PLANT PROTECTION - Article

Bacterial halo blight of coffee crop: aggressiveness and genetic diversity of strains

Karen Wolf Maciel[1]*, Suzete Aparecida Lanza Destefano[1], Luis Otavio Saggion Beriam[1], Irene Maria Gatti de Almeida[1], Flavia Rodrigues Alves Patricio[2], Lucas Mateus Rivero Rodrigues[3], Oliveiro Guerreiro Filho[3]

1.Instituto Biológico - Laboratório de Bacteriologia Vegetal - Campinas (SP), Brazil.
2.Instituto Biológico - Laboratório de Fitopatologia - Campinas (SP), Brazil.
3.Instituto Agronômico - Centro de Café - Campinas (SP), Brazil.

ABSTRACT: Bacterial halo blight, caused by *Pseudomonas syringae* pv. *garcae*, is an important disease of coffee crop occurring in Brazil and other countries. In recent years, outbreaks of this disease have damaged several coffee crops in Brazil. Aggressiveness and genetic diversity of 25 strains of *P. s.* pv. *garcae,* obtained between the years 1958 and 2011, in 23 cities of São Paulo and Minas Gerais states, as well as three strains from Kenya were evaluated in this study. The strains were inoculated on coffee seedlings cultivar Mundo Novo, and their genetic diversity was evaluated by ERIC-PCR, REP-PCR, and their combination. All the strains were pathogenic to the coffee seedlings; the results of pathogenicity tests, in both experiments, could be divided in four aggressiviness classes (highly aggressive; aggressive; moderately aggressive and less aggressive). The Kenyan strains grouped separately from the Brazilian strains with ERIC-PCR and the combination of ERIC- and REP-PCR. The Brazilian strains could be grouped in two sub-clusters, the first including the older strains, obtained from 1958 to 1978, and the other comprising the remaining strains. With a few exceptions, strains isolated from 1997 to 2011, grouped mainly by their region of origin, were predominantly isolated from higher altitude regions, above 800 m. This probably occurred because the climatic conditions that prevail in these regions, characterized by milder temperatures and regular rainfall, are favorable for the coffee crop and for the production of high quality coffee beverage, but can be also favorable to bacterial halo blight.

Key words: *Pseudomonas syringae* pv. *garcae*, *Coffea arabica,* ERIC-PCR, REP-PCR.

INTRODUCTION

Brazil is the world´s largest producer and exporter of coffee. The Arabica coffee (*Coffea arabica* L.) is cultivated mainly in Minas Gerais, and São Paulo States (CONAB 2015). Bacterial halo blight, caused by *Pseudomonas syringae* pv. *garcae,* is an important disease of coffee in Brazil and outbreaks of this disease have occurred in this country in recent years (Rodrigues et al. 2013**;** Zoccoli et al. 2011).

The disease is characterized by lesions on leaves, flowers, pin-head berries, and die-back of twigs and branches. The most characteristic symptom of the disease is the presence of brown necrotic lesions on the leaves, surrounded by a chlorotic halo. Growth and coalescence of the lesions on the leaves cause their drop and subsequent defoliation of branches. At the end of the rainy season, the symptoms are confined to the branches that exhibit sometimes severe die-back. Bacterial halo blight also affects seedlings in nurseries, causing lesions on leaves and die-back of the seedlings (Costa et al. 1957).

This disease was first described in 1955, affecting coffee crops in the county of Garça, State of São Paulo, Brazil (Amaral et al. 1956) and was considered of minor importance for approximately 17 years after its emergence, when only isolated cases were reported (Kimura et al. 1973). From 1973 to 1975, a high incidence of the disease was observed in the state of Paraná in nurseries and coffee plantations that were recovered from frost[4] (Kimura et al. 1973; Mohan 1976). Bacterial halo blight has spread across the country and the pathogen has been detected in the States of Paraná, São Paulo and Minas Gerais (Malavolta Junior et al. 2008). Recently, damages caused by the disease were reported in some regions of the State of São Paulo as well as in regions with irregular topography and high altitudes in the State of Minas Gerais, such as Alto Paranaíba, Triângulo Mineiro and Sul de Minas (Zoccoli et al. 2011; Rodrigues et al. 2013).

The rep-PCR technique has been considered an important tool in phylogenetic studies of bacterial populations and shows high correlation with other more laborious techniques such as AFLP and DNA hybridization (Rademaker et al. 2000). This technique is based on the amplification of three families of repetitive DNA sequences that are present in the genome of several bacterial species, the repetitive extragenic palindromic sequence (REP), the enterobacterial repetitive intergenic consensus (ERIC) and the BOX elements (Louws

[4]Mohan, S.K. (1976). Investigação sobre *Pseudomonas garcae* Amaral et al. em cafeeiros. Congresso Brasileiro de Pesquisas Cafeeiras, Caxambu, 4. (p. 56). Rio de Janeiro: IBC.

et al. 1994). Rep-PCR has been frequently used to assess the genetic diversity of several plant pathogenic bacteria such as *Xanthomonas axonopodis* pv. *juglandis* (Scortichini et al. 2001), *P. syringae* pv. *syringae* (Natalini et al. 2006) and studies of inter-and intraspecificity in strains of *P. syringae* (Vicente and Roberts 2007; Çepni and Gürel 2012; Gašić et al. 2012).

Although bacterial halo blight occurs in Brazil since 1955, it is unknown if the recent outbreaks of the disease are due to changes in the population of the pathogen or to increases in the aggressiveness of new strains or to other unknown factors. Therefore, aggressiveness and genetic diversity of 25 Brazilian strains of *P. syringae*. pv. *garcae* from several coffee producing areas, obtained between the years 1958 to 2011, were investigated in this study.

MATERIAL AND METHODS

Twenty-five strains of *P. syringae*. pv. *garcae,* isolated from coffee crops cultivated in the States of Minas Gerais and São Paulo, Brazil, were investigated in this study. Three strains from Kenya were included in the analysis for comparative purposes, since bacterial halo blight was also reported as an important disease in that country (Kairu 1997). The strains were obtained from the Phytobacteria Culture Collection of Instituto Biológico (IBSBF) and their geographical origin, year of isolation, and climate data of the localities of origin are shown in Table 1.

The aggressiveness of the strains was evaluated in two experiments carried out in September and October of 2015, in a greenhouse located at Centro Experimental Central, Instituto Biológico, Campinas, SP, with seedlings of the susceptible coffee cultivar Mundo Novo. Four pairs of true leaves of each seedling were inoculated with bacterial suspensions (approximately 10^8 CFU·mL^{-1}) prepared by the cultivation of the strains in nutrient agar medium (NA) for 48 h at 28 °C. The inoculations were performed by spraying the suspensions until runoff on leaves that were previously punctured with sterile entomological needles (four punctures per leaf). Three control plants were inoculated with sterile distilled water. After the inoculations, the plants were kept in a moist chamber for 72 h and then transferred to a greenhouse at room temperature.

Table 1. Strains of *Pseudomonas syringae* pv. *garcae* used in this study.

| IBSBF[1] strain number | Geographical origin | | Climate data | | | | Year of isolation |
	County/ State	Country	Average temperature (°C)	Precipitation (mm)	Altitude (m)	Climate classification[4]	
65	Jaú, SP	Brazil	20.8	1277	538	Cfb[5]	1976
75	Pirajú, SP	Brazil	20.7	1257	524	Cfa[6]	1978
152	Ouro Fino, MG	Brazil	19.3	1518	881	Cfb	1978
158	Campinas, SP	Brazil	19.3	1315	678	Cfa	1978
248[P] (= LMG[2]5548)	Garça, SP	Brazil	19.9	1306	684	Cfa	1958
249 (=LMG 5551)	-	Kenya					1962
1293	Guaxupé, MG	Brazil	19.7	1486	881	Cfb	1997
1372	Cristais Paulista, SP	Brazil	19.6	1557	996	Cfb	1998
1373	São João da Boa Vista, SP	Brazi	20.1	1493	764	Cfa	1998
1664	Serra Negra, SP	Brazil	18.1	1512	967	Cfb	2001
2212	Franca, SP	Brazil	19.4	1564	1002	Cfb	2005
2511	Patrocínio, MG	Brazil	21,4	1507	977	Aw[7]	2007
2840	Caconde, SP	Brazil	20.1	1479	788	Cfa	2009
2841	Águas da PrataSP	Brazil	19.3	1595	967	Cfb	2009
2883 (= ICMP[3]4962)	-	Kenya					1973
2998	Carmo de Minas, MG	Brazil	19.5	1516	902	Cfa	2008
2999	Serra Negra SP	Brazil	18.1	1512	967	Cfb	2008
3005	Altinópolis,SP	Brazil	19.7	1545	930	Cfb	2008
3015	Garça, SP	Brazil	19.9	1306	684	Cfa	2008
3019	São Sebastião da Grama, SP	Brazil	19.5	1510	911	Cfb	2009
3022	Bragança Paulista SP	Brazil	18.2	1397	832	Cfb	2009
3024	Serra do Salitre	Brazil	19.8	1590	1241	Cfb	2011
3031	Varginha, MG	Brazil	20.2	1400	865	Cfa	2009
3032	Albertina, MG	Brazil	18.7	1590	1021	Cfb	2009
3037 (= LMG 5549)	-	Kenya					1972
3046	Divinolândia, SP	Brazil	18.8	1576	1057	Cfb	2009
3049	Andradas, MG	Brazil	20.0	1579	872	Cfa	2009
3065	Unaí, MG	Brazil	23.5	1275	563	Aw	2010

[1]IBSBF - Phytobacteria Culture Collection of Instituto Biológico – Brazil (http//www.biologico.sp.gov.br/pdf/catalogo_IBSBF.pdf); [2]LMG – Laboratoriumvoor Microbiology Culture Collection, Rijksuniversitseit, Gent, Belgium; [3]ICMP – International Collection of Microorganisms from Plants, Auckland, New Zealand; [P]Pathovar reference strain; [4]Köppen-Geiger Climate classification (Climate.date.org. 2016); [5]Cfb - Temperate humidity climate with warm summer; [6]Cfa - Temperate humidity climate with a hot summer; [7]Aw -Tropical climate with a dry winter.

The severity of the disease was assessed by the number of lesions and the area affected by the disease in the second and third pairs of leaves of the seedlings at 30 days after the inoculation. To evaluate the area affected by the disease the leaves were photographed and, in order to be measured in the interactive application Leaf Doctor (Pethybridge and Nelson 2015), their photographs were modified using Adobe Photoshop to create a black background that surrounded all the individual leaves. The interactive application Leaf Doctor was used to distinguish diseased from healthy plant tissues and to calculate the percentage of disease severity (Pethybridge and Nelson 2015).

Two independent experiments were carried out in a completely randomized design with four replicates, each replicate represented by one seedling. The results were submitted to analysis of variance and the averages were compared by the Scott Knott test at the level of 5% of probability.

In the molecular characterization, the amplification reactions were conducted with primer sets: ERIC1R (5´ - ATG TAA GCT CCT GGG GAT TCA C - 3´); ERIC2 (5´ - AAG TAA GTG ACT GGG GTG AGC G - 3´); REP1R-I (5´ - III ICG ICG ICA TCI GGC - 3´); REP2-I (5´ - ´ICG ICT TAT CIG GCC TAC - 3´), and BOX A1R (5' CTA CGG CAA GGC GAC GCT GAC G - 3´) (Louws et al. 1994). PCR was conducted in a MyCycle thermal cycler (Bio-Rad) in 25 µL reaction volume containing: 100 ng of genomic DNA; 0.8 µM of ERIC primers; 0.2 mM of deoxynucleoside triphosphates; 2.5 mM of magnesium chloride; and 2.5 units of Taq DNA polimerase (Fermentas) in a reaction buffer. The following cycling conditions were used: one cycle of initial denaturation at 95 °C for 7 min; 30 cycles consisting of 94 °C for 1 min; 52 °C for 1 min; 65 °C for 8 min; and a single final extension at 65 °C for 16 min. REP-PCR reactions were performed in 25 µL volume containing: 100 ng of genomic DNA; 0.5 µM of each primer; 0.2 mM of deoxynucleoside triphosphates; 2.5 mM of magnesium chloride; and 2.5 units of Taq DNA polimerase in a reaction buffer. The amplification conditions included one cycle of initial denaturation at 95 °C for 6 min; 30 cycles consisting of 94 °C for 1 min; 40 °C for 1 min; 65 °C for 8 min; and a single final extension at 65 °C for 16 min.

Aliquots of REP-PCR (8 µL) and ERIC-PCR (10 µL) products were separated by electrophoresis on a 2.5% agarose gel stained with ethidium bromide 10 mg·mL⁻¹ in TAE buffer (0.04 M Tris-acetate/0.001 M EDTA) and then visualized and photographed under UV light using a digital system (Alpha Innotech 2200).

A dendrogram was constructed using fragments ranging from 200 to 1400 bp, scored for presence (1) or absence (0), using the Jaccard (S_J) coefficient and UPGMA (Unweighted Paired Group Method) algorithm from the NTSYS-PC program, version 1.70 (Rohlf 1992).

RESULTS AND DISCUSSION

All *P. syringae* pv. *garcae* strains investigated herein were pathogenic to the coffee seedlings, but 12 of them, obtained from 1978 to 2011, were more aggressive. Considering the leaf area affected by the disease, in the first experiment the strain IBSBF 1293 was the most aggressiveness, followed by the strains IBSBF 65, 1664, 2212, 3046 and 75. In the second experiment, the strains IBSBF 2212 and 3046 were more aggressive followed by the strains IBSBF 1664 and 1293. By observing simultaneously the results of percentage of area affected by the disease and number of lesions per leaves, in both experiments, the isolates can be divided in four categories: HA - highly aggressive; A – aggressive; MA - moderately aggressive and LA - less aggressive. According to this results, the higher aggressive strains were IBSBF 1293, 1664, 2212, e 3046. The aggressiveness classification of all the other strains are described on Table 2. Symptoms of bacterial halo blight were not observed in the control treatments.

This is the first study in which the severity of bacterial halo blight was assessed with the interactive application Leaf Doctor (Pethybridge and Nelson 2015). The use of the application was somewhat laborious because all the leaves needed to be photographed, but it reduced the human error involved in estimating the leaf area affected by the disease and also made possible the experiment be well documented, therefore it can be a valuable tool for assessing this disease. There was a significant correlation between the number of lesions per leaf and the area affected by the disease in the first ($R^2 = 0,776847$) and second experiment ($R^2 = 0,699389$).

Although all strains investigated herein have been obtained from severely affected coffee crops, some strains showed high and others low levels of aggressiveness in the coffee seedlings. Variations in aggressiveness of bacterial strains have already been reported in other pathosystems and are rather expected. Natalini et al. (2006) found variations in the aggressiveness of *P. syringae*. pv. *garcae* strains obtained from a pear (*Pyrus communis*) orchard. Mirik et al. (2011) observed that strains of *P. cichorii* obtained from lettuce (*Lactuca sativa*), tomato (*Solanum lycopersicum*) and *Schefflera arboricola* were more aggressive to the hosts from which they had been isolated, although they were pathogenic to all hosts. In our study, the genetic clustering of the *P. syringae* pv. *garcae* strains showed no correlation with the aggressiveness of the isolates. In contrast, Vicente and Roberts (2007) found that rep-PCR grouped, with some exceptions, the more aggressiveness strains of *P. syringae* pv. *morsprunorum* race 1 isolated from cherry trees. It was observed for *P syringae* pv. *garcae* that aggressiveness can be lost by successive subcultures (Moraes et al. 1975). Furthermore, aggressiveness can vary depending on the bacterial strains and environmental conditions, as demonstrated in this paper.

Table 2. Aggressiveness of *Pseudomonas syringe* pv. *garcae* strains to coffee seedlings cv. Mundo Novo, evaluated by the severity, estimated by the leaf area affected by disease and the number of lesions per leaf.

IBSBF[1] strains	Percentage of leaf area affected by the disease		Number of lesions per leaf		Aggressivity categories
	Experiment 1	Experiment 2	Experiment 1	Experiment 2	
65	15.56 b[2]	10.03 c	3.25 a	3.06 b	Aggressive
75	12.14 b	6.58 c	2.95 a	2.00 b	Aggressive
152	8.04 c	6.73 c	2.31 b	2.00 b	Aggressive
158	5.04 c	7.63 c	2.81 b	2.87 b	Aggressive
248[p]	0.19 e	0.13 d	0.43 d	0.31 d	Little aggressive
249	0.57 d	0.93 d	0.75 c	0.87 c	Moderately aggressive
1293	21.64 a	12.98 b	4.18 a	3.18 b	Highly aggressive
1372	0.59 d	0.51 d	2.37 b	1.16 c	Moderately aggressive
1373	0.06 e	0.11 d	0.62 d	0.43 d	Little aggressive
1664	15.01 b	14.92 b	3.56 a	3.06 b	Highly aggressive
2212	14.60 b	24.10 a	3.93 a	4.31 a	Highly aggressive
2511	5.90 c	3.98 c	3.06 a	2.81 b	Aggressive
2840	1.39 d	3.05 d	2.25 b	3.00 b	Moderately aggressive
2841	7.23 c	5.98 c	2.68 b	3.04 b	Aggressive
2883	4.72 c	6.37 c	1.87 b	1.31 c	Moderately aggressive
2998	0.00 e	0.06 d	0.00 d	0.06 d	Little aggressive
2999	0.10 e	0.55 d	0.37 d	1.43 c	Little aggressive
3005	0.33 d	0.42 d	2.12 b	1.75 c	Moderately aggressive
3015	0.14 e	0.10 d	0.12 d	0.12 d	Little aggressive
3019	0.00 e	0.10 d	0.00 d	0.18 d	Little aggressive
3022	0.40 d	0.46 d	1.25 c	1.56 c	Moderately aggressive
3024	7.03 c	10.14 c	2.22 b	2.62 b	Aggressive
3031	0.26 d	0.20 d	0.93 c	1.00 c	Moderately aggressive
3032	0.11 e	0.20 d	0.62 d	0.68 d	Little aggressive
3037	1.74 d	2.09 d	1.50 c	1.75 c	Moderately aggressive
3046	14.36 b	21.47 a	3.37 a	3.62 a	Highly aggressive
3049	0.42 d	0.18 d	1.29 c	0.56 d	Little aggressive
3065	0.04 e	0.06 d	0.08 d	0.12 d	Little aggressive
Control	0.00 e	0.00 d	0.00 d	0.00 d	
CV(%)	34.68	46.33	36.21	40.81	

[1]IBSBF - Phytobacteria Culture Collection of Instituto Biológico - Brazil. (http//www.biologico.sp.gov.br/pdf/catalogo_IBSBF.pdf); [2]Means were compared by the Scott-Knott test at 5 % probability; [p]Patovar reference strain.

In this study, the strains did not show variations in the BOX-PCR (data not shown) but exhibited variability in the ERIC- and REP-PCR patterns. The genetic relatedness of *P. syringae* pv. *garcae* strains, investigated by ERIC- and REP-PCR, yielded fingerprints with 20 and 24 bands ranging from 1900 bp to 80 bp and 1825 bp to 200 bp, respectively. Banding patterns generated by ERIC- and REP-PCR revealed a high genetic diversity among the strains. The dendrogram constructed with ERIC allowed the separation of the strains into two major clusters with approximately 37% similarity. Cluster I was represented by four strains from the State of São Paulo isolated between the years 1958 and 1978, with altitudes ranging from 524 to 684 m. Cluster II, divided into two subclusters, "a" and "b", allocated the remaining strains with approximately 45% similarity between them. The subcluster "a" was represented by the three strains from

Kenya, and the subcluster "b" was further subdivided into two other subclusters, "i" and "ii", with approximately 53% similarity. The subcluster "ii" allocated strains from the counties of Guaxupé, MG, Andradas, MG, and Varginha, MG, (70% similarity), while the subcluster "i" grouped five strains from Minas Gerais 57% similarity), isolated from 1978 to 2011, and the remaining 12 from the State of São Paulo isolated from 1998 to 2009. One strain from

Albertina, MG, was allocated with São Paulo strains, and the genetic similarity between them probably has occurred due the exchange of vegetal material between these cities which are geographically close (Figures 1 and 2).

Banding patterns generated by REP-PCR also revealed a high genetic diversity among the strains. In this analysis the strains were separated into two clusters: Cluster I was represented only by the IBSBF 2883 strain from Kenya, with
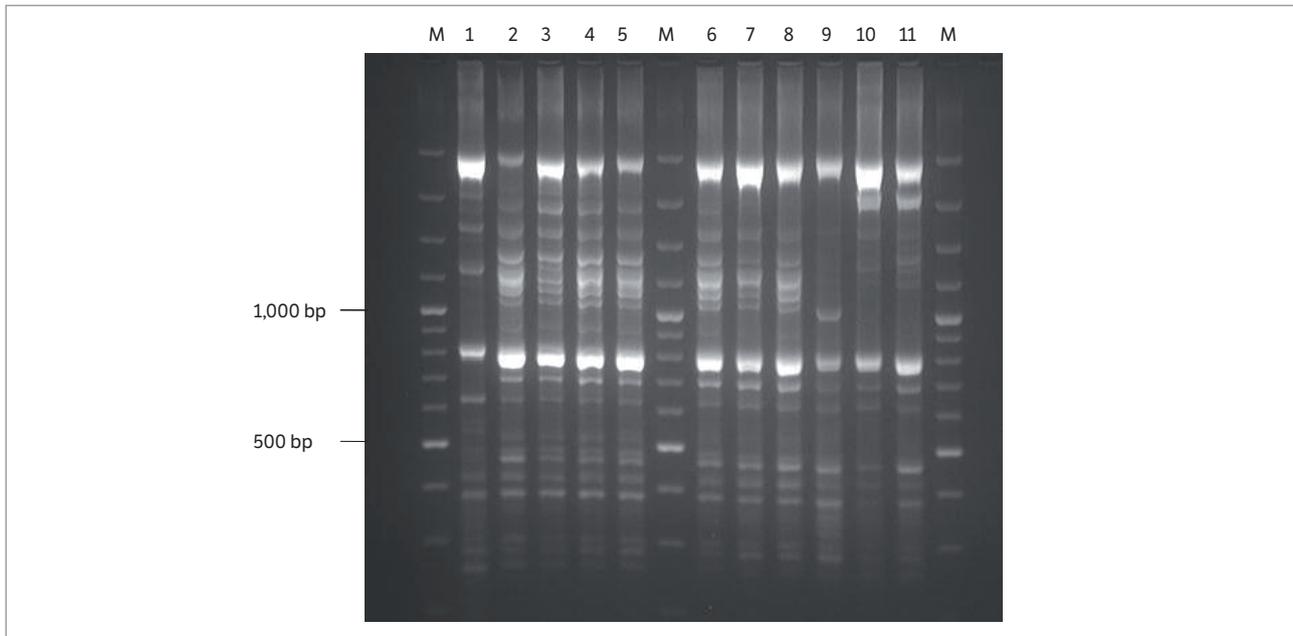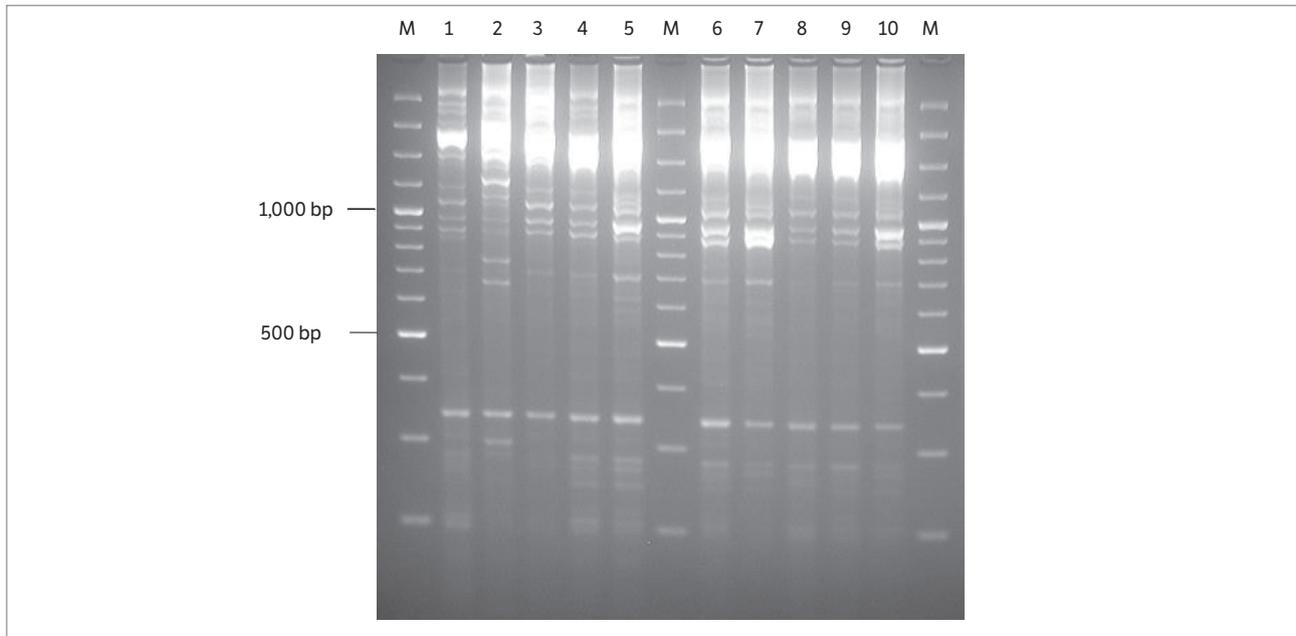

Figure 1. Amplification of DNAs from *Pseudomonas syringae* pv. *garcae* strains with ERIC 1R/ERIC2 primers. (M) Molecular weight marker 100 bp (1) IBSBF 248ᴾ; (2) 2999; (3) 3005; (4) 3015; (5) 3019; (6) 3022; (7) 3032; (8) 3046; (9) 249; (10) 2883 and (11) 3037.
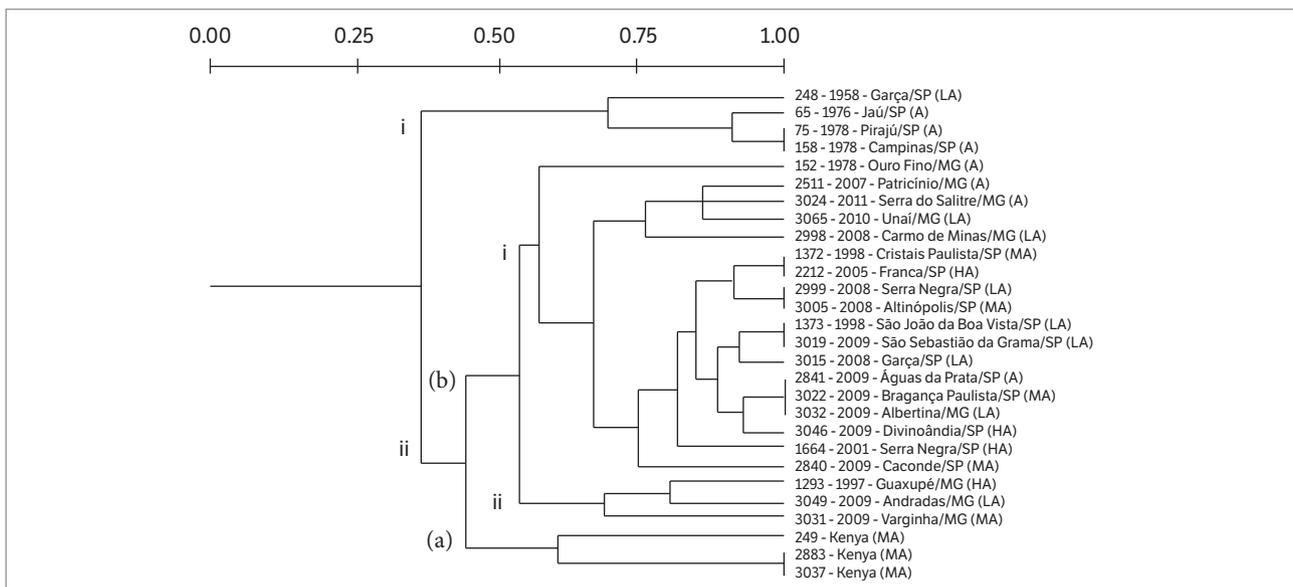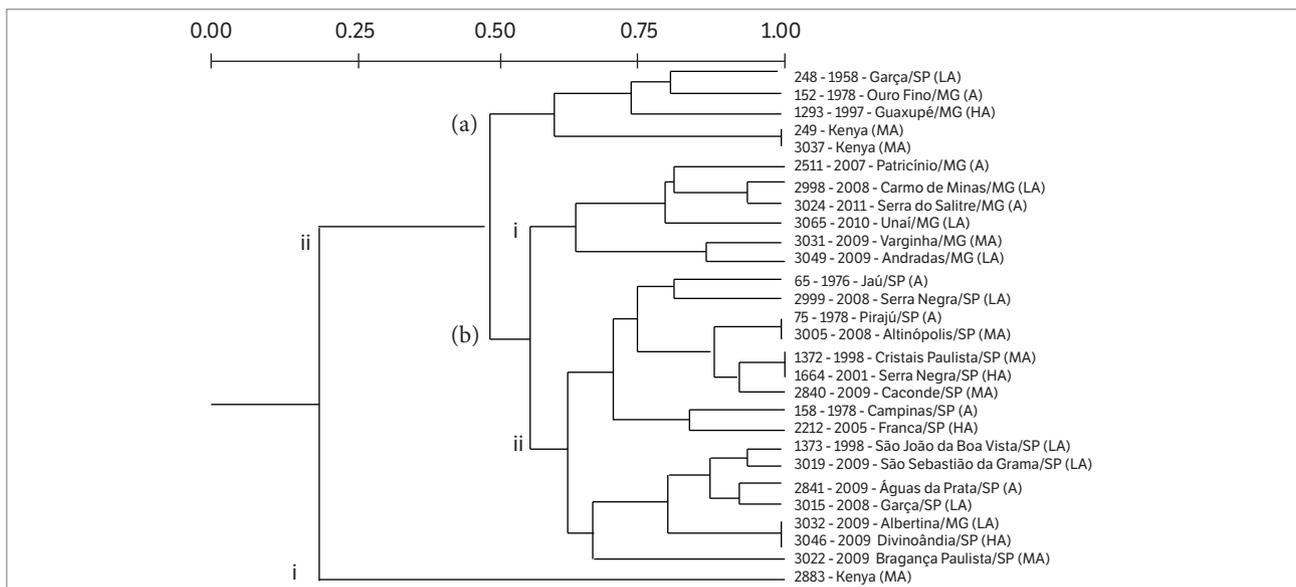

Figure 2. Genetic diversity of *Pseudomonas syringae* pv. *garcae* strains. Dendrogram generated according to the fingerprint of the strains using ERIC 1R/ERIC2 primers, based on the UPGMA method, using Jaccard similarity coefficient (S_J).

approximately 20% similarity to Cluster II, which allocated the remaining strains and was subdivided into subclusters "a" and "b", with about 49% similarity to each other. The subcluster "a" included only five strains with 60% similarity, two from Kenya (IBSBF 249 and IBSBF 3037), the pathotype strain (IBSBF 248[P]), also one strain from Guaxupé, MG, and another from Ouro Fino/MG. The subcluster "b" grouped the remaining 22 strains, which could be further divided into two

other subclusters, "i" and "ii". Subcluster "i" grouped only the strains isolated from Minas Gerais with approximately 63% similarity, while subcluster "ii" only those from São Paulo (similarities ranging 60 to 93%), with exception of Albertina, MG, as also observed in ERIC-PCR analysis. According to this analysis, the strains from São Paulo (cluster II, subcluster "b" and "ii") were mainly separated from those of Minas Gerais (cluster II, subcluster "b" and "i") (Figures 3,4).

Figure 3. Amplification of DNAs from *Pseudomonas syringae* pv. *garcae* strains with REP1R/REP2I primers. (M) Molecular weight marker 100 bp; (1) IBSBF 248[P]; (2) 65; (3) 75; (4) 158; (5) 1372; (6) 1373; (7) 1664; (8) 2840; (9) 2841 and (10) 2999.

Figure 4. Genetic diversity of *Pseudomonas syringae* pv. *garcae* strains. Dendrogram generated according to the fingerprint of the strains using REP1R/REP2I primers, based on the UPGMA method, using Jaccard similarity coefficient (SJ).

In the combined analysis, the strains were separated in two clusters (Figure 5). Cluster I was only composed by the three strains from Kenya, with approximately 37% similarity with the other strains of this study. Cluster II comprised the remaining 25 strains, divided in two subclusters, "a" and "b", with approximately 50% similarity with each other. The subcluster "a" was composed by four strains from the State of São Paulo in the period from 1958 to 1978 (IBSBF 65, IBSBF 75, IBSBF158 and IBSBF 248[P]). The subcluster "b" allocated 21 strains from the States of São Paulo and Minas Gerais and was subdivided in two subclusters, "i" and "ii", with approximately 57% similarity between them. The subcluster "i" was composed only by strains from the state of Minas Gerais and "ii" by those from São Paulo, with exception of IBSBF 3032 from Albertina/MG (Figure 5).

In the combined ERIC- and REP-PCR analysis, strains from Guaxupé/MG, Varginha, MG, and Andradas, MG, obtained from 1997 to 2009, grouped in the same sub-group (Figure 5). These counties belong to the same coffee production region in Sul de Minas, with altitudes varying from 865 to 881 m, Cfb and Cfa climate, annual average temperature of 19,7 to 20,2 ºC; and 1200 to 1500 mm average annual rainfall (Table 1).

The strains from São João da Boa Vista, São Sebastião da Grama, Águas da Prata, Garça, Bragança Paulista, Divinolândia, all from the State of São Paulo, as well as Albertina, from Minas Gerais, grouped in similar sub-groups in the REP-PCR, and in the combined ERIC- and REP-PCR dendrograms. These counties, with the exception of Garça (IBSBF 3015), are relatively close to each other, have altitudes varying from 764 to 1021 m, average temperature from 18,2 to 20,1 ºC, and 1,493 to 1,590 mm annual average rainfall, as well as Cfa and Cfb climate (Table 1).

Strains from Cristais Paulista, Franca and Altinópolis, that are relatively close to each other, as well as Serra Negra and Caconde, that are more distant, grouped in the same sub-group in the combined ERIC- and REP-PCR dendrogram. These counties belong to the same coffee producing region, Mogiana, and show average temperature ranging from 18,1 to 20,1 ºC, average annual rainfall of 1,479 to 1,557 mm, Cfb to Cfa climate classification (Climate date org. 2016) (Table 1).

In this study, the strains did not show variations in the BOX-PCR (data not shown) but exhibited variability in the ERIC- and REP-PCR patterns. Isolates from the same pathovar may have identical REP, BOX and ERIC fingerprints or they may differ in some banding patterns (Louws et al. 1994). Çepni and Gürel (2012) found that ERIC-PCR could not differentiate strains of *P. savastanoi* pv. *savastanoi* from strains of *P. syringae.* pv. *tomato* and *P. s.* pv. *phaseolicola*, but the combination of BOX-PCR with REP-PCR allowed the grouping of
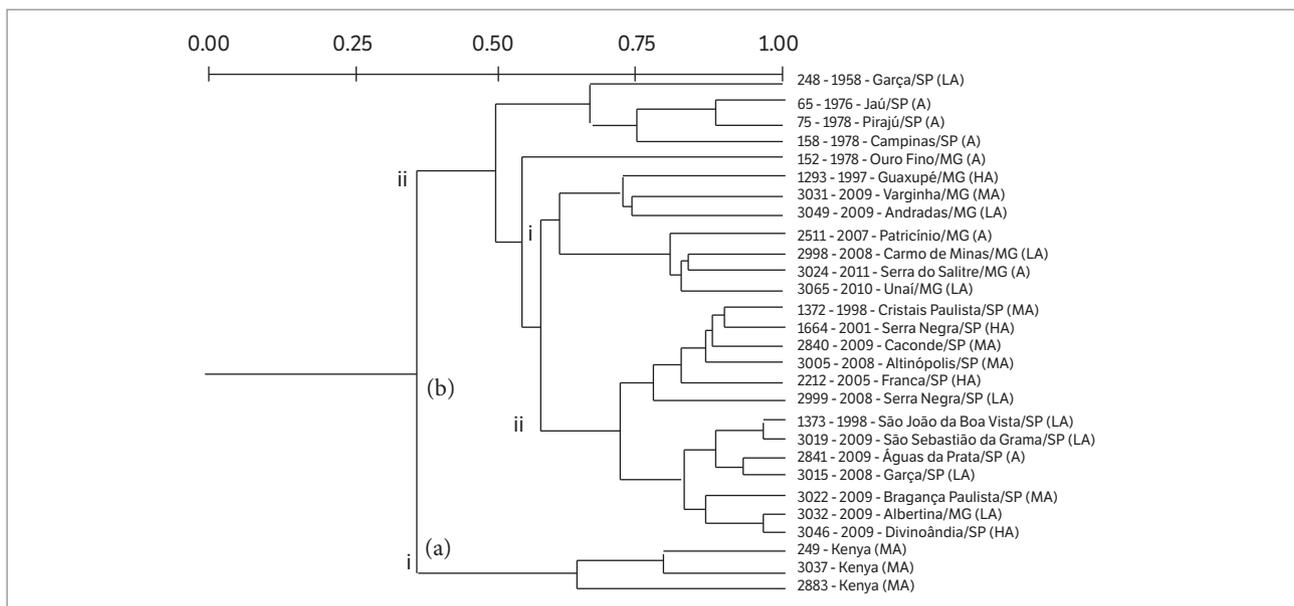
Figure 5. Genetic diversity of *Pseudomonas syringae* pv. *garcae* strains. Dendrogram generated according to the fingerprint of the strains using the primers ERIC-PCR and REP-PCR, based on the UPGMA method, using Jaccard similarity coefficient (S_J).

strains of different species and hosts in diverse clusters. Gašić et al. (2012) demonstrated that this technique was able to differentiate strains of *P. syringae.* pv. *syringae, P. syringae.* pv. *morsprunorum* and *P. syringae.* pv. *persicae* from stone fruits with rep-PCR. On the other hand, Vicente and Roberts (2007), while studying strains of *P. syringae.* pv. *syringae* from cherry trees and other hosts and *P. syringae.* pv. *morsprunorum* isolated from cherry trees, found that strains of *P. syringae.* pv. *syringae* could vary in their rep-PCR patterns, and strains from different hosts grouped together, but strains from *P. syringae.* pv. *morsprunorum* were more aggressive and less variable. Kaluzna et al. (2010) found that BOX-PCR provided a better differentiation of the genetic diversity of *P. syringae.* pv. *syringae* strains and *P. syringae.* pv. *morsprunorum* races 1 and 2 than ERIC-PCR.

In this study, strains from Kenya grouped separately from Brazilian strains in the ERIC- and REP-PCR analysis. The low genetic similarity observed between these strains could be related to their geographic origin. Kairu (1997), previously related differences between strains isolated from Brazil and Kenya, in relation to aggressiveness and biochemical characteristics such as UV fluorescent pigment and bacteriocin production. In a similar pathosystem, Ferrante and Scortichini (2010) also observed that *P. syringae* pv. *actinidiae* strains, the causal agent of an outbreak of bacterial canker on *Actinidia chinensis* (yellow kiwifruit) in central Italy, grouped separately by rep-PCR from strains obtained in other countries.

In this study, the ERIC-PCR technique allows to cluster the strains of *P. syringae* pv. *garcae* by period of isolation, while with the REP-PCR technique the strains were grouped by geographical origin. Similar results were observed for Xanthomonads of tomato by Louws et al. 1994.

The combination of ERIC with REP-PCR showed the genetic relatedness of the Brazilian strains of *P. syringae.* pv. *garcae*. With some exceptions, the strains obtained after the year of 1997 could be grouped by geographic origin. This may have occurred, probably, because nurseries distribute coffee seedlings in the regions where they are produced, and the exchange of seedlings between different regions may occur, but is rather infrequent. As infected seedlings are the main source of spread of bacterial halo blight to new coffee crops, the strains could evolve separately in each region. This is a relevant

information found in this study and implies that control measures aiming to reduce the disease in coffee seedlings nurseries can impact the spread and management of the disease in new coffee crops. Although some of the strains were grouped by aggressiveness classification, such as, in the combined analysis (ERIC/REP-PCR), the Cluster II, subcluster "a" were allocated three strains (IBSBF 65, 75 and 158) as aggressive, and the three strains of Cluster I (IBSBF 249, 2883 and 3037) as moderately aggressive. Even though some strains were grouped, these results could not be correlated. A study with a major number of strains might give a better correlation of aggressiveness and genetic diversity of *P. syringae* pv. *garcae*.

Similar results were observed by Scortichini et al. (2001), while studying strains of *Xanthomonas arboricola* pv. *juglandis* from Persian walnut. The authors observed that, although the genetic similarity of those isolates was high, the strains grouped in three different clusters related to their Italian or Greek geographic origin. Gutiérrez-Barranquero et al. (2013) also reported that the ERIC- and REP-PCR clustered separately *P. syringae* pv. *syringae* strains isolated from mango from strains obtained from other hosts, and also grouped them by their geographic origin.

Since 1997, the majority of the *P. syringae* pv. *garcae* strains (80.9%) was originated from regions near or above 800 m altitude, considered high altitude regions in Brazil. In higher altitude regions, the climatic conditions are very suitable to the coffee crop and are favorable to produce beverage with higher quality. However, in these areas, the milder temperatures and frequent incidence of winds are also favorable for bacterial halo blight. Considering that *P. syringae* pv. *garcae* strains isolated in the last years clustered by their region of origin, recent outbreaks of the disease could be related to nurseries distributing infected seedlings in regions with favorable climatic conditions, but further studies are still necessary to validate this hypothesis.

CONCLUSION

All the *Pseudomonas syringae* pv. *garcae* strains were pathogenic to coffee seedlings but 12, obtained from 1977 to 2011, were more aggressive on *Coffea arabica* cv. Mundo Novo.

In the combined analysis of ERIC- and REP-PCR, the Brazilian strains obtained from 1958 to 1978 grouped separately from the remaining strains and the Kenyan strains grouped separately from the Brazilian strains.

Strains isolated from 1997 to 2011 clustered by their region of origin in the combination of ERIC- and REP-PCR, and were predominately from regions above 800 m.

ACKNOWLEDGMENTS

REFERENCES

Amaral, J. F., Teixeira, C. G. and Pinheiro, E. D. (1956). O bactério causador da mancha aureolada do cafeeiro. Arquivos do Instituto Biológico, 23, 151-155.

Çepni, E. and Gürel, F. (2012).Variation in extragenic repetitive DNA sequences in *Pseudomonas syringae* and potential use of modified REP *primers* in the identification of closely related isolates. Genetics and Molecular Biology, 35, 650-656. http://dx.doi.org/10.1590/S1415-47572012005000040.

Climate Data Org. Dados climáticos para as cidades mundiais; [accessed 2016 February 22]. PT.climate-data.org

Companhia Nacional de Abastecimento (2014). Avaliação da Safra Agrícola Cafeeira 2014 - Terceira Estimativa - Janeiro/2015; [accessed 2016 January 15]. http://www.conab.gov.br/OlalaCMS/uploads/arquivos/15_01_14_11_57_33_boletim_cafe_janeiro_2015.pdf.

Costa, A. S., Amaral, J. F., Viegas, A. P. Silva, D. M., Teixeira, C. G. and Pinheiro, E. D. (1957). Bacterial halo blight of coffee in Brazil. Phytopathologische Zeitschrift, 28, 427-444.

Ferrante, P. and Scortichini, M. (2010). Molecular and phenotypic features of *Pseudomonas syringae* pv. *actinidiae* isolated during recent epidemics of bacterial canker on yellow kiwifruit (*Actinidia chinensis*) in central Italy. Plant Pathology, 59, 954-962. http://dx.doi.org/0.1111/j.1365-3059.2010.02304.x.

Gašić, K., Prokić, A., Ivanović, M., Kuzmanović, N. and Obradović, A. (2012). Differentiation of *Pseudomonas syringae* pathovars originating from stone fruits. Pesticides and Phytomedicine, 27, 219-229. http://dx.doi.org/10.2298/PIF1203219G.

Gutiérrez-Barranquero, J. A., Carrión, V. J., Murillo, J., Arrebola, E., Arnold, D. L., Cazorla, F. M. and Vicente, A. (2013). A *Pseudomonas syringae* diversity survey reveals a differentiated phylotype of the pathovar syringae associated with the mango host and mango toxin production. Phytopathology 103, 1115-1129. http://dx.doi.org/10.1094/PHYTO-04-13-0093-R.

Kaluzna M., Ferrante P., Sobiczewski P. and Scortichini M. (2010). Characterization and genetic diversity of *Pseudomonas syringae* from stone fruits and hazelnut using repetitive-PCR and MLST. Journal of Plant Pathology, 92, 781-787.

Kairu, M.G. (1997). Biochemical and pathogenic differences between Kenyan and Brazilian isolates of *Pseudomonas syringe* pv. *garcae*. Plant Pathology, 46, 239-246. https://doi.org/10.1046/j.1365-3059.1997.d01-218.x.

Kimura, O., Robbs, C. F. and Ribeiro, R. L. D. (1973). Estudos sobre o agente da "mancha aureolada do cafeeiro" (*Pseudomonas garcae* Amaral et al.). Arquivos da Universidade Federal Rural do Rio de Janeiro, 3, 15-18.

Louws, F. J., Fulbright, D. W., Stephens, C. T. and De Bruijn, F. (1994). Specific genomic fingerprints of phytopathogenic *Xanthomonas* and *Pseudomonas* pathovars and strains generated with repetitive sequences and PCR. Applied and Environmental Microbiology, 60, 2286-2295.

Malavolta Junior, V. A., Beriam, L. O. S., Almeida, I. M. G., Rodrigues Neto, J. and Robbs, C. F. (2008). Bactérias fitopatogênicas assinaladas no Brasil: uma atualização. Summa Phytopathologica, 34, 9-88.

Mirik, M., Aysan, Y. and Sahin, F. (2011). Characterization of Pseudomonas cichorii isolated from different hosts in Turkey. International Journal of Agriculture and Biology, 13, 203-209.

Moraes, S. A., Sugimori, M. H., Tomaziello Filho, M. and Carvalho, P. C. T. (1975). Resistência de cafeeiros a *Pseudomonas garcae*. Summa Phytopathologica, 1, 105-110.

Natalini, E., Rossi, M. P., Barionovi, D. and Scortichini, M. (2006). Genetic and pathogenic diversity of *Pseudomonas syringae* pv. *syringae* isolates associated with bud necrosis and leaf spot of pear in a single orchard. Journal of Plant Pathology, 88, 219-223. http://dx.doi.org/ 10.4454/jpp.v88i2.866.

Pethybridge, S. J. and Nelson, S. C. (2015). Leaf Doctor: A new portable application for quantifying plant disease severity. Plant Disease, 99, 1310-1316. http://dx.doi.org/10.1094/PDIS-03-15-0319-RE.

Rademaker, J. L. W., Hoste, B., Louws, F J., Kersters, K., Swings, J., Vauterin, L., Vauterin, P. and Bruijn, F. J. (2000). Comparison of AFLP and rep-PCR genomic fingerprinting with DNA–DNA homology studies: *Xanthomonas* as a model system. International Journal of Systematic and Evolutionary Microbiology, 50, 665-677. http://dx.doi.org/ 10.10466/J.1439-0434.2001.00628.x.

Rodrigues, L.M.R., Almeida, I. M. G., Patrício, F. R. A., Beriam, L. O. S., Maciel, K. W. , Braghini, M. T. and Guerreiro Filho, O. (2013). Mancha aureolada do cafeeiro causada por *Pseudomonas syringae* pv. *garcae*. Boletim Técnico IAC 212. (p. 24). Campinas: Instituto Agronômico.

Rohlf, F. J. (1992). NTSYS-PC Numerical Taxonomy and Multivariate Analysis Sistem Version 1.70. Stony Brook. New York: State University of New York.

Scortichini, M., Marchesi, U. and Di Prospero, P. (2001). Genetic diversity of *Xanthomonas arboricola* pv. *juglandis* (synonyms: *X. campestrispv. juglandis;X. juglandis* pv. *juglandis*) strains from different geographical areas shown by repetitive polymerase chain reaction genomic fingerprinting. Journal of Phytopathology, 149, 325-332. http://dx.doi.org/10.1046/j.1439-0434.2001.00628.x.

Vicente, J. G. and Roberts, S. J. (2007). Discrimination of *Pseudomonas syringae* isolates from sweet and wild cherry using rep-PCR. European Journal of Plant Pathology, 117, 383-392. http://dx.doi.org/10.1007/s10658-007-9107-y).

Zoccoli, D. M., Takatsu, A. and Uesugi, C. H. (2011). Ocorrência de mancha aureolada em cafeeiros na Região do Triângulo Mineiro e Alto Paranaíba. Bragantia, 70, 843-849. https://doi.org/10.1590/s0006-87052011000400017.