ANA BEATRIZ ZACARONI

TAXONOMIC STUDIES OF STRAINS OF *Xanthomonas* spp. IN *Toona ciliata* AND *Cichorium intybus*

LAVRAS – MG

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Tese apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Agronomia/Fitopatologia, área de concentração em Fitopatologia, para a obtenção do título de Doutora.

Orientador

Dr. Ricardo Magela de Souza

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Ficha Catalográfica Elaborada pela Divisão de Processos Técnicos da Biblioteca da UFLA

Zacaroni, Ana Beatriz.
Taxonomic studies of strains of *Xanthomonas* spp. in *Toona ciliata* and *Cichorium intybus* / Ana Beatriz Zacaroni. – Lavras : UFLA, 2012.
76 p. : il.

Tese (doutorado) – Universidade Federal de Lavras, 2012.
Orientador: Ricardo Magela de Souza.
Bibliografia.

1. Cedro australiano. 2. Bacteriose. 3. Radicchio. 4. MLST. 5. Ácidos graxos. I. Universidade Federal de Lavras. II. Título.

CDD – 632.32

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APROVADA em 30 de julho de 2012.

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LAVRAS – MG
2012

EPÍGRAFE

"Você é livre para usar as palavras, mas prisioneiro da escrita".

Ana B. Zacaroni

RESUMO

O presente estudo foi realizado com o objetivo de isolar e identificar o agente etiológico de duas diferentes doenças foliares em cedro australiano (*Toona ciliata* var. *australis*) no Brasil e em radicchio (*Cichorium intybus*) nos Estados Unidos. Os postulados de Koch foram completados para ambos os hospedeiros. Os isolados bacterianos foram submetidos a testes bioquímicos, moleculares (*Multilocus Sequence Typing - MLST*) e à análise de perfis de ácidos graxos. Vinte e cinco isolados bacterianos patogênicos à *T. ciliata* e nove patogênicos à *C. intybus* tiveram fragmentos dos genes *dnaK*, *fuyA*, *gyrB*, *rpoD* sequenciados, juntamente com 31 patovares de *Xanthomonas axonopodis*. Utilizaram-se os primers XdnaK1F/XdnaK1R, XfuyA1F/XfuyA1R, XgyrB1F/XgyrB1R e XrpoD1F/ XrpoD1R para amplificação dos fragmentos que foram sequenciados, preparados e analisados com o uso do Programa *CLC Main Workbench* 6.5. O padrão de bandas dos isolados e daqueles com maior identidade verificada por análises de *MLST* foram comparados por Rep-PCR, utilizando o primer BOX1R. Primers B162, também, foram utilizados para amplificação dos fragmentos genômicos dos isolados patogênicos às plantas de radicchio. Não houve variabilidade entre os isolados bacterianos dentro de cada grupo. Isolados patogênicos à *Toona ciliata* incorporaram ao grupo 9.6 de *Xanthomonas axonopodis* previamente descrito na literatura e àqueles patogênicos à *C. intybus* agruparam com *Xanthomonas hortorum*. Ambos patógenos podem representar um novo patovar dentro de cada espécie. Dados de ácidos graxos, Rep-PCR e B162-PCR suportam os resultados.

Palavras-chave: bacteriose, cedro australiano, radicchio, MLST, ácidos graxos.

ABSTRACT

This study was conducted in order to isolate and identify the etiological agent of two different leaf diseases occurring in Australian cedar (*Toona ciliata* var. *australis*) in Brazil and radicchio (*Cichorium intybus*) in the United States. Koch's postulates were completed for both hosts. The bacterial isolates were subjected to biochemical, molecular (Multilocus Sequence Typing - MLST) tests and analysis of fatty acid profiles. Twenty-five bacterial isolates pathogenic to *T. ciliata* and nine bacterial isolates pathogenic to *C. intybus* had the genomic fragments, *dnaK*, *fyuA*, *gyrB*, and *rpoD* sequenced along with 31 pathovars of *Xanthomonas axonopodis*. The primers XdnaK1F/XdnaK1R, XfuyA1F/ XfuyA1R, XgyrB1F/XgyrB1R, and XrpoD1F/XrpoD1R were used for amplification of fragments that were sequenced, prepared and analyzed using CLC Main Workbench Program 6.5. The banding pattern isolates and those with the highest identity verified by analysis of MLST were compared by Rep-PCR using the primer BOX1R. B162 primers also were used for amplification of genomic fragments of the isolated pathogens of plants of radicchio. There was no variability among bacterial isolates within each group. Pathogenic isolates of *Toona ciliata* incorporated the group 9.6 of *Xanthomonas axonopodis* previously described in the literature and those pathogenic *to C. intybus* clustered with *Xanthomonas hortorum*. Both pathogens may represent a new pathovar within each species. Fatty acids, Rep-PCR and PCR-B162 data support the results.

Keywords: bacteria, australian cedar, radicchio, MLST, fatty acids.

SUMÁRIO

PRIMEIRA PARTE

1 INTRODUÇÃO

A taxonomia de bactérias, que teve início no passado num processo amplamente intuitivo, tem sido alvo de revisão com o advento da taxonomia numérica e técnicas de divergência evolucionária, baseada em informações moleculares dos ácidos nucleicos e proteínas (WAYNE et al., 1987). Desde que o sistema atual de nomenclatura começou a evoluir, durante o período em que as divisões hierárquicas taxonômicas eram ainda vagamente definidas, tornou-se importante reexaminar o sistema antigo sob a ótica dos novos entendimentos taxonômicos. Criou-se um Comitê Internacional para Sistemática de Bactérias, num Workshop de sistemática no Instituto Pasteur, Paris, em 1987. Estirpes de bactérias são normalmente agrupadas dentro de espécies baseadas em similaridades genotípicas e fenotípicas importantes no contexto ecológico (VOS, 2011). Uma das ferramentas mais recentes em taxonomia é a análise multigênica (*Multilocus Sequence Typing* - *MLST*), baseada na identificação de distintos grupos filogenéticos, em esquema de tipagem baseado na sequência de DNA de quatro a 10 locos, em um genoma para identificar e classificar isolados bacterianos, desvendar populações genéticas, avaliar a evolução molecular e epidemiologia dos isolados. Em *MLST*, às sequências alélicas em cada locus são atribuídos números. Isolados que apresentam os mesmos alelos em todos os locos são considerados pertencentes à mesma sequência tipo. Dendrogramas são construídos a partir das diferenças em perfis alélicos multilocus por meio de análise de clusters. No entanto, métodos clássicos de identificação não devem ser descartados, pois a associação de métodos constitui-se em uma maneira eficaz e segura de identificar um organismo.

A reclassificação do gênero *Xanthomonas* mudou signitivamente a taxonomia dos membros das espécies de *X. campestris* (VAUTERIN et al., 1995). Antes de 1995, *X. campestris* consistia de mais de 140 patovares distintos (membros de espécies que diferenciam por patogenicidade) (DYE et al., 1980). Os 62 patovares de *X. campestris,* examinados por Vauterin e colaboradores, foram alocados para 16 genomospécies. A reclassificação de *X. campestris* resultou em: 1) uma lista restrita de patovares de *X. campestris*; 2) elevação de patovares ou geração de novas espécies (ex,. *X. hortorum*); 3) tranferência de isolados de *X. campestris* para outras espécies (ex., *X. axonopodis*); e um grupo de patovares de *X. campestris* de classificação ambígua porque eles não foram avaliados.

A reclassificação introduziu uma particular ambiguidade na taxonomia de *Xanthomonas campestris* pv. *vitians,* agente etiológico da mancha foliar em alface, resultando na transferência do isolado patótipo de *X. campestris* pv. *vitians* para *X. axonopodis* como *X. axonopodis* pv. *vitians* (VAUTERIN et al., 1995). Entretanto, alguns laboratórios documentaram falha de patogenicidade do isolado patótipo (RADEMAKER et al., 2005; SAHIN et al., 2003), desse modo o estatus desse patovar é ambíguo porque nenhum isolado patogênico foi alocado para tal (YOUNG et al., 1996). Um isolado patogênico de *X. campestris* pv. *vitians* foi alocado para *X. hortorum*, entretanto, o nome proposto por Vauterin et al. (1995), *X. hortorum* pv. *vitians,* é invalido porque nenhum patótipo foi designado para o patovar (YOUNG et al., 2012).

Uma nova doença em membro da família Asteraceae foi observada em Monterey County, California em 2002. Dados preliminares não publicados indicam que a mancha foliar de radicchio (*Cichorium intybus*) pode ser causada por *X. hortorum.* Esta doença não tem registro prévio na literatura.

O cultivo de cedro australiano (*Toona ciliata*) está em constante expansão no Brasil despertando cada vez mais o interesse de produtores

florestais. Em 2009, sintomas de mancha foliar foram observados em mudas de cedro australiano e atualmente podem ser encontrados comumente em viveiros no Brasil. Além da redução dos preços, a doença pode também causar perda foliar e morte das mudas.

No presente trabalho objetivou-se isolar e identificar dois grupos diferentes de isolados bacterianos patogênicos ao cedro australiano (*Toona ciliata*) e radicchio (*Cichorium intybus*) por meio de testes bioquímicos, análises fenotípicas e multigênicas além de perfis de ácidos graxos.

2 REFERENCIAL TEÓRICO

2.1 *Análise multigênica - Multilocus Sequence Typing – MLST*

Multilocus Sequence Typing - MLST foi proposta primeiramente em 1998 (MAIDEN et al., 1998), como método universal e definitivo para caracterizar bactérias, utilizando *Neisseria meningitidis* como exemplo, por meio da análise de sequências de nucleotídeos de múltiplos locus que codificam genes vitais ou fragmentos destes (MAIDEN, 2006).

Os autores propuseram que a classificação poderia ser aperfeiçoada, por meio da geração de uma representação do cromossomo, usando *MLST* - concatenação de uma seleção de genes codificadores de proteínas e identificação de diferenças alélicas nos locos dos organismos mais relacionados (MAIDEN et al., 1998). O conceito foi estendido para consideração de mais taxa para incluir gêneros inteiros, usando sequências de genes codificadores de proteínas, chamado de análise de sequência multilocus (*MLSA*) (GEVERS et al., 2005).

Numa recente MLSA de *Xanthomonas*, Fargier e Manceau (2006) investigaram seis house-keeping genes (atpD, dnaK, glnA, gyrB, e rpoD tpiA) e o gene estrutural, fyuA, como base para a diferenciação dentro de *Xanthomonas*.

Destes, quatro sequências de genes, chaperone protein dnaK (dnaK), tonB-dependentre- captor (fyuA), DNA gyrase subunitB (gyrB) e RNA polymerase sigma factor (rpoD) foram congruentes na representação de *Xanthomonas* spp. (YOUNG et al., 2008). Sequências parciais destes genes foram utilizados no presente estudo.

2.2 *Perfil de ácidos graxos*

Os lipídeos ou ácidos graxos são extensas moléculas com esqueleto de carbono cujas funções são estruturar a membrana plasmática e servir de reserva de energia. Em fungos e bactérias, o perfil quali-quantitativo de ácidos graxos, por meio de cromatografia gasosa, pode ser utilizado na diagnose molecular de doenças de plantas (LANOISELET et al., 2005). Um sistema automatizado denominado MD (Microbial Identification System) é capaz de identificar com grande precisão fungos filamentosos, leveduras e bactérias, por meio de análise qualitativa e quantitativa dos ácidos graxos, presentes na parede celular destes microrganismos (YANG et al., 1993). Este sistema constitui-se de um cromatógrafo gasoso, acoplado a um computador, o qual armazena a base de dados dos perfis de ácidos graxos de uma grande coleção de espécimes, além do software que analisa os dados obtidos na leitura do cromatógrafo.

A utilização de perfis de ácidos graxos, para estudos taxonômicos do gênero *Xanthomonas,* foi descrito por Yang et al. (1993). Estes autores mostraram que membros do gênero *Xanthomonas* contêm pelo menos 65 diferentes ácidos graxos, em grandes quantidades e que 50 destes ácidos graxos estão presentes em mais do que 1% das estirpes quando 975 estirpes foram testadas. O gênero *Xanthomonas* caracteriza-se pela presença de muitas cadeias ramificadas e ácidos graxos hidroxi. A grande diversidade de ácidos graxos, encontrados neste taxon, torna a análise de perfis de ácidos graxos uma

ferramenta valiosa para estudar as relações com o gênero *Xanthomonas* e, em particular, para a identificação rápida de estirpes novas e desconhecidas (VAUTERIN; YANG; SWINGS, 1996).

2.3 *Toona ciliata*

O cedro australiano é uma espécie arbórea pertencente à família Meliaceae. Cresce em áreas com precipitação anual entre 800 e 1800 mm com dois a seis meses de seca, apresentando bom desenvolvimento em regiões de 100 a 1500 m de altitude. Necessita de solos com boa drenagem, profundos e eutróficos. Seu cultivo fornece madeira de qualidade para serrarias e indústrias moveleiras, sendo utilizada na fabricação de compensados, aglomerados, móveis, esculturas, entalhes em portas e janelas, na construção de navios e aviões, fabricação de lápis e instrumentos musicais (CENTRO DE INTELIGÊNCIA EM FLORESTAS - CIFLORESTAS, 2009). Além de ser considerada uma madeira nobre, é leve e resistente, permitindo um excelente acabamento e consequente valorização no mercado. Assim, desperta cada vez mais o interesse de produtores florestais.

As doenças florestais são responsáveis, em parte, pela diminuição da produtividade dos plantios comerciais brasileiros. Até o momento, as doenças registradas em plantios e povoamentos florestais têm como agentes etiológicos, principalmente, os fungos, com alguns poucos registros de ocorrência de bactérias e sem registros concretos da ação de vírus ou outros microrganismos (AUER; GOMES; GRIGOLETTI JÚNIOR, 2009).

No entanto, poucos são os relatos na literatura sobre tais doenças. Ferreira (1989) relatou a presença de *Phyllachora balansae* Speg. em cedro rosa (*Cedrela fissilis* Vell.) e cedro australiano (*Toona ciliata*), capaz de levar à senescência precoce das folhas. Hanada, Gasparotto e Ferreira (2005) relataram

pela primeira vez, em *Cedrela fissilis*, lesões portadoras de sinais correspondentes às frutificações dos fungos *Pseudobeltrania cedrelae* e *Colletotrichum gloesporioides*, no entanto, os resultados dos testes de patogenicidade comprovaram apenas *P. cedrelae* como o agente etiológico da doença, embora *C. gloesporioides* tenha estado frequentemente associado.

Entre as poucas bacterioses florestais já relatadas, podem-se citar duas bactérias de maior ocorrência e expressão em território brasileiro, sendo elas *Ralstonia solanacearum* e *Xantomonas axonopodis* pv. *eucalypti*, agentes etiológicos da murcha e de manchas foliares respectivamente, ambas em eucalipto (ALFENAS et al., 2004, 2006; GONÇALVES et al., 2008). Coutinho e Preisig (2002) relataram *Pantoea ananatis* como agente etiológico na morte dos brotos e folhas jovens de híbridos e clones de *Eucalyptus grandis* x *E. nitens* em viveiros de KwaZulu/Natal – África do Sul.

O cultivo de *Toona ciliata* está em constante expansão no Brasil e a ocorrência da doença vem comprometendo a produção de mudas e, consequentemente, reduzindo a produtividade.

2.4 *Cichorium intybus*

Radicchio (*Cichorium intybus*) está ranqueado entre as culturas mais importantes em Monterey County, Califórnia, Estados Unidos, movimentando cerca de US$ 19.531.000,00 e é uma excelente fonte de antioxidantes (LAURITZEN et al., 2012). Em 2002 começou-se a observar manchas foliares em plantas de radicchio em Monterey County, Califórnia. A incidência da doença no primeiro ano resultou em até 10% de plantas de radicchio não colhidas.

No presente estudo identificou-se o patógeno como um membro da espécie *Xanthomonas hortorum* indicando que ele pode estar relacionado a um importante patógeno bacteriano em alface.

3 CONSIDERAÇÕES GERAIS

A classificação taxonômica de um organismo exige o segmento de regras e padrões. Realizar o procedimento de forma correta é de fundamental importância, pois se as regras e os padrões estabelecidos não forem seguidos, o processo de tornar a classificação válida é laborioso. Somando-se a isso, o papel educador de uma correta classificação deve ser prezado para que mais pesquisadores possam utilizar a taxonomia de forma correta gerando nomes válidos e aumentando o círculo de conhecimento das regras e padrões taxonômicos.

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SEGUNDA PARTE – ARTIGOS

ARTIGO 1

Xanthomonas axonopodis cause disease in nurseries of Australian cedar (*Toona ciliata* var. *australis*)

(Plant Disease)
(Versão preliminar)

Zacaroni, A.B., Souza, R.M., Figueira, A.R., Pozza, E.A., Mansur, T.O.F., author6, author7, Bull, C.T.

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Abstract

The aim of this study was to isolate and identify the etiological agent of a foliar disease of Australian cedar (*Toona ciliata* var. *australis*). The isolated colonies were whitish slimy-looking, the bacteria were Gram negative, strictly aerobic, negative for utilization of carbon and nitrogen from arginine, asparagine and did not produce fluorescent pigments on King B medium. They were positive for starch and esculin hydrolysis, glucose as carbon source and gas production. The bacteria induced hypersensitive reaction on tomato and pepper, but not in tabacco. All isolates from Australian cedar had identical DNA fragment-banding patterns generated by rep-PCR using the BOX- A1R primers. Koch' postulates were fulfilled when seedlings inoculated with the cedar isolates developed the symptoms of the original disease and the bacteria reisolated from symptomatic tissue were identical to the isolates used to inoculate seedlings according to rep-PCR. Multilocus Sequence Analysis was conducted to identify the pathogen to the species level. The sequences of fragments of *dnaK*, *fyuA*, *gyrB*, and *rpoD* housekeeping genes of the 25 isolates pathogenic to *Toona ciliata* var. *australis* were identical and clustered with isolates of *Xanthomonas axonopodis* group 9.6. Fatty acid profiles and rep-PCR analysis further supported the placement of the cedar isolates in *Xanthomonas axonopodis*.

Introduction

The Australian cedar (*Toona ciliata* var. *australis*) is a tree species belonging to the family Meliaceae. It originates in Asia and is found as a native plant in Australia, India, Myanmar, Indonesia and Malaysia. Its cultivation provides quality wood for sawmills and furniture industries and it is used in the manufacture of plywood, particle board, furniture, sculptures, carvings on doors and windows, building ships and aircraft, manufacture of pencils and musical instruments. Besides being considered a hardwood, it is lightweight and durable. These characters result in high quality finished products leading to its high value and interest to forest producers.

The forest diseases are responsible, in part, by decreasing the productivity of the commercial plantations. In general, fungal diseases are the primary etiological agents of diseases of plantations and forest; however, there are a few records bacterial diseases and no confirmed records of viruses or other microorganisms causing forest diseases.

Among the few bacterial diseases in forest species, there are two bacterial diseases present and important in Brazil: bacterial wilt caused by *Ralstonia solanacearum* and bacterial leaf spot caused by *Xanthomonas axonopodis* pv. *eucalypti*, both in eucalyptus (3, 4).

In 2009, leaf spot symptoms were observed on cedar seedlings and now the disease is commonly found in nurseries in Brazil (Fig. 1). In addition to reduced sales, the disease causes leaf loss and seedling death. The aim of this study was to isolate and identify the etiological agent of this foliar disease of Australian cedar found in nurseries in Brazil.

Materials and methods

Disease and bacterial isolation

Twenty five isolates were obtained from seedlings of Australian cedar (*Toona ciliata* var. *australis*) in a commercial forest nursery located in South of Minas Gerais state, Brazil. The nursery is the main forest producer of seedlings in Brazil.

Early symptoms of bacterial leaf spot were small water-soaked leaf spots. These lesions are typically defined by leaf veins and angular in the shape. The lesions quickly develop of dark brown to dark irregular blotches. In severe disease, numerous lesions may coalesce, resulting in the collapse and premature fall of the leaflets. Older lesions dry up and become papery in texture (Fig. 1).

Disease symptoms were recorded. Symptomatic tissues from different plants and lesions, outbreaks from 2009 to 2010 were sampled and submitted to bacterial streaming test and those which showed the positive results were isolated from small sections of tissue symptomatic surface disinfested with 0.5% sodium hypochlorite for 1 min followed by 70% ethyl alcohol for 2 min, rinsing in sterile distilled water. And then they were macerated into 40 µl of sterile distilled water. The suspensions were streaked onto 523 medium (5) and incubated at 28 °C for 2 days. After that, single colonies were purified and stored at -80°C.

Physiological characterization of bacteria

The bacteria isolated from Australian cedar seedlings were evaluated for hypersensitivity reaction (HR) in tobacco, tomato and pepper plants and those that were positive for at least one of them were tested for Gram reaction, anaerobic growth, production of fluorescent pigments on King B medium (KB), colony color on Nutrient Agar (NA Difco Laboratories, USA), colony color and mucoid growth on YDC, gas production from glucose as carbon source, utilization of carbon and nitrogen from arginine and asparagine, urease production, presence catalase and starch and esculin hydrolysis as described by

Schaad et al. (20). Isolates of *Pseudomonas syringae* pv. *tomato*, *Xanthomonas axonopodis* pv. *malvacearum*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* and *Ralstonia solanacearum* were used as controls.

Bacterial isolates and growth conditions

The bacterial isolates obtained from Australian cedar and the pathotypes strains of *Xanthomonas axonopodis* obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB) are listed in Table 1. The bacteria were stored at -80°C in a solution of 50% glycerol and 50% nutrient broth (NB Difco Laboratories, USA) and were cultured onto 523 medium for pathogenicity test and Nutrient Agar (NA) for the others.

Pathogenicity test

The inoculation for each of the 25 isolates from Australian cedar was generated from two-day-old lawns of bacteria on 523 medium. Bacteria were suspended in sterile distilled water and adjusted to 0.600 OD at 600_{nm}. The undersides of leaves of four Australian cedar seedlings with three expanded true leaves were spray inoculated until runoff with individual isolates. After the inoculation, the seedlings were kept for 12 h in the greenhouse until completely dry and transferred to a mist chamber for 72 h and then they were maintained in greenhouse until complete evaluation. The negative control seedlings were inoculated with sterile distilled water. Treatments were arranged in four randomized blocks. The greenhouse was maintained at 20 to 32 ^{o}C. The plants were monitored daily for 30 days to check the occurrence of symptoms and isolation of etiological agent. After reisolation, single colonies were purified and submitted to rep-PCR using BOX1R primers to compare to the original band pattern of the strain to confirm the reisolated strain identity.

Multilocus sequence typing - MLST

The 25 bacterial isolates pathogenic to *Toona ciliata* var. *australis* seedlings and 31 pathotype of *Xanthomonas axonopodis* pathovars had the genomic fragments of *dnaK*, *fyuA*, *gyrB*, and *rpoD* amplified by PCR with primers and methodology described by Young et al. (25). The PCR products were sequenced by McLab (South San Francisco, CA) and/or TACGen (Richmond, CA) commercial laboratories and the sequences were prepared and analyzed using the CLC Main Workbench 6.5 software (CLCbio, Aarhus, Denmark). The concatenated sequences were compared and the maximum likelihood trees were built, as well the similarity matrix of those sequences and the sequences of type and pathotypes strains of *Xanthomonas* available in the *Plant Associated and Environmental Microbes Database - PAMDB.*

The lengths of the sequences of four housekeeping genes fragments were modified and, the start and end point of each gene are described in Table 2.

Relationships among cedar isolates evaluated by Rep-PCR

The strains *X. axonopodis* pv. *dieffenbachiae*, *X. axonopodis* pv. *rhynchosiae*, *X. axonopodis* pv. *sesbaniae* and *X. axonopodis* pv. *vignaeradiatae* belonging the 9.6 group of *Xanthomonas axonopodis sensu* Rademaker et al. (17) had their rep-PCR band patterns compared to Australian cedar isolates. The BOX-A1R primers were used (24) to amplify the genomic DNA using published method (16). Amplified DNA fragments were examined in 1.5% agarose gels in 0.5X Tris acetic acid EDTA buffer, TAE. The gels were stained with GelRed and photographed on a UV transilluminator using a digital camera and Kodak Molecular Imaging software (v. 4.5.1, Carestream Health, Inc., Rochester, NY).

Fatty acid profiles

The bacterial isolates from Australian cedar seedlings had their fatty acid profiles analyzed and compared with the 31 *X. axonopodis* pathovars described in Table 1. Fatty acids were methyl-esterified and extracted using a previously published method (13). Fatty acid methyl esters were analyzed with the Sherlock Microbial Identification System Version 6.1 (MIDI Inc., Newark, DE) using an automated GC 6890 Hewlett-Packard gas chromatograph fitted with a 25 X 0.2 mm phenyl methyl silicone-fused silica capillary column, an HP 7683 automatic sampler, and Agilent ChemStation Software (Ver. B.03.02). The mean and standard deviation of the area for each named peak from three (cedar isolates) or four (*X. axonopodis* pathovars) independent replications was reported as a percentage of the total area of all peaks in the chromatogram not including the solvent peak.

Results

Characterization

The isolated colonies were whitish and mucoid on 523 and YDC mediums. The isolates presented hypersensitivity reaction in tomato and pepper plants but not in tobacco plants. The 25 Australian cedar isolates showed no variability among them in the biochemical tests (Table 3).

Pathogenicity test

All isolates obtained from Australian cedar produced leaf spot symptoms on *Toona ciliata*. var. *australis* seedlings. They appeared 15 days after the inoculation (Fig. 1). Small water-soaked leaf spots were the first to appear, bordered by leaf veins and angular in shape. The lesions started with dark brown color and quickly became dark irregular blotches. The seedlings inoculated with the cedar isolates developed the symptoms of the original disease and the bacteria reisolated from symptomatic tissue were identical to the

isolates used to inoculate the seedlings according to rep-PCR banding patterns, thus fulfilling the requirements of Koch's postulates.

Multilocus sequence typing - MLST

The sequences of the four fragments of the housekeeping genes of the 25 isolates pathogenic to *Toona ciliata* var. *australis* showed no variability among them.

Isolates pathogenic to Australian cedar seedlings clustered with isolates of *Xanthomonas axonopodis* group 9.6 defined previously in the literature by Rademaker et al. (17) (Fig. 2 and 3). The group 9.6 is composed by type strains *X. axonopodis* pv. *cajani* and *X. axonopodis* pv. *phaseoli* var. *fuscans*; the pathotype strains *X. axonopodis* pv. *rhynchosiae*, *X. axonopodis* pv. *sesbaniae*, *X. axonopodis* pv. *vignaeradiatae*, *X. axonopodis* pv. *vignicola* and *X. axonopodis* pv. *anarcadii*; and isolates of *X. axonopodis* pv. *aurantifolii* (B,C,D) and *X. axonopodis* pv. *dieffenbachiae* (1, 17).

The cedar isolates DNA sequences shared more than 99% similarity with the isolates of *Xanthomonas axonopodis* group 9.6: *X. axonopodis* pv. *rhynchosiae* (99,25%), *X. axonopodis* pv. *sesbaniae* (99,70%), *X. axonopodis* pv. *vignaeradiatae* (99,66%), *X. axonopodis* pv. *vignicola* (99,59%), *X. axonopodis* pv. *phaseoli* var. *fuscans* (99,89%).

Relationships among cedar isolates evaluated by Rep-PCR

The isolates pathogenic to Australian cedar showed no differences between them on the Rep-PCR electrophoretic profile. However, the BOX-PCR successfully distinguished cedar isolates from *X. axonopodis* pv. *dieffenbachiae* and the pathotypes strains belonging to group 9.6 *sensu* Rademaker et al. (17) *X. axonopodis* pv. *rhynchosiae*, *X. axonopodis* pv. *sesbaniae* and *X. axonopodis* pv. *vignaeradiatae* (Fig. 4).

Fatty acid profiles

The Australian cedar isolates produced the fatty acids: 10:00, 11:0 iso, 11:0 iso 3OH, 11:0 3OH, 13:0 iso, 12:0 iso 3OH, 14:0 iso, 14:00, 13:0 iso 3OH, 13:0 2OH, 15:1 iso F, 15:0 iso, 15:0 anteiso, 15:1 w6c, 16:0 iso, 16:1 w9c, 16:00, 17:0 iso, 17:0 anteiso, 17:1 w8c and 17:1 w6c, represented by 3 cedar isolates in Table 4. The 31 *X. axonopodis* pathovars produced eight of 21 fatty acids that isolates of Australian cedar did. They are: 10:00, 11:0 iso, 11:0 iso 3OH, 14:00, 13:0 iso 3OH, 15:0 anteiso, 16:0 iso, 16:00, but none of those produced exactly the same fatty acids that isolates from Australian cedar did.

Xanthomonas axonopodis pv. *sesbaniae* (NCPPB582) produced 13 of those fatty acid produced by cedar isolates and producing also 10:0 3OH and 12:0 3OH. *X. axonopodis* pv. *rhynchosiae* (NCPPB1827) behaved like *X. axonopodis* pv. *sesbaniae* producing 11:0 3OH instead 14:0 iso and did not produce 15:0 iso F. *X. axonopodis* pv. *vignaeradiatae* (NCPPB2058) also behaved similarly to *X. axonopodis* pv. *sesbaniae* and *X. axonopodis* pv. *rhynchosiae*, producing also 13:0 iso, but not 11:0 3OH, 14:0 iso, 10:0 3OH and 15:0 iso F.

Discussion

This research was conducted to identify the etiological agent of a foliar disease in Australian cedar (*Toona ciliata* var. *australis*) occurring in nurseries in Brazil. Among the few reports of bacteria causing diseases in forest species, *Ralstonia solanacearum* and *Xanthomonas axonopodis* pv. *eucalypti* causing respectively bacterial wilt and leaf spot in eucalyptus plants are present in Brazil (3,4), but no report of bacterial disease in cedar plants in the world is found. The cedar isolates fulfilled the requirements of Koch's postulates and the bacterium was Gram-negative, aerobic, whitish mucoid colony, catalase and glucose

positive, hydrolysis of starch and esculin positive and urease negative. It did not use asparagine and arginine as a source of carbon and nitrogen and did not fluorescent on King's Medium B. No use of asparagine as the sole carbon and nitrogen source, according to Bergey Manual (8) is characteristic of the genus *Xanthomonas*. Despite the yellow pigment is present in most isolates belonging to the genus *Xanthomonas*, there are reports of nonpigmented species of *Xanthomonas*, as *X. campestris* pv. *manihotis*, *X. campestris* pv. *ricini*, *X. albilineans*, *Xanthomonas campestris* pv. *mangiferaeindicae*, *X. campestris* pv. *viticola*, *X. axonopodis* pv. *pedalii* and *X. axonopodis* pv. *phyllanthi* (18, 10, 14, 19).

The bacteria induced hypersensitive reaction on tomato and pepper plants, but did not in tobacco plants. The fact that some non-host plants present positive and negative reaction to HR, or even different behavior among isolates, is described in the literature (6, 7, 21), therefore we can indicate the tomato and pepper plants as ideal for testing the hypersensitivity reaction for isolates pathogenic to Australian cedar. Tessmann et al. (21) obtained negative HR results in tobacco plants after inoculation with nine isolates of *Xanthomonas campestris* isolated from *Alstroemeria caryophyllaea*. While, Nascimento (12) and Marques et al. (11) observed a positive HR result in tomato plants inoculated with *X. campestris* pv. *viticola*. From these characteristics just mentioned, we assigned the bacterium to the genus *Xanthomonas*. The cedar isolates produced the fatty acids that Vauterin et al. (23) reported for *X. axonopodis*, excepting 17:1 w6cf fatty acid. The 17:1 fatty acid was produced by 13 *Xanthomonas axonopodis* pathovars used in this study. The MLST, the BOX-PCR and fatty acid data confirm the result and support the placement of these isolates into one of the six subgroups of *X. axonopodis* defined by Rademaker et al. (17).

The MLST proposed by Maiden et al. (9) is particularly useful in understanding phylogeny because genetic distance can be analyzed based on several independent loci. Amplicons for MLST were generated using a modification of the scheme developed by Young et al. (25) where some nucleotides were withdrawn from the extremities of the sequences and the sequences concatenated were compared to sequences with original length and the maximum likelihood trees were built, as well as the similarity matrix to confirm that modification does not affect the final analysis. The MLST results suggest that cedar isolates belong to *Xanthomonas axonopodis* group 9.6 *sensu* Rademaker et al. (17). Those authors distributed the strains identified as pathovars of *X. axonopodis* in six clusters, corresponding to subgroups 9.1 to 9.6, using primers targeting the conserved repetitive sequences BOX, enterobacterial repetitive intergenic consensus (ERIC), and repetitive extragenic palindromic (REP) (rep-PCR).

The group 9.6 is composed by type strains *X. axonopodis* pv. *cajani* and *X. axonopodis* pv. *phaseoli* var. *fuscans*; the pathotype strains *X. axonopodis* pv. *rhynchosiae*, *X. axonopodis* pv. *sesbaniae*, *X. axonopodis* pv. *vignaeradiatae*, *X. axonopodis* pv. *vignicola* and the recently described *X. axonopodis* pv. *anarcadii*; and isolates of *X. axonopodis* pv. *aurantifolii* (B,C,D) and *X. axonopodis* pv. *dieffenbachiae* (1, 17). Although there are 2 strains of *X. axonopodis* pv. *dieffenbachiae* in the group 9.6, the pathotype strain belong to group 9.4 what justify the distance in the phylogenetic tree (Fig. 3).

The DNA sequences of cedar isolates shared more than 99% similarity with *X. axonopodis* pv. *rhynchosiae*, *X. axonopodis* pv. *sesbaniae*, *X. axonopodis* pv. *vignaeradiatae*, *X. axonopodis* pv. *vignicola, X. axonopodis* pv. *fuscans* supported the hypothesis that the cedar isolates belong to group 9.6 *sensu* Rademaker et al. (17).

ACKNOWLEDGMENTS

The authors gratefully acknowledge the U.S. Agricultural Research Station for the opportunity to work together, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship of the first author.

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Table 1. Strains/isolates names referred to in this study, with the code.

Code	Strains/isolates	Location of isolation	Host of origin
NCPPB466^{Pt}	*Xanthomonas axonopodis* pv. *punicae*	India	*Punica granatum*
NCPPB481^{Pt}	*Xanthomonas axonopodis* pv. *desmodii*	India	*Desmodium diffusum*
NCPPB536^{Pt}	*Xanthomonas axonopodis* pv. *khayae*	Sudam	*Khaya senegalensis*
NCPPB577^{Pt}	*Xanthomonas axonopodis* pv. *desmodiigangetici*	India	*Desmodium gangeticum*
NCPPB578^{Pt}	*Xanthomonas axonopodis* pv. *erythrinae*	India	*Erythrina indica*
NCPPB581^{Pt}	*Xanthomonas axonopodis* pv. *poinsettiicola*	India	*Euphorbia pulcherrima*
NCPPB582^{Pt}	*Xanthomonas axonopodis* pv. *sesbaniae*	India	*Sesbania aegyptiaca*
NCPPB584^{Pt}	*Xanthomonas axonopodis* pv. *tamarindi*	India	*Tamarindus indica*
NCPPB637^{Pt}	*Xanthomonas axonopodis* pv. *cyamopsidis*	India	*Cyamopsis tetragonolobus*
NCPPB761^{Pt}	*Xanthomonas axonopodis* pv. *physalidicola*	Japan	*Physalis alkekengii*
NCPPB840^{Pt}	*Xanthomonas axonopodis* pv. *patelii*	India	*Crotalaria juncea*
NCPPB885^{Pt}	*Xanthomonas axonopodis* pv. *desmodiirotundifolii*	India	*Desmodium rotundifolium*
NCPPB971^{Pt}	*Xanthomonas axonopodis* pv. *maculifoliigardeniae*	USA	*Gardenia* sp.
NCPPB993^{Pt}	*Xanthomonas axonopodis* pv. *lepdzae*	USA	*Lespedeza* sp.
NCPPB994^{Pt}	*Xanthomonas axonopodis* pv. *melhusii*	India	*Tectona grandis*
NCPPB1063^{Pt}	*Xanthomonas axonopodis* pv. *ricini*	Ethiopia	*Ricinus communis*
NCPPB1148^{Pt}	*Xanthomonas axonopodis* pv. *martyniicola*	India	*Martynia diandra*
NCPPB1335^{Pt}	*Xanthomonas axonopodis* pv. *bauhiniae*	India	*Bauhinia racemosa*
NCPPB1337^{Pt}	*Xanthomonas axonopodis* pv. *nakataecorchorii*	India	*Corchorus acutangulus*
NCPPB1786^{Pt}	*Xanthomonas axonopodis* pv. *coracanae*	India	*Eleusine coracana*
NCPPB1827^{Pt}	*Xanthomonas axonopodis* pv. *rhynchosiae*	Sudan	*Rhynchosia memnonia*
NCPPB1833^{Pt}	*Xanthomonas axonopodis* pv. *dieffenbachiae*	Brazil	*Anthurium* sp.
NCPPB2058^{Pt}	*Xanthomonas axonopodis* pv. *vignaeradiatae*	Sudan	*Vigna radiata*
NCPPB2066^{Pt}	*Xanthomonas axonopodis* pv. *phyllanthi*	Sudan	*Phyllanthus niruri*
NCPPB2228^{Pt}	*Xanthomonas axonopodis* pv. *biophyti*	India	*Biophytum sensitivum*
NCPPB2230^{Pt}	*Xanthomonas axonopodis* pv. *fascicularis*	India	*Corchorus fascicularis*

NCPPB2368[Pt]	*Xanthomonas axonopodis* pv. *pedalii*	India	*Pedalium mure*
NCPPB2972[Pt]	*Xanthomonas axonopodis* pv. *betlicola*	India	*Piper betle*
NCPPB2973[Pt]	*Xanthomonas axonopodis* pv. *cassia*	India	*Cassia tora*
NCPPB3086[Pt]	*Xanthomonas axonopodis* pv. *desmodiilaxiflori*	India	*Desmodium laxiflorum*
NCPPB3092[Pt]	*Xanthomonas axonopodis* pv. *clitoriae*	India	*Clitoria biflora*
UFLA1301	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1302	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1303	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1304	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1305	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1306	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1307	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1308	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1309	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1310	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1311	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1312	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1313	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1314	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1315	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1316	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1317	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1318	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1319	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1320	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1321	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1322	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1323	unknown	Brazil	*Toona ciliata* var. *australis*

UFLA1324	unknown	Brazil	*Toona ciliata* var. *australis*
UFLA1325	unknown	Brazil	*Toona ciliata* var. *australis*

[Pt]Pathotype strain. The nomenclature of Vauterin et al. (22) is used.

Table 2. Lengths of the fragments of the gene with the start and end point of each gene, in base pairs.

Gene	begin (bp)	end (bp)	length (bp)
dnaK	89	851	762
fyuA	90	612	522
gyrB	91	778	687
rpoD	90	783	693

Table 3. Results of biochemical tests. *Pseudomonas syringae* pv. *tomato*, *Xanthomonas axonopodis* pv. *malvacearum*, *Pectobacterium carotovorum* subsp. *carotovorum*, *Curtobacterium flaccumfaciens* pv. *flacumfaciens*, *Ralstonia solanacearum*, UFLA1301, UFLA1313, and UFLA1325- Cedar isolates representing the 25 isolates. + = positive, - = negative.

Strains	*P. syringae* pv. *tomato*	*P. carotovorum* subsp. *carotovorum*	*C. flaccumfaciens* pv. *flacumfaciens*	*R. solanacearum*	*X. axonopodis* pv. *malvacearum*	UFLA1301	UFLA1313	UFLA1325
Gram reaction	-	-	+	-	-	-	-	-
Grows anaerobically	-	+	-	-	-	-	-	-
Fluorescent pigment on KB	+	-	-	-	-	-	-	-
Colonies yellow on NA	-	-	+	-	+	-	-	-

Urease	-	-	-	+	-	-	-	-
Starch hydrolysis	-	-	+	-	+	+	+	+
Esculin hydrolysis	+	+	+	-	+	+	+	+
Mucoid growth on YDC	-	-	-	+	+	+	+	+
Glucose utilization	+	+	+	+	+	+	+	+
Fermentation of glucose	+	+	+	+	+	+	+	+
Catalase test	+	+	+	+	+	+	+	+
Utilization of arginine	+	-	-	-	-	-	-	-
Utilization of asparagine	+	+	-	+	-	-	-	-

Table 4. Fatty acids of the bacterial isolates pathogenic to *Toona ciliata* var. *australis* and of the *Xanthomonas axonopodis* pathovars.

Features	UFLA1308	UFLA1310	UFLA1312	NCPPB1335	NCPPB2972	NCPPB2228	NCPPB2973	NCPPB3092
10:00	0.57 ± 0.04	0.55 ± 0.05	0.93 ± 0.51	0.65 ± 0.02	1.27 ± 0.28	0.91 ± 0.08	1.10 ± 0.48	0.73 ± 0.04
11:0 iso	3.89 ± 0.33	3.84 ± 0.40	3.99 ± 0.17	4.32 ± 0.33	4.79 ± 0.92	4.16 ± 0.42	3.34 ± 0.10	4.84 ± 0.34
10:0 3OH						0.36 ± 0.03	0.30 ± 0.03	
11:0 iso 3OH	1.46 ± 0.32	1.54 ± 0.24	1.63 ± 0.10	1.74 ± 0.12	0.37 ± 0.26	1.74 ± 0.24	1.35 ± 0.09	2.09 ± 0.11
11:0 3OH	0.38 ± 0.07	0.36 ± 0.07	0.42 ± 0.08			1.52 ± 0.08		
13:0 iso	0.50 ± 0.14	0.44 ± 0.11	0.51 ± 0.04					
12:0 iso 3OH	1.67 ± 0.24	1.83 ± 0.13	1.97 ± 0.27					
12:0 3OH				2.23 ± 0.14	3.69 ± 0.21	2.33 ± 0.18	2.62 ± 0.45	2.29 ± 0.11
14:0 iso	0.69 ± 0.23	0.63 ± 0.14	0.67 ± 0.22	0.65 ± 0.06	0.40 ± 0.27	0.61 ± 0.06	0.33 ± 0.03	
14:00	1.52 ± 0.09	1.53 ± 0.12	1.63 ± 0.14	1.52 ± 0.12	2.01 ± 0.36	1.95 ± 0.15	1.12 ± 0.12	1.45 ± 0.18
13:0 iso 3OH	3.57 ± 0.70	3.67 ± 0.39	3.95 ± 0.45	3.98 ± 0.44	4.02 ± 0.70	3.36 ± 0.39	2.84 ± 0.52	4.41 ± 0.30
13:0 2OH	0.32 ± 0.07	0.28 ± 0.06	0.35 ± 0.06				0.36 ± 0.08	
15:1 iso F	0.49 ± 0.05	0.42 ± 0.07	0.52 ± 0.13			0.37 ± 0.29		0.34 ± 0.24
15:0 iso	35.78 ± 2.45	35.53 ± 1.90	35.18 ± 1.27	36.12 ± 2.97	28.06 ± 1.87	25.13 ± 2.00	23.98 ± 2.63	33.61 ± 3.12
15:0 anteiso	11.21 ± 0.18	10.89 ± 0.32	10.40 ± 0.35	11.24 ± 0.76	10.01 ± 0.64	10.66 ± 0.95	13.61 ± 1.17	9.29 ± 0.81
15:1 w8c						0.75 ± 0.08		
15:1 w6c	2.13 ± 0.37	1.83 ± 0.31	1.65 ± 0.36		0.52 ± 0.40	1.76 ± 0.13	0.36 ± 0.05	0.51 ± 0.06
16:0 iso	1.82 ± 0.26	1.75 ± 0.15	1.77 ± 0.17	2.33 ± 0.36	2.11 ± 0.56	1.70 ± 0.16	2.45 ± 0.25	1.47 ± 0.19
16:1 w9c	1.18 ± 0.12	1.21 ± 0.15	1.36 ± 0.15	1.19 ± 0.16	1.88 ± 0.35	2.01 ± 0.12	1.71 ± 0.15	2.22 ± 0.17
16:00	2.77 ± 0.71	3.01 ± 0.54	3.27 ± 0.70	3.07 ± 0.80	6.26 ± 1.36	4.70 ± 0.47	4.85 ± 0.40	4.54 ± 0.52
17:0 iso	4.26 ± 1.14	4.63 ± 0.98	4.57 ± 0.82	6.47 ± 0.90	5.46 ± 0.43	1.67 ± 0.18	7.77 ± 0.78	5.03 ± 0.44
17:0 anteiso	0.39 ± 0.11	0.40 ± 0.08	0.40 ± 0.08	0.57 ± 0.12	0.47 ± 0.33		0.76 ± 0.08	0.59 ± 0.10
17:1 w8c	1.98 ± 0.51	1.86 ± 0.35	2.06 ± 0.38	2.10 ± 0.59	1.49 ± 0.14	0.46 ± 0.17	1.67 ± 0.31	0.40 ± 0.31
17:1 w6c	0.71 ± 0.12	0.67 ± 0.05	0.74 ± 0.16	0.54 ± 0.10		0.38 ± 0.05	0.41 ± 0.04	
18:1 w9c				0.60 ± 0.13			0.52 ± 0.07	
17:0 iso 3OH				0.30 ± 0.03				
Summed Feature 3 (16:1 w7c/16:1 w6c)	15.47 ± 0.32	15.65 ± 0.59	15.66 ± 1.48	19.35 ± 0.15	33.31 ± 1.59	21.08 ± 1.02	18.71 ± 0.04	21.24 ± 1.11
Summed Feature 4 (17:1 iso I/anteiso B)	0.38 ± 0.05	0.41 ± 0.09	0.39 ± 0.03					

Summed Feature 8 (18:1 w7c)	0.25 ± 0.07	0.30 ± 0.06		0.83 ± 0.08	1.88 ± 0.17		0.79 ± 0.10	
Summed Feature 9 (17:1 iso w9c)	5.35 ± 0.37	5.49 ± 0.15	5.14 ± 0.19	4.99 ± 0.19	3.71 ± 0.55	2.87 ± 0.38	5.85 ± 0.60	7.49 ± 0.44

Table 4. Continuation…

Features	NCPPB1786	NCPPB637	NCPPB481	NCPPB577	NCPPB3086	NCPPB885	NCPPB1833	NCPPB578
10:00	0.91 ± 0.03	2.07 ± 0.87	0.92 ± 0.07	0.93 ± 0.56	0.95 ± 0.10	1.23 ± 0.34	1.13 ± 0.04	0.84 ± 0.12
11:0 iso	4.22 ± 0.20	1.99 ± 0.11	3.64 ± 0.15	3.61 ± 0.47	3.85 ± 0.14	2.52 ± 0.25	4.19 ± 0.40	3.54 ± 0.37
11:0 anteiso						0.39 ± 0.01		
10:0 2OH		0.44 ± 0.07						
10:0 3OH	0.42 ± 0.08		0.35 ± 0.03	0.41 ± 0.03	0.36 ± 0.03	0.33 ± 0.00	0.39 ± 0.02	0.30 ± 0.03
11:0 iso 3OH	2.03 ± 0.45	0.53 ± 0.06	1.43 ± 0.14	2.00 ± 0.32	1.46 ± 0.05	3.91 ± 0.03	1.58 ± 0.16	1.60 ± 0.20
11:0 3OH	0.49 ± 0.07			0.38 ± 0.08		0.29 ± 0.06	0.54 ± 0.05	0.42 ± 0.09
13:0 iso	0.3 ± 0.04		0.68 ± 0.03					
12:0 3OH	3.17 ± 0.42	5.74 ± 0.49	2.81 ± 0.23	3.79 ± 0.36	2.80 ± 0.35	2.63 ± 0.12	3.55 ± 0.15	2.84 ± 0.35
14:0 iso	0.45 ± 0.05		1.04 ± 0.09	0.41 ± 0.05	0.47 ± 0.04	0.38 ± 0.01		
14:1 w5c			0.44 ± 0.06					
14:00	1.40 ± 0.08	2.77 ± 0.40	4.89 ± 0.72	1.34 ± 0.16	1.77 ± 0.18	1.22 ± 0.07	1.43 ± 0.11	1.32 ± 0.17
13:0 iso 3OH	4.06 ± 0.79	1.73 ± 0.12	3.22 ± 0.27	4.40 ± 0.55	2.98 ± 0.37	2.01 ± 0.08	3.12 ± 0.45	3.59 ± 0.42
13:0 2OH	0.33 ± 0.07					0.77 ± 0.07		
15:1 iso F			0.58 ± 0.07					
15:0 iso	25.64 ± 1.86	14.38 ± 0.74	37.25 ± 2.49	25.76 ± 1.33	33.48 ± 1.71	13.40 ± 1.28	24.55 ± 2.91	27.31 ± 0.95
15:0 anteiso	8.54 ± 0.38	6.44 ± 1.05	7.97 ± 0.52	9.84 ± 0.46	10.17 ± 0.49	23.75 ± 0.52	10.43 ± 0.58	8.75 ± 0.29
15:1 w6c	0.88 ± 0.12		1.07 ± 0.14		0.74 ± 0.03		1.19 ± 0.14	0.68 ± 0.04
16:0 iso	2.35 ± 0.33	2.66 ± 0.42	1.23 ± 0.11	2.27 ± 0.23	2.45 ± 0.42	3.12 ± 0.17	2.52 ± 0.33	1.91 ± 0.14
16:1 w9c	1.99 ± 0.31		1.94 ± 0.19	1.59 ± 0.02		1.96 ± 0.10	1.12 ± 0.05	1.13 ± 0.03
16:1 w5c			0.34 ± 0.06					
16:00	5.56 ± 0.92	13.50 ± 1.33	2.60 ± 0.60	5.28 ± 0.81	3.09 ± 0.62	8.07 ± 0.36	5.78 ± 0.86	4.95 ± 0.30
17:0 iso	6.28 ± 0.18	7.70 ± 0.84	9.35 ± 0.61	5.45 ± 0.62	7.33 ± 0.89	6.68 ± 0.27	7.41 ± 0.67	6.41 ± 0.52
17:0 anteiso	2.59 ± 0.32	0.82 ± 0.24	8.75 ± 0.29		1.58 ± 0.16	0.59 ± 0.07	0.60 ± 0.09	0.62 ± 0.07
17:1 w8c	2.28 ± 0.46	2.57 ± 0.68	2.30 ± 0.66	1.75 ± 0.59	0.73 ± 0.16	1.73 ± 0.28	1.09 ± 0.18	0.79 ± 0.07

17:1 w6c	0.47 ± 0.06	0.48 ± 0.32	0.52 ± 0.35	0.34 ± 0.24		0.45 ± 0.03	0.26 ± 0.01	
17:00	0.30 ± 0.07							
18:1 w9c	0.88 ± 0.11	0.36 ± 0.25	0.48 ± 0.06		0.54 ± 0.37	0.43 ± 0.04	0.62 ± 0.13	0.38 ± 0.06
17:0 iso 3OH						0.25 ± 0.02		
Summed Feature 3 (16:1 w7c/16:1 w6c)	18.97 ± 0.38	19.58 ± 0.70	18.49 ± 0.78	19.14 ± 0.98	17.71 ± 0.56	18.30 ± 0.16	20.03 ± 0.34	19.65 ± 0.40
Summed Feature 4 (17:1 iso I/anteiso B)			0.36 ± 0.24				0.32 ± 0.01	0.33 ± 0.03
Summed Feature 8 (18:1 w7c)	1.04 ± 0.13	0.51 ± 0.34	0.79 ± 0.06		0.63 ± 0.43	0.49 ± 0.05	0.68 ± 0.12	0.47 ± 0.07
Summed Feature 9 (17:1 iso w9c)	3.31 ± 0.10	5.20 ± 0.34	7.18 ± 0.47	4.06 ± 0.09	8.10 ± 0.33	5.06 ± 0.44	5.22 ± 0.36	5.93 ± 0.31

Table 4. Continuation…

Features	**NCPPB2230**	**NCPPB536**	**NCPPB993**	**NCPPB971**	**NCPPB1148**	**NCPPB994**	**NCPPB1337**	**NCPPB840**	**NCPPB2368**
10:00	1.26 ± 0.58	0.71 ± 0.10	0.89 ± 0.08	0.79 ± 0.02	0.63 ± 0.06	0.80 ± 0.13	1.02 ± 0.04	0.96 ± 0.03	0.91 ± 0.13
11:0 iso	4.72 ± 0.40	3.92 ± 0.27	4.31 ± 0.11	4.05 ± 0.24	3.39 ± 0.26	4.50 ± 0.26	4.64 ± 0.23	2.94 ± 0.17	4.97 ± 0.37
11:0 anteiso								0.39 ± 0.05	
10:0 3OH			0.30 ± 0.02	0.35 ± 0.02	0.28 ± 0.01	0.34 ± 0.05	0.40 ± 0.04	0.30 ± 0.03	0.34 ± 0.05
11:0 iso 3OH	1.74 ± 0.15	1.55 ± 0.12	1.61 ± 0.08	1.60 ± 0.15	1.71 ± 0.12	1.86 ± 0.17	1.60 ± 0.03	1.18 ± 0.04	2.06 ± 0.24
11:0 3OH			0.36 ± 0.06					0.30 ± 0.07	
13:0 iso			0.27 ± 0.07	0.28 ± 0.05	0.27 ± 0.04	0.31 ± 0.08	0.25 ± 0.05		0.28 ± 0.07
12:0 iso 3OH								0.28 ± 0.02	0.26 ± 0.07
12:0 3OH	2.73 ± 0.29	2.10 ± 0.15	2.79 ± 0.27	2.81 ± 0.06	2.43 ± 0.17	2.44 ± 0.34	3.60 ± 0.36	2.76 ± 0.17	2.73 ± 0.25
14:0 iso	0.35 ± 0.23		0.54 ± 0.04	0.33 ± 0.03	0.45 ± 0.06	0.51 ± 0.05	0.35 ± 0.04	0.41 ± 0.04	0.59 ± 0.03
14:00	1.67 ± 0.24	0.96 ± 0.11	1.46 ± 0.08	1.37 ± 0.13	1.30 ± 0.01	1.47 ± 0.06	1.64 ± 0.16	1.05 ± 0.06	1.23 ± 0.05
13:0 iso 3OH	4.01 ± 0.34	3.65 ± 0.18	3.57 ± 0.19	3.50 ± 0.36	3.69 ± 0.12	4.08 ± 0.36	3.79 ± 0.40	2.47 ± 0.20	3.95 ± 0.16
13:0 2OH		0.66 ± 0.13	0.29 ± 0.04	0.28 ± 0.03	0.32 ± 0.02	0.30 ± 0.06	0.42 ± 0.04	0.81 ± 0.06	0.28 ± 0.05
15:1 iso F						0.32 ± 0.05			
15:0 iso	30.64 ± 3.30	23.14 ± 1.32	28.30 ± 1.53	27.75 ± 2.07	30.22 ± 1.04	30.78 ± 2.68		15.15 ± 1.52	29.13 ± 2.73
15:0 anteiso	10.07 ± 0.66	17.90 ± 2.33	9.83 ± 0.55	9.36 ± 0.31	10.43 ± 0.99	8.62 ± 0.56	10.83 ± 0.30	24.00 ± 0.65	8.83 ± 0.53
15:1 w6c	0.96 ± 0.10		0.93 ± 0.12	0.28 ± 0.05	0.31 ± 0.07	0.33 ± 0.03	0.51 ± 0.08		0.47 ± 0.06

16:0 iso	1.71 ± 0.28	2.57 ± 0.24	2.54 ± 0.13	1.90 ± 0.26	2.31 ± 0.49	2.14 ± 0.24	1.89 ± 0.36	3.32 ± 0.23	3.14 ± 0.23
16:1 w9c	2.23 ± 0.05	1.68 ± 0.12	1.92 ± 0.18	2.07 ± 0.10	1.78 ± 0.07	2.26 ± 0.15	1.17 ± 0.09	1.91 ± 0.12	1.64 ± 0.05
16:00	5.25 ± 0.63	3.66 ± 0.43	4.92 ± 0.42	5.97 ± 0.64	4.56 ± 0.50	4.45 ± 0.33	4.74 ± 0.57	5.90 ± 0.49	3.73 ± 0.26
17:0 iso	5.89 ± 0.37	6.38 ± 0.51	6.82 ± 0.25	6.86 ± 0.47	9.32 ± 0.68	4.97 ± 1.11	8.57 ± 0.36	5.76 ± 0.59	8.12 ± 0.67
17:0 anteiso	0.43 ± 0.08	0.69 ± 0.09	2.58 ± 0.29	0.59 ± 0.10	0.61 ± 0.09	0.56 ± 0.23	0.63 ± 0.13	0.42 ± 0.21	0.86 ± 0.17
17:1 w8c	1.01 ± 0.34	0.90 ± 0.27	2.18 ± 0.43	1.07 ± 0.21	0.94 ± 0.16	2.80 ± 0.48	1.20 ± 0.33	0.38 ± 0.27	1.69 ± 0.35
17:1 w6c			0.52 ± 0.03	0.31 ± 0.03		1.11 ± 0.23			
18:1 w9c	0.46 ± 0.12		0.78 ± 0.11	0.44 ± 0.08	0.65 ± 0.07		0.69 ± 0.10		0.48 ± 0.05
Summed Feature 3 (16:1 w7c/16:1 w6c)	19.71 ± 0.11	20.47 ± 0.19	17.27 ± 0.12	17.82 ± 0.20	18.31 ± 0.51	20.59 ± 0.57	19.91 ± 0.21	17.27 ± 1.62	17.00 ± 1.87
Summed Feature 4 (17:1 iso I/anteiso B)				0.43 ± 0.03	0.44 ± 0.03	0.35 ± 0.23	0.47 ± 0.02	0.52 ± 0.35	
Summed Feature 8 (18:1 w7c)	0.47 ± 0.16	0.65 ± 0.20	0.99 ± 0.13	0.63 ± 0.13	0.85 ± 0.13		0.73 ± 0.08		0.34 ± 0.23
Summed Feature 9 (17:1 iso w9c)	5.48 ± 0.43	5.82 ± 0.14	4.15 ± 0.20	6.63 ± 0.32	7.70 ± 0.26	6.49 ± 0.40	7.35 ± 0.21	9.83 ± 0.33	6.85 ± 1.04

Table 4. Continuation…

Features	NCPPB2066	NCPPB761	NCPPB581	NCPPB466	NCPPB1827	NCPPB1063	NCPPB582	NCPPB584	NCPPB2058
10:00	0.97 ± 0.08	1.17 ± 0.91	1.07 ± 0.11	0.96 ± 0.10	0.70 ± 0.14	0.85 ± 0.06	0.84 ± 0.12	1.05 ± 0.09	1.00 ± 0.16
11:0 iso	6.00 ± 0.63	3.48 ± 0.29	5.03 ± 0.12	6.30 ± 0.14	4.12 ± 0.47	4.80 ± 0.34	3.97 ± 0.24	2.94 ± 0.10	5.83 ± 0.61
11:0 anteiso								0.26 ± 0.02	
10:0 2OH								0.28 ± 0.03	
10:0 3OH	0.33 ± 0.03		0.40 ± 0.05	0.39 ± 0.02	0.32 ± 0.06	0.31 ± 0.03	0.32 ± 0.03	0.41 ± 0.03	
11:0 iso 3OH	1.96 ± 0.22	1.70 ± 0.12	2.12 ± 0.11	2.92 ± 0.13	2.09 ± 0.22	1.61 ± 0.14	1.86 ± 0.11	1.29 ± 0.09	2.07 ± 0.20
11:0 3OH		0.60 ± 0.07			0.31 ± 0.09				
13:0 iso				0.69 ± 0.05		0.38 ± 0.06			0.35 ± 0.24
12:0 iso 3OH			2.99 ± 0.04					0.29 ± 0.05	
12:0 3OH	3.08 ± 0.36	2.64 ± 0.34		3.29 ± 0.30	2.35 ± 0.29	2.70 ± 0.09	2.32 ± 0.27	2.99 ± 0.25	2.67 ± 0.41
14:0 iso	0.28 ± 0.04		0.30 ± 0.03			0.47 ± 0.02	0.39 ± 0.06	0.81 ± 0.10	
14:00	1.10 ± 0.06	1.57 ± 0.18	1.12 ± 0.04	1.01 ± 0.12	1.15 ± 0.34	1.79 ± 0.34	1.25 ± 0.24	1.89 ± 0.18	1.72 ± 0.47
13:0 iso 3OH	4.30 ± 0.55	3.63 ± 0.22	4.01 ± 0.19	6.14 ± 0.27	4.30 ± 0.37	3.60 ± 0.42	3.72 ± 0.19	2.79 ± 0.14	4.68 ± 0.66

13:0 2OH			0.26 ± 0.04			0.28 ± 0.04		0.64 ± 0.06	
15:1 iso F							0.29 ± 0.05		
15:0 iso	26.96 ± 1.63	28.83 ± 2.65	25.25 ± 1.88	31.96 ± 1.34	28.15 ± 1.45	31.14 ± 1.68	27.20 ± 2.43	21.71 ± 1.08	34.52 ± 1.77
15:0 anteiso	6.44 ± 0.49	10.55 ± 0.68	7.32 ± 0.88	7.40 ± 0.31	11.51 ± 0.85	9.55 ± 0.30	9.43 ± 0.58	16.38 ± 1.06	11.26 ± 1.33
15:1 w8c									
15:1 w6c	0.30 ± 0.04	1.24 ± 0.44	0.26 ± 0.02	0.56 ± 0.00	0.74 ± 0.11	0.52 ± 0.03	0.45 ± 0.03	0.37 ± 0.06	0.96 ± 0.08
16:0 iso	2.15 ± 0.21	1.19 ± 0.20	2.45 ± 0.22	1.10 ± 0.18	1.65 ± 0.33	2.26 ± 0.29	2.38 ± 0.60	3.50 ± 0.11	0.86 ± 0.19
16:1 w9c	1.59 ± 0.08	1.63 ± 0.18	2.00 ± 0.14	0.78 ± 0.53	1.75 ± 0.21	1.44 ± 0.03	2.50 ± 0.05	2.49 ± 0.16	0.78 ± 0.52
16:1 w5c									
16:00	4.36 ± 0.49	3.38 ± 0.55	4.69 ± 0.29	1.77 ± 0.10	4.33 ± 0.98	4.04 ± 0.56	4.77 ± 0.26	6.15 ± 0.34	2.87 ± 0.56
17:0 iso	5.88 ± 0.51	7.76 ± 0.92	4.64 ± 0.57	5.11 ± 0.82					
17:0 anteiso	0.47 ± 0.07	0.63 ± 0.15	0.80 ± 0.10	0.39 ± 0.27					
17:1 w8c	0.98 ± 0.19	1.67 ± 0.37	0.72 ± 0.22	0.77 ± 0.20					
17:1 w6c	0.34 ± 0.02								
18:1 w9c	0.37 ± 0.05	0.50 ± 0.34	0.52 ± 0.09						
Summed Feature 3 (16:1 w7c/16:1 w6c)	18.94 ± 1.25	19.82 ± 0.96	22.14 ± 0.78	16.33 ± 2.75					
Summed Feature 4 (17:1 iso I/anteiso B)	0.36 ± 0.02								
Summed Feature 8 (18:1 w7c)	0.47 ± 0.06	0.53 ± 0.36	0.60 ± 0.09						
Summed Feature 9 (17:1 iso w9c)	5.38 ± 0.51	6.18 ± 0.55	3.47 ± 0.34	6.05 ± 1.25					

Figure 1. Symptoms of leaf spot disease on Australian cedar and bacterial colonies. **A**, **B** - Leaf spot symptoms on Australian cedar nurseries in Brazil. **C** - Bacterial colonies from isolation. **D** - Symptoms of bacterial leaf spot 15 days after the inoculation.

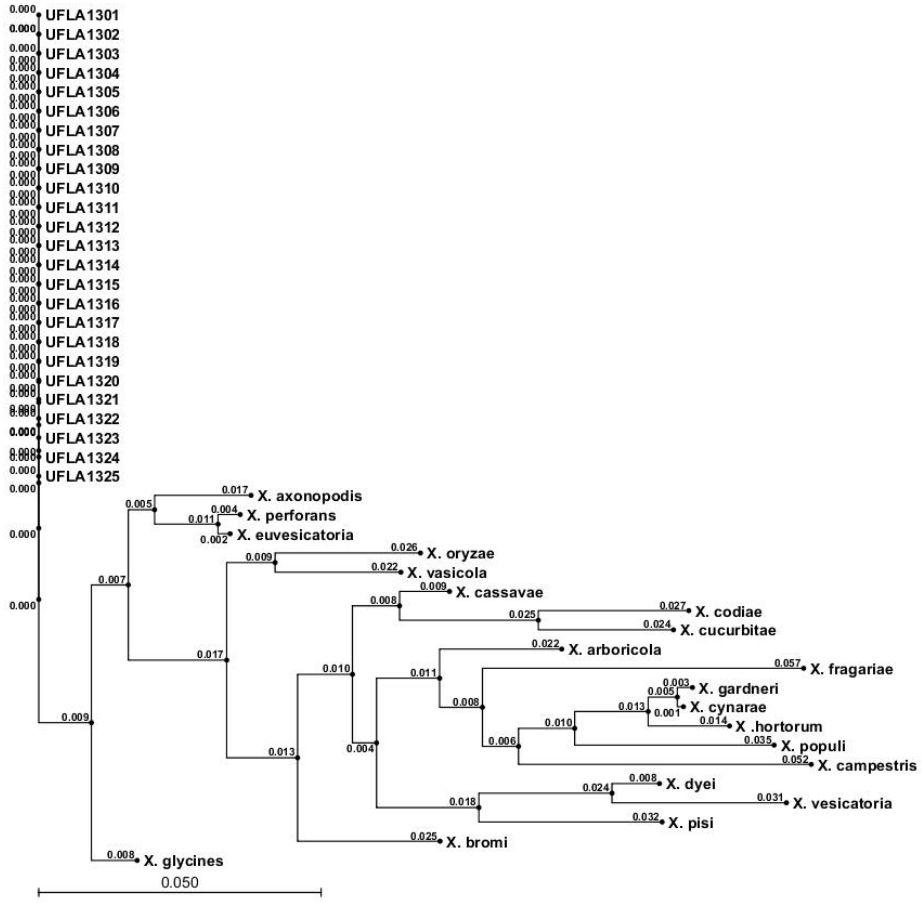

Figure 2. Phylogenetic tree using the Neighbor Joining method of concatenated partial sequences of genes *dnaK*, *fyuA*, *gyrB* and *rpoD*, based on 20 type strains of species of *Xanthomonas* and 25 isolates pathogenic to *Toona ciliata* var. *australis*.

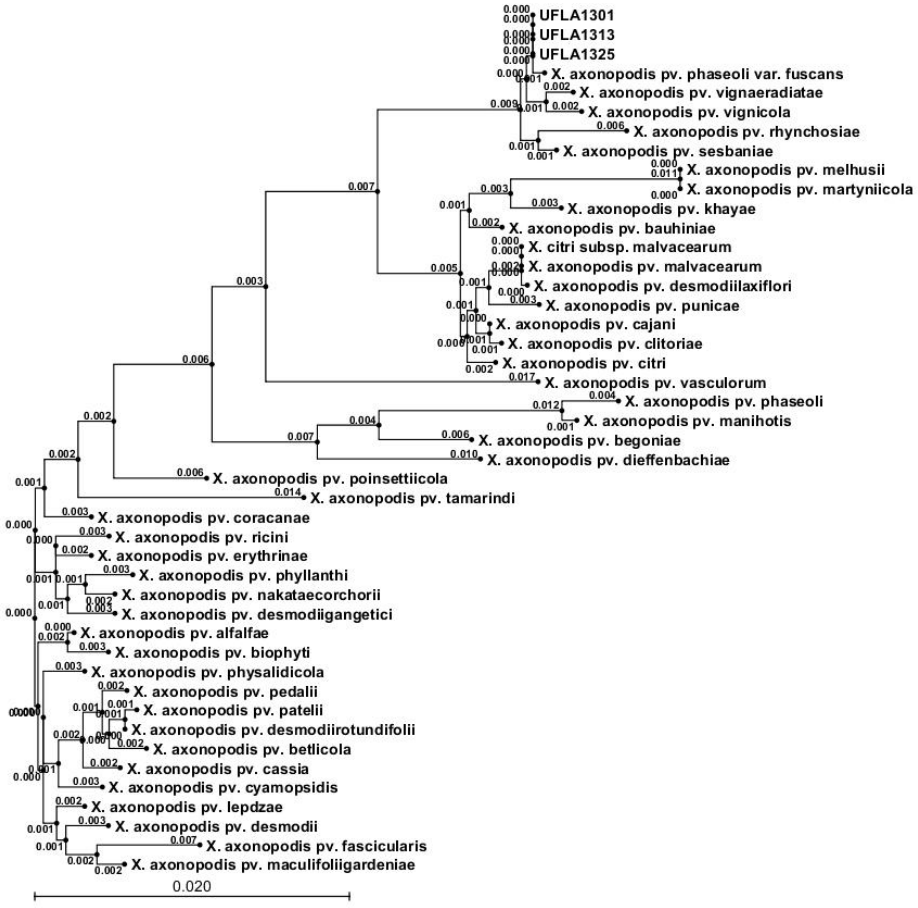

Figure 3. Phylogenetic tree using the Neighbor Joining method of concatenated partial sequences of genes *dnaK*, *fyuA*, *gyrB* and *rpoD*, based on 42 pathovars of *Xanthomonas axonopodis*, and 3 isolates pathogenic to *Toona ciliata* var. *australis* representing the 25 isolates.

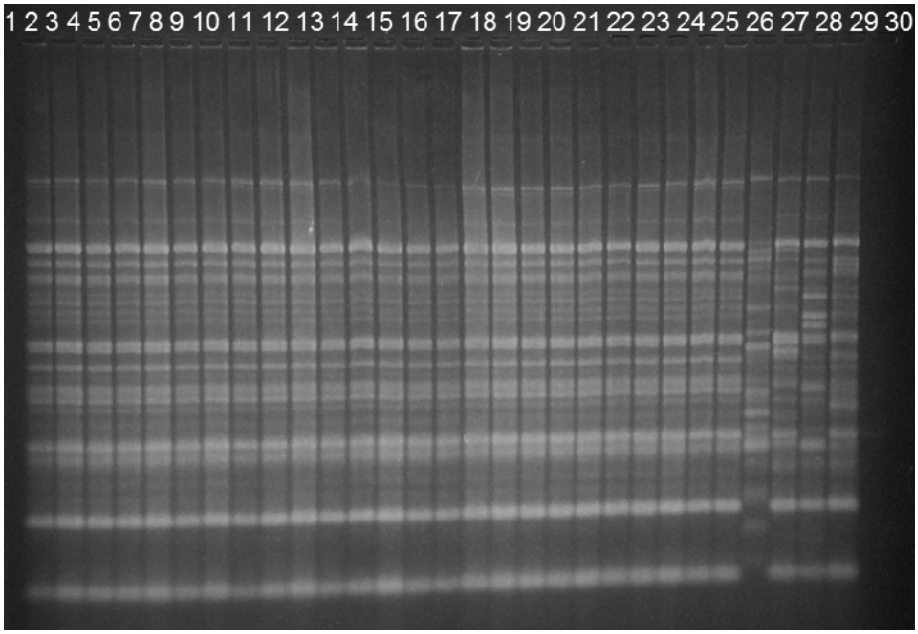

Figure 4. REP-PCR electrophoretic profile of 25 isolates pathogenic to *Toona ciliata* var. *australis* and three pathovars of *X. axonopodis* belonging to the group 9.6 *sensu* Rademaker et al. (17) and *X. axonopodis* pv. *dieffenbachiae*. **1-25 -** Isolates pathogenic to *Toona ciliata* var. *australis*. **26 -** *X. axonopodis* pv. *dieffenbachiae*. **27 -** *X. axonopodis* pv. *rhynchosiae*. **28 -** *X. axonopodis* pv. *sesbaniae*. **29 -** *X. axonopodis* pv. *vignaeradiatae*. **30 -** Negative control.

ARTIGO 2

Novel disease of radicchio and taxonomy of *Xanthomonas campestris* pv. *vitians* strains related to *Xanthomonas hortorum*

Preparado de acordo com as normas da Systematic and Applied Microbiology
(Versão preliminar)

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ABSTRACT

In the beginning of 2002 a new leaf spot disease of radicchio (*Cichorium intybus*) was observed in Monterey County, California. Bacteria forming yellow mucoid colonies were isolated from surface disinfested symptomatic tissue that was macerated and streaked onto sucrose peptone agar medium. Bacteria were Gram negative, did not fluoresce on King's Medium B and used esculin as a carbon source. Nine isolates from symptomatic radicchio had the same DNA fragment-banding pattern generated by repetitive extragenic palindromic sequence polymerase chain reactions (rep-PCR) using the BOXA1R primer. Amplicons for multilocus sequence typing (MLST) were generated using a modification of the scheme developed by Young and colleagues [15] and sequenced by a commercial laboratory. Sequences from the radicchio isolates were compared to sequences of *X. hortorum* pathotypes, *X. campestris* pv. *vitians* and with sequences of type and pathotypes strains available in the Plant Associated and Environmental Microbes Database. The B162 primers and amplification protocol were used to amplify the DNA of the isolates. The genetic distance between the isolates from radicchio and pathotypes of *X. hortorum* were 0.03 or less and MLST analysis indicated that radicchio isolates were members of the species *X. hortorum*. The genomic DNA of isolates pathogenic to radicchio *X. campestris* pv. *vitians* and *X. hortorum* pv. *taraxaci* was amplified by B162 primers. To complete Koch's postulates, freshly grown cultures were suspended in phosphate buffer and inoculated in radicchio plants. The plants treated with isolates from radicchio developed leaf spots similar to those observed in the field. The bacteria isolated from symptomatic tissue on inoculated plants were identical to the original strains. Pathogenicity test with all *X. hortorum* pathotypes were also performed. The range of host was evaluated twice on lettuce cv. Vista Verde plantlets. The isolates from radicchio, *X.*

campestris pv. *vitians*, X. *hortorum* pv. *hederae*, X. *hortorum* pv. *taraxaci* and X. *hortorum* pv. *pelargonii* showed symptoms. These data demonstrate that *X. campestris* pv *vitians* is a novel pathovar of *X. hortorum* which we propose to be called *X. hortorum* pv. *vitians*. Included in this novel pathovar are isolates that cause bacterial leaf spot on radicchio. This is the first report of *X. hortorum* causing a leaf spot disease on radicchio.

Introduction

Reclassification of the genus *Xanthomonas* significantly changed the taxonomy of members of *X. campestris* plant pathogenic species [11]. Prior to 1995, *X. campestris* consisted of more than 140 distinct pathovars (members of species that differ for pathogenicity) [2]. The 62 *X. campestris* pathovars examined by Vauterin and colleagues were allocated to 16 genomospecies. Reclassification of *X. campestris* resulted in: 1) a restricted list of pathovars within *X. campestris*; 2) elevation of pathovars or generation of novel species (e.g., *X. hortorum*); 3) transfer of strains from *X. campestris* to other species (e.g., *X. axonopodis*); and a group of *X. campestris* pathovars of ambiguous classification because they were not evaluated.

The reclassification introduced a particular ambiguity in the taxonomy of *Xanthomonas campestris* pv. *vitians,* the causal agent of bacterial leaf spot of lettuce world-wide. The reclassification resulted in the transfer of the pathotype strain of *X. campestris* pv. *vitians* to *X. axonopodis* as *X. axonopodis* pv. *vitians* [11]. However several laboratories documented the lack of pathogenicity of the pathotype strain (NCPPB 976) [9, 8], thus the status of this pathovar is ambiguous because no pathogenic strains were allocated to it [16]. A pathogenic strain of *X. campestris* pv. *vitians* (NCPPB 2248) was allocated to *X. hortorum*, however, the name proposed by Vauterin and colleagues [11], *X. hortorum* pv. *vitians,* is invalid because no pathotype was designated for the pathovar [14].

Currently *X. hortorum* consists of four legitimately named pathovars *X. hortorum* pv. *carotae*, *X. hortorum* pv. *hederae*, *X. hortorum* pv. *pelargonii* and *X. hortorum* pv. *taraxaci.* Among these four pathovars only *X. hortorum* pv. *taraxaci* was evaluated for pathogenicity on hosts other than the host from which it was isolated. *X. hortorum* pv. *taraxaci* was evaluated on lettuce because the genera *Lactuca* and *Taxacum* are in the Asteraceae family. *Xanthomonas*

hortorum pv. *taraxaci* did not cause symptoms on the three lettuce (*L. sativa*) types tested or prickly lettuce (*L. scariola*). Thus, although it is unlikely that the *X. campestris* pv. *vitians* strains transferred to *X. hortorum* belong to *X. hortorum* pv. *taraxaci*, there are no data to determine if this pathogen represents novel pathovar within this species or if it is a synonym of a previously validly named pathovar.

A novel disease on an additional member of the Asteraceae was observed in the Salinas Valley of California in 2002. Preliminary unpublished data indicated that bacterial leaf spot of radicchio (*Cichorium intybus*) may also be caused by *X. hortorum.* This disease has not been reported in the literature previously.

Radicchio is ranked among the most important crops in Monterey County, California, United States, moving around $ 19,531,000.00 and is an excellent source of antioxidants [3].

The disease incidence in the first year resulted in up to 10% unharvested radicchio because of cap leaf infections or reduced head size if outer wrapper leaves were all removed; outbreaks in subsequent seasons were more limited.

In this manuscript we investigate the taxonomy of the causal agent from this novel disease along with the taxonomy of *X. campestris* pv. *vitians* as a member of *X. hortorum.* We used Multilocus Sequence Typing to confirm placement of the pathogens in *X. hortorum* and to develop hypotheses about what constitutes a pathovar. Pathogenicity test with all *X. hortorum* pathotypes demonstrates that *X. campestris* pv *vitians* is a novel pathovar of *X. hortorum* which we propose to be called *X. hortorum* pv. *vitians* and may include the isolates that cause bacterial leaf spot on radicchio in this novel pathovar.

Materials and Methods

Disease and Bacterial isolation

Beginning in 2002 a leaf spot disease of radicchio (*Cichorium intybus*) was observed in Monterey County, California. The disease began as small lesions and in some cases coalesced into larger, irregularly shaped spots. Lesions were maroon to dark brown in color; in some cases the margins of brown lesions became dark maroon with aging. Each leaf spot was observable from both adaxial and abaxial leaf surfaces. Disease incidence in the first year resulted in up to 10% unharvestable radicchio; outbreaks in subsequent seasons were more limited. Bacteria forming yellow mucoid colonies in NA medium were isolated from surface disinfested symptomatic tissue that was macerated and streaked onto sucrose peptone agar medium.

Bacterial strains and growth conditions

Bacteria used in these experiments are listed in Table 1. Pathotype strains and the strain NCPPB 2248 were received from the National Collection of Plant Pathogenic Bacteria (NCPPB), England. The bacteria were stored at -80°C in a solution of 50% glycerol and 50% nutrient broth (NB Difco Laboratories,USA) and were routinely cultured on Nutrient Agar (NA Difco Laboratories, USA). Bacteria were isolated from plants of radicchio showing bacterial leaf spot symptoms. *Xanthomonas campestris* pv. *vitians* strains were obtained from Salinas, California; Australia; Hawaii; United Kingdom; USA and Canada.

Pathogenicity test on radicchio plants

Two isolates from radicchio (BS0953, BS0954) and the pathotype strains of *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci*, *X. hortorum* pv. *carotae*, *X. hortorum* pv. *pelargonii* and *X. campestris* pv. *vitians* NCPPB 2248 were removed from the -80C freezer on Nutrient Agar and incubated at 27 °C

for two days. Single colonies were sub-cultured on Nutrient Agar for growth in a lawn at 27 °C for three days. After 3 days, bacteria were removed from plates suspended in phosphate buffer 0.01 M, pH 7.0. The solution was adjusted to 0.600 OD at 600 nm using a spectrophotometer and it was artificially inoculated by atomizing the bacterial suspension with a sprayer onto the lower side of the leaf blade to the point of runoff on six radicchio plantlets containing three expanded true leaves. The plants were kept for 4 h in the greenhouse and transferred to a mist chamber for 72 h and then they were maintained at 25 to 32 ^{o}C in greenhouse with drip irrigation until complete evaluation. The negative control was inoculated with 0.01 M Phosphate buffer pH 7.0. The experiment was conducted twice in addition to a preliminary experiment that is not reported here, with three randomized blocks with the treatments represented once in each block. The plants were monitored daily for symptom development.

Symptoms were described and photographed. Symptomatic leaves were surface-sterilized with 0.5 % sodium hypochlorite for 1 min followed by rinsing in sterile distilled water three times. Small (3 X 3 mm) sections of tissue were excised aseptically from bacterial leaf spot margins and macerated in 40 µl of sterile distilled water. The resulting suspensions or a dilution were streaked onto NA and incubated at 24 to 26 °C. After 3 to 5 days, single colonies were purified and were evaluated by rep-PCR using BOX1R primers to compare to the original band pattern of the strain to confirm the reisolated strain identity.

Relationships among *X. hortorum* pathovars and radicchio isolates evaluated by Rep-PCR

The band patterns of the radicchio isolates, *X. hortorum* pathovars, and *X. campestris* pv.*vitians* (NCPPB 2248) were compared by rep-PCR, using BOX1R primers [4] to amplify the genomic DNA as described by Rademaker and colleagues [7]. Amplified DNA fragments were examined by agarose gels

electrophoresis in 1.5% agarose gel in 0.5X Tris acetic acid EDTA buffer, TAE. The gels were stained with GelRed and photographed on a UV transilluminator using a digital camera and Kodak Molecular Imaging software (v. 4.5.1, Carestream Health, Inc., Rochester, NY). DNA fragment banding patterns were compared visually.

Host range evaluation on lettuce plants

All isolates used in the pathogenicity test on radicchio plants and two additional radicchio isolates BS0955 and BS0957 were used to inoculate lettuce cv. Vista Verde seedlings using the same methodology of the previous essay. The lettuce was chosen because is member of the Asteraceae. The experiment conducted twice under the same greenhouse conditions with three randomized blocks with the treatments represented once in each block. The plants were monitored daily for symptom development.

Amplification using B162 primers

The B162 primers and amplification protocol [1], considered to be specific to *X. campestris* pv. *vitians*, were used to amplify the DNA of the isolates described in Table 1. The PCR was carried out in a 40-µl reaction volume and, unless stated otherwise, values for reagents correspond to final concentrations. The 40-µl PCR reaction included 3.00 mM MgCl2, 0.37 µl of each oligonucleotide primer, and 5x commercial Master Mix (1.50 mM $MgCl_2$ 1x buffer mix). The PCR was carried out in a MJ Research DNA-Engine thermo-cycler (MJ Research, Waltham, MA). The amplification profile was 35 cycles of 92°C for 1 min, 59°C for 1.5 min, and 72°C for 1.5 min. Amplified DNA fragments were examined in 1.5% agarose gels in 0.5X Tris acetic acid EDTA buffer, TAE. The gels were stained with GelRed and photographed on a UV transilluminator using a digital camera and Kodak Molecular Imaging

software (v. 4.5.1, Carestream Health, Inc., Rochester, NY). The PCR products were sent to McLab (South San Francisco, CA) and/or TACGen (Richmond, CA) labs to be sequenced and the sequences were prepared and compared using the program CLC Main Workbench 6.5. A basic local alignment search tool-BLAST (http://blast.ncbi.nlm.nih.gov/) comparison with sequences in public databases and was also used and the sequences were compared by alignment.

Multilocus Sequence Typing

The isolates pathogenic to radicchio, *X. campestris* pv.*vitians* (NCPPB 2248) and *X. axonopodis* pv. *vitians* (NCPPB 976) had the genomic fragments of *dnaK*, *fyuA*, *gyrB*, and *rpoD* amplified by PCR with primers and methodology described by Young and colleagues [15] and compared with 31 sequences of *Xanthomonas axonopodis* pathovars (Table 2) and sequences of type and pathotypes strains of *Xanthomonas* available in the site *Plant Associated and Environmental Microbes Database* - PAMDB (http://genome.ppws.vt.edu/cgi-bin/MLST/home.pl). The isolates were cultured 2-3 days on nutrient agar at 27 □C. The genomic DNA was isolated from single colonies using the NucleoSpin® Tissue Kit (Macherey-Nagel, USA) following manufacturer's instructions and label information. The PCR was carried out in a 40-µl reaction volume and, unless stated otherwise, values for reagents correspond to final concentrations. The 40-µl PCR reaction included 1.50 mM MgCl2, 0.50 µl of each oligonucleotide primer, and 2x Bioline ImmoMix (3.00 mM $MgCl_2$ 1x buffer mix). The reaction was carried out in a MJ Research DNA-Engine thermo-cycler (MJ Research, Waltham, MA). The PCR amplifications were performed with initial denaturation at 95 °C for 10min, 30 cycles of denaturation at 94 °C for 30s, annealing at 54 °C for 30s, extension at 72 °C for 1min and final extension at 72 °C for 10min. Amplified DNA fragments were examined in 1.5% agarose gels in 0.5X Tris acetic acid EDTA buffer - TAE, stained with

GelRed and photographed on a UV transilluminator using a digital camera and Kodak Molecular Imaging software (v. 4.5.1, Carestream Health, Inc., Rochester, NY). The PCR products were sequenced by commercial laboratories as described above and the sequences were compared using the program CLC Main Workbench 6.5. The concatenated sequences were compared and the maximum likelihood trees were built, as well the similarity matrix of all sequences and the sequences available in PAMDB.

Bacterial Characterization

API test, Gram and fluorescence on King's Medium B

The bacteria isolated from radicchio plants were tested for Gram reaction, production of fluorescent pigments on King B. The capacity of utilization of 49 carbohydrates using the API® 50 CH test (bioMérieux Inc., Marcy l'Etoile, France) was evaluated for the isolates pathogenic to radicchio plants, *X. hortorum* pathovars, and *X. campestris* pv. *vitians* (NCPPB 2248). The test was set up and evaluated following the manufacturer's instructions.

Characterization of Fatty Acid Methyl Esters

The bacteria isolated from radicchio plants had their fatty acid profiles analyzed. Fatty acids were methyl-esterified and extracted using a previously published method [6]. Fatty acid methyl esters were analyzed with the Sherlock Microbial Identification System Version 6.1 (MIDI Inc., Newark, DE) using an automated GC 6890 Hewlett-Packard gas chromatograph fitted with a 25 X 0.2 mm phenyl methyl silicone-fused silica capillary column, an HP 7683 automatic sampler, and Agilent ChemStation Software (Ver. B.03.02). The mean and standard deviation of the area for each named peak from three independent replications was reported as a percentage of the total area of all peaks in the chromatogram not including the solvent peak.

Results

Pathogenicity test on radicchio plants

The two isolates from radicchio BS953 and BS954, *X. hortorum* pv. *taraxaci* and *X. campestris* pv.*vitians* (NCPPB 2248) showed symptoms 5 days after the inoculation (Fig. 1). The identity of pathogens was confirmed by rep-PCR.

Relationships among *X. hortorum* pathovars and radicchio strains evaluated by Rep-PCR

The nine isolates pathogenic to radicchio showed no differences between them on the Rep-PCR electrophoretic profile. However, the isolates showed differences from those reported by *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci*, *X. hortorum* pv. *carotae*, *X. hortorum* pv. *pelargonii* and *X. campestris* pv. *vitians* (NCPPB 2248) (Fig. 2).

Host range evaluation on Lettuce plants

All strains inoculated on lettuce cv. Vista Verde plantlets showed symptoms, excepting *X. hortorum* pv. *carotae*. The symptoms started to be visible 5 days after inoculation. The symptoms started with a small water-soaked spots that increased to angular lesions quickly becoming black. Symptoms by *X. campestris* pv. *vitians* NCPPB 2248 and the bacterial isolates from radicchio were visibly more severe than *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci* and *X. hortorum* pv. *pelargonii* which in some plants just showed one or two lesions. Even, those isolates that were less severe were consistently reisolated from symptomatic tissues and confirmed by rep-PCR.

Amplification using B162 primers

The B162 primers amplified the DNA of *X. campestris* pv. *vitians* strains, excepting BS3052 and BS3039 from USA and United Kingdom respectively and, the pathotype *X. axonopodis* pv. *vitians* NCPPB 976 (Fig. 3). They amplified also bacterial isolates from radicchio and *X. hortorum* pv. *taraxaci* (Fig. 4).

Multilocus Sequence Typing - MLST

The bacteria isolated from radicchio plants clustered also with *X. hortorum* pv. *hederae, X. hortorum* pv. *pelargonii* and *X. hortorum* pv. *taraxaci*, besides *X. gardneri* and *X. cynarae* in the *Xanthomonas* pathotypes phylogenetic tree (Fig. 5).

The DNA sequences of isolates pathogenic to radicchio shared more than 99% similarity with *X. cynarae* and *X. gardneri*. And from 97.30 to 98.27% with *X. hortorum* pv. *pelargonii*, *X. hortorum* pv. *hederae* and *X. hortorum* pv. *taraxaci*.

Bacterial Characterization

API test, Gram and fluorescence on King's Medium B

The isolates isolated from radicchio were Gram negative and did not fluorescent on King's Medium B. Among the 49 sources of carbon tested by API® 44 CH strips; the isolates pathogenic to radicchio, *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci*, *X. hortorum* pv. *pelargonii* and *X. campestris* pv.*vitians* (NCPPB 2248) used only esculin as a carbon source. The pathotype *X. hortorum* pv. *carotae* showed inconclusive results to the test.

Characterization of Fatty Acid Methyl Esters

All isolates pathogenic to radicchio produced the fatty acids: 11:0 iso 3OH, 12:0 3OH, 14:00, 13:0 iso 3OH, 13:0 2OH, 15:0 iso, 15:0 anteiso, 15:1

w6c, 16:0 iso, 16:1 w9c, 16:00, 17:0 iso, 17:0 anteiso, 17:1 w8c, 17:1 w6c and 18:1 w9c (Table 3). All isolates, except BS0954 also produced the fatty acid 10:00. Additionally, only BS0956 produced the fatty acid 13:0 iso.

The fatty acid dendrogram shared that isolates from radicchio differ to *Xanthomonas hortorum* pathovars and *X. campestris* pv. *vitians* (Fig. 6).

Discussion

This study confirms the ambiguity in the taxonomy of *X. campestris* pv. *vitians* and support the allocation of the pathogenic strain of *X. campestris* pv. *vitians* (NCPPB 2248) in *X. hortorum* pv. *vitians* as proposed by Vauterin and colleagues [11].

The reclassification of the genus *Xanthomonas* significantly changed the taxonomy of members of the plant pathogenic species *X. campestris* [11], resulting in transference of strains from *X. campestris* to *X. axonopodis* including the patotype strain; and a group of *X. campestris* pathovars of ambiguous classification because they were not evaluated. However several laboratories documented the lack of pathogenicity of the pathotype strain (NCPPB 976) on lettuce plants failing to cause disease [9, 8]. A pathogenic strain (NCPPB 2248) of *X. campestris* pv. *vitians* was proposed to be allocated to *X. hortorum* [11]. Stefani and colleagues [10] and Yang and colleagues [13] divided *X. campestris* pv. *vitians* strains into two groups based on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and fatty acid methyl ester (FAME) analysis, respectively. In both cases, the majority of the strains tested fell into one group, with a smaller group containing the pathotype strain NCPPB 976. The separation of *X. campestris* pv. *vitians* into two species: *X. axonopodis* pv. *vitians*, including the pathovar type strain NCPPB 976, and *X. hortorum* pv. *vitians*, including the reference strain NCPPB 2248 proposed by

Vauterin and colleagues [11] based on DNA hybridization and metabolic fingerprinting (Biolog) data was later supported by repetitive extra- genic palindromic (rep-PCR) fingerprinting analysis [12], but the name proposed by Vauterin and colleagues [11], *X. hortorum* pv. *vitians,* is invalid because no pathotype was designated for the pathovar [14].

The isolates pathogenic to radicchio plants fulfilled the requirements of Koch's postulates. *Xanthomonas hortorum* pv. *taraxaci* and *X. campestris* pv.*vitians* (NCPPB 2248) were also able to cause disease on radicchio plants. However, in the host range test with lettuce cv. Vista Verde plants only *X. hortorum* pv. *carotae* showed no symptoms. Symptoms by *X. campestris* pv. *vitians* NCPPB 2248 and the bacterial isolates from radicchio were visibly more severe than *X. hortorum* pv. *hederae*, *X. hortorum* pv. *taraxaci* and *X. hortorum* pv. *pelargonii*. Among the four pathovars of *X. hortorum* only *X. hortorum* pv. *taraxaci* was evaluated for pathogenicity on hosts other than the host from which it was isolated. *X. hortorum* pv. *taraxaci* was evaluated on lettuce because the genera *Lactuca* and *Taxacum* are both in the Asteraceae. *X. hortorum* pv. *taraxaci* did not cause symptoms on the three lettuce (*L. sativa*) types tested and prickly lettuce (*L. scariola*) [5]. Thus, although it is unlikely that the *X. campestris* pv. *vitians* strains transferred to *X. hortorum* belong to *X. hortorum* pv. *taraxaci,* there are no data to determine if this pathogen represents novel pathovar within this species or if it is a synonym of a previously validly named pathovar.

The B162 primers and amplification protocol [1] considered to be specific to *X. campestris* pv. *vitians*, amplified the DNA of bacterial isolates from radicchio, *X. hortorum* pv. *taraxaci* and *X. campestris* pv. *vitians* strains, excepting BS3052, BS3039 and, the pathotype *X. axonopodis* pv. *vitians* NCPPB 976. These results suggest that isolates and strains belong to *Xanthomonas hortorum*, including those worked by Barak and colleagues [1].

The genetic distance between isolates from radicchio and pathotypes of *X. hortorum* were 0.03 or less and MLST analysis indicated that radicchio isolates were members of the species *X. hortorum* supporting the hypothesis that the radicchio isolates belong to *X. hortorum* group. The fatty acid data shared that isolates from radicchio differ to *Xanthomonas hortorum* pathovars and *X. campestris* pv. *vitians*. The symptoms observed on radicchio plants caused by *X. hortorum* pv. *taraxaci* NCPPB 940 and *X. campestris* pv.*vitians* NCPPB 2248 differed visually of symptoms caused by isolates from radicchio and the DNA fragment-banding patterns of isolates from radicchio on Rep-PCR showed no differences between them but differed to pathotypes of *X. hortorum* and *X. campestris* pv. *vitians* NCPPB 2248. However, additional research is needed to determine if this pathogen represents a previously described or novel pathovar of *X. hortorum*.

ACKNOWLEDGMENTS

The authors gratefully acknowledge the U.S. Agricultural Research Station for the opportunity to work together, the Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) and Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) for the scholarship of the first author.

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Table 1. Strains and isolate names referred to in this study, with the code.

Code	Species name	Origin
BS0953	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0954	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0955	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0956	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0957	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0958	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0959	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0960	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0961	Radicchio (*Cichorium intybus*)	Salinas, California (Greenhouse, 2005)
BS0339	*Xanthomonas campestris* pv. *vitians* (Xav Salinas 2/01)	Salinas, California (Barak, unpublished)
BS0340	*Xanthomonas campestris* pv. *vitians* (Xav 98-12 2/01)	Salinas, California (Barak, unpublished)
BS0347	*Xanthomonas campestris* pv. *vitians* (Xcv 5/01)	Salinas, California (Barak, unpublished)
BS2994	*Xanthomonas campestris* pv. *vitians*	Australia
BS2999	*Xanthomonas campestris* pv. *vitians*	Hawaii
BS3039	*Xanthomonas campestris* pv. *vitians*	United Kingdom
BS3052	*Xanthomonas campestris* pv. *vitians*	USA
BS3127 (VT106)	*Xanthomonas campestris* pv. *vitians*	Canada
BS3129 (VT111)	*Xanthomonas campestris* pv. *vitians*	Canada
BS3132	*Xanthomonas campestris* pv. *vitians*	Canada

(ID200707A)		
NCPPB 2248	*Xanthomonas campestris* pv. *vitians*	Zimbabwe
NCPPB 976	*Xanthomonas axonopodis* pv. *vitians*[Pt]	= *Xanthomonas campestris* pv. *vitians* (Brown 1918) Dye 1978.
NCPPB 939	*Xanthomonas hortorum* pv. *hederae*[Pt]	= *Xanthomonas campestris* pv. *hederae* (Arnaud 1920) Dye 1978.
NCPPB 940	*Xanthomonas hortorum* pv. *toraxaci*[Pt]	= *Xanthomonas campestris* pv. *taraxaci* (Niederhauser 1943) Dye 1978.
NCPPB 1422	*Xanthomonas hortorum* pv. *carotae*[Pt]	= *Xanthomonas campestris* pv. *carotae* (Kendrick 1934) Dye 1978b
NCPPB 21464	*Xanthomonas hortorum* pv. *pelargonii*[Pt]	= *Xanthomonas campestris* pv. *pelargonii* (Brown 1923) Dye 1978.

Table 2. *Xanthomonas axonopodis* pathovars.

Code	Strains	Location of isolation	Host of origin
NCPPB466^{Pt}	*Xanthomonas axonopodis* pv. *punicae*	India	*Punica granatum*
NCPPB481^{Pt}	*Xanthomonas axonopodis* pv. *desmodii*	India	*Desmodium diffusum*
NCPPB536^{Pt}	*Xanthomonas axonopodis* pv. *khayae*	Sudam	*Khaya senegalensis*
NCPPB577^{Pt}	*Xanthomonas axonopodis* pv. *desmodiigangetici*	India	*Desmodium gangeticum*
NCPPB578^{Pt}	*Xanthomonas axonopodis* pv. *erythrinae*	India	*Erythrina indica*
NCPPB581^{Pt}	*Xanthomonas axonopodis* pv. *poinsettiicola*	India	*Euphorbia pulcherrima*
NCPPB582^{Pt}	*Xanthomonas axonopodis* pv. *sesbaniae*	India	*Sesbania aegyptiaca*
NCPPB584^{Pt}	*Xanthomonas axonopodis* pv. *tamarindi*	India	*Tamarindus indica*
NCPPB637^{Pt}	*Xanthomonas axonopodis* pv. *cyamopsidis*	India	*Cyamopsis tetragonolobus*
NCPPB761^{Pt}	*Xanthomonas axonopodis* pv. *physalidicola*	Japan	*Physalis alkekengii*
NCPPB840^{Pt}	*Xanthomonas axonopodis* pv. *patelii*	India	*Crotalaria juncea*
NCPPB885^{Pt}	*Xanthomonas axonopodis* pv. *desmodiirotundifolii*	India	*Desmodium rotundifolium*
NCPPB971^{Pt}	*Xanthomonas axonopodis* pv. *maculifoliigardeniae*	USA	*Gardenia* sp.
NCPPB993^{Pt}	*Xanthomonas axonopodis* pv. *lepdzae*	USA	*Lespedeza* sp.
NCPPB994^{Pt}	*Xanthomonas axonopodis* pv. *melhusii*	India	*Tectona grandis*
NCPPB1063^{Pt}	*Xanthomonas axonopodis* pv. *ricini*	Ethiopia	*Ricinus communis*
NCPPB1148^{Pt}	*Xanthomonas axonopodis* pv. *martyniicola*	India	*Martynia diandra*
NCPPB1335^{Pt}	*Xanthomonas axonopodis* pv. *bauhiniae*	India	*Bauhinia racemosa*
NCPPB1337^{Pt}	*Xanthomonas axonopodis* pv. *nakataecorchorii*	India	*Corchorus acutangulus*
NCPPB1786^{Pt}	*Xanthomonas axonopodis* pv. *coracanae*	India	*Eleusine coracana*
NCPPB1827^{Pt}	*Xanthomonas axonopodis* pv. *rhynchosiae*	Sudan	*Rhynchosia memnonia*

NCPPB1833[Pt]	*Xanthomonas axonopodis* pv. *dieffenbachiae*	Brazil	*Anthurium* sp.
NCPPB2058[Pt]	*Xanthomonas axonopodis* pv. *vignaeradiatae*	Sudan	*Vigna radiata*
NCPPB2066[Pt]	*Xanthomonas axonopodis* pv. *phyllanthi*	Sudan	*Phyllanthus niruri*
NCPPB2228[Pt]	*Xanthomonas axonopodis* pv. *biophyti*	India	*Biophytum sensitivum*
NCPPB2230[Pt]	*Xanthomonas axonopodis* pv. *fascicularis*	India	*Corchorus fascicularis*
NCPPB2368[Pt]	*Xanthomonas axonopodis* pv. *pedalii*	India	*Pedalium mure*
NCPPB2972[Pt]	*Xanthomonas axonopodis* pv. *betlicola*	India	*Piper betle*
NCPPB2973[Pt]	*Xanthomonas axonopodis* pv. *cassia*	India	*Cassia tora*
NCPPB3086[Pt]	*Xanthomonas axonopodis* pv. *desmodiilaxiflori*	India	*Desmodium laxiflorum*
NCPPB3092[Pt]	*Xanthomonas axonopodis* pv. *clitoriae*	India	*Clitoria biflora*

Table 3. Fatty acids of the bacterial isolates pathogenic to *Cichorium intybus*.

	Strains pathogenic to *Cichorium intybus*								
Feature	**BS0953**	**BS0954**	**BS0955**	**BS0956**	**BS0957**	**BS0958**	**BS0959**	**BS060**	**BS0961**
10:00	0.92 ± 0.18		0.80 ± 0.07	0.85 ± 0.16	0.80 ± 0.02	0.81 ± 0.06	0.79 ± 0.11	0.56 ± 0.49	0.78 ± 0.05
11:0 iso	3.97 ± 0.41		4.13 ± 0.29	4.27 ± 0.65	4.05 ± 0.21	4.13 ± 0.12	4.12 ± 0.36	2.68 ± 2.32	3.94 ± 0.24
11:0 anteiso	0.26 ± 0.04			0.27 ± 0.06	0.26 ± 0.02	0.25 ± 0.01			0.26 ± 0.03
10:0 3OH	0.36 ± 0.05		0.35 ± 0.03	0.34 ± 0.05	0.35 ± 0.01	0.34 ± 0.06	0.34 ± 0.04		0.31 ± 0.01
11:0 iso 3OH	2.08 ± 0.28	2.20 ± 0.17	2.26 ± 0.15	2.31 ± 0.36	2.21 ± 0.09	2.30 ± 0.07	2.30 ± 0.15	1.45 ± 1.25	2.19 ± 0.07
11:0 3OH	0.47 ± 0.04	0.42 ± 0.02	0.49 ± 0.05	0.49 ± 0.06	0.48 ± 0.02	0.49 ± 0.03	0.45 ± 0.03		0.50 ± 0.04
13:0 iso				0.33 ± 0.06					
12:0 3OH	2.59 ± 0.24	2.58 ± 0.36	2.59 ± 0.18	2.52 ± 0.27	2.46 ± 0.05	2.55 ± 0.08	2.5 ± 0.25	1.69 ± 1.47	2.52 ± 0.16
14:0 iso	0.26 ± 0.02			0.26 ± 0.02	0.26 ± 0.04		0.25 ± 0.01	0.29 ± 0.06	0.26 ± 0.02
14:00	1.13 ± 0.17	1.11 ± 0.21	1.03 ± 0.03	1.11 ± 0.09	1.03 ± 0.04	0.96 ± 0.03	1.05 ± 0.05	1.14 ± 0.13	1.06 ± 0.08
13:0 iso 3OH	2.68 ± 0.28	2.72 ± 0.29	2.94 ± 0.14	2.87 ± 0.23	2.81 ± 0.24	2.86 ± 0.15	2.94 ± 0.31	2.78 ± 0.11	2.80 ± 0.15
13:0 2OH	0.43 ± 0.02	0.44 ± 0.05	0.46 ± 0.03	0.46 ± 0.05	0.46 ± 0.04	0.47 ± 0.04	0.47 ± 0.05	0.44 ± 0.01	0.46 ± 0.04
15:0 iso	24.53 ± 1.85	24.93 ± 2.33	25.44 ± 1.18	25.59 ± 0.48	25.15 ± 1.22	25.04 ± 1.06	25.96 ± 0.97	27.09 ± 3.16	25.16 ± 0.51
15:0 anteiso	15.16 ± 0.33	14.96 ± 0.24	14.94 ± 0.54	14.83 ± 0.36	15.08 ± 0.27	15.36 ± 0.45	14.97 ± 0.31	15.37 ± 0.90	15.07 ± 0.39
15:1 w6c	1.30 ± 0.16	1.29 ± 0.17	1.38 ± 0.05	1.40 ± 0.01	1.38 ± 0.02	1.32 ± 0.04	1.50 ± 0.10	1.54 ± 0.14	1.46 ± 0.11
16:0 iso	1.37 ± 0.05	1.40 ± 0.03	1.29 ± 0.07	1.29 ± 0.13	1.39 ± 0.07	1.39 ± 0.02	1.35 ± 0.10	1.43 ± 0.11	1.37 ± 0.04
16:1 w9c	1.84 ± 0.25	1.82 ± 0.30	1.76 ± 0.07	1.74 ± 0.05	1.74 ± 0.10	1.70 ± 0.07	1.71 ± 0.05	1.80 ± 0.07	1.72 ± 0.02
16:00	3.27 ± 0.94	3.11 ± 0.89	2.74 ± 0.22	2.71 ± 0.20	2.80 ± 0.31	2.77 ± 0.18	2.60 ± 0.30	3.00 ± 0.15	2.94 ± 0.26
17:0 iso	5.55 ± 0.30	5.47 ± 0.27	5.51 ± 0.26	5.24 ± 0.75	5.55 ± 0.61	5.86 ± 0.28	5.39 ± 0.46	5.37 ± 0.29	5.66 ± 0.41
17:0 anteiso	0.87 ± 0.05	0.89 ± 0.04	0.83 ± 0.07	0.81 ± 0.08	0.88 ± 0.08	0.91 ± 0.05	0.86 ± 0.11	0.82 ± 0.05	0.87 ± 0.09
17:1 w8c	2.50 ± 0.11	2.39 ± 0.16	2.46 ± 0.20	2.47 ± 0.24	2.49 ± 0.35	2.56 ± 0.14	2.35 ± 0.16	2.55 ± 0.11	2.58 ± 0.05

17:1 w6c	0.58 ± 0.05	0.57 ± 0.09	0.54 ± 0.07	0.55 ± 0.06	0.58 ± 0.09	0.54 ± 0.04	0.56 ± 0.09	0.61 ± 0.03	0.62 ± 0.04
18:1 w9c	0.49 ± 0.08	0.50 ± 0.07	0.48 ± 0.02	0.43 ± 0.06	0.45 ± 0.06	0.48 ± 0.02	0.45 ± 0.01	0.44 ± 0.01	0.45 ± 0.01
17:0 iso 3OH				0.28 ± 0.01	0.30 ± 0.05				0.30 ± 0.02
Summed Feature 3 (16:1 w7c/16:1 w6c)	20.30 ± 1.66	20.13 ± 2.07	19.48 ± 0.36	19.54 ± 0.38	19.31 ± 0.55	19.13 ± 0.28	19.31 ± 0.60	20.43 ± 1.34	19.30 ± 0.39
Summed Feature 4 (17:1 iso I/anteiso B)	0.48 ± 0.05	0.53 ± 0.05	0.56 ± 0.07	0.49 ± 0.04	0.53 ± 0.03	0.52 ± 0.01		0.51 ± 0.03	0.54 ± 0.07
Summed Feature 8 (18:1 w7c)	0.50 ± 0.08	0.49 ± 0.09	0.46 ± 0.01	0.42 ± 0.05	0.46 ± 0.05	0.49 ± 0.03	0.44 ± 0.04	0.45 ± 0.03	0.44 ± 0.01
Summed Feature 9 (17:1 iso w9c)	5.66 ± 0.72	5.78 ± 0.88	6.04 ± 0.18	5.83 ± 0.34	6.09 ± 0.08	6.33 ± 0.25	6.25 ± 0.55	6.01 ± 0.45	5.95 ± 0.43

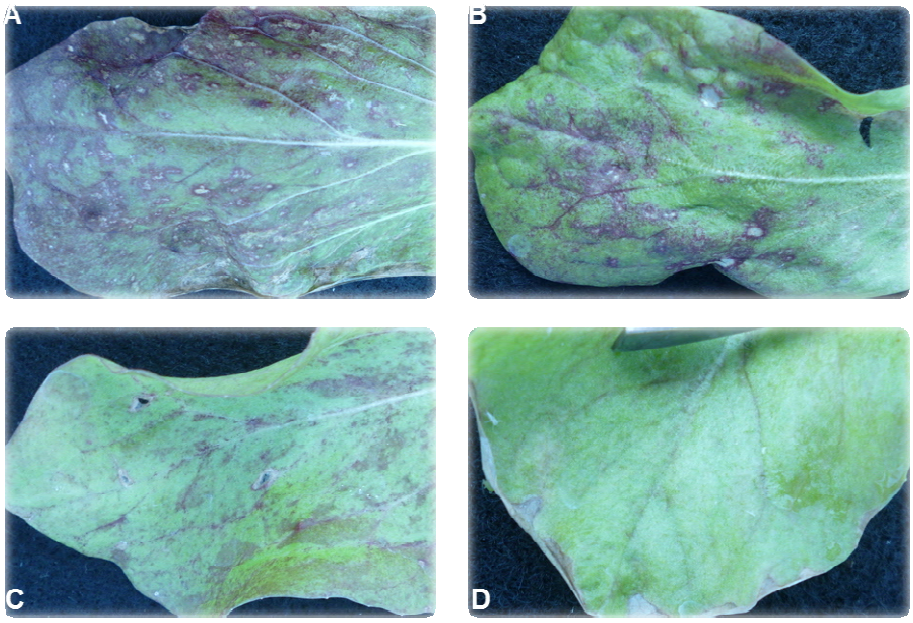

Fig. 1. Radicchio plants showing symptoms of bacterial spot. **A-** Radicchio isolate BS953. **B-** Radichio isolate BS954. **C-** *X. hortorum* pv. *taraxaci* NCPPB 940. **D-** *X. campestris* pv.*vitians* NCPPB 2248.

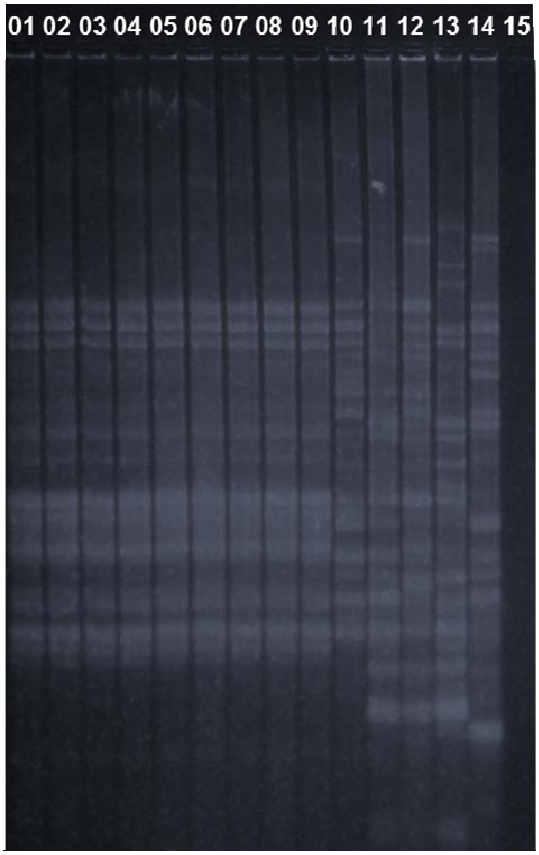

Fig. 2. Electrophoretic profile of isolates pathogenic to *C. intybus*, *X. campestris* pv. *vitians* and pathotypes of *X. hortorum*. **01-09-** Isolates pathogenic to *C. intybus*. **10-** *X. campestris* pv. *vitians* (NCPPB 2248). **11-** *X. hortorum* pv. *hederae*. **12-***X. hortorum* pv. *taraxaci*. **13-** *X. hortorum* pv. *carotae*. **14-** *X. hortorum* pv. *pelargonii*. **15-** Negative control.

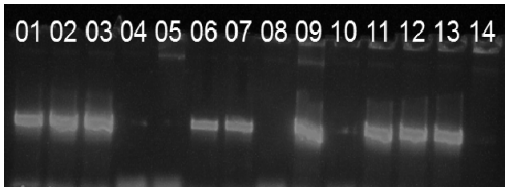

Fig. 3. Banding patterns obtained using the B162 primers. **1-** *X. campestris* pv. *vitians* BS0339. **2-** *X. campestris* pv. *vitians* BS0340. **3-** *X. campestris* pv. *vitians* BS0347. **4-** *Pseudomonas marginalis* pv. *marginalis*. **5-** *X. axonopodis* pv. *vitians* NCPPB 976. **6-** *X. campestris* pv. *vitians* BS2994. **7-** *X. campestris* pv. *vitians* BS2999. **8-** *X. campestris* pv. *vitians* BS3039. **9-** *X. campestris* pv. *vitians.* NCPPB 2248. **10-** *X. campestris* pv. *vitians* BS3052. **11-** *X. campestris* pv. *vitians* BS3127. **12-** *X. campestris* pv. *vitians* BS3129. **13-** *X. campestris* pv. *vitians* BS3132. **14-** Negative control.

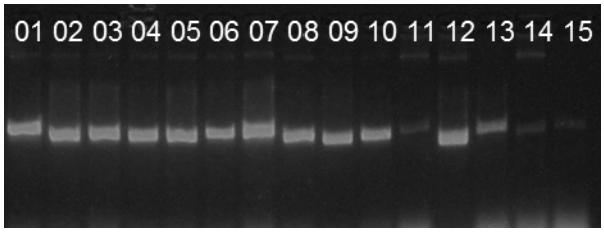

Fig. 4. Banding patterns obtained using the B162 primers. **1-** Radicchio isolate BS0953. **2-** Radicchio isolate BS0954. **3-** Radicchio isolate BS0955. **4-** Radicchio isolate BS0956. **5-** Radicchio isolate BS0957. **6-** Radicchio isolate BS0958. **7-** Radicchio isolate BS0959. **8-** Radicchio isolate BS0960. **9-** Radicchio isolate BS0961. **10-** *X. campestris* pv. *vitians* NCPPB 2248. **11-** *X. hortorum* pv. *hederae* NCPPB 939. **12-** *X. hortorum* pv. *taraxaci* NCPPB 940. **13-** *X. hortorum* pv. *carotae* NCPPB 1422. **14-** *X. hortorum* pv. *pelargonii* NCPPB 21464. **15-** Negative control.

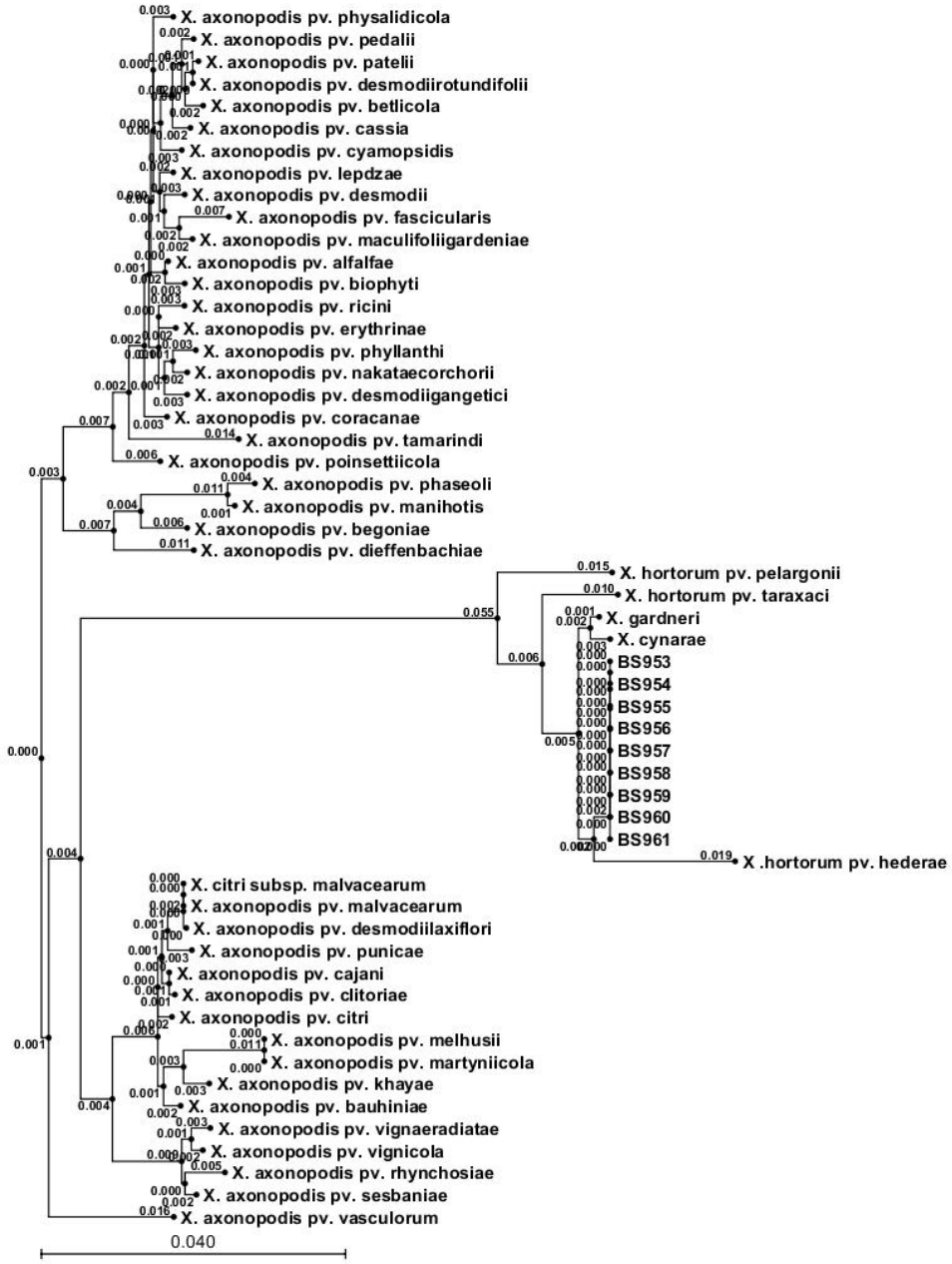

Fig. 5. Phylogenetic tree using the Neighbor Joining method of concatenated partial sequences of genes *dnaK*, *fyuA*, *gyrB* and *rpoD*, based on 50 pathotypes of *Xanthomonas* and 9 strains pathogenic to *C. intybus*.

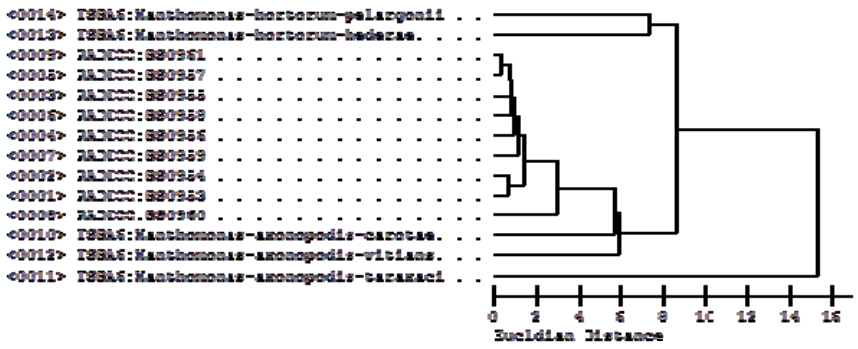

Fig. 6. Dendrogram based on fatty acid methyl ester profiles of radicchio isolates, *Xanthomonas hortorum* pathovars (referred to as *X. axonopodis*) and *X. campestris* pv. *vitians* (referred to as *X. axonopodis*).