European Journal of Plant Pathology **103:** 323–329, 1997.

Morphological and molecular characterization of *Colletotrichum* spp. from citrus orchards affected by postbloom fruit drop in Brazil

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Accepted 4 December 1996

Key words: Colletotrichum acutatum, citrus postbloom fruit drop disease, RAPD

Abstract

Brazilian isolates of *Colletotrichum* spp. from citrus orchards affected by postbloom fruit drop were examined for colony colour, mycelial growth, benomyl-resistance, pathogenicity, and genetic variability by random amplified polymorphic DNA (RAPD) analysis. All isolates were obtained from flowers and persistent calyxes from different citrus hosts from Sao Paulo, Brazil. DNA polymorphisms detected after amplification with random 10-mer primers were used to classify the isolates into two groups. Group I isolates grew rapidly on potato-dextrose agar (PDA) and were sensitive to benomyl, and group II isolates grew slowly on PDA and were benomyl-resistant. *Colletotrichum acutatum* was analyzed by RAPD and had high genetic similarity with group II isolates of *Colletotrichum* from citrus. Probably, the group I is *C. gloeosporioides* and group II is *C. acutatum.*

Introduction

Postbloom fruit drop disease (PFD) of citrus results from blossom infection by the fungus *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. in Penz. (Fagan, 1979). The fungus infects petals, producing peach to orange-coloured necrotic spots, and under favourable conditions produces blossom blight of entire flowers and clusters. After infection, fruitlets drop, but buttons composed of the penduncle, floral disk, calyx and nectaries remain. These persistent buttons are diagnostic for the disease.

Currently, the disease occurs widely throughout the humid tropics and subtropics of the Americas. Schwarz et al. (1978) reported the disease in 1978 in Argentina and Orozco Santos and Gonzales Garza (1986) studied the disease in Mexico. In Brazil the disease has become a problem over the last 4 years, where suitable conditions for fungus proliferation and infection have caused up to 90% yield losses (Prates et al., 1991).

Historically, *C. gloeosporioides* has been described as a variable fungus with many morphological variants (Baxter et al., 1985; Sonoda and Pelosi, 1988). Other authors (Agostini et al., 1992; Bonde et al., 1991; Orozco Santos and Gonzales Garza, 1986) have also noted strains differing in morphology, growth and pathogenicity to Tahiti lime flowers (*Citrus aurantifolia*). Agostini et al. (1992) and Sonoda and Pelosi (1988) indicate that three different strains of *C. gloeosporioides* occur on citrus. The first is a saprophytic, fast-growing, gray (FGG) strain and is not responsible for PFD. The second, originally described by Clausen (1912), causes anthracnose, necrotic spots on leaves, twigs and fruits, of Key lime [*Citrus aurantifolia* (L.) Swingle] and if it is severe, it can blight entire shoots. A third strain, that is reported to cause PFD, is a specialized form of *C. gloeosporioides* (Fagan, 1979) and it is referred to by Agostini et al. (1992) and Sonoda and Pelosi (1988) as the slow-growing, orange (SGO) strain, producing mostly white mycelia with orange conidial masses. The second and the third strains are now considered to be *C. acutatum* (Brown et al., 1996).

Liyanage et al. (1992) found that SGO and FGG strains of *C. gloeosporioides* differed by polymorphisms within ribosomal DNA and chromosome number, and Liyanage et al. (1993) observed differences in cutinase enzymes of these two strains. Brown et al. (1996) confirmed by nucleotide sequence of ribosomal DNA, the classification of the SGO and KLA isolates as *C. acutatum* and the FGG isolates as *C. gloeosporioides*.

In this study we examined growth and colony colour, pathogenicity to detached Tahiti lime flowers, benomyl resistance and random amplified polymorphic DNA (RAPD) markers to analyse the genetic variability of Brazilian *Colletotrichum* isolates from different regions and a range of citrus hosts.

Materials and methods

Isolation and culture conditions. The host, geographic location and source of *Colletotrichum* isolates used in this study, are listed in Table 1. All were obtained from the field ('buttons' or flowers with PFD symptoms), isolated on PDA and single conidium-derived cultures were established in the same medium for all analyses. The *C. acutatum* isolate from strawberry flower was supplied by the Departamento de Defesa Fitossanitária da Faculdade de Ciências Agronômicas-UNESP/Botucatu. For each isolate, a 0.5-cm diameter disk containing mycelium was placed in the center of a PDA plate and maintained in the dark at 24 °C for four days. The radius was measured at the four cardinal points on each plate and averaged, and data were analysed by Tukey's test with $P < 0.01$. After six days colony colour was assessed. Light (white, light orange or orange) or dark (gray, dark gray or black).

Pathogenicity. All isolates from citrus were tested for their ability to infect fresh, mature flowers of Tahiti lime under laboratory conditions. Pathogenicity testing using suspensions containing 1.0×10^5 conidia/ml was as described by Liyanage et al. (1992). Disease symptoms were recorded after 24 and 48 h. A disease index based on a scale of 0 to 3 was developed according to the percentage of infection as follows: 0 = no infection; 1 = from 0 to 40% infection; 2 = from 40% to 70% infection; 3 = over 70% infection.

Benomyl resistance. The reaction of isolates to benomyl (Benlate 50WP; E.I. du Pont de Nemours & Co., Wilmington, DE) was determined by transferring 0.5-cm PDA disks containing mycelium to PDA amended with benomyl at 0, 1, 10, 100, 500 and 1,000 μg/ml added after autoclaving. The isolates were classified as sensitive if they did not grow on 1 μg/ml and resistant if growth occured on 1, 10, 100, 500 and 1,000 μg/ml benomyl. Growth was measured as colony radius after 6 days at 24 °C in the dark.

Table 1. Host, geographic location and source of *Colletotrichum* spp. from citrus used in this study

Isolate	Host[*y]	Location[*z]	Source
1KLCC	Key lime	C.C.S.M.	Buttons
2KLCC	Key lime	C.C.S.M.	Buttons
3KLCC	Key lime	C.C.S.M.	Buttons
4SOCC	Sweet orange	C.C.S.M.	Buttons
8TLCC	Tahiti lime	C.C.S.M.	Buttons
9-VII	Sweet orange	Itapetininga-SP	Buttons
10-2146	Sweet orange	Mogi Guaçu-SP	Flowers
12SOCC	Sweet orange	C.C.S.M.	Buttons
15TLCC	Tahiti lime	C.C.S.M.	Buttons
16TLCC	Tahiti lime	C.C.S.M.	Buttons
17KLFCA	Key lime	FCA	Buttons
18KLFCA	Key lime	FCA	Buttons
19KLTQ	Key lime	Taquari – RS	Flowers
20KLFCA	Key lime	FCA	Flowers
21KLFCA	Key lime	FCA	Buttons
22TLFCA	Tahiti lime	FCA	Flowers

[*y] Sweet orange – Pera sweet orange (*Citrus sinensis* (L.) Osbeck); Key Lime (*Citrus aurantifolia* (L.) Swingle); Tahiti Lime (*Citrus aurantifolia*).
[*z] C.C.S.M. – Centro de Citricultura 'Sylvio Moreira' – Coordeirópolis, SP; FCA – Faculdade de Ciências Agronômicas – UNESP – 'Campus' de Botucatu, SP; Itapetininga – Citrovita farm; Mogi Guaçu – Sete Lagoas Farm; Taquari isolated in 1987 in Rio Grande do Sul.

RAPD analysis. Sixteen isolates of *Colletotrichum* from citrus and one isolate of *C. acutatum* were grown in 200 ml of potato-dextrose broth for 7 days at 24 °C in the dark on a rotary shaker at 150 rpm. DNA was extracted as described by Liyanage et al. (1992). Nineteen ten-mer primers (Operon Technologies Inc. Alameda, CA) were used for RAPD analysis essentially as described by Williams et al. (1990). Controls, in which DNA template solution was replaced by water, were included in all experiments. After amplification, samples were electrophoresed on a 1.7% (w/v) agarose gel, stained and photographed.

Cluster analysis. Comparison of each profile for each primer was carried out on the basis of presence (1) or

Table 2. Colony colour and mycelial growth (cm) of *Colletotrichum* spp. isolates of citrus on PDA at 24 °C, in darkness, and pathogenicity to detached Tahiti lime flowers

Isolate	Colony colour	Colony radius*x (cm)	Pathogenicity*z (24 hours)	Pathogenicity (48 hours)
Control	–	–	0.00 a	0.00 a
10-2146	Dark gray	3.94 a*y	1.30 bcde	1.93 b
1KLCC	White	3.68 ab	1.29 bcdef	1.81 bc
12SOCC	Gray	3.67 ab	1.47 bcd	1.90 b
22TLFCA	Dark gray	3.65 ab	1.39 bcde	1.76 bc
15TLCC	Dark gray	3.64 ab	1.58 b	1.91 b
8TLCC	Dark gray	3.57 b	1.52 bcd	1.93 b
9-VII	Dark gray	3.56 b	1.44 bcd	1.64 cd
21KLFCA	Orange	2.24 c	1.26 cdef	1.86 bc
20KLFCA	Orange	2.16 c	1.10 ef	1.42 d
17KLFCA	Orange	2.12 c	1.45 bcd	1.75 bc
3KLCC	Orange	2.07 cd	1.19 def	1.86 bc
18KLFCA	Dark gray	2.04 cd	1.36 bcde	1.89 b
4SOCC	Dark gray	2.01 cd	1.37 bcde	1.93 b
16TLCC	Black	1.98 cd	1.47 bcd	1.90 b
2KLCC	Light orange	1.96 cd	1.47 bcd	1.94 b
19KLTQ	White	1.81 d	1.56 bc	1.82 bc

*x Values are means of five replicates after 4 days of growth on PDA at 24 °C in dark.
*y Values not followed by the same letter are significantly different ($P < 0.01$) according to Tukey's test.
*z Pathogenicity to Tahiti lime flowers infected by *Colletotrichum gloeosporioides* 24 and 48 h after inoculation (1.0×10^5 conidia/ ml) at 24 °C in 12h dark/12h light regime. Each value is a mean of 5 plates with each plate containing 4 flowers of Tahiti lime. Values within columns followed by the same letter are not significantly different ($P < 0.01$) according to Tukey's test.

absence (0) of amplified products of the same length. Bands of the same length were scored as identical. Analysis were based on the Dice Coefficient, which measures the proportion of common discrete bands among isolates. A dendogram was derived from the distance matrix by the Unweighted Pair-Group Method Arithmetic Average (UPGMA) contained in the computer program package NTSYS-pc 1.7 (Numerical Taxonomy and Multivariate Analysis System) (Rohlf, 1992).

Results

Colony colour and mycelial growth. The isolates of *Colletotrichum* spp. were divided into two main groups (light and dark), based on colony colour on PDA and mycelial growth (Table 2). After four days, isolates belonging to group I had radial growth between 3.94 and 3.56 cm, and those in group II had growth between 2.24 and 1.81 cm. The radial growth of isolates in group I was twice that of those in group II.

Pathogenicity. All isolates caused peach to orange-coloured necrotic spots on the Tahiti lime flowers petals after 24 h. The control had no symptoms after 72 h. All isolates were of similar pathogenicity regardless of the time of incubation (Table 2).

Benomyl resistance. Benomyl completely inhibited the mycelial growth of isolates 1KLCC, 8TLCC, 9-VII, 10-2146, 22TLFCA, 12SOCC and 15TLCC at all concentrations tested (Table 3). In contrast, isolates 2KLCC, 3KLCC, 16TLCC, 4SOCC, 18KLFCA, 19KLTQ, 17KLFCA, 20KLFCA and 21KLFCA grew at all benomyl concentrations tested. These isolates had similar mycelial growth at 1, 10, 100 and 500 μg of benomyl/ml ($P < 0.01$), but at 1,000 μg/ml, growth was reduced (Table 3).

RAPD analysis

Genetic distance among Colletotrichum *spp. isolates*. All nineteen primers revealed polymorphisms useful for the classification of *Colletotrichum* spp. isolates.

Table 3. Radial growth (cm) of colony of *Colletotrichum* spp. from citrus grown on PDA amended with 0, 1, 10, 100, 500 and 1,000 μg benomyl/ml medium

	Colony radius (cm)					
	Concentration (μg/ml)					
Isolates	0	1	10	100	500	1,000
1KLCC[s]	3.415a	0.0b	0.0b	0.0b	0.0b	0.0b
8TLCC[s]	3.095a	0.0b	0.0b	0.0b	0.0b	0.0b
9-VII[s]	3.200a	0.0b	0.0b	0.0b	0.0b	0.0b
10-2146[s]	3.895a	0.0b	0.0b	0.0b	0.0b	0.0b
22TLFCA[s]	3.700a	0.0b	0.0b	0.0b	0.0b	0.0b
12SOCC[s]	3.721a	0.0b	0.0b	0.0b	0.0b	0.0b
15TLCC[s]	3.740a	0.0b	0.0b	0.0b	0.0b	0.0b
2KLCC[r]	1.975a	1.285b	1.134b	1.050b	0.900b	0.745c
3KLCC[r]	1.735a	1.414ab	1.337ab	1.155bc	0.895bc	0.750c
4SOCC[r]	2.115a	1.000b	0.903 b	0.880bc	0.865bc	0.780c
16TLCC[r]	2.015a	1.017b	0.957bc	0.940bc	0.833bc	0.800c
17KLFCA[r]	2.015a	1.639b	1.205c	1.145c	1.045c	0.725d
18KLFCA[r]	1.675a	1.105b	1.051b	1.057bc	0.925bc	0.790c
19KLTQ[r]	1.895a	0.968b	0.854bc	0.730cd	0.600de	0.490e
20KLFCA[r]	1.745a	1.140b	0.950b	0.880b	0.875c	0.655d
21KLFCA[r]	1.840a	1.739b	1.610b	1.095b	0.855b	0.655c

[s] Susceptible to benomyl.
[r] Resistant to benomyl.
Radial growth after 6 days of growth at 24 °C in 12-h light/dark regime. Each value is a mean of four measurements of each five plates. Values followed by the same letter within columns are not significantly different ($P < 0.01$) according to Tukey's test.

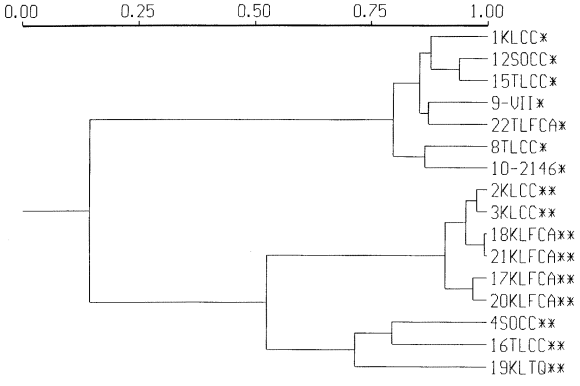

Figure 1. Dendogram of relationships between 16 isolates of *Colletotrichum* spp. of citrus based on UPGMA clustering of the matrix obtained by Dice coefficient. Two main groups: group I (*) and group II (**).

Depending on the isolate-primer combination, 7 to 23 DNA fragments were amplified, ranging in size from 250 to 4,500 bp, but only bands that stained strongly, sized between 350 to 2,500 were considered for analysis. A total of 283 fragments, 280 polymorphic and 3 monomorphic fragments was found. Most isolates could be fingerprinted by RAPD analysis. At a genetic similarity of 0.14, two distinct groups were distinguishable among the 16 *Colletotrichum* spp. isolates (Figure 1). Nine of the primers gave highly conserved and characteristic banding patterns giving fragments specific for a group (Table 4, Figure 2).

Genetic distance between Colletotrichum *spp. and* C. acutatum. A total of 304 fragments, 298 polymorphic and 6 monomorphic, were analyzed and two groups were obtained by the UPGMA method using Dice analysis. The first group contained the two isolates from group I of *Colletotrichum* spp. (10-2146 and 12SOCC) and the second group comprised the two isolates from group II of *Colletotrichum* spp. (18TLCC and 19KLTQ) and the isolate of *C. acutatum*. Similarity between these two groups was 0.13 (Figure 3).

Discussion

The RAPD analyses revealed polymorphisms among Brazilian isolates of *Colletotrichum* spp. and estab-

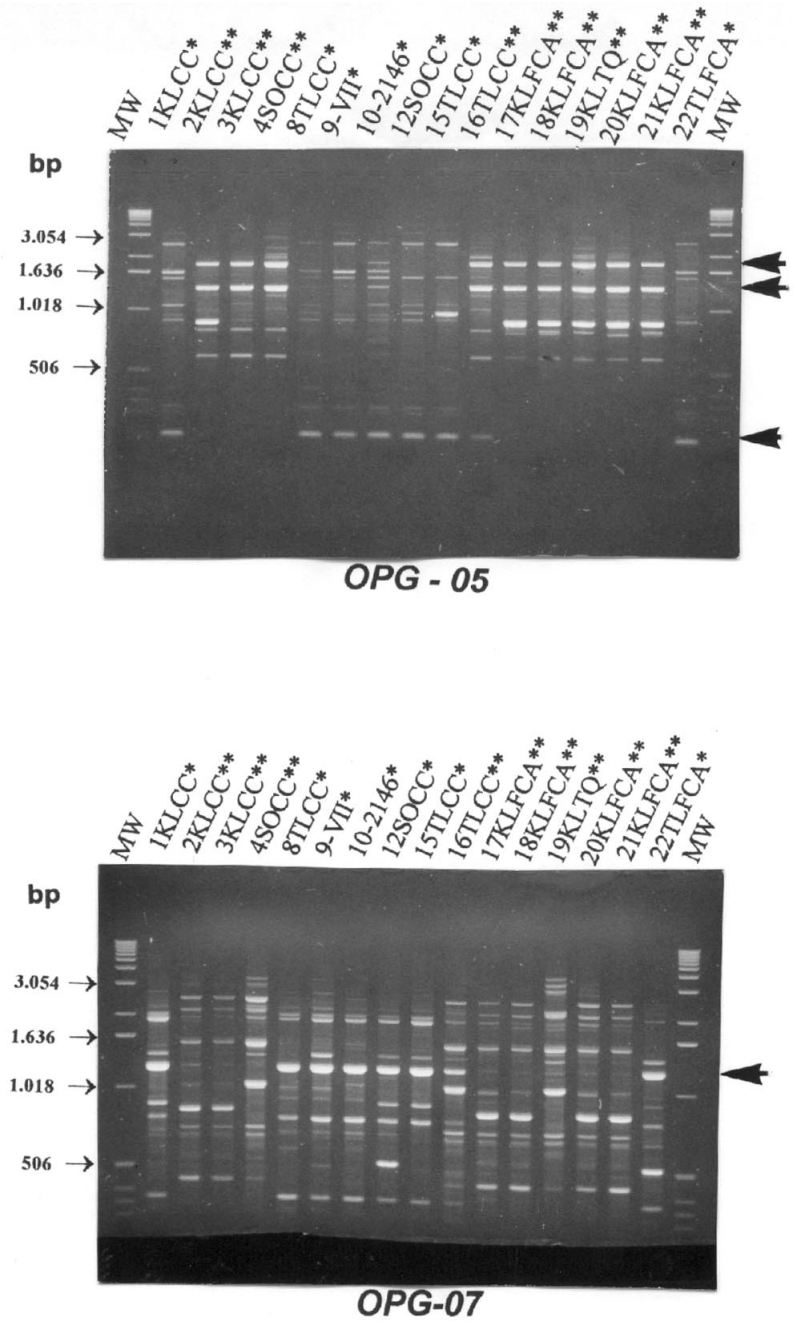

Figure 2. Example of amplification of fragments specific to groups I (∗) and II (∗∗) of *Colletotrichum* spp. isolates of citrus by OPG-05 and OPG-07 primers. MW = molecular marker 1 kb Ladder. Arrows mark group-specific bands.

lished DNA banding patterns useful for group characterization. Isolates were divided into two groups directly related to banding patterns, growth and benomyl resistance but not to pathogenicity and colony colour. Group I was composed of fast-growing isolates, susceptible to benomyl and group II comprised slow-growing isolates which were benomyl-resistant. *C. acutatum* was included in group II on the basis of RAPD banding patterns only. Liyanage et al. (1992, 1993) also found two genetically different popula-

Table 4. Group specific fragment lengths (bp) using nine different primers

Primer	Sequence 5′ to 3′	Group	Fragment length (bp)
OPB – 07	GGTGACGCAG	I	903
		II	531/1665
OPG – 02	GGCACTGAGG	I	1133
OPG – 05	CTGAGACGGA	I	1243/1839
OPG – 07	GAACCTGCGG	I	1040
OPG – 08	TCACGTCCAC	II	227/249
OPG – 09	CTGACGTCAC	I	792/843
		II	419/660/ 897
OPG – 13	CTCTCCGCCA	I	695/1878
		II	868
OPX – 04	CCGCTACCGA	I	1031/2225
		II	1374/3069/5150
OPX – 07	GAGCGAGGCT	I	694/718/1054
		II	1381

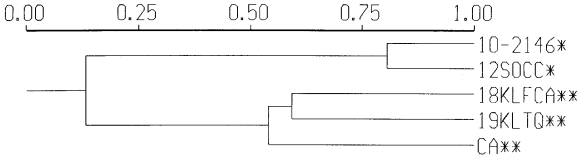

Figure 3. Dendogram of relationships between four isolates of *Colletotrichum* spp. of citrus (10-2146, 12SOCC, 18KLFCA, 19KLTQ) and one isolate of *C. acutatum* (CA) based on UPGMA clustering of the matrix obtained by Dice coefficient. Isolates are divided into two main groups: group I (∗) and group II (∗∗).

tions of *Colletotrichum* spp. in citrus from Florida, using molecular markers, ribosomal DNA, chromosome number and cutinase. Our group I corresponded to the FGG (fast-growing gray colony) and group II to the SGO (slow-growing orange) of *C. gloeosporioides* described by Sonoda and Pelosi (1988).

We could not distinguish the isolates by pathogenicity to detached whole flowers of Tahiti lime in contrast to Sonoda and Pelosi (1988) and Agostini et al. (1992). Because *C. gloeosporioides* colonizes senescent tissues, detached petals apparently have no resistance to infection by any of the strains. We used half the inoculum concentration that Agostini et al. (1992) used and the necrosis was first analyzed after 24 h, whereas the test of Agostini et al. was evaluated after 48 h. However in our study, the petals were blighted completely at 24 h and had dropped at 48 h. Controls showed no necrosis even 72 h after inoculation. Similar results were obtained by Severo et al. (1995) when 1×10^6 conidia/ml from isolates of *Colletotrichum* spp. gray and orange colonies from Brazilian citrus were inoculated onto detached whole flowers of Tahiti lime. Results of Liyanage et al. (1992) were similar to ours, but they applied 100-fold more spores to detached flowers.

We also observed some morphological variations such as colony colour within cultures derived from single spores. The colony colour instability of *C. gloeosporioides* was also observed by Von Arx (1957), Fagan (1979), Deham and Waller (1981), Baxter et al. (1985) and Sonoda and Pelosi (1988), with different coloured sectors being formed. Liyanage et al. (1992) also observed the presence of sectors in colonies from single-spore derived cultures and Agostini et al. (1992) observed differences in the amount of gray pigmentation of the mycelium. SGO isolates formed sectors with white mycelia, but radial growth remained the same with the original isolates.

RAPD analysis revealed that the second group of *Colletotrichum* spp. isolated from Brazilian citrus was genetically very close to the *C. acutatum* isolated from strawberry. Similar results, were obtained by Kuramae-Izioka (unpublished) by cleaving PCR-amplified ribosomal DNA internal transcribed spacers with different restriction enzymes.

Control of *Colletotrichum* spp. in Brazil is attempted by using the fungicide benomyl. However the group II isolates showed high levels of resistance to the fungicide and we also observed one-step mutations to benomyl in some isolates from group I (data not shown). This indicates that there are resistant isolates in Brazilian citrus orchards probably due to the fact that benomyl is one of the most commonly used fungicides in Brazil.

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