Horticultura Brasileira

REFERÊNCIA

MARQUES, Eder; BORGES, Rafaela CF; UESUGI, Carlos H. Identification and pathogenicity of Pseudomonas cichorii associated with a bacterial blight of gerbera in the Federal District. **Horticultura Brasileira**, Vitoria da Conquista, v. 34, n. 2, p. 244-248, abr./jun. 2016. Disponível em: <http://www.scielo.br/scielo.php?script=sci_arttext&pid=S0102-05362016000200244&lng=en&nrm=iso>. Acesso em: 9 mar. 2018. doi: http://dx.doi.org/10.1590/S0102-053620160000200015.

horticultura brasileira **Comunicação científica / *Scientific communication***

MARQUES, E; BORGES, RCF; UESUGI, CH. 2016. Identification and pathogenicity of *Pseudomonas cichorii* associated with a bacterial blight of gerbera in the Federal District. *Horticultura Brasileira* 34: 244-248. DOI - http://dx.doi.org/10.1590/S0102-053620160000200015

Identification and pathogenicity of *Pseudomonas cichorii* associated with a bacterial blight of gerbera in the Federal District

Eder Marques; Rafaela CF Borges; Carlos H Uesugi

Universidade de Brasília, Depto. Fitopatologia, Brasília-DF; eder.marques.08@gmail.com; rafaelafal@hotmail.com; uesugich@unb.br

ABSTRACT

In 2013, leaf samples of gerbera plants showing symptoms of bacterial blight were collected in cut-flower high tunnels, in the settlement of Núcleo Rural Alexandre Gusmão, located in Brazlândia, Distrito Federal, Brazil. Seven isolates obtained were subjected to phenotypic and molecular characterization, including pathogenicity tests, LOPAT, and partial sequencing of the 16S rDNA gene. All isolates were gram-negative, aerobic, oxidase-positive, produced fluorescent pigment, induced hypersensitivity in tobacco leaves, used sorbitol and glutamate and were pathogenic to 24 different plant species. Results of these tests and analysis of the sequences of rDNA showed 100% identity with *Pseudomonas cichorii*. To our knowledge, this is the first report of *P. cichorii* in gerbera in the Federal District.

Keywords: *Gerbera jamesonii*, leaf spot, LOPAT.

RESUMO

Identificação e patogenicidade de *Pseudomonas cichorii* associada ao crestamento bacteriano da gérbera no Distrito Federal

Em 2013, amostras foliares de plantas de gérbera, apresentando sintomas de crestamento, foram coletadas em telados de cultivo de corte, no Núcleo Rural Alexandre Gusmão, localizado em Brazlândia-DF. Sete isolados bacterianos obtidos foram submetidos à caracterização fenotípica e molecular, incluindo testes de patogenicidade, testes LOPAT e sequenciamento parcial do gene 16S rDNA. Todos os isolados foram gram-negativos, aeróbios, oxidase positivos, produziram pigmento fluorescente, induziram reação de hipersensibilidade em folhas de fumo, utilizaram sorbitol e glutamato, e foram patogênicos a 24 diferentes espécies de plantas. Os resultados destes testes e análises de sequências do rDNA mostraram 100% de identidade com *Pseudomonas cichorii*. Para o nosso conhecimento, este é o primeiro relato de *P. cichorii*, em gérbera, no Distrito Federal.

Palavras-chave: *Gerbera jamesonii*, mancha foliar, LOPAT.

(Recebido para publicação em 14 de outubro de 2014; aceito em 22 de setembro de 2015)
(*Received on October 14, 2014; accepted on September 22, 2015*)

The bacterial species *Pseudomonas cichorii*, which was originally isolated from the herbaceous plants *Cichorium intybus* and *Cichorium endivia*, belongs to rRNA homology group I, together with *P. syringae* (Garrity *et al.*, 2005). Recent taxonomic advances, based on biochemical and genotypic processes, demonstrated that the plant pathogenic species *P. cichorii* consists of a cluster of closely related genomic groups with genetic heterogeneity of the species (Trantas *et al.*, 2013).

This bacterium has already been reported causing leaf spot in many plants, and recently it was reported in *Stevia rebaudiana* in Florida (Strayer *et al.*, 2012), associated with leaf blight in soybean in South Korea (Yu, 2012), a new symptom of okra bacterial leaf blight in a local variety of okra (*Abelmoschus esculentus*) in Japan (Inoue *et al.*, 2013) and in Chengdu City in China (Li *et al.*, 2014) causing leaf spot of vegetable sponge (*Luffa cylindrical*).

In Brazil it is considered an opportunistic pathogen in that it infects a wide range of dicotyledons and herbaceous plants, including vegetables and ornamental species. In lettuce, bacterial blight is not a limiting disease, varying with climatic and inoculum densities. Among crucifers, *P. cichorri* causes spots on the leaves and head of cabbage (Amorim *et al.*, 2005). In sunflower, this bacterium causes spot and blight on the stalks, first described in 1981 (Robbs & Almeida, 1981). It has also been found causing leaf spot in mint, *Mentha arvensis* (Maia *et al.*, 1996). Among ornamentals, it mainly occurs in chrysanthemum, philodendron, and African violet. Silva Júnior *et al.* (2009) reported this bacterium affecting the tomato crop in the state of São Paulo for the first time in 2007 and also registered leaf blight on eucalyptus (Gonçalves *et al.*, 2008).

Gerbera is an Asteracea that originates in South Africa and Asia (Radice & Marconi, 1998), and it is among the most extensively exploited flowers in Brazil. In this plant, bacterial blight caused by *P. cichorii* was first described in Apopka, Florida (Miller & Knauss, 1973) and subsequently in Greece (Alivizatos, 1986). Although its occurrence in Brazil has not been clearly

established, it was reported in São Paulo (Malavolta Júnior *et al.*, 1994). According to studies on the cataloguing of gerbera diseases in the state of Paraná from 2004 to 2006, leaf spot was noted when inspections were carried out in properties producing ornamental plants, but in only two commercial pot-plants (Ferronato *et al.*, 2008). In the Federal District of Brazil, leaf spot has been observed in gerbera cultivars for some time. Therefore, the objective of this work was to investigate the causal agent associated with bacterial blight of gerbera and to study its range of hosts.

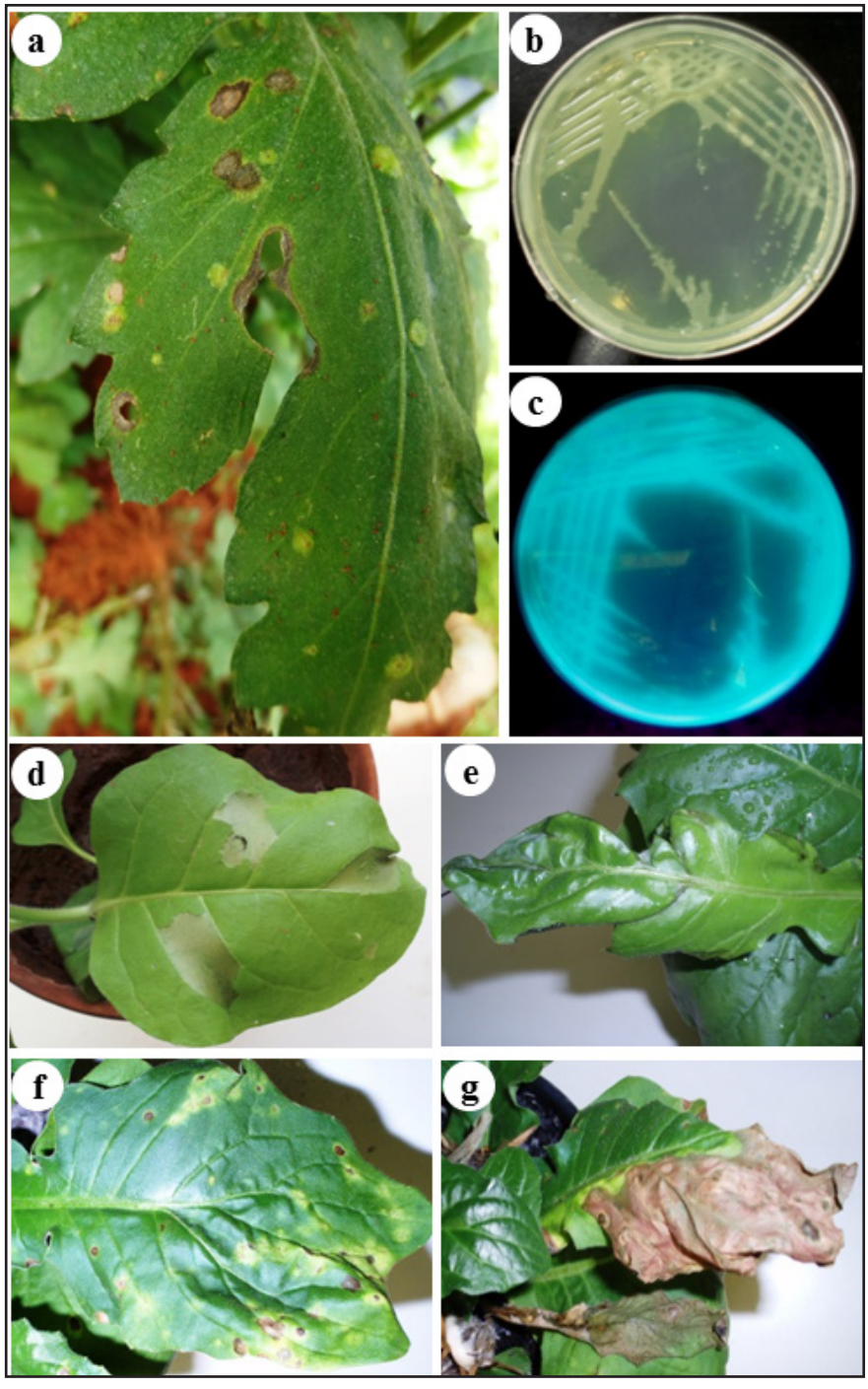
MATERIAL AND METHODS

In February 2013, leaves from different plants showing symptoms of leaf blight were collected in six high tunnels, approximately 8 m wide and 50 m long (8 x 50), from a field of gerbera (directly in the soil) situated at Núcleo Rural Alexandre Gusmão, located in Brazlândia, Federal District, Brazil. Overall, the plants showed 80% infection, especially in lower leaves. The same region produces sunflower, chrysanthemum, and goldenrod.

Segments of symptomatic leaves were surface disinfected, macerated in sterile distilled water and then used for dilution plating on culture media 523 (Kado & Heskett, 1970). Single colonies were selected and submitted to standard morphological, physiological, and/or biochemical tests for bacterial identification according to Garrity *et al.* (2005) and Schaad *et al.* (2001).

Hypersensitivity response (HR) tests were carried out by infiltrating a 10^9 cfu/mL (Scale 7 of McFarland) bacterial suspension on tobacco leaves using a needle-less syringe. For this, the bacterial isolates were grown on 523 solid medium for 48 h and then diluted in sterile distilled water. Characteristic HR symptoms were scored at 24 h. Bacterial inoculation on gerbera plants (pathogenicity test) was carried out by spraying a 10^9 cfu/mL bacterial suspension on the abaxial and adaxial surfaces of the leaves up to the beginning of run-off; following inoculation, plants were incubated for 24

Figure 1. Pathogenicity tests of gerbera bacterial isolates. A) Gerbera plant with necrotic lesions on leaf, observed in the field; b) Whitish colonies producing green pigment; in 523 culture medium; c) Observation of fluorescent pigments in King B medium, under UV light; d) Hypersensitivity response (HR) test in tobacco leaf 16 hours after inoculation; e) Lesions on the edges of gerbera leaves; f) scattered necrotic spot-like lesions, with a depressed center, followed also by leaf chlorosis; and g) Coalescent lesions, leaf blight {teste de patogenicidade com isolados de gérbera. a) Planta de gérbera com lesões necróticas na folha, observada em campo; b) Colônias brancas produzindo pigmento verde, em meio 523; c) Observação do pigmento fluorescente em meio B de King, sob luz UV; d) Reação de hipersensibilidade em folha de fumo 16 h horas após a inoculação (HR); e) Lesões nas margens das folhas de gérbera; f) Lesões necróticas esparsas, com centro deprimido, no limbo foliar; e g) Coalescência das lesões, crestamento}. Brasília, UnB, 2013.



h at 28ºC in a wet chamber and then kept in the greenhouse for the remainder of the experiment. In order to fulfill Koch's postulates, disease symptoms were observed and the pathogen re-isolated in culture medium.

Purification of the total DNA of the bacterial genome was carried out using the Wizard® Genomic DNA Purifications Kit (Promega, Madison, WI), and a partial sequence of the 16S rDNA gene was amplified using the universal primers for bacteria F984 5'-AACGCGAAGAACCTTAC-3' and R1492 5'-CTACGGYTACCTTGTTACGAC-3' (Heuer *et al.*, 1997). The obtained PCR products were purified and sequenced (Macrogen Inc.). The resulting sequences were analyzed using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov). The contiguous sequences were assembled using the DNA Sequence Assembly BASER Software v.4.5.0 (http://www.dnabaser.com/index.html).

For phylogenetic analysis, the sequences were aligned using MAFFT (Katoh & Standley, 2013). The trees were determined using MrBayes v3.2.1 (Ronquist & Huelsenbeck, 2003), which uses the Markov chain Monte Carlo method (MCMC). The analysis was released using the default settings except the appropriate template, which was implemented automatically using "Reversible-jump" MCMC (Huelsenbeck *et al*., 2004). The program ran for 2,000,000 generations and the frequency separation stabilized below 0.008. The first 25% of the trees were discarded ("burn-in") before calculating the consensus tree.

RESULTS AND DISCUSSION

A total of seven gram-negative bacterial isolates (PC1, PC2, PC3, PC4, PC5, PC6, and PC7) were isolated from the symptomatic tissues of gerbera plants collected in the field (Figure 1a). The bacterial isolates were included in the Collection of Plant Pathogenic Bacteria of the Department of Plant Pathology (University of Brasília) with the following designations: UnB 1379, UnB 1380, UnB 1381, UnB 1382, UnB 1383, UnB 1384, and UnB 1385.

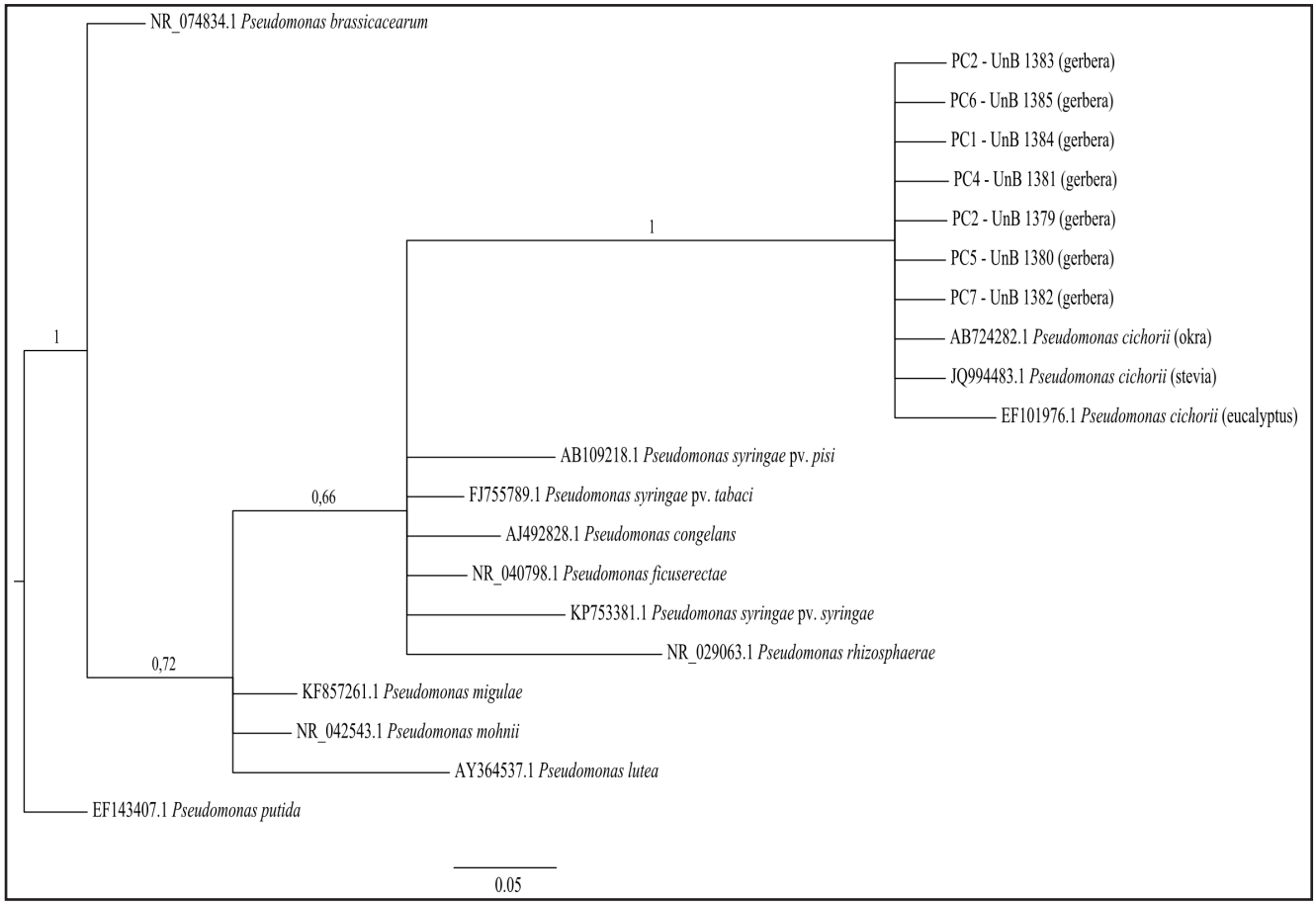
All bacterial isolates had whitish colonies (Figure 1b); positive for production of fluorescent pigments in King B medium (Figure 1c); HR positive (Figure 1d), gram negative, KOH positive, oxidative, negative for hydrolyses of gelatin and mucoid

Table 1. Plants used in the pathogenicity and host tests, in reaction to artificial inoculation of seven bacterial isolates obtained from gerbera in the Federal District (plantas utilizadas no teste de patogenicidade e de hospedeiras, em reação a inoculação artificial com sete isolados bacterianos, obtidos de gerbera no Distrito Federal). Brasília, UnB, 2013.

Common name	Scientific name	Family	Result in test
Amaranth	*Amaranthus* sp.	*Amaranthaceae*	+*
Philodendron	*Philodendron* sp.	*Araceae*	+
Silver potho	*Scindapsus* sp.	*Araceae*	-
Syngonium	*Syngonium podophyllum*	*Araceae*	+
Umbrella tree	*Schefflera arboricola*	*Araliaceae*	-
Chicory	*Cichorium endivia*	*Asteraceae*	+
Chrysanthemum	*Chrysanthemum* sp.	*Asteraceae*	+
Gerbera	*Gerbera jamesonii*	*Asteraceae*	+
Sunflower	*Helianthus annuus*	*Asteraceae*	+
Daisy	*Bellis* sp.	*Asteraceae*	+
Silver ragwort	*Senecio cineraria*	*Asteraceae*	-
Marigold	*Tagetes patula*	*Asteraceae*	+
Zinnia	*Zinnia elegans*	*Asteraceae*	+
Impatiens	*Impatiens walleriana*	*Balsaminaceae*	+
Cabbage	*Brassica oleracea* var. *capitata*	*Brassicaceae*	+
Mustard	*Brassica juncea*	*Brassicaceae*	+
Beetroot	*Beta vulgaris*	*Chenopodiaceae*	+
Squash	*Cucurbita pepo*	*Cucurbitaceae*	+
Soybean	*Glycine max*	*Fabaceae*	+
Pea	*Pisum sativum*	*Fabaceae*	+
African violet	*Saintpaulia ionantha*	*Gesneriaceae*	+
Mint	*Mentha* sp.	*Lamiaceae*	+
Purslane	*Portulaca oleracea*	*Portulacaceae*	+
Coffee	*Coffea arabica*	*Rubiaceae*	-
White eggplant	*Solanum melongena*	*Solanaceae*	+
Datura	*Datura stramonium*	*Solanaceae*	+
Petunia	*Petunia* x hybrida	*Solanaceae*	+
Sweet pepper	*Capsicum annuum*	*Solanaceae*	+
Tomato	*Solanum lycopersicum*	*Solanaceae*	+

Positive result in the test (+), negative result in the test (-) {resultado positivo no teste (+), resultado negativo no teste (-)}.

Figure 2. Phylogenetic tree based on bacterial 16S rDNA gene sequences from seven gerbera isolates and references from GenBank (names preceded by accession number). Estimation is based on Bayesian inference and MAFFT alignment (árvore genética baseada em sequências do gene 16S rDNA de sete isolados de gérbera e referências do GenBank (nomes precedidos do número de acesso). Estimativa baseada na inferência Bayesiana e alinhamento no MAFFT). Brasília, UnB, 2013.



colonies in YDC medium. In the LOPAT test the isolates were: levan negative, oxidase positive, arginine dihydrolase negative and had no pectinolitic activity on potato discs. The reduction of nitrate was negative, as was the utilization of sorbitol, trehalose, cellobiose, D-arabinose, oxalate, tartrate, salicine, succinate, glycerol, saccharose and citrate. On the other hand, the isolates used mannitol and glutamate, in accordance with Garrity *et al.* (2005); and Schaad *et al.* (2001).

In inoculated gerbera plants, the typical symptoms of bacterial blight could be observed (Malavolta Júnior *et al.*, 1994; Ferronato *et al.*, 2008), three days after inoculation. Initially, dark lesions appeared on the edges of the leaves (Figure 1e). These evolved to become necrotic spot-like lesions, with a depressed center distributed across the leaf area. Leaves became chlorotic (Figure 1f), coalescent and dried out (Figure 1g). Stem and flower rotting could also be observed. Plants exhibited irregular lesions on leaf edges with a depressed center (Figure 1f). The isolates were pathogenic to 24 other plants (Table 1). Taking into account the fulfillment of Koch's postulates, altogether, these results confirmed the pathogenicity of the isolates.

Studies that report the occurrence of this bacteriosis in Brazil are scarce (Malavolta Júnior *et al.*, 1994). In this article, most of the plants showed symptoms of infection by the bacterial isolates obtained from gerbera, although the symptoms were more pronounced in gerbera, in which lesions even coalesced and leaves died; however, the plants were still able to recover and bloom again. In the other plants the symptoms were discrete and did not affect their development, for example in tomato, in which the lesions were small and sparse on the leaf. However, this was unlike the symptoms induced by isolates on tomato in the work of Silva Júnior *et al.* (2009), in which the disease appeared in the form of wet irregular lesions that evolved to irregular necrosis of the leaf area.

The biochemical oxidase test (positive) allowed the distinction of gerbera isolates from *P. syringae* group, *P. congelans, P. rhizosphaerae* and *P. lutea*. The arginine dihydrolase test (negative) also differentiated them from *P. putida, P. mohnii* and *P. brassicacearum*. Trehalose distinguished *P. cichorii* (used it) from *P. migulae,* which did not use it (Garryti *et al.*, 2005; Schaad *et al.*, 2001).

Heterogeneity within the species *P. cichorii* and its host range is already recognized (Trantas *et al.*, 2013). Thus the amplification of the 16S rDNA

sequences using universal primers generated a product of approximately 545 bp for the seven isolates tested, with PCR products varying from 508 to 523 bp. The verification of the sequences in *BLASTn* showed identity with various *Pseudomonas* species. The non-fluorescent representatives were *P. lutea*; *P. mohnii, P. ficuserectae,* and *P. rhizosphaerae*. The fluorescent representatives were: *P. syringae*, *P. migulae*, *P. brassicacearum*, *P. congelans,* and *P. putida*. The phylogenetic tree that was generated clarified the identity of isolates (Figure 2) which showed 100% similarity with accessions AB724282.1 (Inoue *et al.*, 2013), JQ994483.1 (Strayer *et al.*, 2012) and EF101305.1 (Gonçalves *et al.*, 2008) of *P. cichorii*, whose original hosts are okra, stevia, and eucalyptus, respectively.

These results based on identification in different tests suggest that the causal agent of bacterial blight of gerbera in the Federal District belongs to the *Pseudomonas cichorii* species. In addition, the isolates were pathogenic to the other 24 plants in artificial inoculations. This bacterium has a wide distribution and range of hosts, and this report is important for cataloging plant-pathogenic bacteria that occur throughout Brazilian territory and in special conditions may become epidemic.

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