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Colletotrichum gloeosporioides, a new causal agent of citrus post-bloom fruit drop

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Abstract Citrus post-bloom fruit drop (caused by *Colletotrichum acutatum*) frequently occurs in the southwestern region of São Paulo State, Brazil. A survey of *Colletotrichum* isolates associated with symptoms of post-bloom fruit drop in São Paulo State showed *C. gloeosporioides* in addition to *C. acutatum*. The objectives of this study were to confirm the identification of *C. gloeosporioides* isolated from symptomatic citrus flowers, to test the pathogenicity of *C. gloeosporioides* isolates, to compare the development of disease caused by *C. gloeosporioides* and *C. acutatum*, and to determine the frequency of *C. gloeosporioides* in a sample of isolates obtained from symptomatic flowers in different regions of São Paulo State. Through the use of species-specific primers by PCR, 17.3% of 139 isolates were *C. gloeosporioides*, and the remaining 82.7% were *C. acutatum*. The pathogenicity tests, carried out in 3-year old potted plants of sweet oranges indicated that both species caused typical symptoms of the disease including blossom blight and persistent calyces. Incubation periods (3.5 and 3.9 days, respectively, for *C. acutatum* and *C. gloeosporioides*) and fruit sets (6.7 and 8.5%, respectively for *C. acutatum* and *C. gloeosporioides*) were similar for both species. The incidences of blossom blight and persistent calyces were higher on plants inoculated with *C. acutatum* than in those inoculated with *C. gloeosporioides*. Conidial germination was similar for both species under different temperatures and wetness periods. Under optimal conditions, appressorium formation and melanisation were higher for *C. gloeosporioides* than for *C. acutatum.* These results indicated that *Colletotrichum gloeosporioides* is a new causal agent of post-bloom fruit drop.

Keywords *Citrus* spp. · Epidemiology · Koch's postulates

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Introduction

Post-bloom fruit drop (PFD) caused by *Colletotrichum acutatum* is one of the most serious citrus diseases in the southwest region of São Paulo State, Brazil. Crop losses due to premature fruit drop may reach 93% in Brazil when flowering coincides with the rainy season (De Goes et al. 2008). All sweet orange varieties cultivated in São Paulo State are susceptible to this disease, and the disease is controlled by use of protectant fungicide sprays (De Goes et al. 2008). This disease was first reported in Belize and is restricted to the American continent (Fagan 1979; Timmer et al. 1994). The pathogen infects the petals of open citrus flowers, causing blossom blight and forming reddish-orange to orange-brown lesions. The pathogen forms salmon-pink acervuli on the lesion which expand rapidly in favourable weather. After petal fall, the newly formed fruit have a pale yellow discolouration and fall, but the calyx remains attached to the branch. This series of events describes the process from where the name "post-bloom fruit drop" was derived. The persistence of the calyces on the branch differentiates PFD from the physiological drop of citrus fruits (Timmer and Brown 2000).

Originally, PFD, post-harvest anthracnose and anthracnose in Key lime were attributed to *Colletotrichum gloeosporioides* (Simmonds 1965; Fagan 1979). Traditionally, *Colletotrichum* species from different hosts have been differentiated based on morphological and cultural characteristics (Sutton 1992). However, the influence of environmental factors on the stability of morphological and cultural features, the existence of intermediate forms and the lack of standardisation of the cultural conditions used in the different studies makes the morphological identification imprecise. The use of molecular tools such as PCR and species-specific primers to the ITS region of the rDNA, for species identification, demonstrated that *C. acutatum* is the causal agent of PFD and anthracnose in Key lime (Brown et al. 1996), whereas *C. gloeosporioides* caused exclusively post-harvest anthracnose. Other molecular tools are used currently to differentiate *Colletotrichum* species and to determine their variability (Sreenivasaprasad and Talhinhas 2005; Talhinhas et al. 2005; MacKenzie et al. 2009).

Recently, a survey of the incidence of PFD in São Paulo State identified *C. gloeosporioides* associated with blossom blight symptoms in sweet oranges by PCR. The objectives of this study were to confirm the identification of *C. gloeosporioides* isolated from citrus flowers with symptoms of PFD and to determine its frequency in a sample of isolates obtained from symptomatic flowers in different regions of São Paulo State; to test the pathogenicity of *C. gloeosporioides* isolates, to compare the effects of temperature and wetness on the development of *C. gloeosporioides* and *C. acutatum* isolates, and to compare the development of the disease caused by both *Colletotrichum* species.

Materials and methods

Isolation and identification

A total of 139 *Colletotrichum* spp. isolates were recovered from flowers of *Citrus sinensis* (L.) Osbeck and *C. latifolia* Tanaka with typical symptoms of blossom blight (Table 1). Flowers were collected from different orchards in São Paulo State, Brazil. The pathogen was isolated on potato dextrose agar (PDA) and the cultures were incubated at 25°C for seven days. From each isolate, a monosporic culture was made and preserved on filter paper at −12°C. DNA extraction from the monosporic isolates was completed by the method described by Junghans et al. (1998). The pellet

Table 1 Number of isolates of *Colletotrichum* sp., *Citrus* spp. where the isolates were obtained, county of origin and frequency of fungicide application in São Paulo State

County of origin	Host	Frequency of fungicide application	Number of isolates of *C. acutatum* [b]	Number of isolates of *C. gloeosporioides*
Barretos	*C. sinensis*	++[a]	34	0
Gavião Peixoto	*C. sinensis*	++	33	1
Taquarituba	*C. sinensis*	++++	32	0
Pedranópolis	*C. sinensis*	–	18	1
Piracicaba	*C. latifolia*	–	10	10

[a] – no fungicide application; ++ fungicide application depending on environmental conditions; ++++ high frequency of fungicide application

[b] – Two isolates from Barretos, Taquarituba, Pedranópolis, and Piracicaba, and four from Gavião Peixoto were used in pathogenicity tests.

was then resuspended in 50 µl of MilliQ water and 0.5 µl RNAse was added. The quantification of DNA was performed on a 0.8% agarose gel stained with ethidium bromide (1 µg/ml) and visually compared with the High DNA Mass Ladder (Invitrogen®, Paisley, United Kingdom) of known concentration. All of the samples were stored at −20°C. All monosporic isolates were identified by PCR using the universal primer ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990) coupled with species-specific primers *Ca*Int2 for *C. acutatum* (5′-GGG GAA GCC TCT CGC GG-3′) (Sreenivasaprasad et al. 1996) and *Cg*Int for *C. gloeosporioides* (5′-GGC CTC CCG CCT CCG GGC GG-3′) (Mills et al. 1992) to amplify a fragment that includes a 494- or 495-bp region containing ITS1, the gene encoding the 5.8S rRNA subunit, and ITS2. The reaction, which contained 100 ng of DNA, was carried out with 25 µl of 10X PCR buffer, 0.5 mM $MgCl_2$, 0.2 mM dNTP, 0.5 µM of each of the oligonucleotides and 0.04 U Taq DNA polymerase (Invitrogen®). Cycling parameters consisted of a 1-min denaturing step at 94°C. The denaturing step was followed by 30 cycles at 94°C for 1 min, 54°C for 1 min, and 72°C for 1 min and with a final single step at 72°C for 1 min. Amplification products were separated in agarose gels (1.5%, wt/vol) in 1× Tris-acetate EDTA buffer (40 mM Tris-acetate, 1 mM EDTA) subjected to electrophoresis at 100 V for 2 h and stained in ethidium bromide (1 µg/ml).

Morphological and cultural characterisation

The reaction of 12 isolates of each species to carbendazim was determined by transferring 0.5-cm diameter PDA disks containing mycelium to Petri dishes with PDA amended with carbendazim at 10 µg ml^{-1} and without carbendazim (control treatment). Five Petri dishes of each monosporic isolate were incubated at 24°C in the dark for seven days, at which point the colonies' diameters were measured. Conidial suspensions of each isolate from the control treatment were observed under optical microscope (1000 x), and the length and width of twenty conidia/replication were determined.

Pathogenicity assays

Pathogenicity tests were performed with 12 monosporic isolates from *C. acutatum* and 12 monosporic isolates from *C. gloeosporioides* (Table 1). The isolates were grown on PDA at 25°C for seven days. Blossoms from 96 potted plants of healthy 3-yr-old Valencia sweet oranges grafted on Rangpur lime maintained in a greenhouse were sprayed with a 25-ml suspension of 10^5 conidia ml^{-1} (four potted plants per isolate) and covered with moistened plastic bags for 48 h. Each cluster contained from 20 to 35 flowers in different stages of development. Controls were established in four potted plants of healthy Valencia sweet oranges by spraying distilled water on the clusters. The flowers of each blossom that remained on the plant after the removal from the humid chamber were counted and evaluated daily, for 15 days, for disease incidence (blossom blight). Incubation period of the disease was estimated as the number of days for the appearance of the first symptoms on the flowers. The percentage of fruits set and persistent calyces (buttons) were evaluated two months after inoculation based on the number of flowers originally present on the plant. The experiments were conducted as a completely randomised design with four replicates, and the experiment was carried out twice.

Effects of temperature and wetness period on the in vitro conidial germination and appressorium formation and melanisation

Colletotrichum acutatum and *C. gloeosporioides* isolates were grown on PDA for 7 days at 24°C prior to spore collection. Spore suspensions were prepared with sterile distilled water and adjusted to 10^5 conidia ml^{-1}. Three drops of a 30-µl conidial suspension of each culture were placed in polystyrene Petri dishes in sealed containers with moistened filter paper. The containers were incubated in growth chambers at 15, 20, 25, 30 and 35°C in the first experiment and at 12, 17, 22, 27 and 32°C in the second experiment for 12, 24, 48 and 72 h. After each interval, 10 µl of lactoglycerol was placed on each drop of the spore suspension, and conidial germination and appressorium melanisation were estimated. Five replicates were used per treatment.

The number of germinated conidia was counted by observing 100 conidia in each droplet under an optical microscope (400 x). The replications were incubated on a random basis at different temperatures.

Data analysis

Disease incidence (blossom blight and persistent calyces) and fruit set in plants inoculated with *C. acutatum* and *C. gloeosporioides* were compared via non parametric tests (dichotomous variables, $p=0.01$), according to the method of Zar (1999). Disease incubation periods from both species were compared

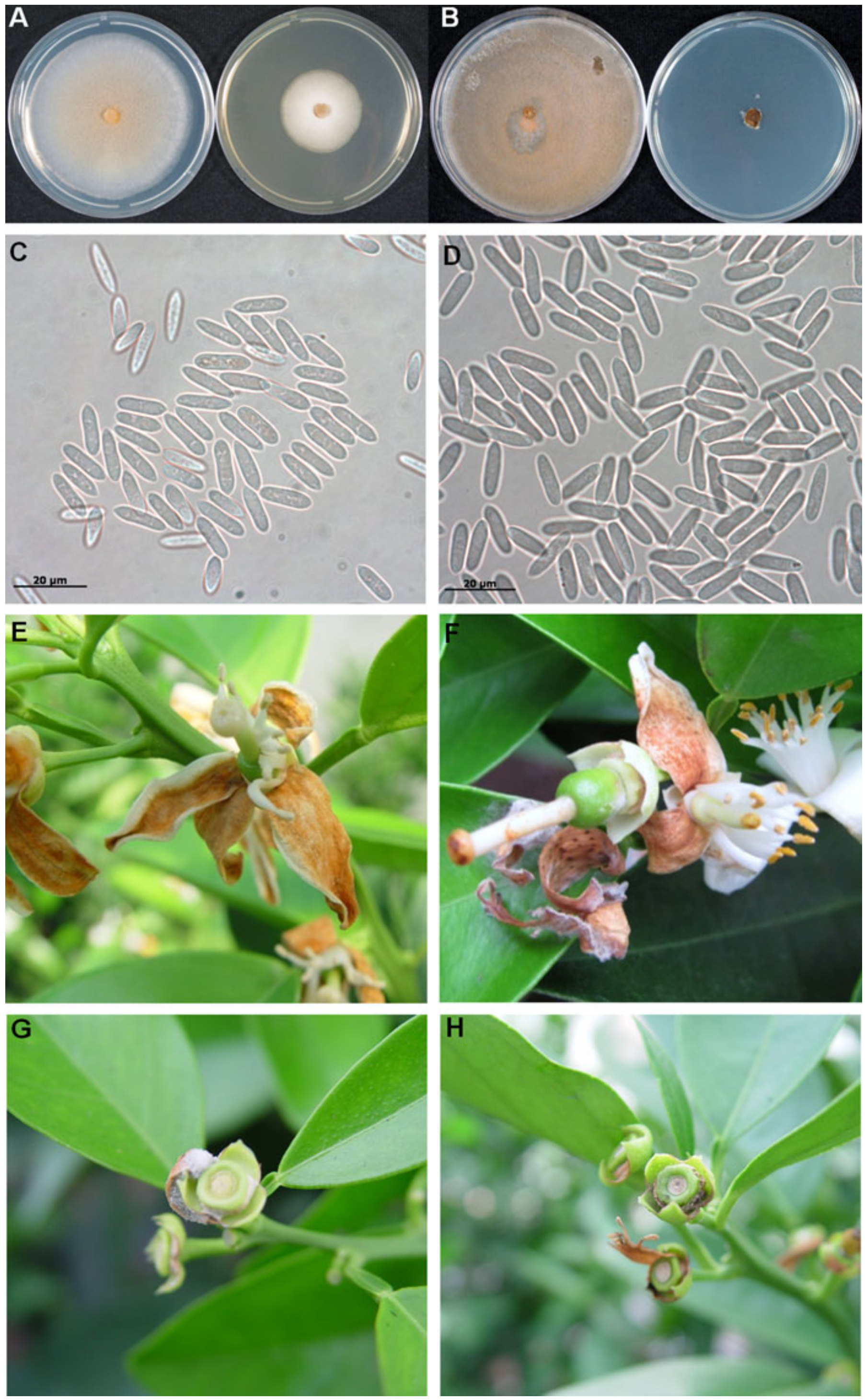

Fig. 1 Mycelial growth of *Colletotrichum acutatum* (**a**) and *C. gloeosporioides* (**b**) on potato dextrose agar amended with carbendazim (right) at 10 μg.ml^{-1} and without carbendazim (left); micrographs of conidial suspensions of *C. acutatum* (**c**) and *C. gloeosporioides* (**d**); symptoms of blossom blight (**e**) and persistent calyces (**g**) caused by *C. acutatum* and by *C. gloeosporioides* (**f**, **h**)

by an F-test after square root transformation. The pooled data from both experiments were combined for analysis.

An extension of the Beta function was fitted by non-linear regression to condial germination, appressorium formation and melanisation (Bassanezi et al. 1998; Christiano et al. 2009): $Y = Y_{opt}\ [(T\text{-}T_{min})/(T_{opt}\text{-}T_{min})]^{B1(Topt\text{-}Tmin)/(Tmax\text{-}Topt)}[(T_{max}\text{-}T)/(T_{max}\text{-}T_{opt})]^{B1}*[1\text{-}B_2*exp(-B_3*W)]$, in which Y is the independent variable, T_{min}, T_{max} and T_{opt} are the lowest, highest and optimal temperatures, and Y_{opt} is the maximum value of each variable. B_1, B_2 and B_3 are the parameters of the model, and W is the wetness period in hours. The shape parameter (B_1) influences the temperature range around T_{opt} in which the curve stays near Y_{opt}. The parameters were compared between themselves via a *t*-test. The software Statistica 7.0 (StatSoft Tulsa, OK) was used for the non-linear regressions.

Results

Isolate identification for *Colletotrichum* spp. using PCR, morphological and cultural characteristics

Of the 139 monosporic isolates, 115 were identified as *C. acutatum* and 24 as *C. gloeosporioides* via PCR (data not shown).

None of the isolates of *C. gloeosporioides* grew on PDA + carbendazim. All *C. acutatum* isolates formed colonies with a 2.9±0.1-cm diameter on PDA with carbendazim (10 ppm) after 7 days of incubation (Fig. 1a, b). Inhibition percentage of mycelium growth in PDA + carbendazim ranged from 51.9 to 57.6%. The length and width of conidia of *C. acutatum* ranged from 10.62 to 17.9 µm and from 3.41 to 3.97 µm, respectively (Fig. 1c). *C. gloeosporioides* conidia ranged from 11.83 to 18.00 µm in length and from 3.28 to 5.81 µm in width (Fig. 1d).

Pathogenicity assays

All isolates of *C. acutatum* and *C. gloeosporioides* inoculated on citrus blossoms caused blossom blight and persistent calyces (Fig. 1e-h). There was no significant difference in incubation period (3.5 and 3.9 days, respectively, for *C. acutatum* and *C. gloeosporioides*) or fruit set (6.7 and 8.5%, respectively, for *C. acutatum* and *C. gloeosporioides*) in plants inoculated with each pathogen (Fig. 2a, d). For both *Colletotrichum* species, two types of flower symptoms

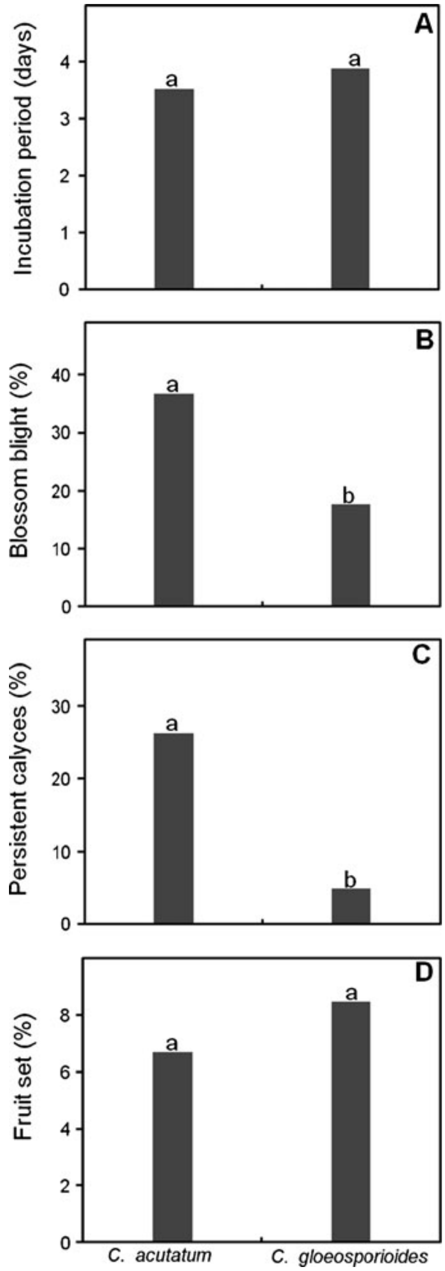

Fig. 2 Postbloom fruit drop incubation period in days (**a**); incidence of citrus blossom blight (percentage of symptomatic flowers 5 days after inoculation) (**b**); persistent calyces (percentage of persistent calyces 60 days after inoculation) (**c**); and fruit set (percentage of fruit set at 60 days after inoculation) (**d**) for *Colletotrichum acutatum* and *C. gloeosporioides* inoculated on flowers on potted plants. Control plants did not develop symptoms

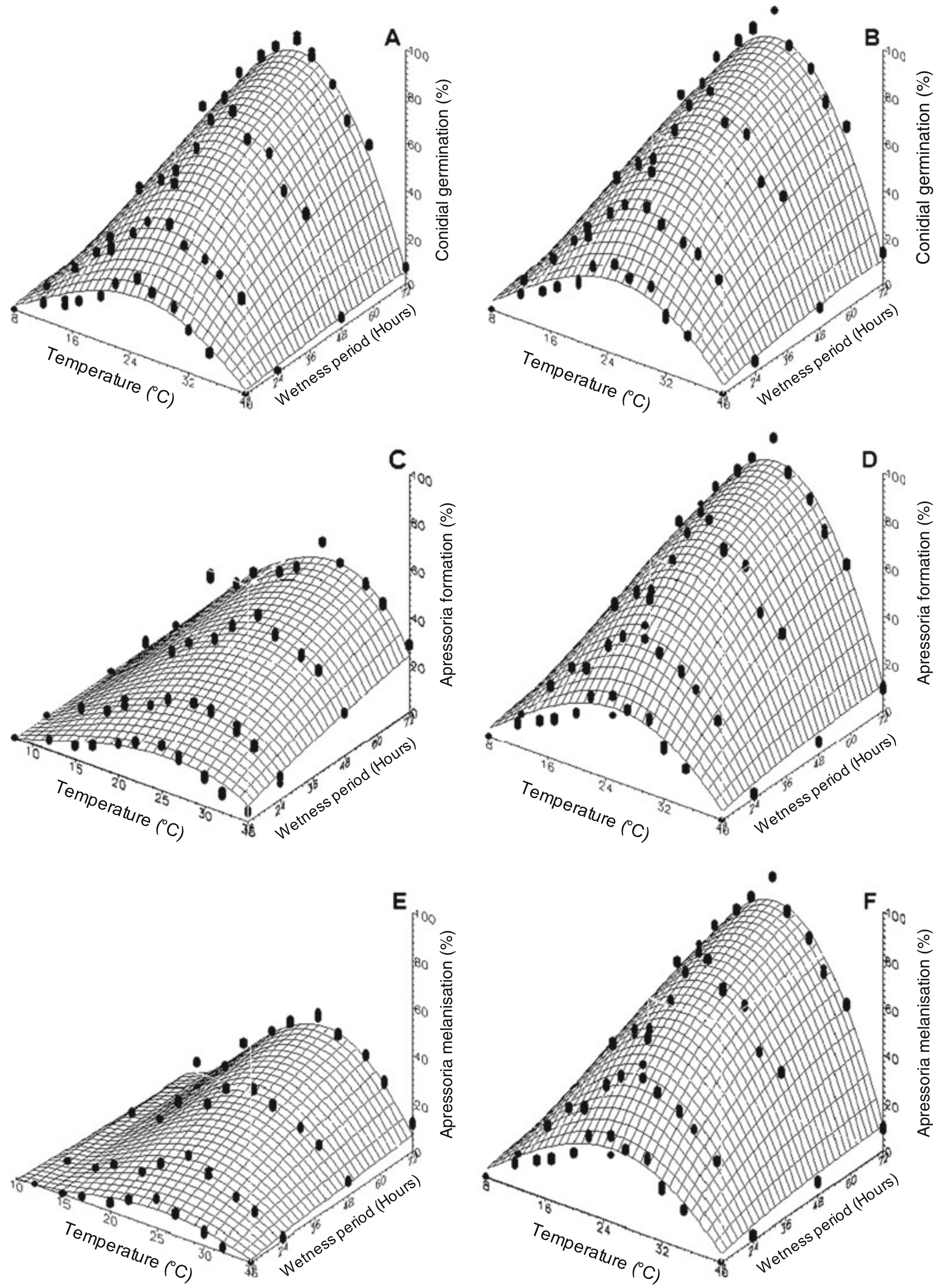

Fig. 3 Response surfaces for conidial germination (**a**, **b**), and formation (**c**, **d**) and melanisation (**e**, **f**) of the appressoria of *C. acutatum* (**a**, **c**, **e**) and *C. gloeosporioides* (**b**, **d**, **f**) as a function of temperature and wetness period described by the equation: $Y(T) = Y_{opt}[(T-T_{min})/(T_{opt}-T_{min})]^{B1(T\mathrm{opt}-T\mathrm{min})/(T\mathrm{max}-T\mathrm{opt})}[(T_{max}-T)/(T_{max}-T_{opt})]^{B1}*[1-B_2*exp(-B_3*W)]$, in which *Y(T)* is conidial germination and formation and melanisation of the appressoria as a function of temperature, *Tmin*, *Tmax* and *Topt* (low, high and optimal, respectively) for conidial germination or formation or melanisation of the appressoria, and *Yopt* is the maximum value of each variable. *B*1 is a shape parameter that influences the temperature range around *T*opt in which the curve stays near *Y*opt. *B2* and *B3* are equation parameters and *W* is the wetness period (hours). Note that X-axis scales in C and E are different from the others

were observed depending on the blossom stage: buds that were still closed at inoculation had blossom blight and anthesis did not occur, the flowers fell with the calyx and peduncle, consequently, persistent calyces were not formed; open flowers at inoculation showed blossom blight with pinkish-orange necrotic lesions and acervuli, followed by persistent calyces. No symptoms were observed on the flowers of the control plants. The pathogens were re-isolated from the petal lesions when they were still attached to the plants. PCR tests confirmed that *C. gloeosporioides* was present on symptomatic flowers inoculated with *C. gloeosporioides* isolates and *C. acutatum* was present on the flowers inoculated with *C. acutatum* isolates. Blossom blight incidence was higher (Fig. 2b) on plants inoculated with *C. acutatum* (36.8%) than in those inoculated with *C. gloeosporioides* (17.6%). The incidence of persistent calyces (Fig. 2c) was also higher for *C. acutatum* (26.2%) than for *C. gloeosporioides* (4.9%).

Effects of temperature and wetness period on in vitro conidial germination and appressorium formation and melanisation

Conidial germination of *C. gloeosporioides* and *C. acutatum* increased as wetness duration increased even at high temperatures (Fig. 3). There were no significant differences in conidial germination of the two *Colletotrichum* species. However, the formation and melanisation of the appressoria of *C. gloeosporioides* occurred at higher levels and over a wider range of temperatures than those of *C. acutatum* (Fig. 3 and Table 2).

Discussion

We concluded that there are two distinct species of *Colletotrichum* causing PFD in citrus: *C. acutatum* and *C. gloeosporioides*. The pathogenicity tests showed that both species of *Colletotrichum* cause blossom blight and premature fruit drop with retention of the calyces on the plant. In anthracnose pathosystems, frequently the same host is infected by different *Colletotrichum* species, and the same pathogen can infect different hosts (Peres et al. 2002a). The pathogen population exhibited high levels of biological variability as exemplified by *C. gloeosporioides* and *C. acutatum* (Sreenivasaprasad and Talhinhas 2005). Until now, PFD was attributed exclusively to *C. acutatum*, whereas *C. gloeosporioides* causes post-harvest anthracnose in citrus fruits and can be found epiphytically on leaves and flowers

Table 2 Parameters and coefficients of determination (R^2) of a generalised Beta function multiplied by the monomolecular model fitted to conidia germination or formation and melanisation of the appressoria of both *C. gloeosporioides* isolates (CG) and *C. acutatum* isolates (CA): $Y(T) = Y_{opt}[(T\text{-}T_{min})/(T_{opt}\text{-}T_{min})]^{B1(Topt\text{-}Tmin)/(Tmax\text{-}Topt)}[(T_{max}\text{-}T)/(T_{max}\text{-}T_{opt})]^{B1}*[1\text{-}B_2*exp(-B_3*W)]$, in which $Y(T)$ is conidial germination, and formation and melanisation of the appressoria as a function of temperature, T_{min}, T_{max} and T_{opt} (low, high and optimal, respectively) for conidial germination and formation and melanisation of the appressoria, and Y_{opt} is the maximum value of each variable. B_1 is a shape parameter that influences the temperature range around T_{opt} in which the curve stays near Y_{opt}. B_2 and B_3 are equation parameters, and W is the wetness period (hours)

Variables	Parameters of the equations							
	Y_{opt}	T_{min}	T_{opt}	T_{max}	B_1	B_2	B_3	R^2
Germination								
CG	100.00	5.72[ns]	25.45[ns]	41.15[ns]	1.08	0.98	0.03	0.98
CA	100.00	6.31	25.30	40.69	1.12	1.02	0.02	0.98
Appressoria								
CG	100.00	6.64[ns]	25.36[a]	39.46[b]	0.93	0.99	0.03	0.98
CA	84.52	7.92	26.32	36.57	0.65	0.96	0.01	0.97
Melanization								
CG	100.00	6.32[b]	25.30[ns]	37.21[ns]	0.81	0.99	0.03	0.98
CA	64.56	12.00	25.66	36.39	1.26	1.03	0.01	0.98

[ns] not significant, [a] significant at $P<0.05$ and [b] significant at $P<0.01$, by *t*-test.

(Brown et al. 1996; Peres et al. 2005). It is possible that epiphytic *C. gloeosporioides* isolates shift into being pathogenic in São Paulo, Brazil. The isolates of *C. gloeosporioides* that cause PFD are apparently less aggressive than those of *C. acutatum*. Disease incidence, assessed by blossom blight or by persistent calyces, was lower when plants were inoculated with *C. gloeosporioides* than with *C. acutatum*. This behaviour could explain the limited occurrence of *C. gloeosporioides* isolates (17.3%) in the survey carried out in citrus-producing regions from the State of São Paulo. The prevalence of *C. acutatum* (82.7%) was similar to that observed for *Colletotrichum* spp. isolates from various olive-growing areas in Portugal using the same molecular tools (Talhinhas et al. 2005). The differences in proportions of *Colletotrichum* species in citrus orchards may also be related to the chemical control of the disease. Benzimidazole-based fungicides are applied commonly in São Paulo State to control PFD. However, only *C. gloeosporioides* is sensitive to these products in vitro. This difference in sensitivity has been helpful to differentiate these species (Peres et al. 2005). In spite of the small sample size, all isolates of *C. gloeosporioides* found in this study were from orchards without or with low frequency of fungicide application (Table 1). This control practice could be acting upon the *C. gloeosporioides* population, thus reducing its presence in the field.

To show the differences between *Colletotrichum* species isolated from flowers with PFD symptoms, different cultural and molecular tools have been used. The species-specific primers for *C. acutatum* and *C. gloeosporioides* have also been used in several studies for identification of populations of these species that are affecting a variety of host plants (Sreenivasaprasad et al. 1996; Talhinhas et al. 2002, 2005).

Some post-bloom fruit drop symptoms are induced by plant hormones (Li, et al, 2003). Natural citrus fruit abscission usually occurs at the base of the peduncle at the attachment to the stem. However, with post-bloom fruit drop, abscission is at the base of the fruit leaving persistent calyces and peduncles (Peres et al. 2005). Our results indicated that the disease identification should not be based exclusively on the presence of persistent calyces because flower infection before anthesis by *C. acutatum* or *C. gloeosporioides* can result in necrosis followed by abscission of the calyx. Thus, the identification of the disease should also take into account symptomatic flowers.

Optimal temperatures for conidial germination were similar to those favourable for appressorium formation and melanisation for both species. These results indicated that both species have similar environmental requirements. The similar in vitro behaviour of these species suggest that the forecast PFD-FAD system developed for *C. acutatum* (Peres et al. 2002b) could be used in preventing the disease caused by *C. gloeosporioides*. However, the intensive use of benzimidazole fungicides could select resistant *C. gloeosporioides* isolates quickly (Peres et al. 2002c).

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