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First report of mixed infection by *Pseudomonas syringae* pathovars *garcae* and *tabaci* on coffee plantations

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ABSTRACT: The bacterial-halo-blight (*Pseudomonas syringae* pv. *garcae*) is disseminated by the main coffee areas in the producing states of Brazil. On the other hand, the disease bacterial-leaf-spot (*Pseudomonas syringae* pv. *tabaci*) was reported only once in coffee seedlings in a sample collected in the State of São Paulo. In mid-2015, samples of coffee leaves with symptoms of foliar lesions surrounded by yellow halo, were collected in coffee plantations in the State of Paraná and fluorescent bacteria producing or not brown pigment in culture medium were isolated and determined as belonging to the Group I of *P. syringae*. Through biochemical, serological and pathogenicity tests, the pathogens were identified as *P. syringae* pv. *garcae* and *P. syringae* pv. *tabaci*, with prevalence of isolates belonging to pathovar *tabaci* and, as well as in certain samples, it was identified simultaneous infection by both etiological agents. Then, this is the first report of associated occurrence of *garcae* and *tabaci* pathovars of *P. syringae* and of the incidence of "bacterial-leaf-spot" under field conditions and in the State of Paraná.

KEY WORDS: *Coffea arabica* L., bacterial-halo-blight, bacterial-leaf-spot.

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INTRODUCTION

The coffee cultivation, especially the Arabica (*Coffea arabica* L.), is the main agricultural activities in Brazil, accounting for approximately 2% of Gross Domestic Product. Factors such as long periods of drought and mismanagement of crops, combined with the occurrence of pests and diseases, are the main factors that result in reduction of grain production.

Among the bacterial pathogens, except for *Xylella fastidiosa*, the causal agent of atrophy of branches of coffee (Paradela Filho et al. 1997), the other phytobacteria described in the literature (*Robbsia andropogonis*, *Pseudomonas cichorii*, *P. syringae* pv. *garcae* and *P. syringae* pv. *tabaci*) cause leaf spot symptoms (Robbs et al. 1974; Lopes Santos et al. 2017; Rodrigues Neto et al. 1981; Rodrigues Neto et al. 2006; Destéfano et al. 2010).

Although atrophy of branches of coffee disease presents greater distribution, mainly due to transmission of the causative agent by psyllid insects, bacterial-halo-blight, caused by *Pseudomonas syringae* pv. *garcae* (Amaral, Teixeira and Pinheiro 1956), is also important to the culture and is widespread in the states of São Paulo (Amaral et al. 1956), Paraná (Mohan et al. 1978) and Minas Gerais (Kimura et al. 1973; Zoccoli et al. 2011), causing serious damage to coffee plantations, mainly those located in mild climates and those subjected to long periods of high relative humidity (Sera 2001; Almeida et al. 2012).

Injury symptoms in coffee leaves, similar to bacterial-halo-blight, can be caused by *P. cichorii*, the causal agent of bacterial-leaf-blight, *R. andropogonis* (syn. *P. andropogonis*), the causal agent of bacterial-brown-spot, and *P. syringae* pv. *tabaci*, the causal agent of bacterial-leaf-spot. It is noted that these 4 bacterial diseases associated with foliar injuries, the pathovars *garcae* and *tabaci* of *P. syringae*, cause very similar symptoms and they are only differentiated by isolations and the resulting cultures subjected to biochemical and/or molecular tests.

In assessing the resistance to bacterial-halo-blight in different field locations in the State of Paraná, Petek et al. (2006) observed the occurrence of disease in a coffee-resistant genotype. From these observations, the authors hypothesized the occurrence of a pathogen related to *P. syringae* pv. *garcae* in the region, inducing symptoms reminding those caused by the bacterial-halo-blight.

Later in 2015, samples of plants from the State of Paraná, showing symptoms of leaf lesions surrounded by yellow halo, were received for analysis. Preliminary tests revealed the presence of bacterial flow, and the isolations obtained followed by artificial inoculations in coffee confirmed the pathogenicity of the isolated bacteria. Thus, this study aimed to characterize and identify the causal agent of the symptoms observed in those coffee plants sampled in the State of Paraná.

MATERIAL AND METHODS

Samples of coffee leaves with symptoms of lesions surrounded by yellow halo collected in fields, between June and July 2015, in the counties of Assaí, Cascavel, Londrina and Mandaguari, Paraná, Brazil, were received and analyzed. Tests to identify the bacteria involved with these symptoms were made in collaboration with the Phytobacteriology Laboratory of the Biological Institute, Campinas, São Paulo, Brazil.

Isolations were made on nutrient agar (NA) culture medium (0.5% peptone, 0.3% meat extract, 0.1% NaCl and 18 g agar·L^{-1} of distilled H_2O and pH 7.0). After 48 h of incubation at 28 °C, morphologically similar colonies to the genus *Pseudomonas,* i.e., convex colonies, smooth, whitish color and irregular borders, were purified and submitted to hypersensitivity test in tobacco detached leaves (HR) (Klement et al. 1964).

For identification of the obtained isolates, morphological, biochemical and serological characteristics were compared with *Pseudomonas* coffee bacterial strains obtained at the Phytobacteria Culture Collection of the Biological Institute, Campinas, São Paulo, Brazil (Table 1).

The isolates were submitted to the following tests:

- Gram reaction: carried out with 3.0% KOH solution (Ryu 1940).
- Pigment production: observed in culture medium NA and in King's B (KB) (King et al. 1954).
- Cultural properties: physiological and biochemical selected tests according to Lelliott et al. (1966), Young and Triggs (1994) and Schaad et al. (2001) for the genus *Pseudomonas*.
- Serological tests: performed by double diffusion in agar, as described by Beriam et al. (2006), and used as antigens in reactions (i) bacterial suspensions in 0.85% NaCl solution, obtained from pure colonies

at 48 h growth at 28 °C in NA; (ii) the extracted protein complex of membrane (CPM). The antigens were tested against antiserum kept in the Antiserum Collection of Phytobacteriology Laboratory of the Biological Institute, produced from isolated pathotype strain of *P. syringae* pv. *garcae* (IBSBF 248) and also against *P. cichorii* (IBSBF 587). Two different forms of antiserum were tested: the antiserum prepared against antigens containing whole cells (AS-248-144 — *P. syringae* pv. *garcae* and AS 1784 — *P. cichorii*) and antiserum prepared against CPM (AS-248-145 — *P. syringae* pv. *garcae* and AS 2310 — *P. cichorii*).

- Pathogenicity tests: inoculations were performed on leaves from the 3 first internodes of seedlings of *C. arabica* cv. IAC 125 RN with bacterial suspensions containing 10^8UFC·mL^{-1} by the abrasive method (Rodrigues et al. 2017). The suspensions were obtained from bacterial growth in NA (48 h, 28 °C) with concentrations adjusted using a spectrophotometer absorbance 0.3 (~ 600 nm) (Lelliott et al. 1966).

RESULTS AND DISCUSSION

The HR test were positive until 48 h after the infiltration of bacterial suspensions into tobacco leaves, confirming the pathogenic character of these isolates. Positive HR isolates were Gram negative and reproduced symptoms of disease when inoculated on seedlings of *C. arabica* cv. IAC 125 RN. The inoculations in coffee plants revealed that 1 isolate of *P. syringae* pv. *tabaci* as well as the 3 isolates coming from State of Paraná induced symptoms similar to those produced by bacterial-halo-blight caused by *P. syringae* pv. *garcae*.

The morphological, physiological and biochemical tests allowed to characterize the isolates, as well as serological reactions (Tables 1, 2, 3, respectively).

In the case of LOPAT tests (Levan, oxidase, proctopectinase activity on potato discs, dihidrolase-arginine and HR) (Lelliott et al. 1966), the isolates were included in the Group I of *P. syringae* (LOPAT + – – – +), which are easily differentiated from *P. cichorii* (Group III, LOPAT – + – – +). Moreover, the separation of *garcae* Brazilian strains and *tabaci* pathovars can be determined by some biochemical tests (e.g., trigoneline, L-tartrate and gelatin hydrolysis) Schaad et al. (2001).

Although, by definition, *P. syringae* pathovars are separated based on pathogenicity of the host range; this characterization is not effective, since some pathovars may have wide host range, as verified in the case of *P. syringae* pv. *tabaci*. Therefore, the identification should be performed based on differential characteristics, which are sufficient to prove the classification at the species level and/or pathovar.

Table 1. Bacterial strains used in this study, its identification, origin and diffusible pigment on nutrient agar and on King's B medium by isolates of *Pseudomonas* pathogenic to coffee, obtained in this study.

IBSBF	Origin	Identification	Host	Also held at	NA	KB
248[P]	Garça, SP	*P. syringae* pv. *garcae*	*C. arabica*	ICMP-588	Brown	Slight
249	Kenya	*P. syringae* pv. *garcae*	*C. arabica*		np	Greenish
587	Machado, MG	*P. cichorii*	*C. arabica*	ICMP-9276; NCPPB 3109	np	ne
2249	Arandu, SP	*P. syringae* pv. *tabaci*	*C. arabica*		np	Bluish
3037	Kenya	*P. syringae* pv. *garcae*	*C. arabica*	LMG-5549; ICMP-5019; NCPPB-2708	np	Greenish
3224	Cascavel, PR	*P. syringae* pv. *tabaci*	*C. arabica*		np	Bluish
3225	Cascavel, PR	*P. syringae* pv. *garcae*	*C. arabica*		np	np
3226	Mandaguari, PR	*P. syringae* pv. *tabaci*	*C. arabica*		np	Bluish
3227	Londrina, PR	*P. syringae* pv. *tabaci*	*C. arabica*		np	Bluish
3241	Assaí, PR	*P. syringae* pv. *garcae*	*C. arabica*		Brown	np

[P]Pathotype strain of the specie. IBSBF = Phytobacteria Culture Collection of the Biological Institute, Campinas, São Paulo, Brazil; NA = Nutrient agar; KB = Media culture B (King et al. 1954); SP = São Paulo State; MG = Minas Gerais State; PR = Paraná State; ICMP = International Collection of Microorganism from Plants, Landcare Research, Manaaki Whenua, Lincoln, New Zealand; NCPPB = National Collection of Plant Pathogenic Bacteria, Food and Environment Research Agency, Department for Environment, Food and Rural Affairs, Sand Hutton, York, YO41 1LZ, England; LMG = Laboratorium voor Microbiologie, Universitseit Gent, Belgium; np = Not present or slight; ne = Not evaluated.

Table 2. Levan production, oxidase activity, potato proctopectinase activity, arginine-dihidrolase utilization, hypertensive reaction on tobacco leaf and biochemical tests of bacterial strains obtained in this study in comparison with other species of *Pseudomonas* species pathogenic to coffee.

Tests	PSG			PST	PC	Isolates under identification				
	248	249	3037	2249	587	3224	3225	3226	3227	3241
Levan	+	+	+	+	–	+	+	+	+	+
Oxidase	–	–	–	–	+	–	–	–	–	–
Pectolytic activity	–	–	–	–	–	–	–	–	–	–
Arginine dihydrolase	–	–	–	–	–	–	–	–	–	–
Tobacco HR	+	+	+	+	+	+	+	+	+	+
Adonitol	–	–	–	–	–	–	–	–	–	–
Lactose	–	–	–	–	–	–	–	–	–	–
Sucrose	+	+	+	+	+	+	+	+	+	+
D-mannitol	+	+	+	+	+	+	+	+	+	+
D-sorbitol	+	+	+	+	+	+	+	+	+	+
Trigonelline	–	–	–	+	ne	+	–	+	+	–
L-tartrate	–	ne	ne	+	ne	ne	ne	+	+	+
Gelatin hydrolysis	–	ne	ne	+	ne	+	–	+	+	–

PSG = *Pseudomonas syringae* pv. *garcae*; PST = *Pseudomonas syrigae* pv. *tabaci*; PC = *Pseudomonas cichorii*; + = Positive result; – = Negative result; ne = Not evaluated.

Table 3. Serological reaction of bacterial strains obtained in this study in comparison with *Pseudomonas syringae* pv. *garcae* and *P. cichorii*.

Bacterial strains	*P. syringae* pv. *garcae*		*P. cichorii*	
	AS 248–144	AS 248–145 (CPM)	AS 1784	AS 2310 (CPM)
248[P]	+	+	–	–
249	+	+	–	–
587	–	–	+	+
2249	–	–	–	–
3037	+	+	–	–
3224	–	–	–	–
3225	+	+	–	–
3226	–	–	–	–
3227	–	–	–	–
3241	+	+	–	–

[P]Pathotype strains of the specie; CPM = Complex protein extracted of the membrane; + = Positive result; – = Negative result.

For identification/differentiation of *P. syringae* pathovars pathogenic to coffee, the observation of diffusible fluorescent pigment through NA and KB medium culture was performed as part of the diagnosis (Table 1). Another feature that may assist in the determination of the causative agent is the production of melanin pigment, since, in most cases, the isolates of *P. syringae* pv. *garcae*, when cultivated on NA medium, produce this pigment, diffusible and causing browning of the medium (Barta and Willis 2005), whereas strains of *P. syringae* pv. *tabaci* do not produce this pigment, but when grown on KB medium, cause large amount of fluorescent pigment, when observed under UV light. Isolates of *P. syringae* pv. *garcae* generally exhibit weak fluorescence on KB medium, when compared to those of *P. syringae* pv. *tabaci*.

As part of the biochemical tests (Table 2), especially the use of trigonelline, L-tartrate and gelatin hydrolysis supported the identification of the isolates. Also, according to the literature (Lelliott et al. 1966; Young and Triggs 1994; Schaad et al. 2001), the use of trigonelline, as well as gelatin hydrolysis, may be variable for *P. syringae* pv. *garcae* strains; however, our results are in accordance with the morphophysiological characteristics as stated by those authors, allowing the differentiation between these 2 bacterial species.

Antigens of 5 isolates of *P. syringae*, originating from coffee plantation in the Paraná State, identified at pathovar level through LOPAT (Table 2), were subjected to the serological test double diffusion in agar, using the 2 previously described antiserum (Table 3). Of these isolates, 2 resulted positive for identity with *P. syringae* pv. *garcae* (3225, 3241) and 3 resulted negative (3224, 3226 and 3227). The results of the serological reactions have been complemented by biochemical tests (Table 2). Thus, it became clear that plants exhibiting visually similar symptoms to lesions caused by *P. syringae* pv. *garcae* were recovered isolates of *P. syringae* pv. *tabaci*.

Results of the mentioned tests confirmed the occurrence of bacteria *P. syringae* pv. *tabaci* on coffee leaves in 3 locations of Paraná (Cascavel, Londrina and Mandaguari). In samples collected on Cascavel, mixed infections were detected, where *P. syringae* pathovars *garcae* and *tabaci* were present in the same plant.

According to the literature, the occurrence of *P. syringae* pv. *tabaci* on coffee was reported in a nursery in Arandu, State of São Paulo, in 2006 (Rodrigues Neto et al. 2006; Destéfano et al. 2010), and, since then, there are no other reports of this bacteriosis in coffee plantations. Thus, this is the first report of bacterial-leaf-spot occurrence caused by *P. syringae* pv. *tabaci* in the Paraná State and in field conditions.

Based on these results, it seems reasonable to assume that the lesions observed by Petek et al. (2006) in coffee plants resistant to *P. syringae* pv. *garcae* present in experimental field located in Londrina, Paraná, were caused by *P. syringae* pv. *tabaci*. At that time, the diagnosis was not confirmed, since isolation and characterization of pathogen(s) involved were not made.

Pathogenicity tests showed high aggressiveness of the obtained *P. syringae* pv. *tabaci* strains when compared to pathotype strains of *P. syringae* pv. *garcae* (IBSBF 248), both in the young and older leaves, considering the third internode. The aggressiveness of *P. syringae* pv. *tabaci* on different leave ages needs to be further investigated, from studies of interaction between susceptible coffee plants and access of diverse nature of the pathogen.

The high incidence of characteristic symptoms in older leaves may suggest the presence of *P. syringae* pv. *tabaci* in the plant material, but the occurrence of bacterial-halo-blight can not be discarded; thus the characterization of the causative agent should be complemented by biochemical, serological and/or molecular tests.

The identification of bacterial-leaf-spot is important from an epidemiological point of view, since coffee is the only natural host of *P syringae* pv. *garcae* (Kimura et al. 1973), and *P. syringae* pv. *tabaci* has a wide host range (Bradbury 1986) and it was detected on more than 17 natural hosts of many botanical families in Brazil (Malavolta Júnior et al. 2008).

The pathovar *tabaci*, originally described as affecting *Nicotiana tabacum* L., is associated with a large number of host plants, and it is possible that some of these plants can act as a source of primary inoculum for coffee. Some of *P. syringae* pv. *tabaci* hosts are usually planted in consortium with coffee, like beans (*Phaseolus vulgaris* L.), soybean (*Glycine max* L.), tobacco (*N. tabacum*) and papaya (*Carica papaya* L.), being also described in the climbers weed plants called morning glory (*Ipomoea acuminata* L., *I. aristolochiaefolia* and *I. cynanchifolia*). These factors, combined with the lack of a correct diagnosis, indicate that the bacterial-leaf-spot may be more widely disseminated and, by the similarity of symptoms mainly caused by *P. syringae* pv. *garcae*, undetected or incorrectly identified.

In addition, it is necessary to highlight the occurrence of mixed infections, caused by both pathovars in the same plant, with characteristic symptoms of bacterial-halo-blight.

Work related to the control and management of the coffee bacterial diseases, as well as breeding programs, must always take into consideration the occurrence of bacterial-leaf-spot caused by *P. syringae* pv. *tabaci*, especially for their epidemiological characteristic, i.e., to introduce numerous host plants of diverse plant families. Since breeding programs of coffee conducted to date have evaluated the resistance only to bacterial-halo-blight, it is essential that these programs aim also to assess the simultaneous resistance to both pathovars.

CONCLUSION

This is the first report of the occurrence of bacterial-leaf-spot, caused by *Pseudomonas syringae* pv. *tabaci* in the Paraná State, as well as of mixed infections caused by *P. syringae* pathovars *garcae* and *tabaci* on coffee.

The bacterial strains obtained are preserved in the Phytobacteria Culture Collection of the Biological Institute, Campinas, São Paulo, Brazil (IBSBF) under the numbers 3224 (*P. syringae* pv. *tabaci*, Cascavel, Paraná), 3225 (*P. syringae* pv. *garcae*, Cascavel, Paraná), 3226 (*P. syringae* pv. *tabaci*, Mandaguari, Paraná), 3227 (*P. syringae* pv. *tabaci*, Londrina, Paraná) and 3241 (*P. syringae* pv. *garcae*, Assaí, Paraná).

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