leaf diseases on tomatoes, such as *Pseudomonas syringae* pv. *tomato*, *P. syringae* pv. *syringae*, *P. cichorii*, *P. marginalis,* and *P. viridiflava* (Beriam, 2007; Maringoni *et al*., 2009).

In 2016, in the states of Paraná and Minas Gerais, Brazil, in commercial staked tomato cultivation isolated plants displayed severe symptoms of pith necrosis. Symptoms were similar to those described by Dimartino *et al*. (2011) and Aiello *et al*. (2013), such as lower leaf withering that progresses upwards, extensive pith necrosis, leaf chlorosis, dark-brown lesions, longitudinal stem cracks, adventitious root growth and premature plant death.

Morphological, physiological and biochemical tests can be used to identify plant pathogenic bacteria. A panel of biochemical (LOPAT) is recommended for the genus *Pseudomonas* (Lelliott & Stead, 1987; Schaad *et al*., 2001). However, these tests are not always sufficient for a precise identification of the species or pathovar associated to a given plant disease. This is because of the great biochemical and genetic diversity of the species; more than 100 species described in genus, as well as the possible emergence of new species or pathovars that have not been previously described for the crop or repositioning of species in the taxon (Garrido-Sanz *et al*., 2016).

For the identification of *Pseudomonas* species, molecular techniques based on genomic analysis can be used, such as PCR with specific primers, already described for *P. syringae* pv. *tomato*, *P. corrugata*, and *P. syringae* pv. *syringae* (Bereswill *et al*., 1994; Catara *et al*., 2000; Hamedan & Harighi, 2014). When this type of identification is not possible, genetic sequencing of the 16S region (16S rRNA) with universal primers (James, 2010), and comparison of allelic mismatches in housekeeping genes by MLSA (Vásquez-Ponce *et al*., 2018) can be applied for bacterial identification. The comparison of nucleotide sequences in the 16S rRNA gene encoding region, and housekeeping genes are useful to study bacterial phylogeny, because the genes are considered preserved among bacteria, but with genetic variability (Yang *et al*., 2016; Vásquez-Ponce *et al*., 2018). Thus, the aim of this study was to identify *Pseudomonas* spp. isolates causing tomato pith necrosis in Brazil.

MATERIAL AND METHODS

This study was conducted at the Laboratory of Plant Bacteriology (LABAC) and at the greenhouse of the Institute of Agricultural Sciences (ICIAG) of the Federal University of Uberlândia (UFU), Umuarama Campus (Uberlândia-MG, Brazil), from November 2016 to December 2019.

Obtained isolates

In 2016, in the states of Paraná and Minas Gerais, Brazil, in open field commercial cultivation areas staked tomatos plants were observed with severe symptoms of pith necrosis and premature death, similar to those described for *Pseudomonas corrugata*. Bacterial isolation was performed from four infected tomato plant tissues with severe necrotic symptoms on the pith region of stem basis (Figures 1A, 1B, 1C) from Araguari-MG (UFU H3, UFU H6); Silvestre-PR (UFU H21); and Uberlândia-MG (UFU H120). All isolates were grown in culture medium 523 (Kado & Heskett, 1970) for two to three days at 28°C.

Biochemical characterization of isolates

All colony isolates were morphologically characterized by visual observation or, when necessary, using a stereomicroscope. Characteristics such as color, shape, edges or margins, elevation, texture or consistency, and optical details were analyzed (Mariano & Silveira, 2005). They were also characterized using the identification key proposed by Schaad *et al*. (2001): Gram with KOH tests, oxidation/fermentation (O/F) and fluorescence in King B medium. The species belonging to the genus *Pseudomonas* were identified using the LOPAT specific tests: levan, oxidase, potato rot, arginine dehydrolase, and hypersensitivity in tobacco plants (Lelliott & Stead, 1987; Schaad *et al*., 2001).

Pathogenicity of isolates

The pathogenicity of the isolates was confirmed by inoculating a bacterial suspension of each isolate into healthy Santa Cruz Kada Gigante tomato plants kept in a greenhouse when they presented two trifoliate leaves. Each bacterial suspension was standardized on a spectrophotometer with an absorbance of 0.2 (540 nm), resulting in an approximate concentration of 1 × 10^8 CFU mL^{-1} (Andrade *et al*., 2013). Inoculation was performed by injecting 100 µL of the bacterial suspension into axillary first true leaves of plantlets at the two leaves stage using a sterile hypodermic syringe. Control plants were mock inoculated with sterile distilled water and all plants were kept in a moist chamber for 24 h before and after inoculation. Six plants were inoculated for each isolate. After 10 days of inoculation, the plants were examined, with stem cross section for the presence of discoloration and pith necrosis. The bacteria were reisolated to fulfill Koch's postulates and characterized using the LOPAT biochemical tests.

DNA extraction

Genomic DNA extractions were performed following the manufacturer's protocol, Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA). The DNA obtained from each isolate was quantified using a NanoDrop 2000/2000c 192 Spectrophotometers apparatus (Thermo Fisher Scientific, Wilmington, DE, USA), with the concentration adjusted to 25 ng μL^{-1}.

Polymerase Chain Reaction with specific primers for Pseudomonas spp.

Polymerase chain reactions were performed with specific primers for *P. corrugata*, *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae.* Primers PC5/1 (5′-CCA CAG GAC AAC ATG TCC AC-3′) and PC5/2 (5′-CAG GCG CTT TCT GGA ACA TG-3′) were used for *P. corrugata* (Catara *et al*., 2000). The final volume of each reaction was 12.5 µL, with the final concentration including 1× buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4), 3 mM $MgCl_2$, 0.2 mM

dNTP, 0.4 µM PC5/1 and PC5/2, 1.25 U of Taq polymerase, and 25 ng of DNA. Amplification was performed in a thermocycler with denaturation at 95°C for 5 min, followed by 30 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, and fragment amplification was visualized at 1,100 bp.

Primers 1 (5′-GGC GCT CCC TCG CAC TT-3′) and 2 (5′-GGT ATT GGC GGG GGT GC-3′) were used for *P. syringae* pv. *tomato* (Bereswill *et al*., 1994). The final volume of each reaction was 12.5 µL, with the final concentration including 1× buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4), 3 mM $MgCl_2$, 0.2 mM dNTP, 2 µM Primers 1 and 2, 1 U of Taq polymerase, and 50 ng of DNA. Amplification was performed in a thermocycler, with 30 cycles at 95°C for 30 s, 60°C for 30 s, and 72°C for 45s, followed by a final extension step at 72°C for 5 min, and fragment amplification was visualized at 650 pb.

Primers PF (5′-GCG CGG TCT TGT TTC GGG GA-3′) and PR (5′-TCT CGC AGC TCC TCG CCC AT-3′) were used for *P. syringae* pv. *syringae* (Hamedan & Harighi, 2014). The final volume of each reaction was 12.5 µL, with the final concentration including 1× buffer (500 mM KCl and 200 mM Tris-HCl, pH 8.4), 3 mM $MgCl_2$, 0.2 mM dNTP, 2 µM Primers PR and PF, 1 U of Taq polymerase, and 50 ng of DNA. Amplification was performed in a thermocycler, with denaturation at 93°C for 3 min, followed by 35 cycles at 93°C for 1 min, 63°C for 1 min, and 72°C for 1 min, followed by a final extension step at 72°C for 6 min, and fragment amplification was visualized at 576 bp.

The final products of the reactions were analyzed using electrophoresis on 1% agarose gel stained with SYBR Safe, and the amplified fragments were visualized using an ultraviolet light transilluminator and photodocumented

Gene sequencing of the 16S region

The 16S region of the isolates was sequenced using PCR with primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′) and 1492R (5′-TAC GGH TAC CTT GTT ACG ACT T-3′) (Martin-Laurent *et al*., 2001). The final volume of each reaction was 50 µL, with the final concentration including 1× buffer, 3 mM $MgCl_2$, 0.2 mM dNTP, 1 µM Primers 27F and 1492R, 1.25 U of Taq polymerase, and 50 ng of DNA. Amplification was performed in a thermocycler, with denaturation at 94°C for 5 min, followed by 40 cycles at 94°C for 1 min, 56°C for 1 min, and 72°C for 2 min, followed by a final extension step at 72°C for 15 min, and fragment amplification was visualized at 1,492 bp.

All PCR products were sent for sequencing at BPI Biotecnologia EPP (Botucatu-SP, Brazil). The obtained sequences were then submitted to the BLAST platform of the National Center for Biotechnology Information (NCBI) and compared with the genetic sequences available in the GenBank database (NCBI, 2019).

RESULTS AND DISCUSSION

The bacterial colonies of the isolates were morphologically characterized in culture medium 523 as circular, shiny, smooth-edged, slightly elevated, transparent and straw-white-colored colonies (Figure 1D). The isolates were Gram-negative, aerobic, fluorescent on King B medium, and were classified as *Pseudomonas* (Schaad *et al*., 2001). From the LOPAT tests, the isolate UFU H120 was classified into group Vb (+ + − + −) as *Pseudomonas fluorescens*, whereas the isolates UFU H3, UFU H6 and UFU H21 were identified into group Va (− + − + −) as *Pseudomonas putida* (Lelliott & Stead, 1987).

Pith necrosis symptoms (Figures 1E, 1F) were observed in the tomato plants 10 days after inoculation, confirming the pathogenicity of the isolates. No symptoms were observed in the mock inoculated control plants.

The genomic DNA of the isolates was not amplified with specific primers for *P. corrugata*, *P. syringae* pv. *tomato*, and *P. syringae* pv. *syringae*. The sequences of the 16S rRNA gene region were compared with those deposited in GenBank. Notably, the isolate UFU H120 was closely aligned with *P. fluorescens* (GenBank no. LC031819.1) with 99.45% similarity and 100% sequence query coverage. The isolates UFU H3, UFU H6, and UFU H21 were closely aligned with *P. putida* (GenBank no. NR_114794.1) with 99.2, 99.53, and 99.64% similarity, respectively, and 100% sequence query coverage.

Pseudomonas putida and *P. fluorescens* are saprophytic bacteria (Lelliot & Stead, 1987) commonly present in the soil in Brazil (Cabral *et al*., 2013; Ferraz *et al*., 2015; Aponte *et al*., 2017; Sousa *et al*., 2019), but until now, no phytopathogenic activity associated with these species has been described, and also have not been described in the Absent Quarantine Pest List (A1) of the Ministry of Agriculture, Livestock and Food Supply (Mapa, 2018). These species have already been reported to cause pith necrosis in Europe (Lo Cantore & Iacobellis, 2002; Saygili *et al*., 2004; Polizzi *et al*., 2007; Dimartino *et al*., 2011), although the bacteria transmission by the seeds was not described up to now. Hence, it is important to investigate this new emergence, because Brazil is one of the top ten producers of tomatoes worldwide (Faostat, 2019) and the pith necrosis caused by *P. fluorescens* or *P. putida* is a serious disease that can significantly decrease crop productivity (Aiello *et al*., 2017).

The symptoms caused by *P. fluorescens* and *P. putida* are identical, as well as those of the pith necrosis caused by *P. corrugata* (Quezado-Duval *et al*., 2007), thus requiring laboratory analysis to differentiate them. Such symptoms include lower leaf wilting that progresses upwards, extensive pith necrosis at the base of the plant, leaf chlorosis, dark-brown lesions, longitudinal stem cracks, adventitious root growth, and premature plant death, which are similar to the symptoms described by Aiello *et al*. (2013).

Generally, *Pseudomonas* spp. are common inhabitants of the aerial parts of plants, soil, and the rhizosphere; act as beneficial antagonistic bacteria against several phytopathogens; and are frequently used in biological control strategies (Lugtenberg & Kamilova, 2009). However, some *P. fluorescens* and *P. putida* isolates may become pathogenic or interact synergistically with other pathogens, increasing the

disease severity (Aiello *et al*., 2017).

Notably, the incidence of pith necrosis in Italy in 2006 was attributed to changes made in cultivation practices after discontinuing the use of methyl bromide. Although the non-use of the pesticide has been environmentally beneficial, to increase the number of saprophytic species in the soil, they were believed to have induced a phytopathogenic behavior in *P. fluorescens* and *P. putida* (Dimartino *et al*., 2011). In addition, several environmental conditions, including high specific humidity, salinity, temperature changes and excessive nitrogen, increase the development of opportunistic species (Lo Cantore & Iacobellis, 2002). Although the bacteria transmission by the seeds has not been described until now, the hypothesis is that the bacteria could be introduced in Brazil by the seeds, but this must be investigated.

Characterization and correct identification of *P. fluorescens* and *P. putida* can improve the management of such disease in the field, especially since the symptoms of pith necrosis in tomato plants can be confused with the symptoms caused by other species of fluorescent *Pseudomonas*, such as *P. mediterranea* and *P. viridiflava*, species described in Brazil. This could help improving the understanding of the pathogenic mechanisms involved in infection (Rodrigues *et al*., 2010; Dimartino *et al*., 2011; Monteiro *et al*., 2019). *Pseudomonas* spp. exhibit a high level of diversity, allowing the occurrence of species or pathovars that have not been previously reported to exhibit phytopathogenicity, as a result of changes in their genome and adaptation to local conditions (Aiello *et al*., 2013).

After a reporting of *X. perforans* causing also pith necrosis in tomato plants (Aiello *et al*., 2013), Torelli *et al*. (2015) have compared the putative coding sequences of the *X. perforans* strain 4P1S2, if unusual pathogenic behavior was due to newly acquired molecular determinants or by environment causes. The authors observed that the genome of strain 4P1S2 has high sequence similarity with that one non-vascular strain, but shows

Figure 1. Pith necrosis in tomato plants, observed in open field, caused by the *Pseudomonas putida* (A, B), and *Pseudomonas fluorescens* (C). Bacterial colony of the *P. putida* isolate UFU H3 in culture medium 523 after three days at 28°C (D). Pith necrosis in tomato plant 10 days after inoculation with *P. putida* isolate UFU H6 (E), and *P. fluorescens* isolate UFU H120 (F). Uberlandia, UFU, 2016-2019.

evidences of an additional sequence, possibly contained on a conjugative plasmid that acts in the horizontal transfer of genes originating from vascular *Xanthomonas* spp. The authors have inferred also, that the ability to cause a vascular disease can be not only due to genome, but due to the environment, because of the coinfection with endophytic microorganisms, since *Pseudomonas* species were also found in the same infection site caused by *X. perforans* (Aiello *et al*., 2013, 2017).

In this sense, the sequencing of housekeeping genes, related to cellular metabolism and essential for the survival of the microorganism, by Multilocus Sequence Typing (MLST) and Multilocus Sequence Analysis (MLSA) has allowed the phylogenetic studies and identification of new species of *Pseudomonas*. The MLST consists of comparing allelic mismatches in housekeeping genes and MLSA in the analysis concatenated sequence of these genes (Sarkar & Guttman 2004; Martens *et al*., 2007; Ribeiro *et al*., 2009; Vásquez-Ponce *et al*., 2018).

The MLST of seven housekeeping genes (acn, cts, gapA, gyrB, pfk, pgi, rpoD) from *P. syringae*, from different patovars and hosts, identified 40 sequences with high congruence, indicating a common evolutionary history and the specific association of the patovars with the host occurs by a small genetic variation (Sarkar & Guttman, 2004). Also with MLST of four housekeeping genes (cts, gapA, gyrB, rpoD) from 216 strains of *P. syringae*, phytopathogenics and saprophytics, 23 clades within 13 phylogroups were identified, demonstrating that the citrate synthase (cts) housekeeping gene can accurately predict the phylogenetic affiliation for more than 97% of strains tested (Berge *et al*., 2014).

In the biochemical, morphological and the molecular characterization (rep-PCR and MLSA the housekeeping genes gyrB, rpoD and rpoB) of 15 isolates of *P. cichorii* cause tomato pith necrosis in Greece, Trantas *et al*. (2013) described a new genomovar of *P. cichorii,* being the strains phylogenetically differentiable but phenotypically indistinguishable. In a similar study, the MSLA of the housekeeping genes aroE, glnS, gyrB, ileS and rpoD, also including the 16S rRNA gene, from *Pseudomonas* isolates from Antartica, identified a new strain of *Pseudomonas mandelii* and three new species from the *Pseudomonas fluorescens* subgroup (Vásquez-Ponce *et al*., 2018).

More in-depth phylogenetic studies, including MLST and MLSA, are needed to compare the *P. putida* and *P. fluorescens* isolates of this study, with isolates of soil opportunists and phytopathogenic, to analyze the DNA changes that enabled them to infect plants. This is considered to be possible, given that the DNA sequences in the 16S rRNA gene and the housekeeping genes, despite having a faster rate of evolution than ribosomal genes, are conserved in bacteria, yet exhibits enough variability and information to clearly show the phylogenetic and evolutionary relationships between the species (Ribeiro *et al*., 2009; Yang *et al*., 2016).

To our knowledge, this is the first occurrence of *P. fluorescens* and *P. putida* causing pith necrosis on staked tomato crops in Brazil. All isolates were deposited in the phytobacteria culture collection at the ICIAG/UFU Laboratory of Bacteriology under the codes UFU H3, UFU H6, UFU H21 (*P. putida*), and UFU H120 (*P. fluorescens*).

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