Ciência e Agrotecnologia
SCIENCE AND AGROTECHNOLOGY
Ciência e Agrotecnologia, 45:e000121, 2021
http://dx.doi.org/10.1590/1413-7054202145000121
Agricultural Sciences
eISSN 1981-1829

Diagnosis of leaf bacterial diseases of coffee reveals the prevalence of halo blight

Diagnóstico de doenças bacterianas foliares de cafeeiro revela prevalência da mancha aureolada

Melina Korres Raimundi[1], Ricardo Magela de Souza[1*], Antônia dos Reis Figueira[1], Gustavo Matheus Silva[1], Ana Carolina de Paula Santos[1], Sarah da Silva Costa Guimarães[1]

[1]Universidade Federal de Lavras/UFLA, Departamento de Fitopatologia, Lavras, MG, Brasil
*Corresponding author: rmagelas@ufla.br
Received in January 5, 2021 and approved in April 5, 2021

ABSTRACT

The diagnosis of foliar bacterial diseases in coffee (*Coffea arabica*), such as halo blight (*Pseudomonas syringae* pv. *garcae*), bacterial leaf spot (*P. syringae* pv. *tabaci*), bacterial blight (*P. cichorii*), and dark leaf spot (*Robbsia andropogonis*), is considered a challenge for plant pathologists. The misidentification has been occurring when the diagnosis is solely based on symptoms and biochemical properties. Thus, the objective of this study was to identify and differentiate species and pathovars of *Pseudomonas* pathogenic to coffee plants, enabling a survey of the occurrence of these bacteria in the main producing regions of Minas Gerais state, Brazil. Firstly, the pathogenicity of the isolates was confirmed by inoculation in *C. arabica* cv. Catuaí Vermelho IAC 99. Then, biochemical analyses, combined with, repetitive element-polymerase chain reaction (rep-PCR) and phylogeny based on *rpoD* gene sequences were used to characterize 84 *Pseudomonas* isolates from coffee crops and nurseries. Based on *rpoD*-phylogeny, 73 isolates were identified as *P. syringae* pv. *garcae*, five as *P. syringae* pv. *tabaci* and six as *P. cichorii.* The rep-PCR results suggest a high genetic variability in populations of *Pseudomonas syringae* pv. *garcae* and *P. cichorii*. This is the first report of the occurrence of bacterial leaf spot (*P. syringae* pv. *tabaci*) in the coffee-producing filed in Minas Gerais State. The findings confirmed the prevalence of *P. syringae* pv. *garcae* in coffee production fields in the State and the generated knowledge will contribute for the development of species-specific primers for the identification and detection of this pathogen.

Index terms: Dark leaf spot; rep-PCR; *rpoD* gene.

RESUMO

A diagnose das bacterioses causadoras de manchas foliares na cultura do cafeeiro (*Coffea arabica*), mancha aureolada (*Pseudomonas syringae* pv. *garcae*), mancha bacteriana (*P. syringae* pv. *tabaci*), crestamento bacteriano (*P. cichorii*) e mancha escura (*Robbsia andropogonis*) é considerada um desafio para os fitopatologistas. A identificação incorreta pode ocorrer quando o diagnóstico é baseado exclusivamente em sintomas e propriedades bioquímicas. Assim, o objetivo com este estudo foi identificar e diferenciar as espécies e patovares de *Pseudomonas* patogênicos ao cafeeiro, possibilitando um levantamento da ocorrência dessas bactérias nas principais regiões produtoras de Minas Gerais, Brasil. Primeiramente, a patogenicidade dos isolados foi confirmada por inoculação em *C. arabica* cv. Catuaí Vermelho IAC 99. Em seguida, análises bioquímicas, combinadas com PCR convencional, reação em cadeia de polimerase de elemento repetitivo (rep-PCR) e filogenia baseada em sequências do gene *rpoD*, foram usadas para caracterizar 84 isolados de *Pseudomonas* de lavouras de café e viveiros em Minas Gerais. Com base na filogenia do gene *rpoD*, 73 isolados foram identificados como *P. syringae* pv. *garcae*, cinco como *P. syringae* pv. *tabaci* e seis como *P. cichorii*. Os resultados de rep-PCR sugerem alta variabilidade genérica nas populações de *Pseudomonas syringae* pv. *garcae* e *P. cichorii.* Este é o primeiro relato da ocorrência de mancha foliar bacteriana (*P. syringae* pv. *tabaci*) em campo de produção de café no Estado de Minas Gerais. Os resultados confirmaram a prevalência de *P. syringae* pv. *garcae* em lavouras de café no Estado e o conhecimento gerado contribuirá para o desenvolvimento de *primers* espécie-específicos para identificação e detecção deste patógeno.

Termos para indexação: Mancha escura; rep-PCR; gene *rpoD*.

INTRODUCTION

Coffee (*Coffea* spp.), one of the most economically important crops worldwide, is consumed in all continents, mainly in temperate countries in the northern hemisphere (United States Department of Agriculture - USDA, 2020b). Brazil is the largest global coffee producer and exporter, with an estimated production of 63.08 million bags benefiting from Arabica and Conilon coffee in the

2020 harvest. The State of Minas Gerais is the main coffee producer in Brazil, with 34.65 million bags, approximately 55% of national production. Arabica coffee accounts for more than 90% of State coffee (Companhia Nacional de Abastecimento - CONAB, 2021, USDA, 2020a).

Bacterial diseases have stood out in recent years among the factors affecting coffee productivity by causing significant losses (Thind, 2020). In Brazil, four foliar bacterial diseases have been described in coffee: halo blight, which is caused by *Pseudomonas syringae* pv. *garcae* (Amaral; Teixeira; Pinheiro, 1956; Young; Dye; Wilkie 1978); bacterial leaf spot, which is caused by *P. syringae* pv. *tabaci* (Yong; Dye; Wilkie, 1978) bacterial blight, which is caused by *Pseudomonas cichorii* (Robbs et al., 1974), and dark leaf spot, which is caused by *Robbsia andropogonis* (Lopes Santos et al., 2017; *syn. Burkholderia andropogonis, P. andropogonis*).

Halo blight was first detected in the producing regions of the states of Paraná, São Paulo and Minas Gerais (Young; Dye; Wilkie 1978; Vale; Zambolim, 1997). This disease is currently considered one of the main bacterial diseases of coffee and has been a limiting factor for coffee growing in cold regions exposed to wind, with high rainfall and at high altitudes in developing or recently pruned fields and/or in nurseries (Zoccoli; Takatsu; Uesugi, 2011). The disease also occurs in Africa, mainly in Kenya, where it is rapidly expanding (Ithiru et al., 2013). The symptoms characterized by dark brown spots with irregular shapes surrounded by a yellow halo can be easily confused with caused by the other bacterial species (Destéfano et al., 2010). The colony morphology and biochemical characteristics including fluorescent pseudomonads, positive for levan sucrase activity, negative for oxidase activity, inability to rot potato, ability to produce arginine dihydrolase, ability to cause a hypersensitive response on tobacco, hydrolysis of gelatin with no accumulation of Poly-β-hydroxybutyrate and the use of same various sugars, of these bacteria are also similar (Schaad; Jones; Chun, 2001). These similarities mainly apply to *P. syringae* pv. *garcae* and *P. syringae* pv. *tabaci,* which may lead to incorrect diagnosis of the disease and thus overestimation of the occurrence of halo blight in the crop and underestimation of the occurrence of bacterial leaf spot. Moreover, correct identification of the etiological agent is clearly a fundamental premise for the development of effective management strategies.

Thus, the repetitive element-polymerase chain reaction (rep-PCR) DNA fingerprint, which includes the repetitive extragenic palindromic sequence-based PCR (REP-PCR), enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) and 154 bp-box elements PCR (BOX-PCR), have been used to differentiate bacterial isolates in study the genetic diversity of plant pathogens (Louws et al., 1994) and accurately identify species and pathovar when type strains are included as references (Tindall et al., 2010). According to Louws et al. (1994), the technique (REP, BOX and ERIC) was effective in distinguishing the different pathovars of *Xanthomonas campestris* and *Pseudomonas syringae*. In the differentiation of the *P. syringae* pathovars, the generated fingerprints were highly characteristic for most of the pathovars tested, and few intra-pathovar variations were noted.

The convenience of sequences of the *rpoD* gene (RNA polymerase sigma^{70} factor) in phylogenetic analyses have been used for rapid and precise identification of species within the *Pseudomonas syringae* pathogen complex, due to its significant phylogenetic information on *Pseudomonas* spp. (Parkinson et al., 2011; Rajwar; Sahgal, 2016; Timilsina et al., 2017; Mulet et al., 2020). This gene is one of the sigma factors that confer promoter-specific transcription initiation on RNA polymerase (Lonetto et al., 1992; Tayeb et al., 2005).

We hypothesize that *Pseudomonas syringae* pv. *garcae* is responsible for the recent outbreak of foliar bacterial diseases in the field coffee in Minas Gerais Brazil. The aim of this study was to confirm the pathogenicity of isolates, to use biochemical analyses and rep-PCR to discriminated the isolates, and phylogeny to identify *P. syringae* species complex strains collected from coffee plants with symptoms of foliar bacterial diseases from several municipalities of the main producing regions of the Minas Gerais State.

MATERIAL AND METHODS

Obtaining pure cultures and pathogenicity test

Samples of leaves and stems collected from seedlings or coffee plants showing typical symptoms of halo spot, from several municipalities in the Minas Gerais State, Brazil, were collected or delivered by coffee producers (one sample per week) to the Laboratory of Plant Bacteriology from the Department of Phytopathology at the Universidade Federal de Lavras - UFLA, mainly in the months of the greatest occurrence of the disease, January, February, March, April and May from 2012 to 2016. The samples were subjected to the exudation test (Mariano; Souza, 2016),

to confirm the bacterial origin, and after isolation in King B medium (King et al., 1954) at 28 °C. After 48 h, colonies were selected and the strains were stored in 15% peptone glycerol broth at −20 °C (Mariano; Souza, 2016).

Preliminary screening for pathogenicity was performed with 161 isolates. Among these isolates, 84 were pathogenic to coffee seedlings and were used for further studies. To determine the pathogenicity of the isolates, healthy coffee seedlings of the *Coffea arabica* cv. Catuaí Vermelho IAC 99 were inoculated with a bacterial suspension prepared in saline solution (0.85% NaCl) from 48 h-old cultures grown on King's B, of each isolate separately. The cell density was adjusted to $A_{600 =}$ 0.2 equivalent to of 10^9 CFU mL^{-1} (Oliveira; Romeiro, 1990). The inoculation method was injection, in which 5 mL of suspension was infiltrated into leaves by a fine hypodermic syringe (Mariano; Souza, 2016). For negative control, leaves were infiltrated with sterile distilled water. The inoculated plants were covered with plastic bags for 24 h to maintain high humidity conditions, and arranged in completely randomized design, then were kept in a greenhouse. After disease symptom development, leaf samples were taken for bacteria re-isolation. For completing Koch's postulates, the bacteria colonies re-isolated were compared with those used for inoculation by phenotypic characters such as fluorescent pigment in KB medium production and oxidase production. Type/reference strains and isolates from other species were also used in the analyses (Table 1).

Biochemical characterization

Eighty-four bacterial isolates were biochemically characterized according to Pérez et al. (2017); Schaad, Jones and Chun (2001) and Barta and Willis (2005). The following tests were used: The Gram reaction, oxidase and arginine dihydrolase tests, the hypersensitivity reaction in tobacco leaves, gelatin hydrolysis, esculin hydrolysis, fluorescent pigment production in King's B medium and the use of sorbitol, sucrose, adonitol and lactose.

DNA extraction

The genomic DNA from the isolates was extracted using the Archive Pure DNA Cell/Tissue extraction kit (5 PRIME), quantified using a spectrophotometer (NanoDrop ND-1000 UV-Vis), adjusted to a concentration of 50 ng/µL by diluting in ultrapure water, and stored at -20 °C prior to use.

rep-PCR DNA fingerprinting

Primer sequences corresponding to repetitive extragenic palin-dromic (REP) DNA sequences (REPIR-I 5' IIICGICGICATCIGGC 3' and REP-2I 5' ICGICTTATGIGGCCTAC 3') (Louws et al., 1994), enterobacterial repetitive intergenic consensus (ERIC) elements (ERIC1R 5' ATGTAAGCTCCTGGGGATTCAC 3' and the BOX elements (BOX-A1R 5 'CTAC GGCAAGGCGACGCTGACG 3') (Louws et al., 1994) were used for DNA fingerprinting of reference strains (Table 1) and isolates this study (Table 2).

Table 1: Reference bacterial strains used in this study.

Strains	Species/pathovar	Host
CFBP 1634	*Pseudomonas syringae* pv. *garcae*	*Coffea arabica*
CFBP 2101	*Pseudomonas cichorii*	*Coffea arabica*
UFLA 160	*Pseudomonas cichorii*	*Lactuca sativa*
IBSBF 2249	*Pseudomonas syringae* pv. *tabaci*	*Coffea arabica*
IBSBF 1972	*Pseudomonas syringae* pv. *tabaci*	*Nicotiana tabacum*
IBSBF 1822	*Pseudomonas syringae* pv. *tabaci*	*Carica papaya*
IBSBF 758	*Pseudomonas syringae* pv. *tabaci*	*Cucumis sativus*
IBSBF 974	*Pseudomonas syringae* pv. *tabaci*	*Desmodium canum*
IBSPF 166	*Robbsia andropogonis*	*Coffea arabica*
UFLA Pst	*Pseudomonas syringae* pv. *tomato*	*Solanum lycopersicum*
UFLA Cmm	*Clavibacter michiganensis* subsp. *michiganensis*	*Solanum lycopersicum*

* CFBP = French Collection of plant pathogenic bacteria, IBSBF = Phytobacteria Culture Collection of the Biological Institute, Campinas, SP, Brazil. UFLA= Universidade Federal de Lavras collection of plant pathogenic bacteria.

Table 2: Strains pathogenic to coffee identified by rep-PCR DNA fingerprint, phylogeny based on *rpoD* gene and biochemical tests and their GenBank accession numbers.

Strains	Origin[1]	*rpoD GenBank accession*
Pseudomonas syringae pv. garcae		
UFLA: 06; 20; 21; 77; 78; 79; 80; 85; 86; **87;** 88; 89 A; 89C; 90; 91A; 97; 98; 99; 101; 102; 103; 104; 105; 106; 107; 109	Nepomuceno	MT006247
UFLA: 81; 82; 83	Lavras	
UFLA: 43; 44; 46; 48; 52; 53; 54; 58; 59; 60; 61; 114; 115; 116; 117; 118; 119; 120; 121; 127; 130; 157; **158**	Três Pontas	MT006259
UFLA 84	Patos de Minas	
UFLA 112	Varginha	
UFLA 113	Vargem Grande	
UFLA: 122; 123	Nova Rezende	
UFLA **125**	São Sebastião do Paraíso	MT006248
UFLA: 126; 154	Santo Antônio do Amparo	
UFLA: 131; 132; 133; 134	Patrocínio	
UFLA: **138**; 139	Elói Mendes	MT006253
UFLA: 148; 149	Ijací	
UFLA: 150; 151	Muzambinho	
UFLA: 152; 153; 156	Santana da Vargem	
Pseudomonas syringae pv. tabaci		
UFLA **69**	Candeias	MT006246
UFLA: **128**; **129**; **142**; **143**	São Sebastião do Paraíso	MT006249; MT006250; MT006254; MT006255
Pseudomonas cichorii		
UFLA **135**	Patrocínio	MT006251
UFLA: **136**; **145**; **146**; **147**;159	Lavras	MT006252; MT006256; MT006257; MT006258

[1] Municipalities in the State of Minas Gerais, Brazil; [2] Genbank number of *rpoD* gene for DNA directed RNA polymerase subunit D generated in this study. Strains in bold were used in phylogeny analyses.

All the reactions were performed in a final volume of 25 µL containing 2.5 µL 10X Taq buffer, 2.0 µL $MgCl_2$ (25 mM), 1 µL dNTP (10 mM), 2 µL each primer (10 µM), 0.5 µL Taq DNA polymerase (5 U/µL) (Invitrogen Life Sciences, São Paulo, Brazil), 2 µL DNA (50 ng/µL) and 13.0 µL nuclease-free water. The thermal conditions for DNA amplification were used according to Louws et al. (1994) in a Therm-1000 Axygen Maxy Gene thermocycler. For the REP-PCR, the conditions were: initial denaturation at 95 °C for 6 min followed by 30 cycles (94 °C for 1 min, 44 °C for 1 min and 65 °C for 1 min), and final extension cycle at 65 °C for 16 min. For the ERIC-PCR, were: initial denaturation at 95 °C for 7 min and 30 cycles (94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min), with a final extension cycle at 65 °C for 16 min. For the BOX-PCR, were: initial denaturation at 95 °C for 2 min and 30 cycles (95 °C for 30 s, 52 °C for 1 min and 72 °C for 5 min) with a final extension cycle at 72 °C for 5 min.

All the rep-PCR products were separated by gel electrophoresis on a 1.5% agarose gel in 1X TBE buffer and stained with GelRed nucleic acid gel stain (Biotium, Hayward, USA). The electrophoretic profiles were used to compare representatives of the 84 bacterial

isolates pathogenic to coffee plants, and reference strains (Supplementary Figs. S1-S3). The similarity matrix was constructed using the Dice coefficient. Dendrograms were obtained using the unweighted pair-group method with the arithmetic mean (UPGMA) clustering algorithm using PAST software version 2.17 (Hammer; Harper; Ryan, 2001).

PCR amplification and DNA sequencing

Based on rep-PCR clusters, were selected four isolates with genetic profiles similar to that of the *P. syringae* pv. *garcae* reference strain, five isolates with genetic profiles similar to that of the *P. syringae* pv. *tabaci* reference strain and five isolates identified as *P. cichorii*. The primers PsrpoD FNP1 (5' TGAAGGCGARATCGAAATCG CCAA 3') and PsrpoDnprpcr1 (5' YGCMGWCAGC TTYTGCTGGCA 3') were used to amplify the *rpoD* gene (Parkinson et al., 2011). The reaction was performed in a volume of 25 µL containing 2.5 µL 10X Taq buffer, 1.5 µL $MgCl_2$ (25 mM), 1 µL dNTP mix (10 mM), 1 µL each primer (10 µM), 0.25 µL Taq DNA polymerase enzyme (5 U/µL) (Invitrogen Life Sciences, São Paulo, Brazil), 1 µL of DNA (50 ng/µL) and 16.75 µL nuclease-free water. Amplification was performed in a Therm-1000 Axygen Maxy Gene thermocycler using the following cycles: initial denaturation at 94 °C for 2 min and 34 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 30 s, with a final extension cycle at 72 °C for 5 min. The amplified products (700 bp) were analyzed on 1.0% agarose gel stained with GelRed nucleic acid gel stain (Biotium, Hayward, USA), in 1X TBE buffer. Amplified fragments were cleaned with Wizard® SV Gel and a PCR Clean-Up System (Promega, São Paulo SP, Brazil). Bidirectional DNA sequences were generated by Macrogen, USA, using the same primers as for PCR amplification.

Phylogenetic analyses

Consensus sequences were assembled from forward and reverse sequences by using SeqAssem ver. 07/2008 (SequentiX - Digital DNA Processing, DE). The consensu *rpoD* sequences were compared with those already available in GenBank using the BLAST. Sequences of species type strains (STS) and pathovar type strains (PTS) in *Pseudomonas syringae* species complex were obtained from NCBI GenBank (http://www.ncbi.nlm.nih.gov) and included in the analyses. The sequences were aligned using Clustal-W as implemented by MEGA7.0 program (Kumar et al., 2008; Thompson; Higgins; Gibson, 1994) and manually adjusted to allow maximum sequence similarity. The resulting dataset consisted of 163 parsimony-informative positions in 585bp. Maximum Likelihood (ML) phylogenetic tree was constructed with the MEGA7.0 program (Kumar et al., 2008; Thompson; Higgins; Gibson, 1994) using the nucleotide substitution models Tamura 3-parameter (T92) with gamma distribution and by assuming that a certain fraction of sites is evolutionarily invariable (G+I). The tree topologies obtained from ML analyses was evaluated with 1000 bootstrap replications. *Pseudomonas lutea* (LMG 21974) was used as outgroup taxa. The DNA sequences generated in this study have been deposited in NCBI GenBank.

RESULTS AND DISCUSSION

Bacterial isolation and pathogenicity confirmation

A total of 161 bacterial isolates with a positive result in exudation test were obtained from coffee plants and seedlings from various municipalities in the State of Minas Gerais. From this, 84 isolates had confirmed pathogenicity (Table 2) due to the production of typical symptoms such as necrotic lesions with or without a yellow halo in coffee seedlings five days after inoculation. The symptoms induced by isolates this study were similar with produced by reference strains. On a subsequent isolation from symptomatic inoculated leaves, the colonies were morphologically similar to the ones isolated for the first time. Overall, they displayed a rounded shape with irregular margins and were small to medium in size, with only five of them showing strong fluorescence in King's B medium, which were identified as *P. syringae* pv. *tabaci*, and only six isolates oxidase-positive, which were identified as *P. cichorii*, the other 73 isolates were identified as *P. syringae* pv. *garcae*. Thus, Koch's postulates were completed.

Due to the similarity of the symptoms caused mainly by *P. syringae* pv. *garcae* and *P. syringae* pv. *tabaci*, bacterial leaf spot in coffee plants may be incorrectly diagnosed as halo blight, since *P. syringae* pv. *tabaci* is associated with a large number of host plants, and some of these plants may act as a source of primary inoculum for coffee. This factor may be linked to the study by Petek et al. (2006); Sera, Sera and Fazuoli (2017) who reported the occurrence of a pv. *garcae*-like pathotype in field conditions in the state of Paraná, suggesting that the lesions observed by these authors in resistant coffee plants in an experimental field located in the municipality of Londrina were caused by *P. syringae* pv. *tabaci*. The results obtained

by Rodrigues et al. (2017a) also suggested that the lesions observed by Petek et al. (2006); Sera, Sera and Fazuoli (2017) were caused by *P. syringae* pv. *tabaci.*

Biochemical characterization

The 84 isolates pathogenic to coffee plants were Gram negative and arginine dihydrolase negative, hydrolyzed esculin, and induced the hypersensitivity in tobacco leaves, since these isolates were obtained from coffee plants. Most of the isolates were negative for oxidase and positive for gelatin hydrolysis. The exceptions were *P. cichorii* (CFBP 2101), UFLA 135, UFLA 136, UFLA 145, UFLA 146, UFLA 147 and UFLA 159 for oxidase and *P. cichorii* (CFBP 2101), UFLA 135, UFLA 136, UFLA 145, UFLA 146, UFLA 147, UFLA 159, UFLA 21, UFLA 48, UFLA 60, UFLA 61, UFLA 79, UFLA 85, UFLA 87, UFLA 98 and UFLA 102 for gelatin hydrolysis (Table 3). Most of the isolates produced acid from sorbitol and sucrose, except for UFLA 44, and for *P. cichorii* (CFBP 2101), UFLA 135, UFLA 136, UFLA 145, UFLA 146, UFLA 147 that failed to produce acid from sorbitol and sucrose, respectively. Only one isolate, UFLA 159, did not use either one of the two sugars. Based on in these results, the isolates UFLA 135, UFLA 136, UFLA 145, UFLA 146, UFLA 147 and UFLA 159 showed the same biochemical pattern as the reference strain *P. cichorii* CFBP 2101 (Table 3). *P. cichorii* can be differentiated from *P. syringae* pv. *garcae* and *P. syringae* pv. *tabaci* through biochemical tests (levan, oxidase, gelatin hydrolysis and the use of trehalose, sucrose, sorbitol, cellobiose, trigonelline and L (+) tartrate) (Schaad; Jones; Chun, 2001). Adonitol was not used by any isolate, and lactose was used only by isolates *P. syringae* pv. *tabaci* (IBSBF2249), UFLA 142 and UFLA 143 (Table 3).

Isolates UFLA 69, UFLA 128, UFLA 129, UFLA 142 and UFLA 143 showed the same biochemical profile as reference strain *P. syringae* pv. *tabaci* (IBSBF2249), and showed strong fluorescence in King's B medium, while most *P. syringae* pv. *garcae* isolates exhibit weak fluorescence in this medium (Table 3). An important characteristic for diagnosing the two pathogens to coffee plants is the strong fluorescence produced by isolates of *P. syringae* pv. *tabaci* in King's B medium when observed under UV light. *P. syringae* pv. *garcae* isolates produce melanin pigment, which causes darkening of the medium. However, the melanin pigment production has been not observed for *P. syringae* pv. *tabaci* (Barta; Willis, 2005).

In LOPAT tests (levan + oxidase + potato rot + arginine + tobacco hypersensitivity), the reference strain IBSPF 166 of *R. andropogonis,* presents negative results (Table 3) as described by Schaad, Jones and Chun, (2001), confirming that it does not belong to the group of bacteria producing fluorescent pigments, and can be differentiated from other bacteria pathogenic to coffee. In the present study, none of the isolates obtained from coffee plants from Minas Gerais exhibited biochemical or molecular

Table 3: Biochemical properties of bacterial strains pathogenic to coffee in Minas Gerais.

Biochemical properties	Isolates			
	pv. garcae[a]	*pv. tabaci*[b]	*P. cichorii*[c]	*R. andropogonis*[d]
Fluorescent pigment on KB medium	-	+	+	-
Oxidase	-	-	+	-
Arginine dihydrolase	-	-	-	-
Tobacco HR	+	+	+	-
Gelatin hydrolisis	+[1]	+	-	-
Utilization of:				
Sucrose	+	+	-	-
Sorbitol	+[2]	+	-	-
Lactose	-	+[3]	-	-
Adonitol	-	-	-	-

Positive (+) = 80% or more were tested positive; Negative (-) = 80% or more were tested negative. [a] *P. syringae* pv. *garcae* reference strain CFBP 1634 and 73 isolates, [b] *P. syringae* pv. *tabaci* reference strain IBSBF2249 and UFLA 69, 128, 129, 135, 136, 142, 143, [c] *P. cichorii* reference strain CFBP 2101 and UFLA 135, 136,145, 146, 147, 159, 160. [d] *R. andropogonis* reference strain IBSPF 166. [1]: Except UFLA 21, 48, 60, 61, 79, 85, 87, 98 e 102. [2]: Except UFLA 44. [3]: Except UFLA 69, 128 e 129.

characteristics similar to those of *R. andropogonis*; therefore, we can confirm that this bacterium was not involved on the outbreaks in the field (Table 3).

Some biochemical characteristics evaluated were not consistent, which made it impossible to identify some isolates due to intraspecific variability (Table 3). According to Rodrigues et al. (2017a), the discrimination of these pathogens from coffee can be difficult, since biochemical tests, mainly the use of trigonelline, and gelatin hydrolysis that supported for the identification of species, can be variable for *P. syringae* pv. *garcae* strains. Therefore, the discrimination of isolates was performed with repetitive element-polymerase chain reaction (rep-PCR), and for accurate identification of isolates was used the phylogeny based on *rpoD* gene sequences.

rep-PCR

According analysis of total DNA fingerprinting patterns using each of the three techniques (REP-, ERIC- and BOX-PCR) the majority of strains grouped with reference strain of the *P. syringae* pv. *garcae* (CFBP 1634). This cluster exhibited six sub-clusters with similarity of 95 to 100%, regardless of their municipality of origin (Figure 1). Of these 73 isolates, 26 were from the municipality Nepomuceno, 23 were from Três Pontas, four were from Patrocínio, three from Patos de Minas and three from Santana da Vargem, Nova Rezende, Santo Antônio do Amparo, Elói Mendes, Ijací and Muzambinho had two isolate seach. Finally, Lavras, Varginha, Vargem Grande and São Sebastião do Paraíso had one isolate each (Figure 1).

The isolate UFLA 69 from the municipality Candeias and isolates UFLA 128, UFLA 129, UFLA 142 and UFLA 143 from the São Sebastião do Paraíso exhibited genetic profiles unique to that of the reference strain *P. syringae* pv. *tabaci* from *Coffea arabica* (IBSBF2249) and a similarity of 100% (Figure 1).

The genetic heterogeneity among the isolates UFLA 135 from the municipality Patrocínio, isolates UFLA 136, UFLA 145, UFLA 146, UFLA 147, UFLA 159 from the municipality Lavras and references isolates of *P. cichorii,* UFLA160 and CFBP2101 was visualized by different banding patterns, resulting in different clusters (Figure 1). This result represents the first report on the genetic variability of *P. cichorii* from coffee.

Belan et al. (2016) used BOX-PCR combined with biochemical and pathogenicity tests to detect the presence of *P. syringae* pv. *garcae* in coffee seeds collected from symptomatic plants, therefore they reported, for the first time, the possibility of transmission of the bacteria in seeds. Recently, Maciel et al. (2018) used ERIC-PCR and REP-PCR to study the genetic diversity of isolates of *P. syringae* pv. *garcae* and group them by place of origin and the date of isolation, which indicated the possible spread of the bacterium through diseased plant material. According to Tindall et al. (2010), rep-PCR can allow accurate identification of isolates in their correct species and pathovar when type isolates/pathotypes are included in comparisons.

Phylogenetic analysis using the *rpoD* locus

In phylogenetic tree based on partial *rpoD* sequences obtained using ML, three clades corresponded to *P. syringae* pv. *garcae, P. syringae* pv. *tabaci* and *P. cichorii.* The isolates UFLA 87, UFLA 125, UFLA 138 and UFLA 158 closed with reference strain *P. syringae* pv. *garcae* (CFBP 1634)**,** while the isolates UFLA 69, UFLA 128, UFLA 129, UFLA 142 and UFLA 143 grouped with reference strains of *P. syringae* pv. *tabaci* from the hosts *Coffea arabica* (IBSBF2249), *Nicotiana tabacum* (IBSBF1972), *Phaseolus vulgaris* (IBSBF703), *Cucumis sativus* (IBSBF758), *Carica papaya* (IBSBF1822) and *Desmodium canum* (IBSBF974). The isolates UFLA 135, UFLA 136, UFLA 145, UFLA 146, UFLA 147 grouped with isolates of *P. cichorii* used by Parkinson et al. (2011) and Timilsina et al. (2017) and the reference strain CFBP 2101 (Figure 2).

The *rpoD* gene sequence was selected for identifications at the coffee strains because it has been proven in previous publications that it is a good and reliable tool with discriminating power of the *Pseudomonas* species (Parkinson et al., 2011; Rajwar; Sahgal, 2016; Mulet et al., 2020). Parkinson et al. (2011) demonstrate that the classification of strains using one housekeeping gene can be sufficient, since the phylogroups using the seven-locus (*rpoD, gyrB, acnB, cts, gap, pgi, pfk*) study were maintained in the phylogeny using the *rpoD* locus alone. According to Rajwar and Sahgal (2016), *rpoD* has a housekeeping function, making it less susceptible to some forms of lateral gene transfer, and a large enough size (760 bp) to contain phylogenetic information, which is sufficient to carry the information that will differentiate among the *Pseudomonas* species. Moreover, *rpoD* gene displays important characteristics as an ecological marker, including the universal presence in all prokaryotes, and the presence of slowly and quickly evolving regions (Rajwar; Sahgal, 2016). This gene provides rapid and accurate identifications at the species level, and it can be used for the design of probes and primers of differing specificities (Mulet et al., 2009; 2020).

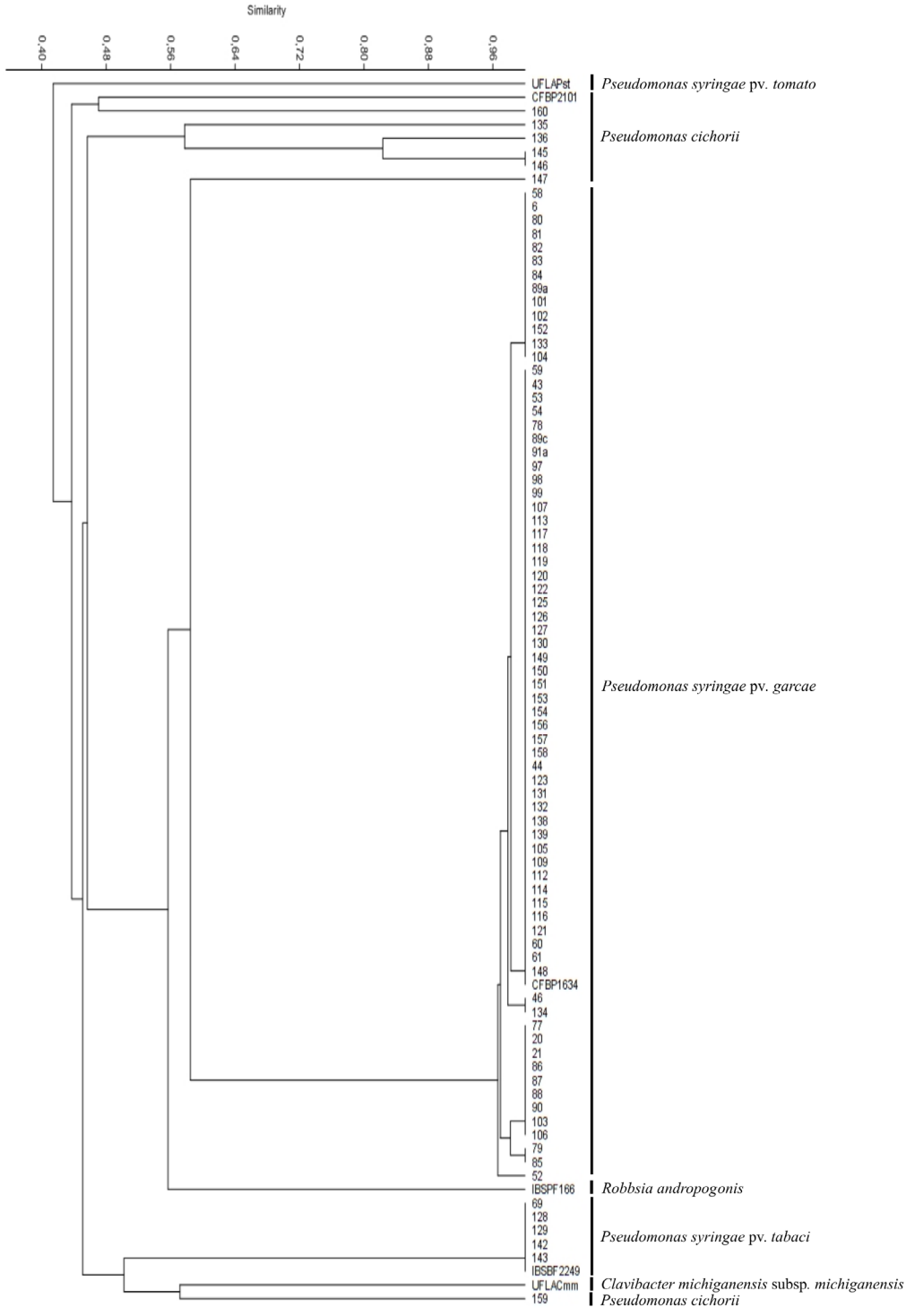

Figure 1: Dendrogram constructed by combined data set of REP, ERIC and BOX primer sets using Unweighted Pair-Group (UPGMA) cluster analysis, based on Dice similarity coefficient in PAST v. 2. Percentages of similarity are shown above the dendrogram.

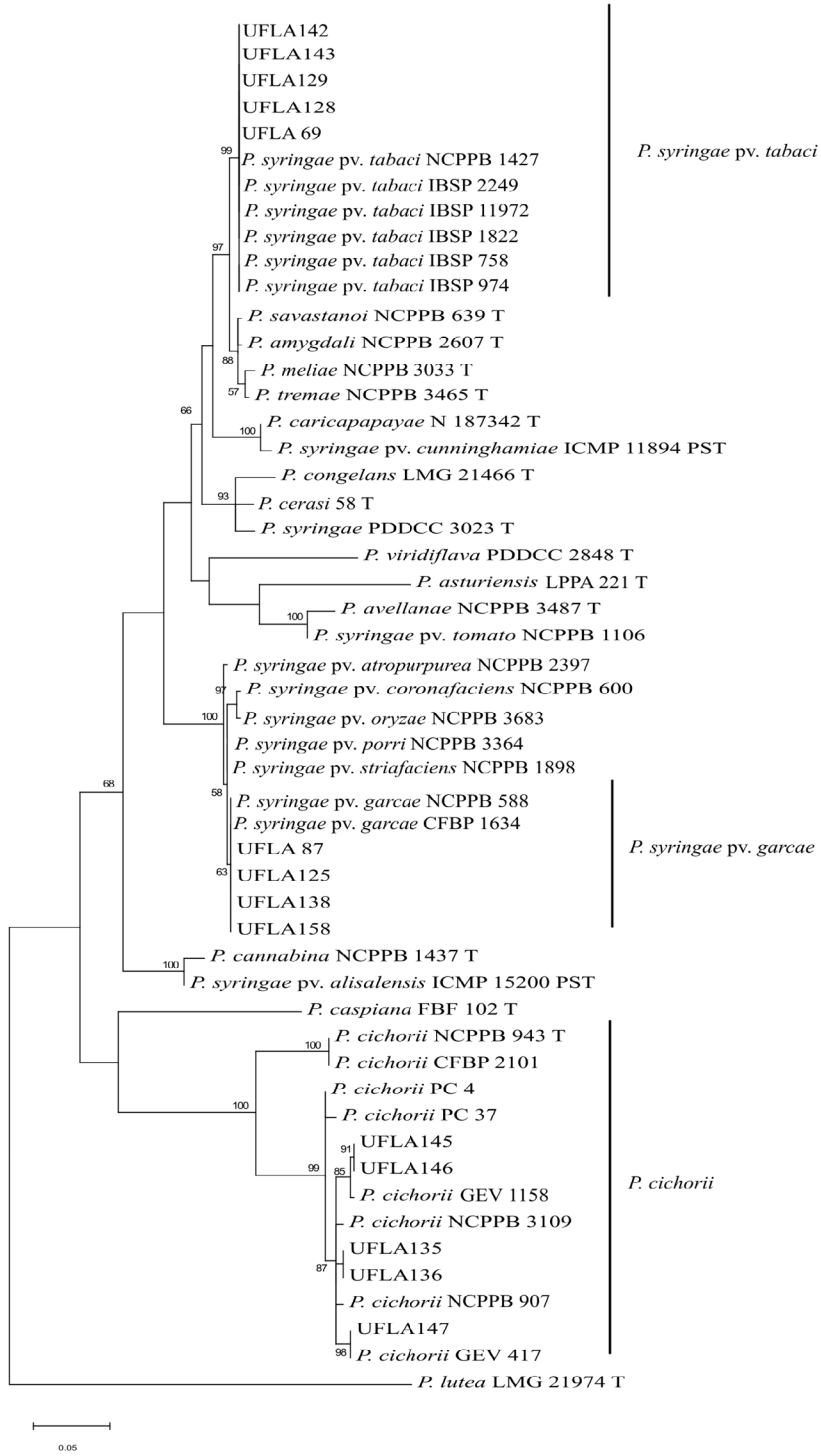

Figure 2: Maximum likelihood tree based on the *rpoD* gene sequence of the strains from assigned to the *Pseudomonas syringae* species complex including the pathovar reference strains and the *Pseudomonas* isolates pathogenic to coffee plants in Minas Gerais. Percentage bootstrap values of more than 50% (from 1000 replicates) are indicated at the nodes.

Pseudomonas syringae was divided into genomospecies determined by DNA:DNA hybridization (Gardan et al., 1999). Sarkar et al. (2006) defined five main phylogroups within this species, using the *rpoD* locus. Then Parkinson et al. (2011) analyzed also the *rpoD* locus and redefined into up to 13 so called phylogroups and showed that *P. syringae* complex comprises over 67 pathovars defined according to their pathogenic ability. These results showed corresponding between the genomospecies and the phylogroups. Based on phylogenomics analysis, *P. syringae* species complex comprises 15 recognized bacterial (Young, 2010; Gomila et al., 2017). *P. syringae* pv. *tabaci* belong to species *P. amygdali, P. syringae* pv. *garcae* belong to species *P. coronafaciens*, and the species *P. cichorii* was confirmed (Gomila et al., 2017). Dutta et al. (2018) proposed *Pseudomonas coronafaciens* sp. nov. as a new species in genus *Pseudomonas*, however, so far it has not been included in the Approved List of Bacterial Names and is not recognized as a valid species name (Parte et al., 2020). Therefore, *Pseudomonas syringae* pv. *garcae* should be used.

The rep-PCR dendrogram, based on the similarity between isolates, showed congruence in comparison with the phylogenetic tree based on *rpoD* sequences. The rep-PCR-clusters correspond to species phylogenetic, which they were identified by *rpoD*-phylogeny as *P. syringae* pv. *garcae, P. syringae* pv. *tabaci* and *P. cichorii*, belonging to the *P. syringae* species complex. *P. syringae* pv. *garcae* was the most common bacteria found in the coffee-producing fields of Minas Gerais State, and showed high variability genetic based on rep-PCR, which can generate races, considering the coffee restriction, and biotypes. *P. cichorii* strains showed also high variability genetic based on rep-PCR, which reflects on phenotypic plasticity of pathogenicity-related traits. This bacterium causes bacterial blight in coffee, it was first observed in Brazil in 1974, occurring in a nursery and field in the Minas Gerais State and, later, in a nursery, in the State of São Paulo (Rodrigues et al., 2017b). *P. cichorii* is economically important for a wide range of host plants around the world, such as lettuce, yams, among others (Cottyn et al., 2011).

The present study is the first report of the natural occurrence of bacterial leaf spot in coffee plants caused by *P. syringae* pv. *tabaci* under field conditions in the State of Minas Gerais. This species/pathovar as a coffee pathogen in Brazil was first detected in nurseries in the municipality of Arandu in the State of São Paulo in 2006 (Destéfano et al., 2010), Since then, the presence of this bacterium has been reported in several municipalities in the State of Paraná, as well as occurring simultaneously with *P. syringae* pv. *garcae* in the same sample of plant material (Rodrigues et al., 2017b).

CONCLUSIONS

The results obtained from biochemical characterization, molecular analysis and pathogenicity test allow confirmation of the predominance of *P. syringae* pv. *garcae* in coffee fields and nurseries in different municipalities of Minas Gerais State. Moreover, *P. syringae* pv. *tabaci* was found in coffee plants in fields in two municipalities of Minas Gerais, Candeias and São Sebastião do Paraíso and of *P. cichorii* in Lavras and Patrocínio. These results show that these pathogens are being disseminated and introduced in coffee-producing areas that were previously exempt from their presence. This reinforces the importance of phytosanitary inspections, germplasm transit control and seedling certification. These findings will contribute to future epidemiological studies of bacterial diseases of coffee, as well as will allow the design of specific primers for PCR diagnosis of its prevalent causal agent and of monitoring of the diseases.

ACKNOWLEDGMENTS

The authors thank the Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq, the Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES and the Instituto Nacional de Ciência e Tecnologia do Café – INCT Café, for providing scholarships to the authors and for supporting and funding this study.

REFERENCES

AMARAL, J. F.; TEIXEIRA, C.; PINHEIRO, E. D. A bacterium causing halo blight of coffee. **Arquivos do Instituto Biológico**, 23:151-155, 1956.

BARTA, T. M.; WILLIS, D. K. Biological and molecular evidence that *Pseudomonas syringae* pathovars *coronafaciens, striafaciens* and *garcae* are likely the same pathovar. **Journal of Phytopathology**, 153(7-8):492-499, 2005.

BELAN, L. L. et al. Occurrence of *Pseudomonas syringae* pv. *garcae* in coffee seeds. **Australian Journal of Crop Science**, 10(7):1015-1021, 2016.

COMPANHIA NACIONAL DE ABASTECIMENTO - CONAB. **Bienalidade positiva e clima favorecem produção histórica de café**. Café, safra 2019-2020. 2021. Available in: <http://www.conab.gov.br/BoletimCafe.pdf>. Access in: January, 19, 2021.

COTTYN, B. et al. Development of a real time PCR assay for *Psedonomas cichorii*, the causal agent of midrib rot in greenhouse-grow lettuce, and its detection in irrigating water. **Plant Pathology**, 60(3):453-461, 2011.

DESTÉFANO, S. A. L. et al. Bacterial leaf spot of coffee caused by *Pseudomonas syringae* pv. *tabaci* in Brazil. **Plant Pathology**, 59(6):1162-1163, 2010.

DUTTA, B. et al. *Pseudomonas coronafaciens* sp. nov., a new phytobacterial species diverse from *Pseudomonas syringae*. **PLoS ONE**, 13(12):e0208271, 2018.

GARDAN, L. et al. DNA relatedness among the pathovars of *Pseudomonas syringae* and description of *Pseudomonas tremae* sp. nov. and *Pseudomonas cannabina* sp. nov. (ex Sutic and Dowson 1959). **International Journal of Systematic and Evolutionary Microbiology**, 49(2):469-478, 1999.

GOMILA, M. et al. Clarification of taxonomic status within the *Pseudomonas syringae* species group based on a phylogenomic analysis. **Frontiers in Microbiology**, 8:2422, 2017.

HAMMER, O.; HARPER, D. A. T.; RYAN, P. D. PAST: Paleontological statistics software: Package for education and data analysis. **Palaeontologia Electronica**, 4(1):4-9, 2001.

ITHIRU, J. M. et al. Methods for early evaluation for resistance to bacterial blight of coffee. **African Journal of Agricultural Research**, 8:2450-2454, 2013.

KING, E. O.; WARD, M. K.; RANEY, D. E. Two simple media for the demonstration of pyocyanin and fluorescin. **The Journal of Laboratory and Clinical Medicine**, 44(2):301-307, 1954.

KUMAR, S. et al. MEGA: A biologist-centric software for evolutionary analysis of DNA and protein sequences. **Briefings in bioinformatics**, 9(4):299-306, 2008.

LONETTO, M. et al. The sigma 70 family: Sequence conservation and evolutionary relationships. **Journal of Bacteriology**, 174(12):3843-3849, 1992.

LOPES SANTOS, L. et al. Reassessment of the taxonomic position of *Burkholderia andropogonis* and description of *Robbsia andropogonis* gen. nov., comb. nov. **Antonie van Leeuwenhoek**, 110(6):727-736, 2017.

LOUWS, F. J. et al. Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. **Applied and Environmmental Microbiology**, 60(7):2286-2295, 1994.

PARTE, A. C. et al. List of prokaryotic names with standing in nomenclature (LPSN) moves to the DSMZ. **International Journal of Systematic and Evolutionary Microbiology**, 70:5607-5612, 2020.

MACIEL, K. W. et al. Bacterial halo blight of coffee crop: Aggressiveness and genetic diversity of strains. **Bragantia**, 77(1):96-106, 2018.

MARIANO, R. L. R.; SOUZA, E. B. **Manual de práticas em fitobacteriologia**. 3. ed. Recife: EDUFRPE, 2016. 234p.

MULET, M. et al. An rpoD based PCR procedure for the identification of pseudomonas species and for their detection in environmental samples. **Molecular and Cellular Probes**, 23(3-4):140-147, 2009.

MULET, M. et al. *Pseudomonas* species diversity along the danube river assessed by *rpod* gene sequence and MALDI-TOF MS analyses of cultivated strains. **Frontiers in Microbiology**, 11:2114, 2020.

OLIVEIRA, J. R.; ROMEIRO, R. S. Reação de folhas novas e velhas de cafeeiro a infecção por *Pseudomonas cichorii* e *P. syringae* pv. *garcae*. **Fitopatologia Brasileira**, 15(1):355-356, 1990.

PARKINSON, N. et al. Rapid phylogenetic identification of members of the *Pseudomonas syringae* species complex using the *rpoD* Locus. **Plant Pathology**, 60(2):338-344, 2011.

PÉREZ, C. D. P. et al. Nitrogênio e potássio na intensidade da mancha aureolada do cafeeiro em solução nutritiva. **Coffee Science**, 12(1):60-68, 2017.

PETEK, M. R. et al. Selection of progenies of *Coffea arabica* with simultaneous resistance to bacterial blight and leaf rust. **Bragantia**, 65(1):65-73, 2006.

RAJWAR, A.; SAHGAL, M. Phylogenetic relationships of fluorescent pseudomonads deduced from the sequence analysis of 16S rRNA, *Pseudomonas-specific* and *rpoD* genes. **3 Biotech**, 6:80, 2016.

ROBBS, C. F. et al. Crestamento bacteriano das folhas: Nova enfermidade do cafeeiro (*Coffea arabica* L.) incitada por *Pseudomonas cichorii* (Swingle) Stapp. **Arquivos da Universidade Federal Rural do Rio de Janeiro**, 4(2):1-5, 1974.

RODRIGUES, L. M. R. et al. First report of mixed infection by *Pseudomonas syringae* pathovars *garcae* and *tabaci* on coffee plantations. **Bragantia**, 76(4):543-549, 2017a.

RODRIGUES, L. M. R. et al. Aggressiveness of strains and inoculation methods for resistance assessment to bacterial halo blight on coffee seedlings. **Journal of Phytopathology,** 165(2):105-114, 2017b.

SARKAR, S. F. et al. Comparative genomics of host-specific virulence in *Pseudomonas syringae*. **Genetics**, 174(2):1041-1056, 2006.

SCHAAD, N. W.; JONES, J. B.; CHUN, W. **Laboratory guide for identification of plant pathogen bacteria**. Saint Paul: Editora American Phytopathological Society, 2001. 373p.

SERA, G. H.; SERA, T.; FAZUOLI, L. C. IPR 102 - Dwarf arabica coffee cultivar with resistance to bacterial halo blight, **Crop Breeding and Applied Biotechnology**, 17(4):403-407, 2017.

TAYEB, L. A. et al. Molecular phylogeny of the genus *Pseudomonas* based on *rpoB* sequences and application for the identification of isolates. **Research in Microbiology**, 156(5-6):763-773, 2005.

THIND, B. S. **Phytopathogenic bacteria and plant diseases**. Boca Raton, FL: CRC Press, 2020. 372p.

THOMPSON, J. D.; HIGGINS, D. G.; GIBSON, T. J. Clustal W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. **Nucleic Acids Research**, 22(22):4673-4680, 1994.

TINDALL, B. J. et al. Notes on the characterization of prokaryote strains for taxonomic purposes. **International Journal of Systematic and Evolutionary Microbiology**, 60(1):249-266, 2010.

TIMILSINA, S. et al. A novel phylogroup of *Pseudomonas cichorii* identified following an unusual disease outbreak on tomato. **Phytopathology**, 107(11):1298-1304, 2017.

UNITED STATES DEPARTMENT OF AGRICULTURE - USDA. **Annual report coffee annual Brazil**. Brazil. 2020a. Available in: <https://apps.fas.usda.gov/psdonline/circulars/coffee.pdf >. Access in: January, 20, 2021.

UNITED STATES DEPARTMENT OF AGRICULTURE - USDA. **Coffee:** World markets and trade foreign agricultural service. 2020b. Available in: <https://apps.fas.usda.gov/psdonline/circulars/coffee.pdf>. Access in: January, 20, 2021.

YOUNG, J. M.; DYE, D. W.; WILKIE, J. P. A proposed nomenclature and classification for plant pathogenic bacteria. **New Zealand Journal of Agricultural Research**, 21(1):153-177, 1978.

YOUNG, J. M. Taxonomy of *Pseudomonas syringae*. **Journal of Plant Pathology**, 92(1):5-14, 2010.

VALE, F. X. R.; ZAMBOLIM, L. **Controle de doenças de plantas:** Grandes culturas. 1.ed. v.2, Visconde do Rio Branco, MG: Suprema, 1997. 1128p.

ZOCCOLI, D. M.; TAKATSU, A.; UESUGI, C. H. Ocorrência de mancha aureolada em cafeeiros na Região do Triângulo Mineiro e Alto Paranaíba. **Bragantia**, 70(4):843-849, 2011.