Brazilian Journal of Microbiology (2009) 40: 678-684
ISSN 1517-8382

LOW GENETIC DIVERSITY AMONG PATHOGENIC STRAINS OF *ERWINIA PSIDII* FROM BRAZIL

Ana C. O. Teixeira[1], Abi S. A. Marques[2], Marisa A. S. V. Ferreira[1]*

[1] Departamento de Fitopatologia, Universidade de Brasília, Brasília DF, Brasil; [2]Embrapa Recursos Genéticos e Biotecnologia, Brasília DF, Brasil

Submitted: September 01, 2008; Returned to authors for corrections: January 07, 2009; Approved: May 03, 2009.

ABSTRACT

Erwinia psidii causes bacterial disease of guava in Brazil. Phenotypic and molecular characterization through rep-PCR fingerprinting of 42 strains from different geographical regions showed that *E. psidii* populations in Brazil have a low level of genetic diversity and suggest that contaminated plant material is the main source for pathogen dissemination in the country.

Key words: guava bacterial blight, *Psidium guajava*, rep-PCR fingerprinting

Brazil is one of the world´s major producers of guava (*Psidium guajava* L.). Three states, São Paulo, Minas Gerais and Pernambuco, account for more than 80% of the country´s production. Bacterial disease caused by *Erwinia psidii* is one of the most important phytosanitary problems of this crop in Brazil (17). This disease can reduce yield significantly and control measures are not effective. The bacterium affects branches and twigs of guava trees, causing dieback. Leaves, blossoms and fruits are also affected. The pathogen was first detected in São Paulo, in 1982, and described as a new species, *Erwinia psidii* sp.nov. (17). The disease was later reported in Minas Gerais in 1993 (18), Espírito Santo and Paraná in 2000 (2, 14) and Distrito Federal in 2001 (21). Currently, *E. psidii* is one of the most important pathogens affecting guava in central Brazil. It causes severe yield losses, leading, in many cases, to growers abandoning the crop. In 1999, yield losses of up to 85% were reported (4). Surveys carried out in 2001 showed that the pathogen was present in 56% of the properties (12).

Several DNA-based techniques have shown great usefulness in disease diagnosis and in detecting diversity in bacterial populations (10). Repetitive-PCR (rep-PCR) has proved to be a reliable technique for genomic analysis, able to reveal intraspecific diversity in bacteria. Repetitive PCR employs primers that are complementary to the repetitive elements REP, ERIC and BOX, which are highly conserved among most gram-negative bacteria (10, 22). Using these primers, reproducible fingerprints can be obtained, as shown in studies with important human and plant pathogens such as *Salmonella* (15), *Pseudomonas* spp. (9,11), *Xanthomonas*

***Corresponding Author. Mailing address:** Universidade de Brasília, Departamento de Fitopatologia, Campus Universitário Darcy Ribeiro. CEP 70910-900 Brasília, DF; (61) 3272-1793. E-mail marisavf@unb.br

spp. (9,16) and *Erwinia amylovora* (1).

The occurrence of *E. psidii* is limited to Brazil and very little is known about its origin and genetic diversity. The objectives of this study were to characterize phenotypically a set of strains collected in central Brazil (DF), to evaluate the genetic diversity among strains collected in different regions and to investigate the origin of *E. psidii* in central Brazil.

Forty-two strains were used in this study (Table 1). Twenty-eight were collected from guava trees with blight and dieback symptoms in Brazlândia, DF. Other 14 strains, previously identified as *E. psidii*, were obtained from IBSBF culture collection and used as reference. For long-term storage strains were kept in 20% glycerol at -20 °C, in sterile distilled water, and on YDC and 523 (5) slants covered with mineral oil. Strains in water, YDC or 523 were maintained at 10 °C. Phenotypic tests were performed to characterize 28 bacterial strains isolated from guava in DF. The minimal list of identification tests proposed by Marques *et al.* (12), which allows the differentiation of *E.psidii* from all known species belonging to the 'amylovora' group species, was performed. The tests were: potassium hydroxide solubility test, O/F, catalase activity, acid production from raffinose and mannitol, nitrate reduction and hypersensitive test on tobacco leaves (HR), as described by Lelliott and Stead (7) and Schaad *et al.* (19). Pathogenicity tests were carried out using detached shoots from adult guava plants cv. Paluma, according to Marques *et al.* (12). Symptom observation was done twice a day for 15 days and re-isolation of the pathogen was attempted. Stem and leaves were surface washed with common detergent and rinsed with distilled water, macerated and streaked onto 523 plates. Three tests (KOH solubility, O/F and catalase activity) were conducted to confirm bacterial identity.

Table 1 *Erwinia psidii* strains used in this study

Strain [a]	Origin[c]	Collection year
Emb.C-076.1, 082.2	orchard 1/ Brazlândia-DF	2002
Emb.C-133.1, 134.1, 140.1,142.2	orchard 2 /Brazlândia-DF	2002
Emb.C-148.2, 150.1, 151.2, 153.1	orchard 3/ Brazlândia-DF	2002
Emb.C-294.1, 295.3,296.1	orchard 4 / Brazlândia-DF	2002
Emb.C-338.2, 342.1, 343.2, 345.2	orchard 5/ Brazlândia-DF	2002
Emb.C-400.3	orchard 6/ Brazlândia-DF	2002
Emb.C-421.2	orchard 7/ Brazlândia-DF	2002
Emb.C-424.1, 432.1, 435.2, 439.3	orchard 8/ Brazlândia-DF	2002
UnB1286, 1285	orchard 9/ Brazlândia-DF	2004
UnB 1287	orchard 2/ Brazlândia-DF	2005
UnB 1288	orchard 11/ Brazlândia-DF	2005
UnB 1289	orchard 12/ Brazlândia-DF	2005
IBSBF 435 [b], 446	São Paulo	1982
IBSBF 493	Itariri-SP	1984
IBSBF 454 (ICMP 8430), 453 (ICMP 8429)	Valinhos-SP	1983
IBSBF 1347 (ICMP 13701)	Brazlândia-DF	1997
IBSBF 1461	Urupês-SP	1999
IBSBF 1480	Santa Tereza-ES	2000
IBSBF 1523	Carlópolis-PR	2000
IBSBF 452	Valinhos-SP	1983
IBSBF 1575 (Emb.A-18.7)	Brazlândia-DF	2000
IBSBF 1576 (Emb.B-67.1), 1577 (74.1), 1579 (78.1)	Brazlândia-DF	2001

[a]Emb.-Plant Pathogenic Bacteria Collection, Brasília, DF, Brazil; UnB- Universidade de Brasília, Brasília, DF, Brazil; IBSBF-Phytobacteria Culture Collection of Instituto Biológico, Campinas, SP, Brazil.
[b]IBSBF 435: *Erwinia psidii* type strain (= NCPPB 3555; ICMP 8426; LMG 7039; ATCC 49406)
[c]DF= Distrito Federal; SP= São Paulo; ES=Espírito Santo; PR=Paraná.

Total genomic DNA from all 42 strains was extracted following the protocol described by Li and DeBöer (8) except that the bacterial cultures were grown in 523 broth for 20-24 hours at 28 ºC and then pelleted by centrifugation in a 1.5-mL microtube. DNA was precipitated with isopropanol for 3 hours, pelleted and washed twice with 70% ethanol, air-dried and dissolved in 50 µL of sterile distilled water. DNA samples were maintained at –20 °C until used. Genomic profiles of all strains were compared by rep-PCR, following Louws *et al.* (9). Each reaction was composed of 1X PCR buffer (500 mM KCl, 100 mM Tris HCl); 1.5 mM $MgCl_2$; 200 µM dNTPs, 2 µM of each primer, 2.5 U of *Taq* polymerase and 30 ng of bacterial DNA in a final volume of 25 µl. Reactions were carried out in a PTC-100™ thermocycler (MJ Research Inc.). With BOX and ERIC primers the following program was used: 95 °C/7 min, followed by 30 cycles of 94 °C/1 min, 53 °C/1 min for BOX or 52 °C/1 min for ERIC-PCR, and 65 °C/ 8 min. With REP primers the program was: 95 °C/7 min, 35 cycles of 94 °C/ 1 min, 44 °C/ 8 min and 65 °C/ 8 min. A final extension step (65 °C/15 min) was included in all programs. Negative water controls were included in all experiments. PCR products (15 µl) were separated by electrophoresis on 1.5% agarose gels in 0.5X TBE buffer for 4 hours at 80 V. Following ethidium-bromide (0.5 µg/ml) staining, gels were photographed (Eagle Eye™- II Stratagene®) and the size of each fragment was determined by comparison with a 100 bp-DNA Ladder (Invitrogen). Analysis was based on the presence or absence of each DNA fragment, regardless of its intensity. Values of 1 and 0 were attributed to presence or absence of a specific fragment, respectively. Results for each primer were analysed separately and then combined, using the MVSP 3.1 program (6), employing Jaccard´s similarity coefficient and cluster analysis with UPGMA (unweighted pair-group method using arithmetic averages).

The 28 strains collected from guava trees in 11 orchards in DF showed phenotypic characteristics of *E. psidii* (17). They were gram-negative, able to metabolize glucose fermentatively and catalase-positive. Nitrate reduction was not detected and acid was produced by all strains from mannitol but not from raffinose. For all strains, there was no visible hypersensitive reaction on infiltrated tobacco leaves within 24 hours. All 28 strains induced symptoms on detached guava shoots. The pathogen was successfully reisolated from inoculated plants, thus confirming the pathogenicity of guava strains from DF. Variation in aggressiveness was detected, since symptoms were visible from 1 to 12 days after inoculation, depending on the strain. The majority of strains (24) were considered moderately aggressive, with visible symptoms showing from 5 to 8 days after inoculation. Three were weakly aggressive inducing symptoms after 9 days and only one strain (Emb.C432-1) was classified as highly aggressive with visible symptoms 24 hours after inoculation. It was possible to find strains with variable levels of aggressiveness in the same orchard.

Amplification from genomic DNA using REP, ERIC and BOX primers allowed assessment of the intraspecific variation among the strains studied. Each reaction was repeated twice and independently, and the same genomic profiles were obtained. Amplification with BOX primer produced 23 distinct bands varying from 380 to 2,642 bp. REP-PCR generated 24 distinct bands from 280 to 2,100 bp, and ERIC-PCR generated 20 fragments from 480 to 2,787 bp. The genomic fingerprints revealed very limited polymorphism among the strains (Fig.1). The resulting dendrogram (Fig.2) showed similar clustering patterns to the ones obtained with each separate primer and revealed more than 87% similarity among the strains. The dendrogram showed that strain UnB 1285 (DF, 2004) was the most divergent. Two other strains, UnB 1287 and Emb.C343-2,

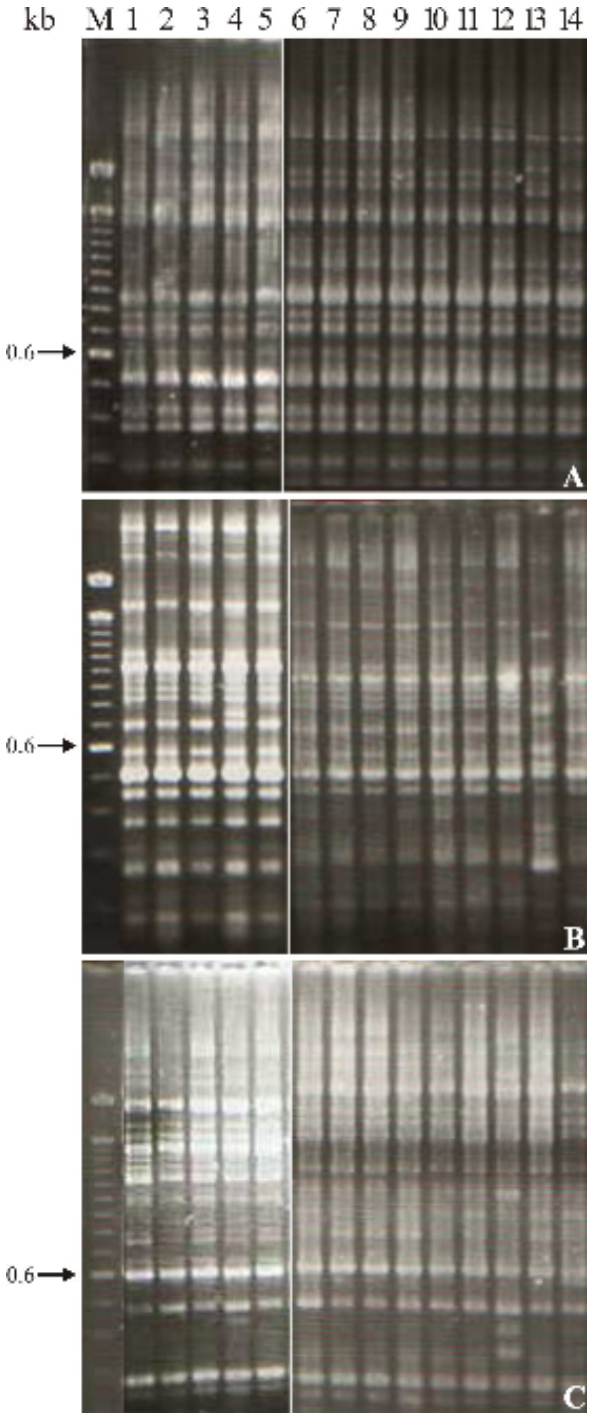

Figure 1. Repetitive PCR (rep-PCR) fingerprints of *Erwinia psidii* strains from Brazil, obtained with: (A) BOX; (B) ERIC and (C) REP primers. M-100 bp-DNA Ladder (Invitrogen); 1-IBSBF 435 (Type strain); 2-IBSBF 446; 3-IBSBF 453; 4-IBSBF 454; 5-IBSBF 493; 6-Emb.C421-2; 7- Emb.C424; 8-Emb.C432-1; 9- Emb.435-2; 10- Emb.C439-3; 11-UnB 1286; 12-UnB 1285; 13-UnB1287; 14-UnB1288.

both from DF, could also be distinguished from the main group at 90% similarity level. This larger group contained the majority (92.8%) of the strains and, within this group, two subgroups sharing 92% similarity could be separated. The first subgroup comprised three strains, two from DF (1997 and 2001) and one collected in São Paulo in 1983 (IBSBF 454). The 36 remaining strains in the second subgroup shared similarity values from 91.8 to 100%. This subgroup contained the type strain (IBSBF435) and strains from DF, ES, PR and SP. Five clusters of strains sharing 100% similarity could be detected. Strains from DF had identical profiles to strains from São Paulo and Paraná. The strain from Espírito Santo (IBSBF 1480) was genetically identical to the strain from Valinhos, SP (IBSBF 452). The type strain shared 97.5% similarity with strain Emb.C148-2, collected in DF in 2002.

Genetic diversity in a pathogen population can be determined by rep-PCR and may contribute to a better understanding of the population structure and dynamics (9, 10). In this study, rep-PCR revealed limited polymorphism in the *E. psidii* population from four regions. No correlation between rep-PCR groups and state of origin, collection year or pathogenicity was evident. Among the primers tested, REP primers generated more polymorphic DNA bands, with the largest number (eight) of genomic profiles. Other studies have also showed differences in resolution for each primer. For example, REP and ERIC were more discriminative in

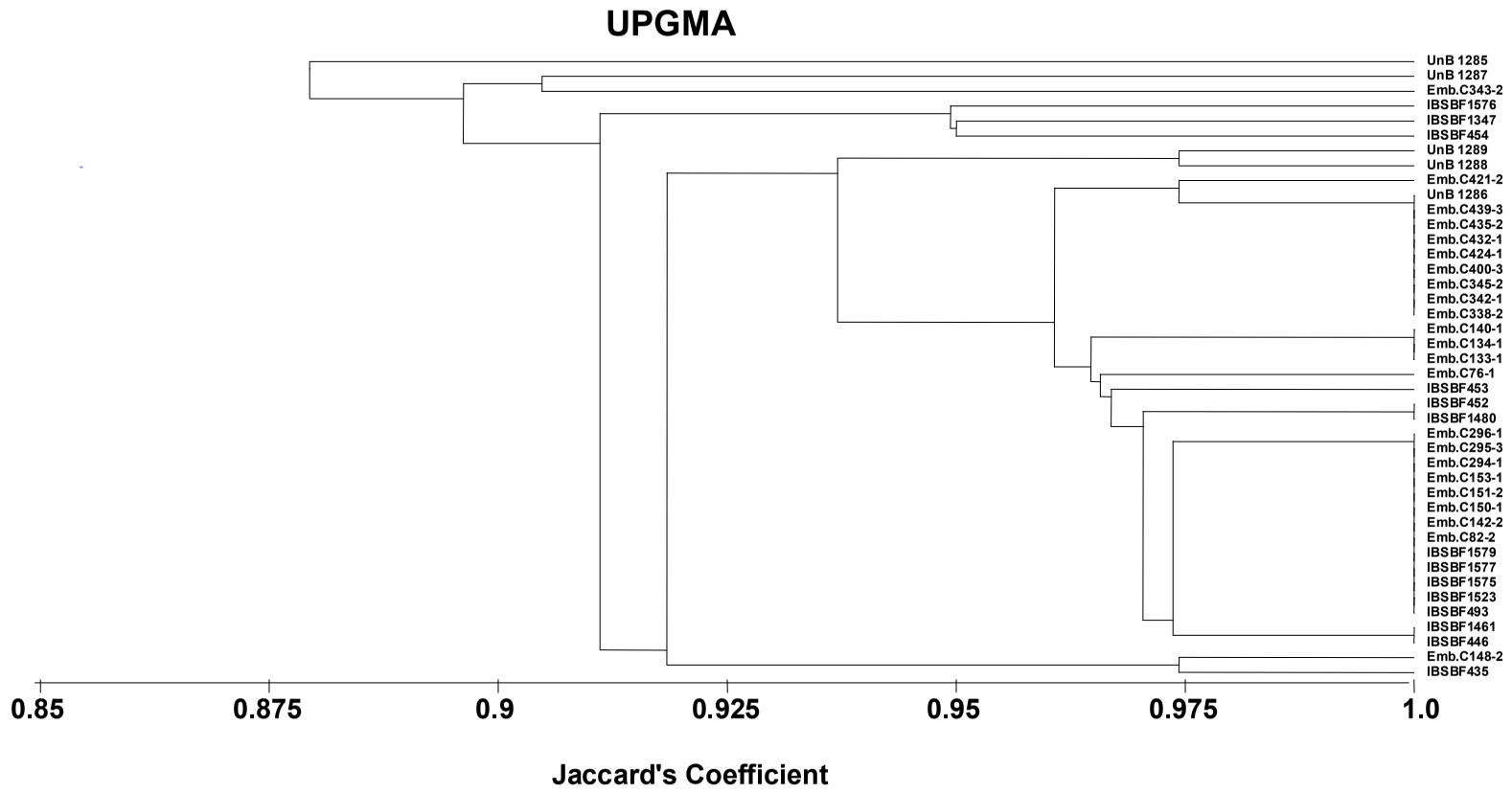

Figure 2. UPGMA dendrogram based on combined rep-PCR (BOX, REP and ERIC) fingerprints of Brazilian strains of *Erwinia psidii*.

showing variability in *E. amylovora* than BOX primers (1). A high level of genetic similarity among the Brazilian strains of *E. psidii* was evident, in particular among those from DF, from where a larger number of strains were collected. Similarity among strains was over 87% and strains from different geographic origins shared 100% similarity, with identical DNA profiles. High similarity was observed among strains from different trees collected in the same orchard, however, strain UnB 1287 collected in 2005, shared less than 90% similarity with four strains, collected in 2002 in the same area (orchard 2). This might suggest that variations in the bacterial population would be likely to occur in the same orchard over time.

E. psidii was first reported in the guava-producing areas of DF in 2001, almost 20 years after its first report in SP. Here, rep-PCR analysis revealed that the DF strains are genetically similar to those from Paraná, Espírito Santo and São Paulo. This suggests that the bacterium, initially detected in São Paulo, was disseminated to other regions through contaminated propagating plant material.

Repetitive-PCR-based analysis has also revealed homogeneity in *E. amylovora* strains from fruit trees (apple and pear) and from different geographical regions (13). Korean strains of *E. pyrifoliae*, a species related to *E. amylovora,* also showed identical patterns with REP and ERIC-PCR, and only one polymorphic band with BOX-PCR (20). The same low level of polymorphism was found in the present study with *E. psidii,* a member of the same phylogenetically related cluster or true erwinias (3). This homogeneity suggests a low frequency of recombination in the *E. psidii* population, as observed with *E. amylovora* strains collected worldwide. It may also suggest a recent adaptation of an original local population from indigenous *Psidium* species, such as *P. cattleyanum* ("red araçá") to common guava (*P. guajava*), which is native to tropical America. *E. psidii* has a narrow host range, limited to a few species in the Myrtaceae family (17). Pathogen specialization towards a plant host or a narrow ecological niche may correspond to genetic homogeneity and to the distribution of repetitive sequences in the genome (23).

The rep-PCR fingerprints of *E. psidii* described here showed several common DNA fragments among the strains. They represent an interesting source of target sequences for *E. psidii*-specific primers that would be useful for developing a PCR protocol for early detection of this pathogen in asymptomatic plants. To our knowledge, this is the first molecular variability study in *E. psidii*. The genomic profiles of this pathogen can be used as tools for epidemiological studies, disease diagnosis and for monitoring pathogen dissemination and new disease outbreaks.

ACKNOWLEDGEMENTS

The authors thank J. Rodrigues Neto (Instituto Biológico de São Paulo, Campinas, SP) and Carlos Uesugi (Universidade de Brasília, Brasília, DF) for providing strains and Wesley R. Souza for assistance in preparing Fig 1. Financial support: CNPq.

RESUMO

Baixa diversidade genética entre estirpes patogênicas de *Erwinia psidii* no Brasil

Erwinia psidii é o agente causal da seca-dos-ponteiros ou bacteriose da goiabeira no Brasil. A caracterização fenotípica e molecular através de rep-PCR de 42 estirpes patogênicas de diferentes regiões mostrou que as populações de *E. psidii* no Brasil têm um baixo nível de diversidade genética e sugere que material de propagação infectado é a principal fonte de disseminação do patógeno para novas áreas no país.

Palavras-chave: bacteriose, seca-dos-ponteiros, *Psidium guajava*, rep-PCR

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