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Colletotrichum abscissum: Detection on symptomless citrus leaves and symptomatic citrus flowers using high-resolution melting analysis

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High-resolution melting (HRM) analysis has been recently applied for the diagnosis of plant diseases. This cost-effective method is advantageous over standard PCR as it does not require

DNA band visualization or the use of probes necessary for quantitative PCR. *Colletotrichum abscissum* causes postbloom fruit drop of citrus (PFD) and survives asymptomatically on vegetative tissue. Quiescent appressoria on citrus leaves are the primary source of inoculum between flowering periods. Early PFD symptoms may be misidentified as physical damage on citrus flowers. Our objectives were to validate an HRM-based method to rapidly identify *C. abscissum* on citrus leaves and flowers. We screened seven previously published primer pairs and concluded that the most effective set of primers was CaITS-F and CaITS-R815. We evaluated three different DNA extraction methods, two with a purification step (DNeasy Plant Mini and PowerSoil kits) and a faster method without a purification step (buffer GEB2). We inoculated citrus leaves with *C. abscissum* conidial suspensions of varying concentrations and HRM detected as few as 200 *C. abscissum* spores using DNeasy Plant Mini or DNeasy PowerSoil kits for DNA extraction. As expected, samples extracted with GEB2 buffer reduced HRM sensitivity. We further collected field samples from areas with high and low *C. abscissum* inoculum levels. The HRM method could detect the pathogen in high inoculum areas from both leaves and flowers regardless of the extraction method used. In summary, we describe a new molecular diagnostic tool to detect *C. abscissum* on citrus during its asymptomatic and symptomatic stages.

Keywords

appressoria, asymptomatic detection, *Colletotrichum acutatum*, *Colletotrichum gloeosporioides*, inoculum detection

1 Introduction

Colletotrichum abscissum and *C. gloeosporioides* cause postbloom fruit drop of citrus (PFD) in tropical and subtropical regions of the Americas (Lima et al., 2011; McGovern et al., 2012; Pinho et al., 2015). *C. abscissum* belongs to the *C. acutatum* species complex and is the most prevalent species causing PFD (Silva et al., 2017). *C. gloeosporioides* accounted for 24% of PFD isolations in a recent survey in Brazil (Silva et al., 2017) and has been found in Bermuda (McGovern et al., 2012). Although *C. gloeosporioides* was never shown to be the causal agent of postbloom fruit drop, *C. gloeosporioides* is commonly isolated from citrus flowers in Florida, especially from decaying flowers. *C. gloeosporioides*, besides causing PFD and anthracnose, is a common fungal epiphyte on citrus depending on environmental conditions and host tissue availability (Brown et al., 1996; Lima et al., 2011). Typical PFD symptoms on citrus flowers are characterized by peach-to-brown lesions on petals and filaments (Timmer et al., 1994). PFD-affected flowers develop into fruit, but these fall off trees prematurely. PFD may cause severe yield losses of up to 100% under favourable environmental conditions, but may not be a problem for several seasons, especially in Florida (Dewdney, 2017; Timmer & Zitko, 1993). When flowers are not present, the pathogen survives on leaves and twigs for long periods without visible symptoms (Zulfiqar et al., 1996). Besides favourable weather, high inoculum levels of *C. abscissum* also represent an increased risk for severe PFD outbreaks (Peres et al., 2002; Timmer & Zitko, 1993).

As *C. abscissum* does not cause symptoms on leaves, it is difficult to detect and/or quantify pathogen inoculum in citrus groves (MacKenzie et al., 2010; Peres et al., 2005). In addition, natural flower senescence or lesions caused by abiotic factors such as physical damage may be mistakenly identified as early PFD symptoms to those not familiar with the disease or

with the citrus crop (Timmer, 1993). Pathogen isolation from asymptomatic leaves is difficult and laborious (MacKenzie et al., 2010), so molecular techniques could be a viable option to detect *C. abscissum* on vegetative tissue. Molecular techniques could also be valuable tools in future efforts to provide certification for citrus nurseries assuring their plants are free of, or do not present high levels of, *C. abscissum* inoculum (Ghosh et al., 2018, 2020). Additionally, a fast, cost-effective and high-throughput technique could greatly aid plant disease diagnosticians to identify PFD on flowers.

Although it has been reported that *C. acutatum* introduction from strawberry nurseries occurs (Peres et al., 2005), there is less information regarding *C. abscissum* movement from citrus nurseries to citrus groves. Recently, a quantitative PCR (qPCR)-based method to detect *C. abscissum* on citrus leaves was developed in Brazil (Pereira et al., 2019). Researchers detected *C. abscissum* DNA in 73% of the surveyed nursery samples collected from budwood increase blocks using a qPCR-based diagnostic method (Ciampi-Guillardi et al., 2021). However, they were not able to isolate the pathogen from the *C. abscissum*-positive citrus samples due to the difficulty of isolation from symptomless leaves (Muñoz, 2018). New molecular techniques could aid in pathogen detection on nursery trees and identify the pathogen during its asymptomatic interaction with citrus leaves that does not result in host colonization (Mackenzie et al., 2010; Savi et al., 2019).

DNA-based methods for pathogen detection have been developed in the last 20 years and used by many plant pathologists (Kang et al., 2010; Kokane et al., 2020; Motghare et al., 2018). The HRM technique was introduced in 2002 by a collaboration between the University of Utah and industry (Reed et al., 2007) and, since then, it has been used for genetic studies in humans. In agricultural sciences, this high-throughput method was initially used for various planting

breeding programs (Lee et al., 2012, 2017; Taheri et al., 2017), and recently has been deployed to detect, among other species, *C. acutatum* and *C. gloeosporioides* from strawberry plant without the requirement of using a probe for pathogen detection, as is the case for qPCR (Forcelini et al., 2018; Rahman et al., 2019; Wang et al., 2021).

As HRM is a simple, fast and efficient molecular method for species differentiation, it could be adapted for citrus plants by using the correct DNA extraction method and set of primers for specific pathogens, such as *C. abscissum*. PCR can be combined with the HRM technique in the same system, which can evaluate 384 samples simultaneously (Vossen et al., 2009). In this system, DNA extraction is performed separately before PCR, and DNA analysis is performed in a closed-tube assay by adding a dye before PCR, without requiring DNA separation by electrophoresis. Currently, there are different dyes available for such a purpose, and one of them is LC-Green Plus (Idaho Technology Inc.), which fluoresces in the presence of double-stranded DNA.

PCR products of samples are heated through a range of temperatures to generate a melting curve. The fluorescence is measured and is stronger at low temperatures. As the temperature increases in the reaction, the fluorescence slowly declines due to the denaturation of double-stranded DNA into single strands (Reed et al., 2007; Vossen et al., 2009). Because different genetic sequences melt at slightly different rates, the resulting melting curves can be used to separate different pathogen species.

The use of HRM analysis for application in citrus has been previously reported. An HRM-based method was developed for genotyping *Alternaria* spp. pathogenic to citrus using genomic DNA (Garganese et al., 2018). HRM analysis is also used for citrus breeding, aiding in

the screening for host resistance to *A. alternata* (Arlotta et al., 2020). Distefano et al. (2012) used the HRM technique to discriminate citrus cultivars and concluded that this method is more efficient than traditional electrophoresis-based approaches. Therefore, this technique has been used in citrus and other hosts affected by *Colletotrichum* spp., which shows its potential to detect *C. abscissum*.

Recently, good results have been obtained from crude DNA extracts used in HRM analysis (Reed et al., 2007; Wang et al., 2021). Crude DNA extracts do not go through any purification steps during extraction and may contain PCR inhibitors. It is important to note that HRM analysis using crude DNA samples becomes even faster, easier and cheaper than other molecular techniques because of the exclusion of DNA purification before PCR (Miles et al., 2015; Xin et al., 2003). However, leaf tissues contain metabolites that can interact with enzymes such as DNA polymerases, inhibiting their biological function. Consequently, PCR on crude DNA samples may not be possible due to complete absence of amplification of target sequences (Lee et al., 2017). The presence of such extracts may also interfere with the sensitivity of the method. Due to this difficulty, some studies have been conducted to improve the DNA extraction from crude samples (Han et al., 2010; Lee et al., 2017; Wang et al., 2021; Xin et al., 2003). Even if a purification step is followed, HRM has advantages over qPCR and traditional PCR, such as not requiring the use of probes or separation by electrophoresis (Reed et al., 2007).

Given the potential of asymptomatic citrus leaves from nursery trees to act as *C. abscissum* inoculum source to initiate PFD epidemics in orchards, and the need to screen these plants in a fast and reliable way, our objective was to adapt, develop and test HRM for the detection of *C. abscissum* on asymptomatic citrus leaves, where the fungus survives as appressoria, using crude and purified DNA samples. We also aimed to validate the technique by

collecting citrus leaf and flower samples in the field, extracting DNA with or without a purification step, and possibly detecting *C. abscissum*.

2 Materials and methods

2.1 Isolates and genomic DNA extraction

C. abscissum and *C. gloeosporioides* isolates were collected from citrus flowers, leaves and fruit in Brazil and the USA. Some strawberry isolates were used as a reference as they were used in previous work to develop the HRM technique for diagnosis of strawberry diseases (Table 1; Forcelini et al., 2018; Wang et al., 2021). Other fungal, oomycete and bacterial species that are commonly associated with citrus plants as either endophytes or pathogens were also used, to test primer specificity (Table 1). Genomic DNA extracted from citrus leaves was also tested against primer specificity (Table 1). Species identifications were previously done by conventional PCR (Hu et al., 2014; Lima et al., 2011; Silva et al., 2017; Wang et al., 2019). Single-spore cultures of the isolates were maintained on filter paper in a sterile envelope with silica gel at −20°C. Isolates were transferred to potato dextrose agar (PDA) and grown at room temperature (about 24°C) under constant light for 7 days until DNA extraction.

One hundred milligrams of fungal mycelia was collected from each plate for DNA extractions. Genomic DNA of bacterial pathogens was previously extracted by the staff of the Citrus Research and Education Center (Lake Alfred, FL, USA). Extractions were performed using the FastDNA kit (MP Biomedicals, LLC). All tested and control DNA samples were quantified by NanoDrop 8000 spectrophotometer (Thermo Scientific) and diluted to a final concentration of 10 ng/µl. Diluted DNA was stored at −20°C until further use. DNA samples of

C. abscissum were diluted to 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 100 fg/µl. The serially diluted samples were used to determine the sensitivity of the HRM assays using the set of primers AcG1-F1 and AcG1-R1, and CaITS-F and CaITS-R815 (Table 2).

2.2 Primers and crude DNA extraction

We tested five primer sets originally developed for the detection of *C. acutatum* sensu lato and two developed for *C. gloeosporioides* sensu lato (Table 2). All primer sets were used on genomic DNA by HRM analysis. For crude DNA extraction, approximately 0.1 g of asymptomatic leaf tissue from each sample was taken and put in a firm plastic bag (16 × 10 cm). One millilitre of ELISA grinding buffer GEB2 (Agdia, Inc.) was added to the bag. The bag contents were macerated with a mortar to obtain an extract (Miles et al., 2015). The final volume of the extract (approximately 800 µl) was transferred to a microtube and stored at −22°C until required.

2.3 PCR and high-resolution melting analysis

PCR amplification and HRM analysis were performed in a total sample volume of 10 µl with the LightCycler 480 System II (Roche Life Science). Each reaction contained 5 µl of 2× AccuStart II PCR ToughMix (QuantaBio), 0.5 µl of 10 µM of each forward and reverse primer, 0.5 µl of 10× LCGreen Plus Dye (BioFire Defense), 2.5 µl of molecular water and 1 µl of a 10 ng/µl sample of genomic DNA or 1 µl of crude plant DNA extract. The crude DNA extract was diluted with molecular biology-grade water (1:10) before PCR as this dilution previously led to significantly higher fluorescence signals from crude DNA samples extracted with GEB2 due to the decrease in PCR inhibitors (Wang et al., 2021).

All PCR and HRM assays were set up in 384-well plates (LightCycler 480 Multiwell Plate 384, White). PCRs were developed following the protocol of Forcelini et al. (2018) and

Wang et al. (2021), as described below. Each DNA sample was preincubated at 95°C for 3 min; followed by 35 cycles of denaturation at 95°C for 20 s, annealing at 60°C for 30 s and extension at 72°C for 30 s. HRM conditions were: 95°C for 1 min, 40°C for 1 min, 6 °C for 1 s and 95°C continuously. Afterward, DNA samples were cooled at 40°C for 30 s (Forcelini et al., 2018; Wang et al., 2021). HRM analyses were conducted with three technical replications per sample, and each sample generated a melting curve that was analysed using the LightCycler 480 system II melting curve software. Each experiment was performed twice.

2.4 HRM analysis using genomic DNA for assay specificity and sensitivity

Experiments using genomic DNA were conducted for different purposes. Initially, we tested the performance of primers on PCRs using DNA samples of single isolates. After these initial experiments, the same concentration of DNA from *C. abscissum* and *C. gloeosporioides* isolates were mixed, as both are common fungi surviving on leaf surfaces. After the verification of each primer set, we tested the sensitivity of primers to detect *C. abscissum* in samples containing different DNA concentrations. DNA samples were diluted as previously described in this section to 10 ng/µl, 1 ng/µl, 100 pg/µl, 10 pg/µl, 1 pg/µl and 100 fg/µl. Additional specificity of the selected primer set was evaluated using pure cultures of other citrus pathogens and saprophytic isolates (Table 1). Again, three technical replications were performed per sample, and experiments were conducted twice.

2.5 HRM analysis using genomic DNA mixed with crude citrus leaf extracts

Crude citrus DNA extracts were prepared using GEB2 and following the protocol described above for crude DNA samples. The crude plant extract was mixed with pure genomic DNA to test the sensitivity of the method in the presence of host metabolites.

2.6 HRM analysis using DNA extracted by different methods

Newly expanded citrus leaves (cultivar Valencia) were wiped with sodium hypochlorite (0.6%), 70% ethanol and sterile distilled water 1 day before inoculation. Twenty microlitre droplets of *C. abscissum* suspensions of 0, 10^4, 10^5, 10^6 and 5×10^6 spores/ml were placed in previously marked 1 cm^2 sites on the freshly expanded leaves of citrus plants located in a greenhouse. The isolate used to prepare the suspension was OCO-ARC-4 (Peres et al., 2004). The calculated number of spores placed in each droplet based on each concentration was, respectively, 0, 200, 2,000, 20,000 and 100,000 spores. Three leaves were inoculated per spore concentration in each experiment. The droplets were left to dry naturally in the greenhouse for about 2 h. Once the droplets were dry, leaves were sprayed with sterile distilled water, bagged and kept under moist chamber conditions for 48 h. After that, leaf discs were excised from the plants and placed into vials for DNA extraction.

Three extraction methods were tested: DNA extraction using the GEB2 buffer, as described above; purified DNA extraction using the DNeasy PowerSoil kit (Qiagen) and following the protocol provided by the manufacturer; and purified DNA extraction using the DNeasy PlantMini kit. Crude DNA extracts obtained with GEB2 buffer were diluted (1:10) as previously described. After DNA extraction, purified DNA samples were obtained using the DNeasy PowerSoil and DNeasy PlantMini extraction kits. The quality and concentration of the purified DNA samples were obtained by spectrophotometry by absorbance at 260/280 nm using a Nanodrop 1000 (Thermo Scientific). After DNA samples were obtained, HRM analyses were carried out to evaluate the presence and the strength of the fluorescence peaks corresponding to each sample. The primer pair used in this step was CaITS-F/CaITS-R815 (Table 2). This

experiment was conducted twice (two different PCR reactions done with samples extracted at different times) with three technical replications per experiment.

2.7 HRM analysis using citrus leaves and flower samples collected from commercial citrus groves

Leaves and flowers were collected from two commercial Valencia citrus orchards in Fort Meade, FL, USA in 2021. Twenty leaves and 20 flowers were collected at each location, totalling 80 samples. For each location, 10 leaf and 10 flower samples were extracted by the GEB2 buffer method or the DNeasy PowerSoil extraction kit protocol as described above. Leaf and flower samples were collected in the commercial orchards following a zigzag pattern. The two orchards were approximately 10 km apart and have different histories of PFD occurrence. The flowers of one orchard were showing severe symptoms of PFD during an early bloom, and in the other, very few flowers with PFD symptoms were present. In addition, PFD incidence on trees was much higher in the field with severe PFD symptoms (Gama et al., 2022). As these fields are in close proximity, our hypothesis was that disease inoculum levels were the underlying factor for differences in symptom severity and incidence, as inoculum levels are one of the most important factors for differences in observed PFD levels (Timmer & Zitko, 1993). The orchard with higher disease levels was planted in 1994, whereas plants in the field with fewer symptoms were planted in 2007, and possibly inoculum built up to more significant levels throughout the years in the older orchard. The area with the older plants is henceforth referred to as the high inoculum area, whereas the field with the newer trees is referred to as the low inoculum area.

A 2.5 cm^2-piece of each sample, corresponding to approximately 0.1 g of the sample, was removed from the leaves to prepare crude DNA extract for subsequent PCR and HRM analysis.

No surface sterilization was performed on leaf samples because *C. abscissum* survives on leaf surfaces. The remaining leaf tissue was stored at 4°C until required for pathogen isolation if the sample was positive for *C. abscissum* according to the analysis. Upon detection by the HRM analysis, leaves from that specific sample were retrieved and cut into one additional 2.5 cm^2 strip each. Twenty microlitre droplets of citrus floral extracts (Gama et al., 2021) were transferred and spread with a plastic rod onto the leaf samples to induce appressorial and conidial formation and germination to facilitate *C. abscissum* isolation. Briefly, purified citrus flower extracts were obtained by freeze-drying flower samples for 3 days, grinding them into powder, extracting the samples with water and vacuum filtering them. Following floral extract application, leaves were kept on plate dishes with moistened filter paper for 24 h at room temperature. After that period, the leaf strips were placed in 10 ml Falcon tubes with sterile distilled water, vortexed for 1 min, and 200 µl of the resulting suspension was transferred to 100 mm-plates with *C. abscissum* isolation medium adapted from MacKenzie et al. (2010). *C. abscissum*-like colonies were transferred to half-strength PDA, single-spored, preserved on filter paper and kept in sterilized envelopes at −20°C. For symptomatic flower samples, mucilage of PFD lesions was transferred directly to the selective medium. No isolation was attempted on asymptomatic citrus flowers.

2.8 Statistical analysis

Generalized linear mixed models were fit to peak relative −(d/dT) fluorescence values data using PROC GLIMMIX of SAS (v. 9.4; SAS Institute) with a normal distribution, based on pattern of residuals and assuming normality due to our sample size. DNA extraction method, inoculum concentration (at sample inoculation, not after dilution following crude DNA extraction), plant tissue used for extraction and sampling location (high or low inoculum area) were considered fixed effects, while experiment and treatment replications were considered random effects. The

interaction between inoculum concentration and DNA extraction method was considered as fixed effect for the comparison of extraction methods. Fixed effects were considered significant at $\alpha < 0.05$. Significant differences were assessed using the LSMEANS statement of SAS. Data visualization was performed in the LightCycler 480 software and using the R software (v. 4.04) and the *ggplot2* package (Wickham, 2016).

3 Results

3.1 HRM analysis using genomic DNA for assay specificity and sensitivity

Different melting temperatures (Tm) and melting curve shapes were observed from HRM primers AcG1-F1 and AcG1-R1 (Table 2) which identified and differentiated citrus isolates of *C. abscissum* from *C. gloeosporioides* (Figure 1a). HRM performed with these primers generated two distinct melting curves with melting temperatures of about 88°C and 86°C for *C. abscissum* and *C. gloeosporioides* isolates, respectively (Figure 1a). When AcG1-F1 and AcG1-R1 were used, HRM analysis could reliably detect up to 1 pg/µl of genomic DNA from *C. abscissum* and *C. gloeosporioides* isolates, although we detected a weak fluorescence peak in 100 fg/µl DNA samples (Figure S1).

Using the AcG1-F1 and AcG1-R1 primers, when purified DNA of *C. abscissum* was mixed with *C. gloeosporioides* DNA in the same reaction, the melting curves of the mixed DNA showed the same profile as the curve resulting from HRM in *C. gloeosporioides* DNA alone (Figure 1b). The melting curve of *C. abscissum* species complex was identified in the HRM analysis when only *C. abscissum* DNA was in the reaction. As *C. gloeosporioides* is not the main

target of our assays, we decided to test different primers (Table 2) that could identify *C. abscissum* DNA when it is mixed with *C. gloeosporioides* DNA.

Some primer sets showed limitations to detect genomic DNA of the PFD pathogens as soon as the first assays were carried out. The pairs of primers ACUT-F1 and ACUT-R2, CaInt2 and ITS4 and CaITS-F and CaITS-R699 (Table 2) were not chosen because when *C. abscissum* DNA was mixed with *C. gloeosporioides* DNA, the resulting melt curve showed an inflexion similar to those referring to *C. gloeosporioides* DNA alone in the reaction (data not shown). HRM analysis did not differentiate whether there was only *C. gloeosporioides* in the mixture or *C. abscissum* in addition to *C. gloeosporioides* DNA.

The primer pair which best identified the *C. abscissum* species complex was CaITS-F and CaITS-R815 (Table 2), showing a melting curve with one peak in HRM analysis (Figure 2a). Melting temperatures of the tested fungal isolates was about 84.8°C and the selected primers did not amplify *C. gloeosporioides* species complex, even when *C. abscissum* genomic DNA was mixed with genomic DNA of *C. gloeosporioides* (Figure 2a).

We analysed the melting curve profiles when genomic DNA of *C. gloeosporioides* isolated from different citrus organs (flowers, fruit or leaves) were mixed with *C. abscissum* DNA in PCRs with the primer pair CaITS-F and CaITS-R815 (Figure 2b). Regardless of the organ from which *C. gloeosporioides* was isolated, when we mixed the DNA of *C. gloeosporioides* with *C. abscissum* DNA, we only observed the characteristic peak for *C. abscissum* (Figure 2b). We also assessed the sensitivity of the primer pair CaITS-F and CaITS-R815, which was able to detect as low as 10 pg/µl of DNA, being 10 times less sensitive than the primer pair AcG1-F1 and AcG1-R1 assessed in this study (Figure 2c).

Primers CgInt and ITS4 (Mills et al., 1992; Table 2) were the best HRM primers for *C. gloeosporioides* species identification (Figure 2d). They amplified *C. gloeosporioides* DNA, and there was no amplification of *C. abscissum* using these primers. Tested isolates of *C. gloeosporioides* (Table 1) showed two melting peaks, and even in a mixture of *C. abscissum* and *C. gloeosporioides* DNA, this pair of primers only amplified *C. gloeosporioides* (Figure 2d).

Besides sensitivity, we also tested the specificity of the CaITS-F and CaITS-R815 primer pair against common citrus pathogens and endophytes (Table 1). Based on the absence of fluorescent peaks, no amplification was observed for any of the nontarget species that are commonly associated with citrus, nor on citrus DNA (Figure S2).

3.2 HRM analysis using genomic DNA mixed with crude citrus leaf extracts

We mixed genomic DNA with crude extracts obtained from macerating healthy citrus leaves with GEB2 buffer to determine how much inhibition would be posed by the extract. Slightly lower peaks were observed when crude extracts were mixed with genomic *C. abscissum* DNA alone (Figure 3a) or in mixture with *C. gloeosporioides* genomic DNA (Figure 3b).

3.3 HRM analysis using DNA extracted by different methods

Details on significance of fixed effects are shown in Table S1. There were significant differences among the fluorescent peaks obtained by HRM assay depending on the method used to extract DNA from the samples ($p < 0.0001$). Mean fluorescence of samples processed by DNeasy PowerSoil was significantly greater than that of the DNeasy Plant Mini kit, and the lowest fluorescence was found in DNA samples extracted by the GEB2 buffer (Figure 4). No fluorescence was observed in samples processed with GEB2 buffer and inoculated with 200 and 2000 conidia, nor with 80% of the replicates inoculated with 20,000 conidia extracted with

GEB2 buffer (Figure 4). Fluorescence was observed in all samples extracted with the DNeasy kits, from 200 to 100,000 spores, and even in some of the mock inoculated samples—28% of samples extracted with DNeasy PowerSoil and 50% of samples extracted with DNeasy Plant Mini kit. The interaction between concentration of inoculated conidia and extraction kit was significant ($p < 0.0001$). Within the same number of inoculated spores, the effect of extraction method on fluorescence was significant for 200, 2000, 20,000 and 100,000 spores, and the mock-inoculated treatments (Figure S3; $p = 0.0008$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$ and $p = 0.033$, respectively). Overall, the higher fluorescence within the same number of inoculated conidia was observed in samples extracted with the DNeasy PowerSoil extraction kit.

3.4 HRM analysis using citrus leaf and flower samples collected from commercial citrus groves

All flower samples collected in the high inoculum area showed typical PFD symptoms, whereas none of the flowers in the low inoculum area had symptoms. As expected, none of the leaves had symptoms of *C. abscissum* infection. Details on significance of the fixed effects are shown in Table S2. Fluorescence peaks were significantly different between samples collected from the evaluated orchards, with the samples from the high inoculum area being higher ($p < 0.001$). The extraction method and citrus organ (flower or leaf) were also a significant factor on fluorescence ($p < 0.001$), being higher for DNeasy PowerSoil and for citrus flowers, respectively. We systematically observed higher fluorescence in samples extracted using the PowerSoil kit, regardless of tissue and inoculum levels (Figure 5; $p = 0.0005$ for flower samples in the high inoculum area and $p < 0.0001$ for the remaining comparisons). In more detail, the interaction between sample tissue, sampling location and extraction method was also significant ($p < 0.001$). DNA samples from leaves extracted with the GEB2 buffer also tested positive for *C. abscissum*

in the high inoculum area (Figure 5). We detected *C. abscissum* in DNA samples extracted with PowerSoil in asymptomatic citrus flowers in the low inoculum area (Figure 5). However, in DNA samples extracted with GEB2 we only identified two positive samples on leaves in the low inoculum area. *C. abscissum* was detected in 9 of 10 leaf samples in the high inoculum area in samples extracted with the DNeasy PowerSoil kit (Figure 5). The use of GEB2 buffer on flowers always yielded positive results for *C. abscissum* in the high inoculum area (Figure 5). All flowers collected from the high inoculum field were showing PFD-like symptoms. HRM analysis of DNA samples extracted with GEB2 buffer detected *C. abscissum* in the three replications of 1 of the 10 leaf samples collected from the low inoculum citrus orchard (10%), while all the 10 leaf samples collected from the same area and extracted with DNeasy PowerSoil tested positive for *C. abscissum* (Figure 5). Asymptomatic flower samples collected from the low inoculum area and extracted with GEB2 were all negative, whereas those extracted with DNeasy PowerSoil were all positive (Figure 5). There were nine positive leaf samples of all the leaf samples collected from the high inoculum orchard, and we were able to isolate the pathogen using traditional techniques and the floral extracts for all those samples (i.e., we isolated *C. abscissum* from the same nine leaf samples). However, for the low inoculum orchard, we were able to isolate the pathogen from two of 10 positive samples (i.e., we isolated *C. abscissum* from 20% of our HRM-positive leaf samples).

4 Discussion

In this study, we evaluated the detection of the PFD causal agent, *C. abscissum*, the most prevalent species in citrus orchards (Silva et al., 2017), using the HRM technique. We developed

a system that could identify the pathogen at low concentrations of purified DNA. In addition, *C. abscissum* could also be detected directly from asymptomatic citrus leaves when at least 20,000 spores were present in a sample using the GEB2 buffer. When DNA extraction protocols that included a purification step (DNeasy Plant Mini or PowerSoil) were used, we were able to identify as few as 200 *C. abscissum* spores, the lowest concentration tested. The development of a simple and rapid system to detect *C. abscissum* on asymptomatic leaves would improve the diagnostics for this disease. Although the initial cost for implementing HRM is high, mostly because of the equipment cost, if a laboratory routinely applies the technique instead of qPCR or standard PCR with a separation step (gel electrophoresis), HRM will be less costly in the long run due to the fewer reagents required. The time for traditional PCR will be at least 1 h longer than HRM due to the separation step (gel electrophoresis). Regarding cost, Noh et al. (2017) stated that the cost of HRM per 5 µl reaction was $0.10. Because we used 10 µl, it is fair to calculate a cost of $0.20 per reaction. The cost for HRM is basically the same as conventional PCR, minus the cost and time for the electrophoresis step. As conventional PCR normally requires at least 20 µl, a price of $0.40 per reaction is a good estimate. The cost for qPCR not counting the equipment would be similar to HRM; however, qPCR requires probes that are usually more expensive than traditional primers.

The HRM technique is an effective, economical and efficient molecular diagnostic method that has been used in many scientific fields in recent years, from medicine to plant breeding (Lee et al., 2017; Reed et al., 2007; Vossen et al., 2009). In plant pathology, HRM has already been successfully tested for the detection of *C. acutatum* and *C. gloeosporioides* species complexes from strawberry (Forcelini et al., 2018; Wang et al., 2021). It has also been routinely used in the diagnostic laboratory of the University of Florida for timely diagnosis of strawberry

fungal diseases (Wang et al., 2021). Thus, HRM could soon be a technology used in nurseries and groves to assist growers to identify and manage the disease in citrus-producing regions of the Americas, similar to the diagnostic assays offered for Florida strawberry growers (Wang et al., 2021). We also attempted isolations from asymptomatic leaves and confirmed the viability and presence of *C. abscissum* in all the positive samples from the high inoculum area. Conversely, we could not confirm the presence of *C. abscissum* on all positive leaf samples from the low inoculum area, possibly due to the lower concentration of conidia on these samples. Previous attempts to isolate the pathogen from leaf samples collected in citrus nurseries were also unsuccessful, possibly also owing to low inoculum levels (Muñoz, 2018).

The CaITS-F and CaITS-R815 pair of primers were chosen over the others due to their ability to identify *C. abscissum* in mixed DNA samples with *C. gloeosporioides*. *C. gloeosporioides* is a common citrus epiphyte and causes PFD less often than *C. abscissum* (Lima et al., 2011; Silva et al., 2017). Although AcG1-F1 and AcG1-R1 primers generated different melting temperatures and melting curve shapes for *C. abscissum* and *C. gloeosporioides* from citrus, with a similar pattern to that of a previous HRM study (Forcelini et al., 2018), the primers failed to differentiate species when *C. gloeosporioides* and *C. abscissum* DNA were mixed. The peaks associated with *C. gloeosporioides* were dominant over the *C. abscissum* peaks in mixed DNA samples when we used the AcG1-F1 and AcG1-R1 primers, adopted by Forcelini et al. (2018).

Based on our results, we recommend two different approaches depending on the purpose of using the HRM technique. If samples are to be processed to assure nursery trees are free or have low *C. abscissum* inoculum levels, we recommend that leaf or asymptomatic flower samples are subjected to DNA extraction procedures with a purification step. In our study,

DNeasy kits were effective in purifying DNA samples and increased HRM sensitivity. For diagnosis of PFD occurring on symptomatic flowers, the use of the quicker extraction method using the GEB2 buffer is effective in providing a reliable molecular diagnosis. In addition, *C. abscissum* was detected on DNA of field leaf samples extracted with GEB2 buffer followed by a 1:10 dilution. Therefore, to identify areas with high *C. abscissum* inoculum levels for disease risk assessment purposes, leaf sample processing using the GEB2 buffer is sufficient, quicker and cheaper than the standard DNA extraction that includes a purification step. The use of GEB2 is very advantageous due to its low cost, ease of use and quick extraction process. The use of DNeasy in the field may lead to unnecessary management practices by growers, as the presence of the pathogen at low levels probably does not pose a threat to production if environmental conditions are not extremely conducive for disease development based on our experience with PFD.

One of the most important variables for PFD epidemics is inoculum levels (Timmer & Zitko, 1993). Unfortunately, there is no study that investigates the infection efficiency of *C. abscissum* (i.e., the relationship between the number of lesions produced per number of infection units). As *C. abscissum* has a remarkable capacity of surviving on leaves and twigs, and as HRM using GEB2 was able to detect the pathogen in our field sample assays, we hypothesize that our greenhouse plants might have had a low level of *C. abscissum* on the leaves after surface disinfestation, which could be the reason the HRM technique detected *C. abscissum* DNA on mock-inoculated leaves processed using the DNeasy kits.

Recently, the detection and quantification of *C. abscissum* (Muñoz et al., 2018; Pereira et al., 2019) and *C. gloeosporioides* (Pereira et al., 2019) on citrus leaves have been successfully performed by qPCR. Although qPCR is a more specific and sensitive technique for the detection

of the PFD pathogens than conventional PCR (Ciampi-Guillardi et al., 2021; Mills et al., 1992; Sreenivasaprasad et al., 1996), it requires the use of specific probes, and it is uncertain whether a quick DNA extraction method without a purification step (like our GEB2 buffer methodology) would work with qPCR. In HRM analysis, the presence of TaqMan probes and prior pathogen DNA extraction could be skipped for some purposes, enabling the detection of the pathogen spores directly from flowers or leaves. As the technique uses a simple protocol, it is a useful tool for rapid detection of PFD-affected plants in nurseries and citrus orchards. The HRM method is less sensitive in detecting *C. abscissum* than qPCR. In our study, we were able to detect as low as 200 conidia when samples were extracted using either DNeasy extraction kit (Plant Mini or PowerSoil), whereas qPCR was able to detect as few as 10 conidia using the modified CTAB method on *C. abscissum* colonies (Pereira et al., 2019). However, *C. abscissum* DNA extraction from inoculated leaves is different than DNA extraction from colonies, as leaves contain more inhibitors that hamper the PCR, therefore the direct comparison between the two methods is not possible.

Besides being useful for diagnostics and application in nurseries, HRM analysis may facilitate more detailed epidemiological studies of the disease in the field by detecting the pathogen on symptomless citrus leaves. Many hypotheses have been raised about *C. abscissum* dispersal. The spatial distribution of PFD-infected plants in the field does not follow the expected pattern for pathogens with splash dispersal, which is thought to be the main dispersal mechanism of *C. abscissum* (Gasparoto et al., 2017). The HRM technique will serve as a useful tool to evaluate the dispersal of *C. abscissum* from symptomless nursery plants to the field by the detection of the pathogen on citrus leaves in the nursery and/or recently planted orchards.

However, the sampling methodology should be adapted to the size of the field or nursery of interest for a reliable inoculum level assessment.

The sets of primers tested in this study were used in previous studies with *C. acutatum* sensu lato and/or *C. gloeosporioides* sensu lato (Debode et al., 2009; Forcelini et al., 2018; Garrido et al., 2009; Mills et al., 1992; Sreenivasaprasad et al., 1996). It is noteworthy that, except for AcG1-F1 and AcG1-R1 (Forcelini et al., 2018), it was the first time they were used in HRM analysis. Previously, these primer sets were used in more routinely applied techniques such as conventional and/or qPCR with purified DNA. For this reason, we found some difficulties in using them with our diagnostic method. The set of primers chosen as the most suitable for the detection of *C. abscissum* and *C. gloeosporioides* by HRM analysis were CaITS-F and CaITS-R815, and CgInt and ITS4, respectively. These primer sets can be used in two different reactions when DNA has not been previously extracted and purified, for the diagnosis of *C. abscissum* and *C. gloeosporioides*, respectively.

The HRM technique was effective in detecting *C. abscissum* and *C. gloeosporioides* from genomic DNA samples and *C. abscissum* from asymptomatic citrus leaves and symptomatic and asymptomatic flowers. The technique was able to detect concentrations as low as 100 fg/µl of *C. abscissum* purified DNA and at least 200 spores on inoculated leaves using DNeasy extraction kits. Furthermore, we were able to detect the pathogen in leaf samples collected from the field. This positive result from all but one of the *C. abscissum*-positive leaf samples from the high inoculum area and three leaf samples from the low inoculum area was later confirmed by isolating the pathogen on a semiselective medium. This was the first time that a PCR-based method used to detect *C. abscissum* on asymptomatic leaves could be confirmed by subsequent pathogen isolation. We hope that the technique described here is used to assist growers to

diagnose and evaluate the risk for PFD in their groves wherever this disease is a problem in the Americas.

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Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Supporting Information

Additional Supporting Information can be found in the online version of this article at the publisher's website.

Figure S1 High-resolution melting (HRM) analysis using the AcG1-F1 and AcG1-R1 primers to determine the technique sensitivity for the detection of *Colletotrichum abscissum* (a, isolate Ca 142) and *C. gloeosporioides* (b, isolate Cg 83) from citrus. DNA concentrations for each pathogen were: 10 ng/µl (blue), 1 ng/µl (red), 100 pg/µl (green), 10 pg/µl (pink), 1 pg/µl (grey) and 100 fg/µl (yellow).

Figure S2 High-resolution melting (HRM) analysis using the CaITS-F and CaITS-R815 primers to determine the specificity of the technique for the detection of *Colletotrichum abscissum* isolate (Ca 142) from citrus (red). No DNA amplification was detected for nontarget species (*Xanthomonas alfalfae* subsp. *citrumelonis*, *X. citri* subsp. *citri*, *Phytophthora nicotianae*, *P. palmivora*, *Alternaria alternata*, *Diaphorthe citri*, *Zasmidium citri-griseum*, *Elsinoë fawcettii*, *Phyllosticta capitalensis (endophyte)*, *C. gloeosporioides* and *Citrus sinensis*) using these primers (blue).

Figure S3 Mean relative fluorescence values (−d/dT) from *Citrus sinensis* leaf samples inoculated with 200, 2000, 20,000 and 100,000 *Colletotrichum abscissum* conidia and mock inoculated samples extracted with DNeasy Plant Mini kit (blue), DNeasy PowerSoil kit (orange) and grinding buffer GEB2 (grey). The effect of the extraction method on relative fluorescence was significant for 200, 2000, 20,000 and 100,000 conidia and mock-inoculated samples (p = 0.0008, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, and $p = 0.033$, respectively). Lowercase letters denote statistical differences among extraction methods within the same number of inoculated *C. abscissum* conidia.

Table S1 Effects of extraction method, conidial concentration and the interaction between extraction method and conidial concentration on relative (−d/dT) fluorescence values in

experiments assessing DNeasy Plant Mini, DNeasy PowerSoil and grinding buffer GEB2 DNA extraction methods and 0, 200, 2000, 20,000 and 100,000 *Colletotrichum abscissum* conidia inoculated on *Citrus sinensis* leaves.

Table S2 Effects of extraction method, inoculum levels, host tissue and all possible two and three factor interactions on relative (−d/dT) fluorescence values in experiments using DNA extracted from *Citrus sinensis* leaf and flower samples to assess the presence of *Colletotrichum abscissum* using DNeasy PowerSoil and grinding buffer GEB2 DNA extraction methods.

Figure legends

Figure 1 High-resolution melting (HRM) analysis using (a) the AcG1-F1 and AcG1-R1 primers for detecting the genomic DNA of *Colletotrichum abscissum* (red, isolate Ca142) and *C. gloeosporioides* (blue, isolate RP2) isolates and (b) for examining the interference of mixed DNA (*C. acutatum* plus *C. gloeosporioides* species complex DNA) on the HRM analysis. *C. abscissum* DNA (red, Ca142 and PFD16), *C. gloeosporioides* (blue, Cg83 and RP2) and DNA from both pathogens (green, Ca142 + Cg83 and PFD16 + RP2) present in the HRM reactions. The light blue lines represent water controls used in the reaction.

Figure 2 High-resolution melting (HRM) analysis (a) using the CaITS-F and CaITS-R815 primers for the detection of *Colletotrichum abscissum* (red, isolates Ca142 and PFD16), *C. gloeosporioides* (blue, isolates Cg 83 and RP 2) and DNA of both citrus pathogens in mixture (green); (b) using the CaITS-F and CaITS-R815 primers for the detection of *C. abscissum* isolates from citrus flowers (red, isolates Ca142 and PFD16) and *C. gloeosporioides* isolates from citrus flowers (blue, isolates Cg 83 and RP 2), citrus fruit (pink, isolate GRF-LAF-FR) and

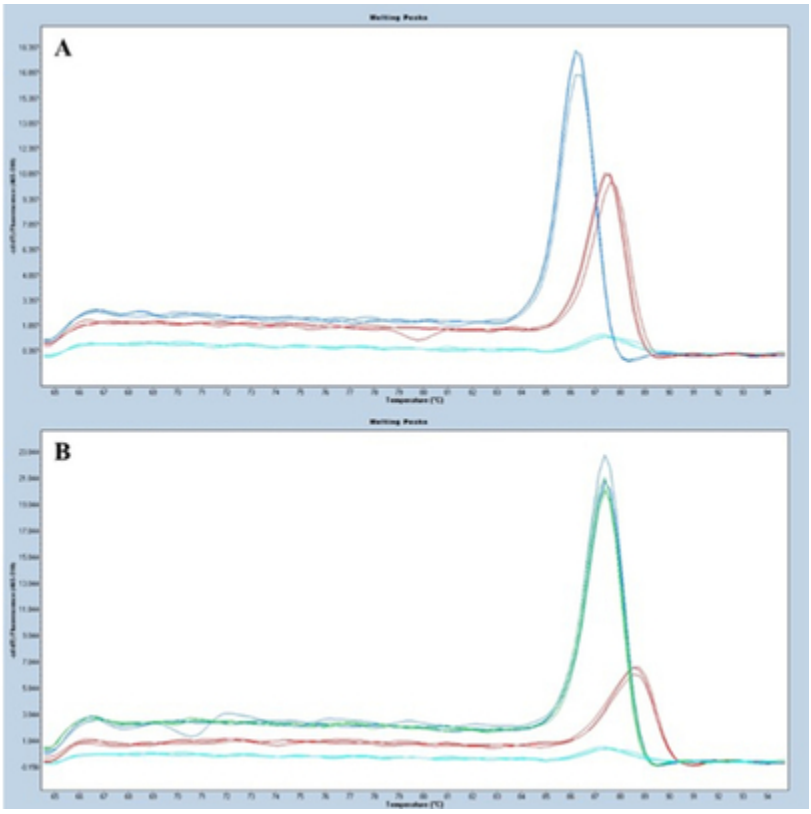
citrus leaves (grey, isolate STL-14A), and mixed DNA of *C. abscissum* and *C. gloeosporioides* (green, isolates Ca142 and Cg 83, respectively); (c) using the CaITS-F and CaITS-R815 primers to determine the technique sensitivity for the detection of a *C. abscissum* isolate (Ca 142) from citrus. DNA concentrations were: 10 ng/µl (blue), 1 ng/µl (red), 100 pg/µl (green), 10 pg/µl (pink), 1 pg/µl (yellow) and 100 fg/µl (grey); and (d) using the CgInt and ITS4 primers for the detection of *C. abscissum* (red, isolates Ca142 and PFD16), *C. gloeosporioides* (blue, isolates Cg 83 and RP 2) and DNA of both citrus pathogens in mixture (green). Control samples (molecular water) are represented by the light blue colour.

Figure 3 High-resolution melting (HRM) analysis (a) using the CaITS-F and CaITS-R815 primers for the detection of *Colletotrichum abscissum* (isolate Ca 142) with (blue) or without (red) crude DNA extract of citrus leaves in the reaction; and (b) detection of *C. abscissum* (isolate Ca 142) and *C. gloeosporioides* (isolate Cg 83) DNA in mixture with (yellow) or without (green) crude DNA extract of citrus in the reaction.

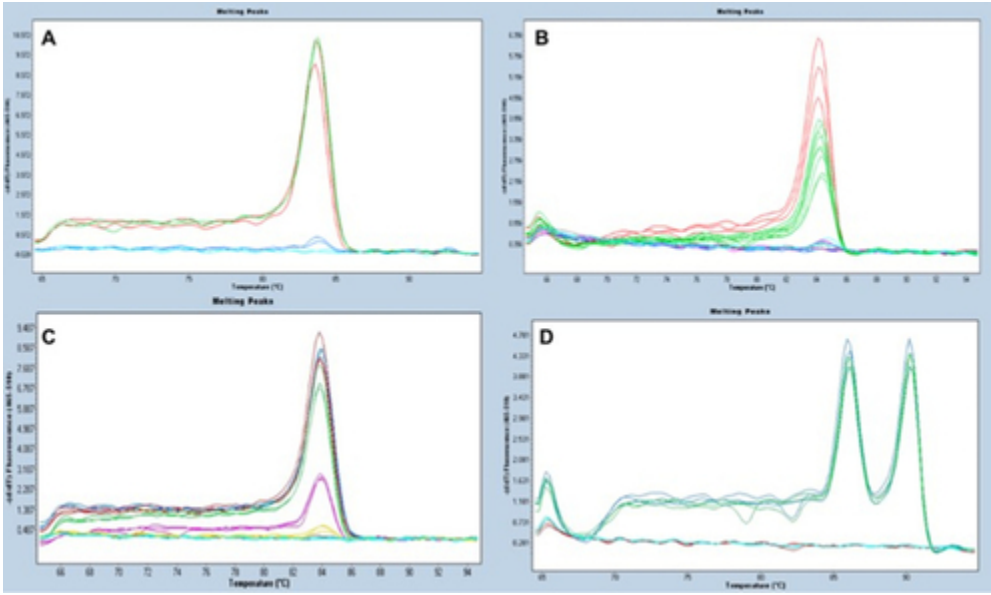
Figure 4 Fluorescence peaks obtained by high-resolution melting (HRM) assays from DNA of citrus leaves inoculated with different conidial concentrations of the *Colletotrichum abscissum* isolate OCO-ARC-4 using the primer pair CaITS-F and CaITSR-815 and three extraction methods: DNeasy Plant Mini, DNeasy PowerSoil and GEB-2 extraction buffer. Mean fluorescence obtained by the three different extraction methods were compared by adjusting generalized mixed models to the data, specifying a normal distribution, considering the extraction method, number of conidia used in the inoculation and their interaction as fixed effects and experiment as a random effect. The effects of the extraction method, the number of *C. abscissum* conidia and the interaction of both factors on fluorescence was significant ($p < 0.001$). The concentration on the *x* axis reflects the original concentration at the moment of

inoculation. DNA extraction using GEB2 buffer followed an additional 10-fold dilution step to reduce PCR inhibitors.

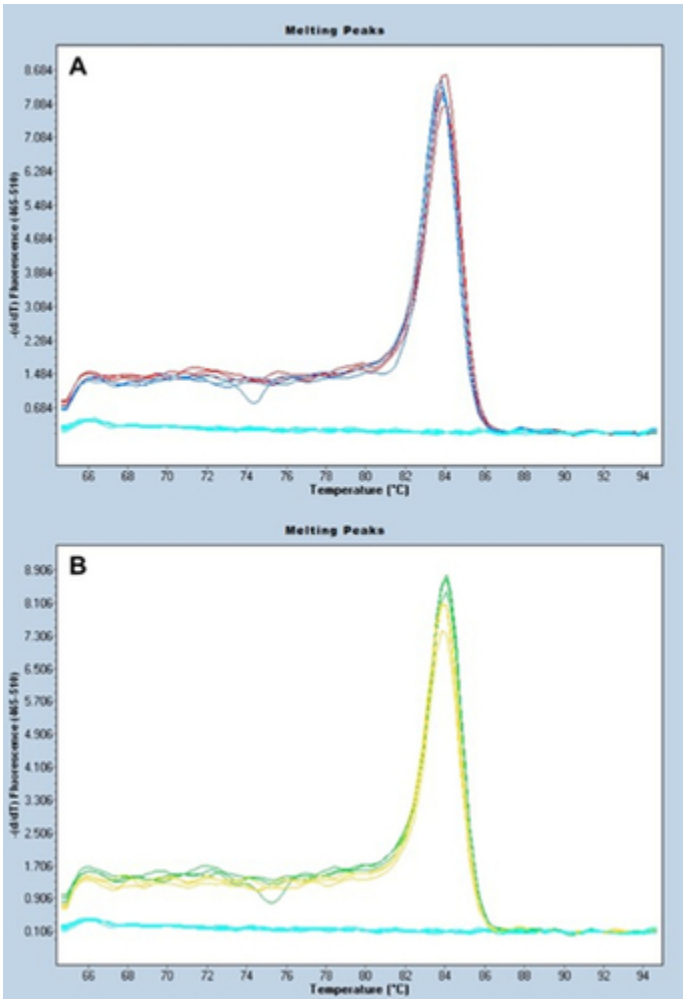
Figure 5 Fluorescence peaks obtained by high-resolution melting (HRM) assays from DNA samples obtained using two different extraction methods, DNeasy PowerSoil and GEB2 buffer. Leaf and flower samples were collected from two different orchards, one with high inoculum (left panel) and another with low inoculum (right panel) using the primer pair CaITS-F and CaITSR-815 and two extraction methods: DNeasy PowerSoil and GEB-2 extraction buffer. The effect of the extraction method on each combination of organ (flower or leaf) and inoculum level (high or low) was assessed by adjusting generalized linear mixed models to the data, considering the extraction method as a fixed effect and sample as a random effect in SAS. The effect of extraction method was significant in all comparisons ($p = 0.0005$ for flower samples in the high inoculum area and $p < 0.0001$ for the remaining comparisons). Letters denote statistical differences between the two extraction methods within the same sample type and inoculum levels.



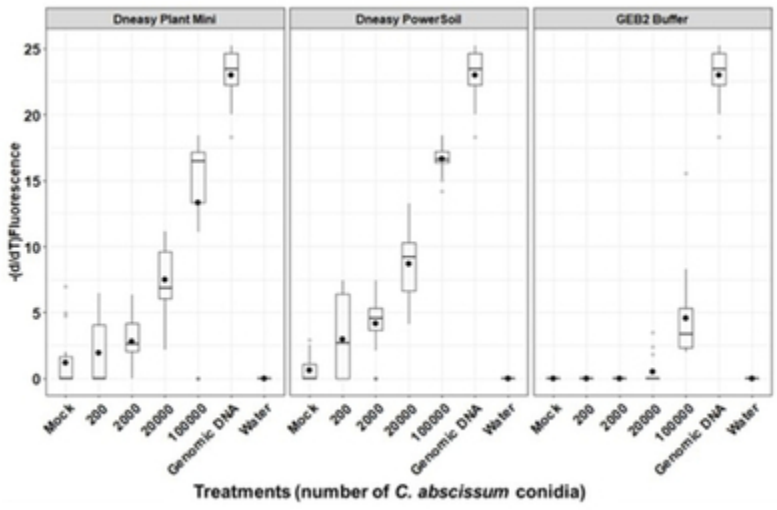
68x68mm (150 x 150 DPI)



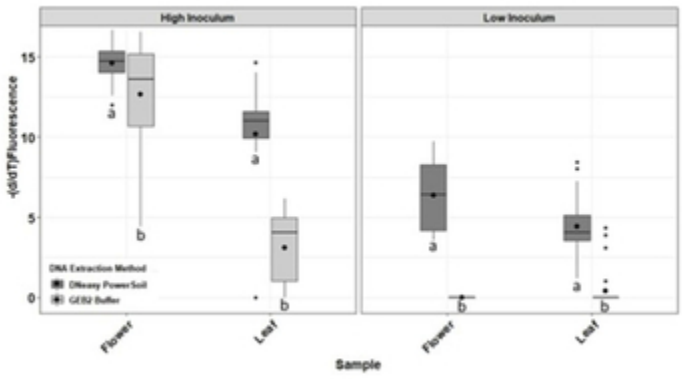
83x49mm (150 x 150 DPI)



57x84mm (150 x 150 DPI)



67x45mm (150 x 150 DPI)



63x37mm (150 x 150 DPI)

Table 1 Isolates used for DNA extraction and PCR, followed by HRM (high-resolution melting) tests performed to detect pathogenic *Colletotrichum abscissum* on asymptomatic citrus leaves

Isolate	**Species**	**Host**	**Origin**	**Collection**[a]
Ca 142[b]	*Colletotrichum abscissum*	Citrus flower	Brazil	Fundecitrus
PFD 16[b]	*C. abscissum*	Citrus flower	USA	GCREC/UF
OCO-ARC-4[b]	*C. abscissum*	Citrus flower	USA	GCREC and CREC/UF
Ca 14-686[c]	*Colletotrichum nymphaeae*	Strawberry fruit	USA	GCREC/UF
Cg 83[b]	*Colletotrichum gloeosporioides*	Citrus flower	Brazil	Fundecitrus
RP 2[b]	*C. gloeosporioides*	Citrus flower	Brazil	ESALQ/USP
Cg 00-182[c]	*Colletotrichum siamense* (*C. gloeosporioides* sensu lato)	Strawberry fruit	USA	GCREC/UF
Xalf	*Xanthomonas alfalfae* subsp. *citrumelonis*	Citrus	USA	CREC/UF
Xcc	*Xanthomonas citri* subsp. *citri*	Citrus	USA	CREC/UF
SB 4	*Phytophthora nicotianae*	Citrus	USA	CREC/UF
P 99-59-1	*Phytophthora palmivora*	Citrus	USA	CREC/UF
CHUR-DAN-RIH	*Alternaria alternata*	Citrus	USA	CREC/UF

PC.STL.SS	*Diaporthe citri*	Citrus	USA	CREC/UF
MC conserve3	*Zasmidium citri-griseum*	Citrus	USA	CREC/UF
Russel 15	*Elsinöe fawcettii*	Citrus	USA	CREC/UF
Ggn 1055	*Phyllosticta capitalensis* (endophyte)	Citrus	USA	GCREC/UF
STL-14A	*C. gloeosporioides*	Citrus leaf	USA	GCREC/UF
GRF-LAF-FR	*C. gloeosporioides*	Citrus fruit	USA	GCREC/UF
–	*Citrus sinensis*	Citrus leaf	USA	GCREC/UF

[a]Fundecitrus: Fund for Citrus Protection (Araraquara, SP, Brazil). GCREC/UF: Gulf Coast Research and Education Center/Strawberry Pathology Laboratory/University of Florida, Wimauma, FL, USA. ESALQ/USP: Epidemiology Lab/University of São Paulo, Piracicaba, SP, Brazil. CREC/UF: Citrus Research and Education Center/University of Florida, Lake Alfred, FL, USA.

[b]Tested isolate.

[c]Reference isolate.

Table 2 List of primers tested for specificity and sensitivity in this study

Target[a]	Gene ID	Primer	Sequence (5′–3′)	Fragment size (bp)	Reference
Ca	ITS	AcG1-F1	CCTGTTCGAGCGTCATTTC	87	Forcelini et al. (2018)
		AcG1-R1	GGTCCGCCACTACCTTT		
		CaITS-F	GATCATTACTGAGTTACCGC	84	Debode et al. (2009)
		CaITS-R815	GCCCACGAGAGGCTTC		
		CaITS-F	GATCATTACTGAGTTACCGC	84	Debode et al. (2009)
		CaITS-R699	GCCCGCGAGAGGCTTC		
		ACUT-F1	CGGAGGAAACCAAACTCTATTTACA	91	Garrido et al. (2009)
		ACUT-R2	CCAGAACCAAGAGATCCGTTG		
		CaInt2	GGGGAAGCCTCTCGCGG	496	Sreenivasaprasad et al. (1996)
		ITS4	TCCTCCGCTTATTGATATGC		
Cg	ITS	CgInt	GGCCTCCCGCCTCCGGGCGG	497	Mills et al. (1992)
		ITS4	TCCTCCGCTTATTGATATGC		
	CAL	Cg-CAL-F2	GCGAGGCTTTCAAGGT	93	Oliveira (2020)
		Cg-CAL-R1	CATGAAGCCGTTGTTGTCGC		

[a]Ca, *Colletotrichum acutatum* species complex; Cg, *Colletotrichum gloeosporioides* species complex.