University of São Paulo
“Luiz de Queiroz” College of Agriculture

Detection and quantification of *Colletotrichum abscissum* from leaves of budwood
increase block and citrus nursery plants by real time PCR

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Dissertation presented to obtain the degree of Master of
Science. Area: Plant Pathology

Piracicaba
2018

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DEDICATION

Juanjo and Violeta

Tankara ambesa and Sol de mi vida

You are everything for me

Eternal gratitude

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GRATIDÃO ETERNA.

EPIGRAPH

OM BHUR BHUVA SVAH

TAT SAVITUR VARENYAM

BHARGO DEVASYA DHIMAH

DHYOYO NAH PRACHODAYAT

Contemplemos a luz que ilumina o plano mortal, astral e divino

Meditamos sobre o esplendor brilhante do Deus Sol

Que ele ilumine nosso intelecto, nos guiando e inspirando

para que possamos fazer a ação correta na hora certa

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RESUMO

Deteção e quantificação de *Colletotrichum abscissum* em folhas de borbulheiras e mudas de citros por PCR em tempo real

O Brasil é o maior produtor de citros do mundo e possui uma grande participação no mercado global de citros. No entanto, várias doenças afetam a cultura, sendo uma delas a podridão floral dos citros (PFC). PFC ganhou importância no Estado de São Paulo pelo deslocamento de áreas de citros para regiões com condições climáticas mais favoráveis para a doença. A identificação precisa do agente causal do PFC foi realizada, tendo sido renomeado como *Colletotrichum abscissum*. A origem do inóculo inicial ainda é um enigma para as epidemias de PFC e as hipóteses do que o inóculo inicial poderia estar presente no material de propagação já foram discutidas, mas nunca foram demonstradas. O objetivo deste trabalho foi detectar e quantificar *Colletotrichum abscissum* em folhas de borbulheiras e mudas de citros por meio de qPCR. Neste trabalho, foram utilizadas quatro fazendas comerciais de citros do Estado de São Paulo, Brasil, com borbulheiras e viveiros de mudas de citros das variedades laranja Pera e Valência. *C. abscissum* foi detectado em borbulheiras e em mudas em ambas as variedades (Valência e Pêra) nas quatro fazendas amostradas. Das 122 amostras de folhas de borbulheiras, 89 (73%) foram positivas para *C. abscissum*. Das 175 amostras de folhas de mudas de citros, 129 (73%) foram detectadas com o patógeno. A maioria das amostras positivas de borbulheiras e mudas de citros continham 10 a 200 e 10 a 400 conídios de *C. abscissum*, respectivamente. Com os métodos utilizados, não foi possível isolar o fungo do material vegetativo. Esta descoberta sugere um novo tipo de dispersão a longas distâncias de *C. abscissum* no ciclo de podridão floral dos citros por meio do material de propagação. A confirmação de *C. abscissum* nas borbulheiras e mudas de citros levaria à atualização da regulamentação para a produção de mudas de citros certificadas e à busca de novas estratégias de controle do patógeno.

Palavras-chave: Podridão floral dos citros; Doenças da laranjeira; Técnicas moleculares; Viveiros

ABSTRACT

Detection and quantification of *Colletotrichum abscissum* in leaves of budwood increase block and citrus nursery plants by real time PCR

Brazil is the largest citrus producer in the world and has a large global citrus market share. However, several diseases affect the crop, being postbloom fruit drop (PFD) one of them. PFD has gained importance in São Paulo State for the displacement of citrus areas to regions with weather conditions more favorable for this disease. The accurate identification of the causal agent of the PFD has been performed and it was renamed as *Colletotrichum abscissum*. The origin of the initial inoculum is still an enigma for PFD epidemics and the hypotheses that the initial inoculum could be present in propagation material have been discussed but it has never been demonstrated. The objective of this work was to detect and quantify *Colletotrichum abscissum* from citrus leaves of budwood increase block and citrus nursery plants by qPCR. Four commercial citrus farms from São Paulo State, Brazil with budwood increase block and citrus nursery plants of Pera and Valencia sweet orange varieties were used for this work. *C. abscissum* was detected in budwood increase block and in nursery plant in both varieties (Valencia and Pera) at the four farms sampled. Out of 122 budwood increase block samples, 89 (73%) were positive for *C. abscissum*. From nursery plants, out of 175 samples, 129 (73%) were detected with the pathogen. The majority of the positive samples of budwood increase blocks and nursery plants contained 10 to 200 and 10 to 400 conidia of *C. abscissum*, respectively. With the methods used was not possible to isolate the fungus from vegetative material. This finding suggests a new long distances dispersion type of *C. abscissum* in the cycle of postbloom fruit drop by propagation material. Confirmation of *C. abscissum* in budwood increase block and nursery plants would lead to update regulations for the production of certified citrus nursery trees and searching for new control strategies of the pathogen.

Keywords: Postbloom fruit drop; Citrus disease; Molecular techniques; Nurseries

1. INTRODUCTION

Currently, Brazil is the largest citrus producer, followed by China and United States. Brazilian agribusiness is responsible for 56% of world orange juice production. In the growing season 2016/2017, orange production was about 14.67 millions of tons (FAVA & TROMBIN, 2017; MAPA, 2018). These records reflect unequivocally the economic importance of citrus to Brazil.

São Paulo state is the major citrus producer in Brazil. The North, Northeast and Center of the State account for 60 to 70% of Brazilian citrus production. In the last decade, citrus industry has been migrated from traditional areas to new ones at southwest of the state (NEVES et al., 2010). However, climatic conditions of southwest region during the flowering period in the winter months favors the occurrence of explosive epidemics of postbloom fruit drop disease (PFD), which can cause losses up to 80% when control measures are not used (GOES et al., 2008).

Postbloom fruit drop is distributed in several countries of the American continent. The first observation of the disease occurred in 1957 in Belize (FAGAN, 1979), where losses of 65% were recorded after the premature fruit drop (DENHAM, 1979). In Brazil, the first report of this disease occurred in 1979 in Rio Grande do Sul (Porto et al., 1979). Subsequently, it was reported in the state of São Paulo in 1991 (FEICHTENBERGER, 1991). In Florida, the disease was first observed during the spring of 1983 (SONODA & Hebb, 1991). PFD has caused serious impacts on citrus production in these countries, with sporadic and sudden occurrence when there is rain associated with flowering (TIMMER et al., 1994; SILVA JR et al., 2014).

The accurate identification of the causal agent of the PFD has been performed by molecular data and it was recently renamed as *Colletotrichum abscissum* (CROUS et al., 2015). Besides this species, LIMA et al. (2011) and MCGOVERN et al. (2012) reported *Colletotrichum gloeosporioides* also as causal agent of PFD. However, *Colletotrichum abscissum* is most important because it is responsible for approximately 80 % of the disease incidence (LIMA et al., 2011, TIMMER & PERES, 2015).

The disease affects mainly newly formed flowers. However, it may also affect the pistil (stigma and stylet) (FEICHTENBERGER et al., 2005; LIN et al., 2001). Petals symptoms are

characterized by brown or orange lesions, with masses of conidia involved by mucilage (FAGAN, 1984; TIMMER et al., 1994) and the pistil by lesions of brown or black coloration (LIN et al., 2001). The newly formed fruits have a yellow discoloration and fall prematurely. The basal discs, calyces and peduncles increase in size and keep firmly attached to the branches, forming typical structures related to the disease that are called "starlets" (FEICHTENBERGER, 1991).

Although the leaves do not present symptoms, they are considered source of primary inoculum of the disease, because they harbor structures of the pathogen which can survive between flowering seasons (AGOSTINI & TIMMER, 1994; ZULFIQAR et al., 1996).

PEREIRA (2013) verified that the survival of *C. abscisum* in citrus leaves is strongly influenced by precipitation. The fungus can survive under greenhouse conditions for up to ten months on the leaves with intermittent precipitation. On the other hand, it can survive only three months without precipitation. In the field, the fungus can survive seven months in periods with higher rainfall and only for three months in seasons with lower rainfall. Furthermore, this author showed that the pathogen does not colonize leaf tissues, and the penetration peg emitted by the appressorium is limited to the pectic layer between the cuticle and the outer periclinal wall of the epidermal cells, where it is believed that the pathogen remove nutrients for its survival on the leaf surface. This finding disagrees with the work of TIMMER & PERES (2015), which describes that quiescent infections occur in leaf tissues.

These results could explain the sudden and explosive character of the PFD epidemics, however, they do not explain the severe epidemics occurring in new orchards with no history of the disease (TIMMER, 1994), where theoretically there would not have inoculum in the leaves. As an example of this controversy, the epidemics in Florida in 1988, 1993, 1994 and 1998 (TIMMER & BROWN, 2000) and in São Paulo in 1977, 1990 and 1993 (PORTO, 1993) were associated with above-average precipitation during flowering season, but it was not possible to identify the origin of the initial inoculum.

Epidemics with high progress rates eventually occur in nature, but are always associated with pathogens with a short latent period and with long distance dispersal ability. In spite of the short latent period of *C. abscisum* in citrus flowers (ZULFIQAR et al., 1996; LIMA et al., 2011), the dispersion of this pathogen occurs mostly at short distances associated with

rain-splashes (BAILEY & JEGER, 1992; TIMMER & PERES, 2015). This type of dispersion is typical of aggregate distribution of diseased plants in the field and therefore does not explain the random distribution of PFD reported by SILVA-JUNIOR et al. (2014). For *C. abscisum*, the distribution should be aggregate, even at the beginning of epidemics. According to the authors, the distribution of diseased trees did not correlate with the presence of symptomatic trees nearby. Likewise, CIAMPI-GUILLARDI et al. (2014) verified the occurrence of identical haplotypes of *C. abscisum* in orchards separated by distances of up to 400 km, without spatial continuity between the points sampled.

These reports suggest a dispersion of *C. abscisum* at long distances (BURDON et al., 1989) or previous presence of the inoculum in the trees, even in the case of new orchards with no previous history of PFD. Possible citrus endophytic colonization by *C. abscisum* could explain these facts, but this hypothesis was completely ruled out in the paper by WACULICZ-ANDRADE et al. (2017).

The dispersion of PFD at long distances is an old enigma and has already been thought to be carried out using pollinating bees (TIMMER et al., 1994; GASPAROTO et al., 2017). Citrus pollen can also be infected by *C. abscisum* (MARQUES et al., 2013), making bee dispersal more likely. However, *Apis mellifera*, the main citrus pollinating bee, has a maximum distance of action of 14 km (ZURBUCHEN et al., 2010). Such distance would explain dispersion between orchards located in the same region, but does not explain the dispersion of 400 km found by CIAMPI-GUILLARDI et al. (2014), leading these authors to suggest that the initial inoculum of the epidemics could be present in the nursery plants.

The origin of the initial inoculum is still an enigma for PFD epidemics and the hypotheses already tested do not explain all the situations that have occurred in the past. From the analysis of two recent papers on PFD (SILVA-JUNIOR et al, 2014; CIAMPI-GUILLARDI et al, 2014) and in the light of the work done by Pereira (2013), the hypothesis that the initial inoculum could be present in nursery plants proves to be quite plausible.

Detection and identification of *C. abscisum* has been performed by classical diagnostic methods, such as isolation of the pathogen in semi-selective culture medium like CaIM (PERES et al., 2004) and Martin medium (Frare et al., 2016) and by molecular methods like conventional PCR using specific primers; in most of the cases flower samples with

symptoms of the disease have been used (MILLS et al., 1992; SREENIVASAPRASAD et al., 1996; WARD et al., 2003; GARRIDO et al., 2009). However, the challenge in this patosystem is the detection of the pathogen in asymptomatic vegetative material. Therefore, more specific, sensitive and accurate methods are necessary to detect and quantify the pathogen in this type of material.

To overcome this problem, molecular techniques such as real-time or quantitative polymerase chain reaction (qPCR) can be useful in this task. The qPCR is currently considered the most efficient technique for detection of phytopathogens, since it allows quantifying the amount of target nucleic acid in the sample, with higher assay sensitivity and specificity than conventional detection methods (CAPOTE et al., 2012; LUCHI et al., 2016). This technique allows monitoring of the reaction during the amplification process by using a fluorescence signal, which increases proportionally with the number of amplicons produced and the number of targets present in the sample (WITTWER et al., 1997; KURIAN et al., 1999; COCKERILL and SMITH, 2002).

Compared to conventional PCR, qPCR has many advantages, including: 1) rapid cycling, which reduces DNA amplification time significantly; 2) the system does not require the use of post-PCR delayed electrophoresis to determine amplification results, avoiding the risk of contamination and reducing assay, work, and material costs; 3) to reduces the risk of cross-contamination of DNA amplification by subsequent DNA detection in the same tube; 4) Real-time PCR can allow quantification of DNA, which may be useful in determining levels of pathogen infestation in the samples; 5) The system allows the analysis of 96 or multiples of that number at the same time. This feature facilitates the robotization of nucleic acid extraction and preparation stages of Master Mix, reducing the time necessary for the procedure. This is particularly interesting in situations where rapid diagnosis is necessary or when there is a considerable amount of samples; 6) the use of fluorophores and different probes may allow mutliplex PCR to detect two or more pathogens in the same reaction (WITTWER et al., 2001; CAPOTE et al., 2012).

Studies involving the detection of pathogens by qPCR and/or multiplex qPCR indicate the potential of the method for several patosystems. TaqMan® primers and probes were used to detect *Diaporthe phaseolorum* and *Phomopsis longicolla* in soybean seeds, which provided faster results when compared to other detection methods tested (ZHANG et al., 1999). This

method was also used to detect *Phytophthora ramorum* and *Phytophthora pseudosyringae* in oak (*Quercus agrifolia* and *Lithocarpus densiflorus*) species in California (TOOLEY et al., 2006), *Verticillium dahliae* causing verticillium wilt in potato (ATALLAH et al., 2007) and *Colletotrichum acutatum* in strawberry leaves (DEBODE et al., 2007).

The accurate and efficient detection of *Colletotrichum abscisum* associated with budwood increase block and citrus nursery plants is an important step towards the definition of PFD control strategies based on the principle of exclusion, which refers to the prevention of the entry of a pathogen in an area still uninfested (AGRIOS, 2005; CAPOTE et al., 2012; BERGAMIN & AMORIM, 2011).

The aim of this study is to detect and quantify *Colletotrichum abscisum* in citrus leaves of budwood increase block and citrus nursery plants by qPCR.

2. MATERIAL AND METHODS

2.1. Detection and quantification of *Colletotrichum abscisum* from leaves of budwood increase block and citrus nursery plants

2.1.1 Multiplex real-time PCR assay

C. abscisum LFN-1 isolate was cultivated in potato dextrose media (PDA) at 25°C with photoperiod of 12 h. For DNA extraction of the pathogen, mycelium plugs were collected from the colonies and ground in sterilized sand with a mortar and pestle. Commercial DNeasy Plant Mini Kit (Qiagen) was used for fungal genomic DNA extraction; the procedures for DNA extraction were according to the manufacturer's instructions. Quality and concentration of DNA obtained was measured in NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA samples were diluted to 10 ng/uL, placed in microtubes and stored at -20 °C for further use in qPCR.

Citrus DNA (*Citrus sinensis*) was extracted from plants generated by tissue culture to guarantee complete exclusion of any pathogen, using the same DNA extraction protocol described before. DNA extraction of these samples was performed in a distinct laboratory to avoid possible contamination with any traces of the pathogen.

For multiplex real-time PCR assay, ACUT-F1 and ACUT-R2 primers and TaqMan probe ACUT-PB specific for *C. abscisum* were used in qPCR. The probe was labeled with fluorescent reporter dye 6-FAM (6-carboxyfluorescein) at the position 5', and with quencher fluorescent dye TAMRA (6-carboxy-tetramethyl-rhodamine) at the position 3' (Table 1) (Garrido et al., 2009).

As a positive internal control, a set of primers and probes specific for citrus, based on cytochrome oxidase gene (COX) was used in the qPCR (Table 1) (Li et al., 2006). These primers and probe were efficient in the detection of host DNA of a 10⁻⁷ concentration (Li et al., 2006).

Table 1. Set of primers and probes used in qPCR detection of *Colletotrichum abscisum* in citrus tissues by TaqMan system.

Target	Primer/Probe	Sequence 5' - 3'
<i>Colletotrichum abscissum</i>	ACUT-F1 ^a	CGGAGGAAACCAAACCTCTATTTACA
	ACUT-R2 ^a	CCAGAACCAAGAGATCCGTTG
	ACUT-PB ^a	CGTCTCTTCTGAGTGGCACAAGCAAATAATTAAA
<i>Citrus</i>	COX-F ^b	GTATGCCACGTCGCATTCCAGA
	COX-Rr ^b	AATGCCCTTAGCAGTTTTGGC
	COX-Pp ^b	ATCCAGATGCTTACGCTGGA

^a Garrido et al., 2009.

^b Li et al., 2006.

The sets of primers and probes cited above were first tested separately for each target species (fungus and plant), in singleplex amplification reactions. qPCR for *C. abscissum* detection was carried out in total volume of 12 µL per reaction containing 30 ng of DNA sample, 4.8 µM of each primer, 6 µL of Path-ID™ qPCR Master Mix (Thermo Fisher Scientific) and 2.4 µM of TaqMan probe. As well, qPCR for citrus DNA detection was carried out in total volume of 12 µL per reaction containing 30 ng µL of DNA sample, 3.6 µM of each primer, 6 µL of Path-ID™ qPCR Master Mix (Thermo Fisher Scientific) and 1.8 µM of TaqMan probe.

The thermocycler Applied Biosystems 7500 Fast Real-Time PCR System (Thermo Fisher Scientific) was programmed for one cycle at 95 °C for 10 minutes, followed by 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. This volume of reaction has been effective in detecting pathogenic fungi by qPCR using TaqMan system (PEREIRA, 2013; RAMIRO, 2015).

After having adjusted the concentrations for each target species, the sets of primers and probes were tested in a multiplex qPCR using the set combination for a simultaneous amplification of the pathogen and the host in the same reaction. A mixture of pathogen and plant DNA (1.5 µL of each, at 10 ng/µL) was used to check the double amplification of both targets. Multiplex qPCR was performed in total volume of 12 µL per reaction containing 3 µL of DNA, 4.8 µM of each primer and 2.4 µM of TaqMan probe for *C. abscissum*, 3.6 µM of each primer and 1.8 µM of TaqMan probe for citrus DNA and 6 µL of Path-ID™ qPCR Master Mix. The thermocycler Applied Biosystems 7500 Fast Real-Time PCR System was programmed as mentioned before.

Once the multiplex qPCR proved to be efficient in simultaneously amplifying the pathogen and the host DNA, it was used to detect the presence of *C. abscissum* DNA in leaves samples of budwood increase blocks and citrus nursery plants.

2.1.2 Standard curve

A standard curve was designed to quantify conidia number of *C. abscissum*. Fragments of colonized filter paper with *C. abscissum* LFN-1 were placed in culture medium composed of PDA and grown at 25°C under constant light for 5 to 7 days.

Conidia were washed from culture plates with sterile water, filtered through four layers of cheesecloth, and diluted to 7×10^5 conidia/ml. From this suspension of conidia, six dilutions were taken containing 1000, 700, 500, 300, 100 and 10 conidia in 200 µl of sterile water. Concentration of each dilution was checked under the microscope using the Neubauer Chamber (hemocytometer). Five repetitions of each dilution of conidia from isolate LFN-1 of *C. abscissum* were used for the assay.

Commercial DNeasy Plant Mini Kit (Qiagen) was used for fungal genomic DNA extraction of each conidia suspension dilution; the procedures for DNA extraction were according to the manufacturer's instructions. Quality and concentration of DNA obtained was measured in NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA samples were stored at -20 °C for further use in qPCR. Amplification reaction were carried out in total volume of 12 µL per reaction containing 3 µL of conidia dilution DNA, 4.8 µM of each primer for *C. abscissum*, 6 µL of Path-ID™ qPCR Master Mix (Thermo Fisher Scientific) and 2.4 µM of TaqMan probe for *C. abscissum*. Three technical replicates of qPCR reactions were performed for each DNA sample to establish a linear regression curve between the threshold cycle (Ct) and conidia number.

2.1.3 Sampling, detection and quantification of *Colletotrichum abscissum* from leaves of budwood increase block and citrus nursery plants

Four commercial citrus farms from São Paulo State, Brazil were evaluated in this study. Budwood increase block (BIB) and citrus nursery (N) plants of Pera and Valencia

sweet orange varieties were sampled for the assays. A total of seven budwood increase block plots and ten citrus nursery plots were sampled. Ten to 35 plants were randomly selected in each budwood increase block plots and citrus nursery plots. Then, a mixed sample composed of six random leaves was taken above the grafting zone from each plant. Sampling dates, total of samples per plots and total of sampled leaves per plots of budwood increase block and citrus nursery can be observed in table 2 and 3, respectively.

Table 2. Sampling dates, total of samples and total of sampled leaves per plots from budwood increase block of three citrus farms.

Farm	Variety	Budwood increase block Plots	Sampling date	Total of samples/plot	Total of sampled leaves/plot
F1	Valencia	BIB 1	January 2016	18	108
	Valencia	BIB 2	January 2016	18	108
	Pera	BIB 3	October 2016	10	60
F2	Valencia	BIB 4	January 2016	37	222
	Pera	BIB 5	October 2016	10	60
F3	Valencia	BIB 6	January 2016	19	114
	Pera	BIB 7	January 2016	10	60
Total				122	732

Farm	Variety	Nursery Plots	Sampling date	Total of samples/plot	Total of sampled leaves/plot
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F1	Valencia	N 1	October 2016	10	60	Table 3. Sampling dates, total of samples and total of sampled leaves per plots from nursery of four citrus farms.
	Pera	N 2	October 2016	10	60	
F2	Valencia	N 3	January 2016	28	168	
	Pera	N 4	January 2016	19	114	
F3	Valencia	N 5	January 2016	19	114	
	Pera	N 6	January 2016	10	60	
F4	Valencia	N 7	January 2016	19	114	
		N 8	January 2016	20	120	
	Pera	N 9	January 2016	21	126	
		N 10	January 2016	19	114	
Total				175	1.050	

Samples were placed in plastic bags, forwarded to the Mycology Laboratory-ESALQ/USP for further processing. Each leaf of the sample of budwood increase block

and citrus nursery plants was divided into two parts. One part was stored at 8 °C for one week for sample preparation to obtain DNA and then analyze by qPCR. Another part was stored at -80 °C for a future isolation of *C. abscissum* and subsequent determination of pathogenicity.

Freezing method was used for sample preparation according to Pereira (2013). A half of the leaf was inserted into a plastic bag with 200 µL of PBS spread on the surface of the leaf and submerged in liquid nitrogen for 20 seconds. The ice layer formed was scraped off from the leaf surface and added to 200 µL of PBS in plastic tubes of 1.5 mL. After, sample extract was centrifuged at 655 g for 1 minute, 200 µL of supernatant was transferred to microtubes and stored at -20 °C.

Sample extracts were submitted to DNA extraction using the commercial kit DNeasy Plant Mini Kit™ (Qiagen), according to the manufacturer's instructions. DNA quality and concentration was checked in a spectrophotometer NanoDrop™ 2000 (Thermo Fisher Scientific, Wilmington, USA). DNA samples were diluted to 10 ng.µl⁻¹, placed in microtubes and stored at -20 °C for further use in qPCR.

A volume of 3 µL of sample extracts DNA was used in the multiplex qPCR, maintaining the same amplification conditions of multiplex qPCR assay. qPCR was performed in total volume of 12 µL per reaction containing 3 µL of DNA sample, 4.8 µM of each primer and 2.4 µM of TaqMan probe for *C. abscissum*, 3.6 µM of each primer and 1.8 µM of TaqMan probe for citrus, DNA and 6 µL of Path-ID™ qPCR Master Mix. The thermocycler Applied Biosystems 7500 Fast Real-Time PCR System was programmed for one cycle at 95 °C for 10 minutes, followed by 45 cycles at 95 °C for 15 seconds and 60 °C for 60 seconds. Three technical qPCR replicates were performed for each DNA sample to ensure accurate detection of the pathogen.

Quantification of conidia number in the leaves samples was obtained by interpolation of Ct values of each sample with the conidia number established in the standard curve.

2.2 Isolation of *Colletotrichum abscisum* from leaves of budwood increase block and citrus nursery plants

Semi-selective medium for *C. abscisum* CaIM (Peres et al., 2002) was used with modifications (0.1 g of Streptomycin + 0.25 g of Ampicilin + 0.2 mL of Tergitol + 0.06 g of Rovral 50WP + 0.1 g of Benomyl 50 WP + 17 g of Agar + 12 g of potato dextrose agar (PDA) diluted in 1 L of deionized water). Plates were incubated at 23 °C under continuous light until pathogen growth.

Three attempts were made to isolate the fungus from vegetative material on the semi-selective medium above described. For the first attempt, it was used citrus leaves samples previously stored at -80 °C. For the second attempt, it was used citrus leaves samples previously stored at 8 °C. The third attempt was also done with leaves stored at 8 °C, but nystatin at 20 µg/mL was added to the medium to avoid yeast growth.

Two methods of sample preparation were used in all attempts: leaf surface washing and leaf fragments (Figure 1 A/B). For the leaf washing method, 600 µl of sterile sucrose solution (2.5% w/v) was sprayed on the surface of each half of leaf which were kept in humid chambers for 24 hours to stimulate secondary conidiation (MACKENZIE et al.; 2010). Then, the solution was collected in sterile microtubes with the help of a sterile brush. Three hundred microliters of the suspension were distributed with a Drigalski loop on each plate with culture medium. For the fragment leaf method, 20 to 25 leaf discs (6 mm) of each half leaf were randomly taken using a sterile cork borer and placed in culture medium. In both methods, five to ten samples were used from each budwood increase block plot and nursery plot and three plates with culture medium were used for each sample.

The possible colonies of *Colletotrichum* sp. that grew in the culture medium were isolated from colonies of other fungi, transferred to new plates containing PDA, grown at 25°C under continuous light for three to five days, and submitted to molecular identification by convencional PCR.

Figure 1. Methods to isolate *Colletotrichum abscissum* from leaves of budwood increase block and citrus nursery plants: leaf surface washing (A) and leaf fragments (B).

For DNA extractions, mycelium plugs were collected from the edges of fungal colonies and ground in sterilized sand with a mortar and pestle. Commercial DNeasy Plant Mini Kit (Qiagen) was used for fungal genomic DNA extraction; the procedures for DNA extraction were according to the manufacturer's instructions. Quality and concentration of DNA obtained was measured in NanoDrop™ 2000 spectrophotometer (Thermo Fisher Scientific, Wilmington, USA). DNA samples were diluted to 10ng/μL, placed in microtubes and stored at -20 °C for further use in PCR.

After DNA extraction, a PCR was performed using the primers pair Calnt2/ITS4 for identifying *C. abscissum* (SCREENIVASAPRASAD et al. 1996). Amplification reactions were performed using 25 μL of total volume, with 20 ng μL of DNA, 2.5 μL of 10X PCR buffer, 1.5 mM of MgCl₂, 0.2 Mm of dNTP Mix, 0.5 μM of each primer and 1 U of Taq DNA polymerase.

The thermocycler Techne TC-512 (Techne Inc., Burlington, EUA) was programmed for 95° C for 2:30 minutes, followed by 35 cycles of 35 seconds at 95° C, 60 seconds at 55° C and 60 seconds at 72° C, ending with 60 seconds at 72° C.

To check amplification, PCR products were submitted to electrophoresis in agarose gel (1%) and buffer 1X Tris-acetate-EDTA (0.04 M Tris-Acetate, 0.0001 M EDTA). The gel into the buffer was submitted to electric potential of 75V for 45 minutes. After this period, the gel was observed in a UV chamber for bands visualization and image register. SYBR Safe™ DNA gel Stain (Invitrogen Corporation) was used for gel coloration.

3. RESULTS

3.1. Multiplex Real-time PCR

The amplification efficiency of the specific primers and probes sets, tested separately for the detection of *C. abscisum* and citrus DNA, was high for both species. Thus, it was possible to combine them to detect simultaneously the two targets at the same reaction in a multiplex qPCR.

Samples containing only *C. abscisum* DNA and citrus DNA showed a single amplification curve when submitted to multiplex qPCR (Figure 2 A/B). Samples with a mixture of *C. abscisum* and citrus DNA showed two amplification curves (Figure 2 C). C_T mean of three reactions can be observed in Table 4.

The preliminary tests revealed that there was no difference in the amplification levels of both target species in the multiplex reaction when compared to singleplex reactions. Therefore, multiplex qPCR proved adequate for the simultaneous detection of either the pathogen or the host in the same reaction, using a combination of specific primers and probes for each species.

The use of primers and probes to detect citrus DNA was useful as an internal control of the amplification reactions. Using this approach, it was possible to distinguish low quality DNA samples, which did not amplify in the qPCR for both targets, from those with high quality DNA but free from the pathogen, which amplified only the host DNA (Figure 2A).

Figure 2. Amplification by multiplex qPCR of samples with only *Citrus sinensis* DNA (A), only *Colletotrichum abscisum* DNA (B) and a mixture of *C. abscisum* and *C. sinensis* DNA (C).

Table 2. Cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus.

Sample	C_T of multiplex qPCR	
	<i>Colletotrichum abscissum</i>	Citrus
<i>Colletotrichum abscissum</i>	34.97 ± 0.93	ND ^a
Citrus	ND	27.00 ± 0.26
<i>Colletotrichum abscissum</i> + Citrus	35.15 ± 1.19	27.87 ± 0.70
Negative Control	ND	ND

^a ND indicates no detection; there was no amplification of the fungus or citrus DNA.

3.2. Standard curve

Correlation between C_t values and DNA from different quantities of conidia was linear ($y = -0.019x + 39.486$, $R^2 = 0.9972$), providing a range of the pathogen detection from 10 to 1000 conidia (Figure 3).

Figure 3. Standard curve for quantification of conidia number by qPCR using specific primers and probes for detection of *Colletotrichum abscissum*.

3.3. Detection of *Colletotrichum abscisum* from leaves of budwood increase block and citrus nursery plants

C. abscisum was promptly detected in budwood increase block in both varieties (Valencia and Pera) at the three farms sampled. An image from the amplification multiplex real-time PCR for detection of *Colletotrichum abscisum* and citrus from one budwood increase block plot can be observed in figure 4.

Figure 4. Amplication by multiplex real-time PCR for detection of *Colletotrichum abscisum* and citrus DNA from one sample of budwood increase block plot 3, Valencia variety, Farm 2.

Out of 122 samples, 89 (73%) were positive for the pathogen. In Farm F1 and F2, the percentage of positive samples varied from 72% up to 100%; F3 showed the lowest positive values detected (50%). In all plots analyzed, the detection percentage was equal or higher than 50% (Table 5).

Table 5. Percentage of leaves samples detected with *Colletotrichum abscisum* by qPCR from budwood increase blocks in three citrus farms.

Farm	Variety	Budwood increase block Plots	Rate (Posivite samples/ Total of samples)	Percentage of positive samples (%)
F1	Valencia	BIB 1	13/18	72
	Valencia	BIB 2	13/18	72
	Pera	BIB 3	8/10	80
F2	Valencia	BIB 4	30/37	81
	Pera	BIB 5	10/10	100
F3	Valencia	BIB 6	10/19	53
	Pera	BIB 7	5/10	50

The pathogen was also detected in all the four farms with sampled nursery plants and in the two citrus varieties under study. An image from the amplification multiplex real-time PCR for detection of *Colletotrichum abscisum* and citrus from one nursery plot can be observed in Figure 5.

Figure 5. Amplification by multiplex real-time PCR for detection of *Colletotrichum abscisum* and citrus DNA from one sample of nursery plot 3, Valencia variety, Farm 2.

Out of 175 samples, 129 (73%) were positive for the pathogen. The nursery plots showed percentages of positive samples ranging 50 to 96% (Table 6).

Table 6. Percentage of leaves samples with *Colletotrichum abscisum* by qPCR from nursery plants in four citrus farms.

Farm	Variety	Nursery plots	Rate (Posivite samples/ Total of samples)	Percentage of positive samples (%)
F1	Valencia	N 1	7/10	70
	Pera	N 2	6/10	60
F2	Valencia	N 3	27/28	96
	Pera	N 4	14/19	74
F3	Valencia	N 5	10/19	53
	Pera	N 6	9/10	90
F4	Valencia	N 7	18/19	95
		N 8	10/20	50
	Pera	N 9	18/21	85
		N 10	10/19	52

3.4. Quantification of *Colletotrichum abscisum* in leaves samples of budwood increase blocks and citrus nursery plants

The majority of the positive samples from budwood increase blocks (90%) contained 10 to 200 conidia of *C. absicissum*. A few samples (10%) showed 200 to 400 conidia, while no samples showed over 400 conidia (Figure 6). Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus in each sample of budwood increase block plot can be observed in appendix A, B, C, D, E, F and G.

Figure 6. Quantity of *Colletotrichum abscissum* conidia per sample from budwood increase block plots (BIB) of three farms estimated by qPCR. F= 'Farm', V= 'Valencia', P= 'Pera'.

The majority of the positive citrus nursery plants samples (96%) showed 10 to 400 conidia per sample; 59% of the samples showed between 10 to 200 conidia, 37% of the samples showed between 200 to 400 conidia and just 4% of the samples showed over 400 conidia of *C. abscissum*. Compared with the budwood increase block samples, a four-fold increase in the amount of positive samples in the range of 200 to 400 conidia was observed in citrus nursery plants samples (Figure 7). Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus in each sample of budwood increase block plot can be observed in appendix H, I, J, K, L, M, N, O, P and Q.

Figure 7. Quantity of *Colletotrichum abscissum* conidia per sample from citrus nursery plots (N) of four farms estimated by qPCR. F= 'Farm' V='Valencia', P='Pera'.

It can be seen a high variation in the number of conidia per sample. This seems to be common for the majority of citrus budwood increase blocks and nurseries sampled. Also, there was not possible to correlate the variation in the number of conidia in the leaves with the citrus varieties. However, in nursery plants it was more common to detect conidia in the ranges above 200 conidia per sample in most of the sampled nurseries when compared with plants from budwood increase blocks, suggesting that nursery plants are more contaminated with the pathogen than budwood increase blocks plants.

3.5. Isolation of *Colletotrichum abscissum* from leaves of budwood increase block and citrus nursery plants

The two first attempts to isolate *C. abscissum* from citrus samples failed due to the predominance of a redish yeast (unidentified), making it impossible the isolation of the pathogen (Figure 8 A/B), either in samples of budwood increase blocks or nurseries.

Figure 8. Isolation of *Colletotrichum abscissum* from leaves of budwood increase block and citrus nursery plants: leaf surface washing (A) and leaf fragments (B) methods after 5 days of incubation.

Therefore, a third attempt of isolation of the pathogen using semi-selective medium with nystatin was performed for the inhibition of yeast growth. Although a decrease in the yeast growth has been observed, it was not completely eliminated. In addition, nystatin hindered the growth of *C. abscissum* in the plates (Figure 9).

Figure 9. Leaves fragments of budwood increase block and citrus nursery plants on semi-selective medium with nystatin for isolation of *Colletotrichum abscissum*.

Using this approach, it was possible to isolate only two fungal strains (F1 and F2), with morphological characteristics similar to typical *Colletotrichum* colonies, which were subjected to DNA extraction and conventional PCR with specific primers as previously described, including the isolate LFN-1 of *C. abscissum* as a control. Nevertheless, the molecular identification was negative for *C. abscissum*, since the amplification failed for both strains. (Figure 10).

Figure 10. Electrophoresis gel (1% agar) of fragments of part of the ITS gene of isolates submitted to conventional PCR with primers pairs Calnt2/ITS4 for identification of *Colletotrichum abscissum*.

4. DISCUSSION

This is the first report of detection of *C. abscissum* in the leaves of sweet orange plants from budwood increase blocks and nurseries. The fungus was detected by qPCR in all citrus budwood increase blocks and nurseries sampled, as well as, in the two most important varieties of sweet orange for Brazil.

Previous works showed that *C. abscissum* remains epiphytically on the surface of citrus trees in commercial plantations, producing new conidia when rains occur, but without causing quiescent infections and without producing symptoms in the leaves and branches (PEREIRA, 2013). At flowering state, this inoculum reaches the petals and triggers the onset of the epidemic (AGOSTINI & TIMMER, 1994; ZULFIQAR et al., 1996). The results of this study indicate that the pathogen survives in budwood increase block and nursery plants too, in which it might play an important role as source of primary inoculum in commercial orchards. Different researchers pointed out this hypothesis in the past (PERES et al. 2005), however, it was never demonstrated before.

Occurrence of epidemics related to primary inoculum originating from seedlings has recently been reported in South Africa for Pinus cancer caused by *Fusarium circinatum* (SANTANA et al., 2015). The authors consistently related the haplotypes present in commercial plantations to those occurring in seedlings nurseries, what allowed them to identify the origin of the initial inoculum of the epidemics.

Another case is oak death disease caused by *Phytophthora ramorum* in United State forests. The researchers monitored the epidemic, sampled symptomatic oak trees and studied the populations to infer the pathogen genetic history. They concluded that the primary inoculum was related to nursery plants, which eventually spread the disease to several regions where oak was planted (KAMVAR et al. 2015).

Detection of the pathogen in leaves of budwood increase block and nursery plants of sweet orange could suggest a new dispersion type of *C. abscissum* in the cycle of postbloom fruit drop by propagation material.

The production of citrus nursery plants involves grafting of desired variety buds on rootstocks. These buds are removed from citrus matrices or budwood increase blocks plants,

which can harbor *C. abscisum* as quiescent appresoria. Thus, these buds can generate nursery plants already containing the inoculum, which would be perpetuated epiphytically on this propagation material, taking it into commercial farms.

On the other hand, *C. abscisum* shows short distance dispersion, which is associated with aggregated distribution of PFD on the orchards. However, the dispersion of PFD by propagation material could be a reason of the random distribution of the disease in the field as reported by SILVA-JUNIOR et al. (2014). According to the authors, the distribution of diseased trees did not correlate with the presence of symptomatic trees nearby.

Therefore, propagation material could be an important dispersion agent of the pathogen over long distances, during the orchard installation. This fact could explain the severe epidemics of PFD occurring in new orchards with no history of the disease (TIMMER, 1994) and the occurrence of identical haplotypes of *C. abscisum* in orchards separated by distances of up to 400 km, without spatial continuity between the points sampled (CIAMPI-GUILLARDI et al. 2014).

In this study, the amount of conidia of *C. abscisum* per sample was quantified and, in general, it was observed less than 400 conidia of the pathogen per sampled leaf. Budwood increase blocks and nursery plants are always inside greenhouse with strictly controlled conditions, which leads to the hypothesis that the pathogen survives in the vegetative parts with a limited reproduction.

It was observed less amount of conidia in samples from budwood increase block than in nursery plants. This could be explained because budwood increase block plants are kept inside of more controlled facilities than nursery plants. In these facilities there are less entry of workers, which use special clothing and the plants are more carefully treated, with less manipulation, drip irrigation and, use of disinfested tools. This makes these facilities more efficient in terms of crop protection.

The higher amount of spores found in nursery plants can be attributed to the fact that these plants have been exposed to more steps during its stabilization and development inside nurseries, such as grafting and pruning. Also, inside the nurseries, there is a higher density of plants than in the budwood increase blocks, which, associated with sprinkler irrigation may favor the pathogen reproduction and dispersion.

Although there are no records about the relation between the numbers of *C. abscissum* conidia on leaf surface with the initial of PFD epidemics. It is important to remember the ability of the fungus to produce secondary conidia, which can increase the total number of conidia on leaf surfaces under favorable conditions of rainfall events contributing with the inoculum levels on the fields (LEANDRO et al. 2001).

In view of the results of this study, it is important to perform other tests to confirm the presence of this pathogen in the budwood increase block and nursery plants. It is necessary to find a way to isolate the fungus and then to prove its pathogenicity, since unfortunately it was not achieved with the methods used in this work.

The State Department of Agriculture of São Paulo created the “Rules for Production of Certified Nursery Trees” with the purpose of establishing an increase block protected due to problems related to soil and others grafted transmissible pathogens like *Xylella fastidiosa* and different virus (ALVES-CARVALHO et al. 2001). Confirmation of *C. abscissum* in budwood increase block and nursery plants would lead to update this regulation and searching for new control strategies of the pathogen.

New control strategies can be implemented in order to avoid the dispersion of the pathogen in non-infected areas, for example: drip irrigation inside of nurseries, eradicator chemical applications in citrus matrices and budwood increase blocks, as well in nursery plants before being planted in the fields.

As well, the methodology presented in this study can be employed as a protocol for the evaluation of plots in nursery plants aiming the detection of *C. abscissum*, but it is necessary to continue testing different sampling and way to process the samples for improving the pathogen detection.

Tools like qPCR turns out to be very convenient for pathogen detection in diseases like PFD, where the casual agent survives in leaves and tissues without symptoms and is able to initiate the infection process when susceptible tissues appear. This technique has already been used to detect *C. acutatum* in plants with asymptomatic leaves in strawberry nurseries (CALIFORNIA STRAWBERRY COMMISSION, 2017).

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APPENDICES

Appendix A. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 1, Valencia variety, farm 1.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
BIB 1.1	ND*	ND	ND	28.93	0.69
BIB 1.2	127	37.06	0.98	25.75	0.37
BIB 1.3	ND	ND	ND	25.47	0.57
BIB 1.4	ND	ND	ND	29.08	0.04
BIB 1.5	173	36.21	0.87	26.47	0.11
BIB 1.6	156	36.52	2.15	26.85	0.18
BIB 1.7	ND	ND	ND	27.43	0.58
BIB 1.8	129	37.04	1.61	26.20	0.52
BIB 1.9	165	36.34	0.80	27.33	0.17
BIB 1.10	129	37.04	1.89	25.06	0.55
BIB 1.11	129	37.03	1.20	26.74	0.37
BIB 1.12	121	37.18	1.41	27.55	0.24
BIB 1.13	112	37.37	1.50	26.91	0.38
BIB 1.14	166	36.33	0.18	24.82	0.34
BIB 1.15	113	37.35	0.00	26.11	0.33
BIB 1.16	66	38.23	0.16	27.79	0.22
BIB 1.17	129	37.03	0.88	27.20	0.52
BIB 1.18	ND	ND	0.00	32.47	6.75
Citrus leaf with <i>C. abscissum</i> control		28.46	0.04	32.58	0.04
Citrus leaf without <i>C. abscissum</i> control		ND	ND	23.80	0.05
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix B. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 2, Valencia variety, farm 1.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
BIB 2.1	156	36.52	0.89	25.10	0.16
BIB 2.2	ND*	ND	ND	24.50	0.56
BIB 2.3	181	36.05	0.65	23.53	0.35
BIB 2.4	69	38.18	0.76	22.67	0.16
BIB 2.5	ND	ND	ND	23.25	0.18
BIB 2.6	ND	ND	ND	25.12	0.09
BIB 2.7	45	38.64	2.80	25.54	0.12
BIB 2.8	185	35.98	0.79	22.96	0.15
BIB 2.9	136	36.90	0.91	24.73	0.09
BIB 2.10	ND	ND	ND	25.61	1.17
BIB 2.11	199	35.70	1.33	25.63	0.11
BIB 2.12	129	37.03	1.29	22.79	0.11
BIB 2.13	196	35.76	0.55	25.01	0.19
BIB 2.14	131	37.01	0.17	25.51	0.34
BIB 2.15	164	36.36	0.38	26.93	0.05
BIB 2.16	130	37.01	1.84	25.98	0.18
BIB 2.17	130	37.02	0.74	26.81	0.07
BIB 2.18	ND	ND	ND	27.48	1.09
Citrus leaf with <i>C. abscissum</i> control		28.46	0.04	29.38	0.10
Citrus leaf without <i>C. abscissum</i> control		ND	ND	26.93	0.05
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix C. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 3, Pera variety, farm 1.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
BIB 3.1	83	37.92	0.05	26.86	0.10
BIB 3.2	304	33.72	2.01	27.43	0.06
BIB 3.3	287	34.04	0.47	26.05	0.42
BIB 3.4	ND*	ND	ND	26.69	0.18
BIB 3.5	137	36.87	0.84	24.75	0.12
BIB 3.6	188	35.92	1.02	26.39	0.06
BIB 3.7	ND	ND	ND	27.83	0.14
BIB 3.8	219	35.32	2.65	28.11	0.10
BIB 3.9	136	36.89	0.78	28.48	0.07
BIB 3.10	246	34.81	0.96	25.46	0.89
Citrus leaf with <i>C. abscissum</i> control		28.60	0.29	24.52	0.22
Citrus leaf without <i>C. abscissum</i> control		ND	ND	24.80	0.84
Negative control		ND	0.00	ND	NDD

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix D. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 4, Valencia variety, farm 2.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
BIB 4.1	125	37.11	2.43	26.38	0.48
BIB 4.2	110	37.39	2.06	27.26	0.31
BIB 4.3	202	35.65	0.58	28.06	0.80
BIB 4.4	72	38.12	0.78	27.13	0.11
BIB 4.5	126	37.09	0.76	27.16	0.21
BIB 4.6	97	37.64	1.52	25.76	0.60
BIB 4.7	ND*	ND	ND	31.16	6.41
BIB 4.8	24	39.03	1.86	28.71	0.49
BIB 4.9	ND	ND	ND	28.78	6.20
BIB 4.10	302	33.75	0.47	26.81	0.43
BIB 4.11	207	35.54	0.74	25.12	0.41
BIB 4.12	104	37.51	0.65	25.69	0.59
BIB 4.13	100	37.58	0.39	24.31	0.40
BIB 4.14	72	38.13	1.12	27.14	0.86
BIB 4.15	153	36.59	0.57	26.16	0.69
BIB 4.16	ND	ND	ND	26.95	0.79
BIB 4.17	168	36.29	0.29	25.06	0.20
BIB 4.18	86	37.86	0.95	25.01	0.35
BIB 4.19	ND	ND	ND	23.52	0.29
BIB 4.20	54	38.45	0.12	22.72	0.39
BIB 4.21	168	36.29	0.41	23.36	0.39
BIB 4.22	196	35.77	0.08	23.03	0.33
BIB 4.23	79	37.99	0.47	24.44	0.36
BIB 4.24	30	38.93	0.53	23.73	0.24
BIB 4.25	148	36.67	0.48	23.95	0.14
BIB 4.26	70	38.15	0.41	21.84	0.17
BIB 4.27	43	38.67	0.91	23.06	0.16
BIB 4.28	109	37.41	0.69	20.74	0.08
BIB 4.29	109	37.41	1.51	21.97	0.11
Citrus leaf with <i>C. abscissum</i> control		28.46	0.04	27.12	2.14
Citrus leaf without <i>C. abscissum</i> control		ND	ND	20.98	0.04
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Continuation Apendix D. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus from budwood increase block plot 4, Valencia variety, farm 2.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
BIB 4.30	ND*	ND	ND	25.58	0.20
BIB 4.31	10	39.32	2.96	23.49	0.29
BIB 4.32	11	39.28	1.31	25.18	0.28
BIB 4.33	12	39.26	2.46	23.63	0.13
BIB 4.34	ND	ND	ND	24.30	0.71
BIB 4.35	130	37.01	0.62	25.38	0.81
BIB 4.36	ND	ND	ND	27.33	1.05
BIB 4.37	168	36.29	0.29	25.06	0.20
Citrus leaf with <i>C. abscisum</i> control		28.46	0.04	27.12	2.14
Citrus leaf without <i>C. abscisum</i> control		ND	ND	20.98	0.04
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix E. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 5, Pera variety, farm 2.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
BIB 5.1	112	37.36	0.95	23.68	0.66
BIB 5.2	80	37.97	1.36	23.03	0.67
BIB 5.3	158	36.49	0.96	22.98	1.79
BIB 5.4	94	37.70	1.77	23.03	0.67
BIB 5.5	76	38.04	0.89	23.78	0.97
BIB 5.6	115	37.30	0.64	24.16	0.25
BIB 5.7	96	37.66	0.83	23.10	0.62
BIB 5.8	315	33.51	1.14	24.93	0.10
BIB 5.9	148	36.67	1.36	24.58	0.82
BIB 5.10	38	38.77	0.34	25.81	0.85
Citrus leaf with <i>C. abscissum</i> control		28.60	0.29	27.73	0.76
Citrus leaf without <i>C. abscissum</i> control		ND	ND	22.76	0.92
Negative control		ND	ND	ND	ND

Appendix F. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus from budwood increase block plot 6, Valencia variety, farm 3.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
BIB 6.1	ND*	ND	ND	25.28	0.23
BIB 6.2	ND	ND	ND	23.98	0.30
BIB 6.3	ND	ND	ND	27.22	0.24
BIB 6.4	ND	ND	ND	23.28	0.16
BIB 6.5	ND	ND	ND	25.69	0.12
BIB 6.6	ND	ND	ND	26.13	0.07
BIB 6.7	14	39.22	1.06	25.16	0.32
BIB 6.8	ND	ND	ND	26.38	0.07
BIB 6.9	123	37.14	0.95	22.84	0.06
BIB 6.10	ND	ND	ND	25.84	0.84
BIB 6.11	51	38.52	1.05	22.47	0.12
BIB 6.12	77	38.02	0.60	22.51	0.08
BIB 6.13	63	38.29	0.12	22.99	0.50
BIB 6.14	116	37.28	0.77	22.40	0.21
BIB 6.15	142	36.79	0.53	23.11	0.06
BIB 6.16	70	38.16	0.95	20.59	0.03
BIB 6.17	ND	ND	ND	25.75	0.06
BIB 6.18	62	38.30	1.02	25.70	0.40
BIB 6.19	51	38.52	0.00	24.03	0.34
Citrus leaf with <i>C. abscissum</i> control		28.60	0.29	27.73	0.76
Citrus leaf without <i>C. abscissum</i> control		ND	ND	23.93	0.21
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix G. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus from budwood increase block plot 7. Pera variety. farm 3.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
BIB 7.1	ND*	ND	ND	26.23	0.06
BIB 7.2	11	39.40	0.81	26.03	0.12
BIB 7.3	63	38.29	0.12	27.96	0.07
BIB 7.4	ND	ND	ND	24.99	0.04
BIB 7.5	116	37.28	0.77	27.43	0.14
BIB 7.6	ND	ND	ND	28.36	1.19
BIB 7.7	77	38.02	1.22	29.05	0.35
BIB 7.8	ND	ND	ND	25.91	0.39
BIB 7.9	10	39.45	1.16	26.87	1.05
BIB 7.10	ND	ND	ND	26.53	0.52
Citrus leaf with <i>C. abscisum</i> control		27.97	0.00	26.56	0.30
Citrus leaf without <i>C. abscisum</i> control		ND	ND	23.93	0.21
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix H. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 1. Valencia variety. farm 1.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
N 1.1	ND	ND	ND	30.27	0.15
N 1.2	304	33.72	2.01	28.56	0.04
N 1.3	93	37.72	3.30	27.44	0.22
N 1.4	178	36.10	0.80	27.43	0.06
N 1.5	287	34.04	0.47	28.83	0.54
N 1.6	91	37.75	0.26	27.26	0.05
N 1.7	163	36.39	0.76	27.14	0.18
N 1.8	153	36.58	1.96	29.93	0.14
N 1.9	ND	ND	ND	27.15	0.29
N 1.10	ND	ND	ND	26.14	0.06
Citrus leaf with <i>C. abscisum</i> control		28.60	0.29	24.52	0.22
Citrus leaf without <i>C. abscisum</i> control		ND	ND	24.80	0.84
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix I. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 2. Pera variety. farm 1.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
N 2.1	ND*	ND	ND	25.21	0.62
N 2.2	188	35.92	1.01	26.55	0.27
N 2.3	96	37.67	0.22	27.96	0.18
N 2.4	ND	ND	ND	26.19	0.17
N 2.5	ND	ND	ND	25.67	0.20
N 2.6	ND	ND	ND	27.44	0.22
N 2.7	137	36.89	ND	25.53	0.09
N 2.8	130	37.02	ND	26.90	0.42
N 2.9	130	37.01	2.03	25.50	0.47
N 2.10	287	34.04	0.47	25.88	0.26
Citrus leaf with <i>C. abscisum</i> control		28.60	0.29	24.52	0.22
Citrus leaf without <i>C. abscisum</i> control		ND	ND	24.80	0.84
ND		ND	ND	ND	0.00

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix J. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus nursery block plot 3. Valencia variety. farm 2.

Samples	Conidia Number	<i>C. abscissum</i>		Citrus	
		C _t	Standard deviation	C _t	Standard deviation
N 3.1	198	35.72	0.55	26.68	0.16
N 3.2	254	34.65	0.64	26.60	0.21
N 3.3	251	34.71	0.33	25.94	0.30
N 3.4	186	35.96	0.69	27.14	0.16
N 3.5	211	35.48	0.41	24.95	0.26
N 3.6	184	36.00	0.95	25.60	0.24
N 3.7	214	35.41	0.36	25.12	0.26
N 3.8	222	35.28	0.62	26.22	0.87
N 3.9	151	36.62	0.53	25.46	0.18
N 3.10	92	37.73	0.92	26.31	0.13
N 3.11	209	35.52	1.01	27.18	0.06
N 3.12	ND*	ND	ND	30.82	7.56
N 3.13	115	37.31	2.27	25.99	0.13
N 3.14	237	34.98	1.87	24.17	0.08
N 3.15	112	37.36	1.74	24.09	0.18
N 3.16	273	34.29	0.89	24.38	0.11
N 3.17	174	36.18	0.58	22.66	0.09
N 3.18	289	34.00	0.45	27.11	0.15
N 3.19	185	35.98	5.63	23.26	0.19
N 3.20	228	35.15	1.03	23.97	0.20
N 3.21	346	32.91	0.39	21.41	0.12
N 3.22	165	36.35	0.34	22.47	0.17
N 3.23	364	32.56	4.29	22.76	0.09
N 3.24	98	37.62	1.75	25.51	0.32
N 3.25	196	35.76	1.44	22.63	0.16
N 3.26	177	36.11	0.61	23.16	0.05
N 3.27	221	35.29	1.34	24.44	0.09
N 3.28	146	36.71	3.77	22.60	0.44
Citrus leaf with <i>C. abscissum</i> control		28.46	0.04	32.58	0.04
Citrus leaf without <i>C. abscissum</i> control		ND	ND	23.82	1.01
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix K. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus nursery block plot 4. Pera variety. farm 2.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
N 4.1	ND*	ND	ND	35.86	1.80
N 4.2	132	36.98	0.03	30.31	0.11
N 4.3	160	36.45	1.83	27.61	0.05
N 4.4	62	38.30	0.10	27.92	0.10
N 4.5	145	36.74	1.15	26.12	0.08
N 4.6	ND	ND	ND	28.07	0.44
N 4.7	ND	ND	ND	27.40	0.14
N 4.8	39	38.75	0.82	28.64	0.44
N 4.9	101	37.57	0.89	28.99	0.86
N 4.10	ND	ND	ND	25.66	0.07
N 4.11	53	38.47	0.57	26.19	0.07
N 4.12	119	37.23	1.19	27.41	0.03
N 4.13	ND	ND	ND	27.25	0.06
N 4.14	152	36.60	0.15	25.18	0.20
N 4.15	244	34.85	0.62	28.31	0.17
N 4.16	143	36.77	1.14	28.77	0.33
N 4.17	144	36.74	0.36	28.27	0.03
N 4.18	246	34.81	0.18	27.73	0.07
N 4.19	191	35.85	0.90	26.78	0.03
Citrus leaf with <i>C. abscissum</i> control		27.53	0.18	31.58	0.12
Citrus leaf without <i>C. abscissum</i> control		ND	ND	26.56	1.01
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix L. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus nursery block plot 5. Valencia variety. farm 3.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
N 5.1	622	27.68	13.54	27.50	0.03
N 5.2	ND*	ND	ND	27.14	0.51
N 5.3	ND	ND	ND	26.47	0.75
N 5.4	256	34.62	2.89	28.51	0.35
N 5.5	ND	ND	ND	30.71	0.02
N 5.6	978	14.46	9.05	26.43	0.27
N 5.7	743	25.37	10.57	26.02	0.50
N 5.8	211	35.47	2.23	26.74	0.21
N 5.9	87	37.83	4.42	27.23	0.91
N 5.10	ND	ND	ND	27.12	0.51
N 5.11	ND	ND	ND	26.24	0.33
N 5.12	304	33.70	0.07	26.33	0.26
N 5.13	ND	ND	ND	26.55	0.15
N 5.14	272	34.31	0.01	26.43	0.27
N 5.15	253	34.68	4.45	24.07	0.15
N 5.16	ND	ND	ND	26.03	0.25
N 5.17	ND	ND	ND	27.93	0.07
N 5.18	316	33.49	0.81	26.63	0.10
N 5.19	ND	ND	ND	25.95	0.16
Citrus leaf with <i>C. abscissum</i> control		27.53	0.18	24.02	0.26
Citrus leaf without <i>C. abscissum</i> control		ND	ND	23.88	0.50
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix M. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 6. Pera variety. farm 3.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
N 6.1	212	35.46	2.22	26.45	0.14
N 6.2	307	33.66	0.82	25.70	0.56
N 6.3	317	33.47	1.21	24.02	0.26
N 6.4	292	33.94	3.18	27.93	0.21
N 6.5	319	33.42	0.87	24.39	0.10
N 6.6	302	33.75	1.20	26.15	0.05
N 6.7	225	35.21	2.40	27.46	0.09
N 6.8	266	34.44	1.60	28.89	0.77
N 6.9	ND*	ND	ND	28.97	0.36
N 6.10	267	34.42	0.82	27.38	1.02
Citrus leaf with <i>C. abscisum</i> control		28.60	0.29	24.02	0.26
Citrus leaf without <i>C. abscisum</i> control		ND	ND	23.88	0.50
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Apendix N. Conidia number and cycle threshold (C_t) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 7. Valencia variety. farm 4.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
N 7.1	188	35.92	0.61	28.95	0.21
N 7.2	204	35.61	0.55	29.33	0.99
N 7.3	167	36.30	2.19	31.64	1.09
N 7.4	312	33.55	0.52	28.61	0.30
N 7.5	187	35.93	0.18	32.54	0.66
N 7.6	273	34.30	0.13	31.16	0.05
N 7.7	165	36.34	1.32	33.52	0.80
N 7.8	131	36.99	0.09	30.32	0.90
N 7.9	171	36.23	1.16	34.81	0.26
N 7.10	178	36.10	1.80	29.18	0.31
N 7.11	227	35.18	0.55	31.72	0.03
N 7.12	169	36.27	0.27	33.32	0.69
N 7.13	236	35.01	0.46	32.88	0.10
N 7.14	130	37.01	0.59	34.96	0.52
N 7.15	175	36.17	0.46	28.76	0.88
N 7.16	ND*	ND	ND	32.24	0.75
N 7.17	228	35.15	0.53	32.66	0.13
N 7.18	162	36.42	1.32	32.33	0.85
N 7.19	234	35.04	0.38	29.94	1.06
Citrus leaf with <i>C. abscisum</i> control		26.08	0.43	28.76	0.69
Citrus leaf without <i>C. abscisum</i> control		ND	ND	23.68	0.18
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix O. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 8. Valencia variety. farm 4.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		C_t	Standard deviation	C_t	Standard deviation
N 8.1	136	36.90	0.38	23.21	0.52
N 8.2	ND*	ND	ND	25.94	0.18
N 8.3	174	36.18	1.20	27.11	1.26
N 8.4	74	38.09	1.54	29.41	1.01
N 8.5	130	37.02	1.41	22.36	0.82
N 8.6	140	36.82	1.35	21.19	0.50
N 8.7	169	36.28	1.01	27.79	0.09
N 8.8	194	35.80	0.47	27.52	0.76
N 8.9	ND	ND	ND	24.99	5.21
N 8.10	ND	ND	ND	26.58	2.26
N 8.11	ND	ND	ND	26.58	2.26
N 8.12	ND	ND	ND	25.49	0.77
N 8.13	162	36.42	1.32	32.33	0.85
N 8.14	ND	ND	ND	32.36	7.40
N 8.15	ND	ND	ND	24.38	3.49
N 8.16	236	35.01	0.46	32.88	0.10
N 8.17	ND	ND	ND	20.45	0.64
N 8.18	ND	ND	ND	22.81	0.73
N 8.19	ND	ND	ND	32.74	2.63
N 8.20	177	36.13	0.46	29.07	0.50
Citrus leaf with <i>C. abscisum</i> control		27.09	0.18	28.27	0.03
Citrus leaf without <i>C. abscisum</i> control		ND	ND	23.68	0.18
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix P. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscissum* and citrus nursery block plot 9. Pera variety. farm 4.

Samples	Conidia Number	<i>C. abscissum</i>		<i>Citrus</i>	
		C _t	Standard deviation	C _t	Standard deviation
N 9.1	ND*	ND	ND	27.87	1.47
N 9.2	288	34.01	0.21	29.11	0.36
N 9.3	75	38.07	0.97	27.77	0.37
N 9.4	128	37.05	0.12	29.37	0.38
N 9.5	ND	ND	ND	32.16	1.59
N 9.6	144	36.75	0.40	24.89	0.95
N 9.7	ND	ND	ND	33.32	0.70
N 9.8	60	38.35	1.62	26.86	0.68
N 9.9	175	36.17	1.07	30.70	0.78
N 9.10	149	36.66	0.61	30.92	0.25
N 9.11	100	37.58	1.62	28.06	0.17
N 9.12	606	27.97	2.60	26.92	0.27
N 9.13	174	36.18	0.24	28.52	0.39
N 9.14	129	37.03	1.88	29.76	0.60
N 9.15	177	36.12	0.94	26.50	1.04
N 9.16	145	36.73	0.76	28.05	1.17
N 9.17	179	36.08	0.13	29.10	0.12
N 9.18	162	36.41	0.96	27.68	0.07
N 9.19	141	36.80	0.44	29.54	0.21
N 9.20	130	37.01	1.20	28.11	0.35
N 9.21	134	36.94	1.69	30.08	0.30
Citrus leaf with <i>C. abscissum</i> control		28.46	0.04	28.30	0.23
Citrus leaf without <i>C. abscissum</i> control		ND	ND	21.45	0.16
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.

Appendix Q. Conidia number and cycle threshold (C_T) of multiplex qPCR for detection of *Colletotrichum abscisum* and citrus nursery block plot 10. Pera variety. farm 4.

Samples	Conidia Number	<i>C. abscisum</i>		<i>Citrus</i>	
		Ct	Standard deviation	Ct	Standard deviation
N 10.1	ND*	ND	ND	29.51	0.16
N 10.2	54	38.46	3.82	25.61	0.68
N 10.3	ND	ND	ND	27.55	0.28
N 10.4	135	36.93	4.16	31.95	0.61
N 10.5	130	37.02	2.19	28.90	0.36
N 10.6	91	37.76	0.11	31.27	0.24
N 10.7	143	36.77	1.37	27.86	0.29
N 10.8	128	37.06	0.37	28.48	0.11
N 10.9	ND	ND	ND	28.58	0.56
N 10.10	148	36.68	0.54	29.38	0.38
N 10.11	ND	ND	ND	31.30	0.25
N 10.12	157	36.51	2.06	32.29	0.34
N 10.13	ND	ND	ND	30.51	0.65
N 10.14	167	36.32	1.52	29.84	0.67
N 10.15	ND	ND	ND	28.49	0.10
N 10.16	70	38.16	0.72	30.06	0.11
N 10.17	ND	ND	ND	31.97	2.52
N 10.18	ND	ND	ND	30.67	0.12
N 10.19	ND	ND	ND	28.55	1.56
Citrus leaf with <i>C. abscisum</i> control		27.09	0.18	28.27	0.03
Citrus leaf without <i>C. abscisum</i> control		ND	ND	24.98	0.28
Negative control		ND	ND	ND	ND

* ND indicates no detection; there was no amplification of the fungus or citrus DNA.