Pseudomonas syringae pv. *tabaci* in papaya seedlings

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ABSTRACT

Beriam, L.O.S.; Almeida, I.M.G.; Destéfano, S.A.L.; Grabert, E.; Balani, D.M.; Ferreira, M.; Rodrigues Neto, J. *Pseudomonas syringae* pv. *tabaci* in papaya seedlings. ***Summa Phytopathologica,*** v.32, p. 21-26, 2006.

The natural occurrence of *Pseudomonas syringae* pv. *tabaci* causing leaf spot symptoms in papaya seedlings is reported. The pathogen was identified through biochemical, physiological, serological, and molecular assays and artificial inoculations in papaya plants. It was also shown that the strains were pathogenic to bean and tobacco plants. The restriction patterns obtained with *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II, *Hinf* I, *Sau* 3A I and *Taq* I of the PCR-RFLP of 16S-23S DNAr were identical to the *P. s.* pv. *tabaci* patterns. Primers corresponding to *hrpL* gene of *P. syringae* were also tested and the results grouped the papaya strains with *P s.* pv. *tabaci*. Bacterial strains were deposited at Coleção de Culturas IBSBF, Instituto Biológico, Campinas, Brazil, under access numbers 1687 and 1822.

Additional keywords: papaya bacterial diseases, 16S-23S rDNA.

RESUMO

Beriam, L.O.S.; Almeida, I.M.G.; Destéfano, S.A.L.; Grabert, E.; Balani, D.M.; Ferreira, M.; Rodrigues Neto, J. *Pseudomonas syringae* pv. *tabaci* em plântulas de mamoeiro. ***Summa Phytopathologica,*** v.32, p. 21-26, 2006.

É relatada a ocorrência natural de *Pseudomonas syringae* pv. *tabaci* causando sintomas de lesões foliares em plântulas de mamoeiro. O patógeno foi identificado por meio de testes bioquímicos, fisiológicos, serológicos e moleculares, além de ensaios de patogenicidade em plantas de mamoeiro, feijoeiro e fumo. Os padrões de restrição obtidos com as enzimas *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II, *Hinf* I, *Sal* 3A I e *Taq* I, utilizando-se a técnica de PCR-RLFP da região espaçadora 16S-23S do DNA ribossômico, foram idênticos àqueles apresentados para *P. s.* pv. *tabaci*. *Primers* correspondentes ao gene *hrpL* de *P. syringae* foram também testados e os resultados obtidos permitiram agrupar as linhagens isoladas de mamão com *P. s.* pv. *tabaci*. Linhagens bacterianas estão depositadas na coleção de culturas IBSBF, Instituto Biológico, Campinas, sob n. 1687 e 1822.

Palavras-chave adicionais: bacteriose do mamoeiro, 16S-23S DNAr.

Brazil is the most important producer of fresh papaya (*Carica papaya* L.) in the world, with a planted area of approximately 30,000 ha and is responsible for an annual production of 1,6 millions of ton of fresh papaya. The main region of exportation is located at the State of Espírito Santo which represents 87.6% of the total exported fruits (24).

Papaya production may be affected by many factors such as the occurrence of phytopathogenic agents, like bacterial diseases, which could causes serious losses. Several bacterial species could infect papaya plants. In 1956, Robbs (19) reported a bacterial disease in Brazil causing symptoms of water soaked and angular spots on papaya leaves, naming the pathogen as *Pseudomonas caricapapayae.* Nelson & Alvarez (13) described in 1976 a disease causing symptoms of "purple stain" in papaya fruits in Hawaii, which causal agent was identified as *Erwinia herbicola.* In 1979, *Erwinia cypripedii* causing black rot on seedlings, trees and fruits of papaya was observed in Taiwan (10). Two other bacterial diseases caused by *Erwinia* spp. and called "erwinia mushy canker" and "erwinia decline" occurring respectively in trees and seedlings of papaya were

reported by Trujillo & Schroth (23) in Hawaii. Other diseases induced by *Erwinia* sp. were also described by Webb (25) in Saint Croix, U.S. Virgin Islands, causing canker on papaya trees and by Frossard et al. (5) who reported a papaya disease caused by *Erwinia* belonging to the "amylovora" group.

An *Erwinia* strain associated with Papaya Ringspot Virus inducing symptoms of bud rot and causing severe damage on papaya plants was described by Robbs et al. (20) in the South of Brazil and further investigations identified it as *E. carotovora* subsp. *atroseptica*[1] .

Recently, the causal agents of the diseases described by Trujillo & Schroth (23), Web (25) and Frossard et al. (5) were identified as a new bacterial species named *Erwinia papayae* (6).

Enterobacter cloacae was another bacterial species described in papaya, inducing symptoms of internal yellowing of fruits in Hawaii (14). Strains of *E. cloacae* were also isolated from papaya fruits in Brazil[1].

In September 2001 seedlings of papaya cv. Golden showing symptoms of brown colored leaf spots, with, sometimes surrounded by a diffuse chlorotic halo, that may progress to large necrotic areas, were observed in commercial nurseries located at Linhares county, State of Espírito Santo, Brazil. Bacterial strains that belong to *Pseudomonas syringae* group (LOPAT Ia) were isolated from these necrotic lesions in a preliminary study (2).

The objective of the present study was to identify these papaya strains at pathovar level through biochemical, serological, pathological, and molecular tests.

MATERIAL AND METHODS

Pathogen isolation

Small pieces of diseased leaf tissues were excised from the lesions and macerated in sterile distilled water. The resultant suspension was streaked on plates containing Nutrient Agar (NA) (11) or King's B (9) media and then incubated at 28^{0}C for 48h. Individual colonies were cultured and used in hypersensitivity tests on tobacco leaves.

Bacterial strains

Besides the papaya isolates, other bacterial strains were included in this study for comparative purposes (Table 1). The strains were recovered from freeze-dried cultures and grown on NA at 28^{0}C for 48h.

Table 1. Bacterial strains used in this study

Strains	Accession Number		Host	Origin
	IBSBF	Other Collection Cultures		
Pseudomonas caricapapayae	361	NCPPB-3439	papaya	Brazil
	821	ICMP-7496	papaya	Brazil
P. syringae pv. not determined	1687		papaya	Brazil
	1822		papaya	Brazil
P. syringae pv. *garcae*	248^P		coffee	Brazil
P. syringae pv. *lachrymans*	961		cucumber	Brazil
	1258^P		cucumber	Brazil
P. syringae pv. *mori*	1419^P	ICMP-4331^P	*Morus alba*	Hungary
P. syringae pv. *pisi*	1418^P	ICMP-2452^P	pea	New Zealand
P. syringae pv. *syringae*	375	ICMP-2842	tomato	Yugoslovia
	451^P	ICMP-3023	*Syringa vulgaris*	United Kingdon
P. syringae pv. *tabaci*	758		cucumber	Brazil
	761	NCPPB-2617	bean	Brazil
	974		*Desmodium canum*	Brazil

^{P} Pathovar reference strain
IBSBF- Phytobacteria Culture Collection of Instituto Biológico, Campinas, SP, Brazil
ICMP - International Collection of Micro-organisms from Plants, Auckland, New Zealand
NCPPB - National Collection of Plant Pathogenic Bacteria, Harpenden, England

[1] Robbs,.C.F. Data not published.

Pathogenicity assays

Papaya seedlings cv. Golden, tobacco (*Nicotiana tabacum* L.), bean (*Phaseolus vulgaris* L.) and poinsettia (*Euphorbia pulcherima* Willd.) plants were inoculated by infiltration with bacterial cell suspensions of papaya strain IBSBF 1687 containing ca. 10^8cfu/mL from 48-72h-old NA cultures, under moisture chamber conditions. Besides leaves, fruits, stems and flowers of papaya were also inoculated. Negative controls were inoculated with sterile distilled water. All the inoculated and controls were maintained in a greenhouse (25 - 30°C) and examined daily for disease development. Papaya plants were also inoculated with *P. caricapapayae* strain IBSBF 361 for comparative purposes.

Biochemical and physiological assays

Biochemical tests for the identification at pathovar level were carried out according to Young & Triggs (27) and Schaad et al. (21).

Serological assays

Bacterial suspensions (ca. 10^9 cfu/mL) obtained from 48 h-old NA cultures as well as membrane protein complex (MPC) (22) were used as antigens. Microscopy slides for double diffusion tests were prepared with 3 mL of 1% purified agar in phosphate buffered saline 0.1M, pH 7 with 200 ppm sodium azide. Papaya bacterial strains were tested with antisera against *P. syringae* strains [*P.s* pv. *syringae* (AS-375), *P. s.* pv. *tabaci* (AS-761), *P. savastanoi* pv. *phaseolicola* (AS-736) and *P. s.* pv. *lachrymans* (AS-961)] obtained from the Antisera Collection of the Laboratório de Bacteriologia Vegetal (LBV), Instituto Biológico, Campinas, SP, Brazil. All the antigen fractions were also tested against normal serum.

DNA extraction and amplification

Genomic DNA from papaya strain (IBSBF-1687), *P. syringae* pv. *garcae* (IBSBF 248^P), *P. s.* pv. *lachrymans* (IBSBF 1258^P), *P. s.* pv *mori* (IBSBF 1419^P), *P. s.* pv. *pisi* (IBSBF 1418^P), *P. s.* pv. *syringae* (IBSBF 451^P), *P. s.* pv. *tabaci* (IBSBF 758) and *P. caricapapayae* (IBSBF-361) were extracted (16) and the concentrations were estimated by comparison of the intensity of fluorescence emitted by known concentrations of the bacteriophage lambda DNA in an ethidium bromide-stained 0.6% agarose gel. Amplification of the 16S-23S spacer region was carried out using the primers pHr (12) and p23Suni322-anti (8). All PCR reactions were performed in a total volume of 25 μL using 100 ng of genomic DNA, 1.0 U *Taq* polymerase (Amersham Biosciences), 1 X *Taq* buffer, 200 μM dNTPs mixture, and 0.4 μM each primer. The PCR protocol consisted of a denaturating cycle of 95°C for 2 min, followed by 25 cycles at 94°C for 1 min, 60°C for 30 s and 72°C for 3 min, and a final extension of 72°C for 5 min, in a thermocycler (GeneAmp PCR system 9700; Perkin-Elmer Corporation, Norwalk, Conn).

The primer set pshrp1F/2R, corresponding to *hrpL* gene of *P. syringae* pathovars *morsprunorum, pisi* and *syringae* (4) was also tested. PCR was performed under the same conditions of the spacer regions and the amplifications were carried out by using an initial denaturation step of 95°C for 2 min, followed by 25 cycles at 94°C for 1 min, 55°C for 30 s and 72°C for 1 min, and a final extension period of 72°C for 3 min. The amplification fragments were observed by electrophoresis in 1% agarose gels in 1X TAE buffer (0.04M tris-acetate, 0.001M EDTA). The gels were stained with 0.1μg/μL of ethidium bromide and photographed under UV light using the Alpha Innotech 2200 Digital System.

PCR-RLFP of the 16S-23S spacer region and *hrpL* gene

PCR products (5 μL) were digested individually with each of the following restriction endonucleases *Afa* I, *Alu* I, *Dde* I, *Hae* III, *Hpa* II, *Hinf* I, *Sau* 3A I and *Taq* I under conditions specified by the manufacturer (Amersham Biosciences) and the restriction fragments separated by electrophoresis in 3% agarose gels using 1X TAE buffer. The gels were stained with ethidium bromide and visualized under UV. The molecular weights of the fragments were determined by comparison with a 100 bp DNA ladder (Amershan Biosciences).

RESULTS AND DISCUSSION

Cultural, morphological, physiological and biochemical tests previously carried out by Beriam et al.(2) showed that the papaya strains belong to *Pseudomonas syringae* species. In this study, these strains were investigated in order to classify them at the pathovar level.

P. syringae causes diseases in a large number of plants and according to Young et al. (26) this species includes more than 50 pathovars, circumscribed on the basis of distinct host range. In addition, Young & Triggs (27) showed that physiological and biochemical determinative tests could be used to differentiate *Pseudomonas syringae* at the pathovar level.

In this study, the papaya strains were pathogenic to tobacco, bean, and poinsettia. Bean and tobacco are natural hosts of *P.s.* pv. *syringae* and *P. s.* pv. *tabaci*, but only *P. s.* pv. *tabaci* was also described causing disease in poinsettia (18), suggesting that the papaya strains could be allocated as *P. syringae* pv. *tabaci*. The determinative tests described in the literature (27) for identification at the pathovar level were very useful in this study (Table 2), corroborating the results of pathogenicity assays.

Another evidence that reinforced the identification of the papaya strains as *P. s.* pv. *tabaci* was the presence of precipitin bands only between the papaya isolates and *P. s.* pv. *tabaci* antisera in the results of the serological assays (Figure 1).

Besides biochemical, serological and pathological results, the molecular tests also confirmed the papaya strains as *P. s.* pv. *tabaci.* The amplification of the 16S-23S spacer region of different pathovars of *P. syringae* (*P.s.* pv. *garcae*, *P. s.* pv. *lachrymans*, *P.s.* pv. *mori*, *P. s.* pv. *pisi*, *P. s.*pv. *syringae* and *P. s.* pv. *tabaci*) resulted in a single product for all strains. The size of the product was approximately 1.1 kilobase (kb). Only fragments ranging from 90 to 1100 base pairs (bp) obtained from restriction endonucleases experiments were considered for analysis. Several restriction enzymes were tested, but only *Dde* I yielded distinct profiles for each pathovar tested, which allowed to group the papaya strains with *P. s.* pv. *tabaci* (Figure 2 and Table 3).

The amplifications with the pshrp 1F/2R primers set were carried out with *P.s.* pv. *syringae* (IBSBF 451^T), *P.s.* pv.*tabaci* (IBSBF 758 and 974) and the papaya strain (IBSBF 1687) and yielded a fragment about 450 bp. No amplification was observed with *P. caricapapayae* strains. In the *Alu* I, *Hae* III, *Hpa* II, *Hinf* I, and *Taq* I digestions, *P.s.* pv. *syringae* could clearly be differentiated from *P. s.* pv. *tabaci* while the papaya strains showed identical profiles to *P.s.* pv. *tabaci* (Figure 3 and Table 4).

Table 2. Determinative tests for the papaya isolates and some *Pseudomonas* pathovars

Tests	papaya isolates	*Pseudomonas syringae* pv. *syringae* (IBSBF-375)	*Pseudomonas syringae* pv. *tabaci* (IBSBF 761)
O/F	Oxid.	Oxid.	Oxid.
Gram reaction	-	-	-
Oxidase	-[1]	-	-
Arginine dihydrolase	-	-	-
Levan production	+	+	+
Potato soft rot	-	-	-
Nitrate reduction ($NO_3 \longrightarrow NO_2$)	-	-	-
Aesculin hydrolysis	+	+	+
Indol production	-	-	-
Gelatin hydrolysis	+	-	+
Production of acid from carbohydrates and related carbon sources:			
L(+)arabinose, D-fructose, erithrytol	+	+	+
D(+)galactose, m-inositol, D(+) mannose	+	+	+
D(-)sorbitol, sucrose	+	+	+
adonitol, D(-)arabinose	-	-	-
cellobiose, inulin, D(+)maltose	-	-	-
salicin, D(+) trehalose	-	-	-
Utilization of organic acids (sodium salts) and aminoacids:			
betaine	+	+	+
DL-homoserine	-	-	-
D(-)tartrate	-	-	-
L(+)tartrate	+	-	+
gluconate, malonate, succinate	+	+	+
benzoate	-	-	-
DL-lactate	-	+	-

[1] - negative result; + positive result

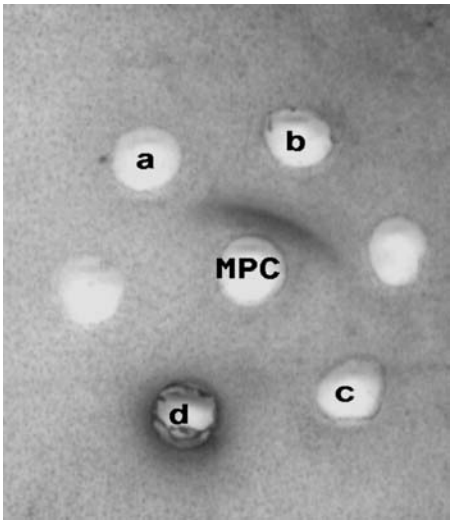

Figure 1. Serological relationship between antisera against *Pseudomonas syringae* pv. *lachrymans* (a) *P.s.* pv. *tabaci* (b), *P. s*. pv. *syringae* (d) and *P. savastanoi* pv. *phaseolicola* (c) strains and Membrane Complex Protein (MCP) of *P. syringae* from papaya.

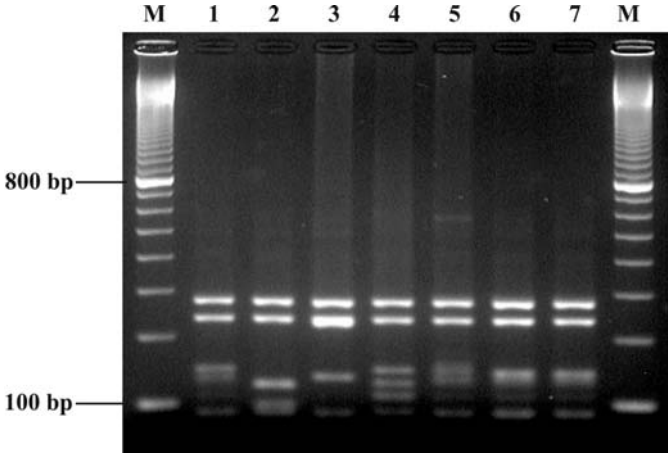

Figure 2. Restriction analysis of the 1.1 kb fragment of the 16S-23S rDNA spacer regions from different pathovars of *Pseudomonas syringae*. **(M)** 100 bp Marker (Amersham Biosciences); **(1)** *P.syringae* pv. *garcae* (IBSBF 248^P); **(2)** *P.syringae* pv. *lachrymans* (IBSBF 1258^P); **(3)** *P.syringae* pv. *mori* (IBSBF 1419^P); **(4)** *P.syringae* pv. *pisi* (IBSBF 1418^P); **(5)** *P.syringae* pv. *syringae* (IBSBF 451^T); **(6)** *P.syringae* pv. *tabaci* (IBSBF 758); **(7)** papaya strain (IBSBF 1687).

Specific primers have been widely used as a rapid method for identification of phytopathogenic bacteria like *Erwinia amylovora* (1), *P. savastanoi* pv. *phaseolicola* (17), *Xanthomonas albilineans* (15), *X. axonopodis* pv. *citri* (7), and others. In this study, the pshrp 1F/2R primer set exhibited specificity, discriminating *P.s.* pv. *syringae* and *P.s.* pv. *tabaci* from *P. caricapapayae.* Although the amplification has occurred for both *P.s.* pv. *syringae* and *P.s.* pv. *tabaci*, the restriction profiles clearly differentiated these pathovars, confirming the identification of the papaya strains as *P.s.* pv. *tabaci*.

According to Bradbury (3), strains of *P. s.* pv. *tabaci* can be transmitted by seeds. Denardin[2] isolated *P.s.* pv. *tabaci* from papaya seed lots. Herein, the seed infection probably could be the source of primary inoculum since the papaya seedlings showed cotyledonary leaf lesions, suggesting bacterial seed transmission, which represents an important vehicle of dissemination of the disease over considerable distances.

Table 3. PCR-RFLP profiles of the spacer region 16S-23S from different pathovars of *P. syringae* produced by *Dde* I digestion.

Strains	Fragments (bp)
P.s. pv. *garcae* (IBSBF 248^{P})	290, 240, 170, 160, 110, 90
P.s. pv. *lachrymans* (IBSBF 1258^{P})	290, 240, 130, 100, 90
P.s. pv. *mori* (IBSBF 1419^{P})	290, 240, 160, 90
P.s. pv. *pisi* (IBSBF 1418^{P})	290, 240, 170, 140, 110, 90
P.s. pv. *syringae* (IBSBF 451^{T})	290, 240, 180, 160, 110, 90
P.s. pv. *tabaci* (IBSBF 758)	290, 240, 160, 150, 110, 90
Papaya strain (IBSBF 1687)	290, 240, 160, 150, 110, 90

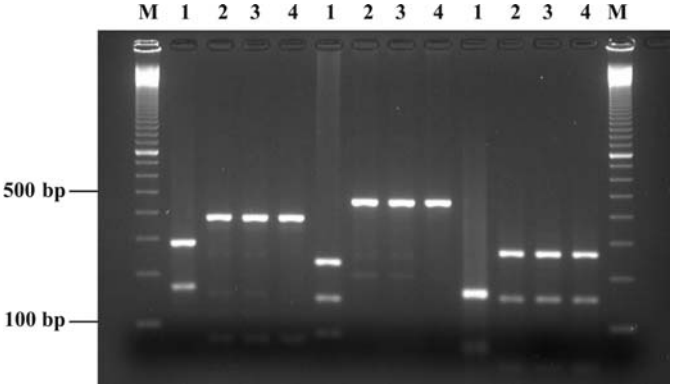

Figure 3. Restriction patterns of the 450 bp fragment of the *hrpL* gene from (1) *Pseudomonas syringae.* pv. *syringae* (IBSBF 451P), (2) *P.s.* pv. *tabaci* (IBSBF 758), (3) *P.s.* pv. *tabaci* (IBSBF 974), (4) papaya strain (IBSBF-1687) digested with *Hae* III, *Hinf* I and *Taq* I. (M) 100 bp Marker.

[2] N.Denardin (Universidade de Passo Fundo/RS). Data not published.

Table 4. PCR-RFLP profiles produced by digestions of the *hrp L* gene with different restriction enzymes.

Enzymes	Fragments (bp)	Strains
Afa I	450	*P.s.* pv. *syringae* (IBSBF 451^{T}), *P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Alu I	200, 180	*P.s.* pv. *syringae* (IBSBF 451^{T})
	250, 200	*P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Dde I	460	*P.s.* pv. *syringae* (IBSBF 451^{T}), *P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Hae III	290, 190	*P.s.* pv. *syringae* (IBSBF 451^{T})
	380, 80	*P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Hpa II	400	*P.s.* pv. *syringae* (IBSBF 451^{T})
	380	*P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Hinf I	250, 150, 90	*P.s.* pv. *syringae* (IBSBF 451^{T})
	440	*P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Mbo I	320, 150	*P.s.* pv. *syringae* (IBSBF 451^{T}), *P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)
Taq I	170, 50	*P.s.* pv. *syringae* (IBSBF 451^{T})
	280, 150, < 50	*P.s.* pv. *tabaci* (IBSBF 758 and 974), papaya strain (IBSBF 1687)

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