

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

Inoculum monitoring, fruit susceptibility to infection and fungicide efficacy for citrus black spot control during fruit development in sweet orange orchards

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Thesis presented to obtain the degree of Doctor
in Science. Area: Plant Pathology

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2021**

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*To my parents, Sebastião and Dalva;
My sisters Joziane and Glaucia*

I dedicate

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"In adversity, some give up, while others exceed records"

Ayrton Senna

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RESUMO

Monitoramento de inóculo, suscetibilidade de frutos à infecção e eficiência de fungicidas para o controle da pinta preta dos citros durante o desenvolvimento do fruto em pomares de laranja doce

Phyllosticta citricarpa produz ascósporos e picnidiósporos, os quais apresentam importância na epidemiologia da pinta preta dos citros no Brasil. Entretanto, a quantificação dos dois tipos de inóculos nos pomares é pouco estudada. Além disso, a suscetibilidade de frutos a *P. citricarpa*, bem como o período crítico para o controle da pinta preta, têm sido reportados como variáveis em diferentes áreas onde a doença ocorre no mundo. Portanto, esse estudo tem como objetivos: (i) monitorar e quantificar ascósporos e picnidiósporos em pomares comerciais; (ii) determinar o período de suscetibilidade de frutos de laranja doce à *P. citricarpa* por meio de inoculação artificial de esporos em diferentes estágios de desenvolvimento dos frutos em pomares comerciais; e (iii) avaliar a eficiência de oxicloreto de cobre e da piraclostrobina aplicado em diferentes fases de desenvolvimento dos frutos para o controle da pinta preta em pomar comercial. O monitoramento de inóculo de *P. citricarpa* foi realizado em dois pomares de laranja 'Valencia' durante duas safras no estado de São Paulo (SP), Brasil, usando mudas de laranja como armadilha de esporos combinadas com análises de PCR em tempo real. Nas armadilhas mantidas sob a copa de árvores o número máximo de cópias ITS do patógeno detectadas foi 407 por cm², enquanto nas armadilhas mantidas fora da copa foi ~60 cópias/cm². O maior número de cópias foi extraído principalmente entre outubro e março, com picos de amplificação entre novembro e fevereiro. Entre março e julho, menos de 20 cópias/cm² foram detectadas nas armadilhas. O número de cópias foi positivamente correlacionado com o número de dias chuvosos (≥ 5 mm) e com a duração do molhamento foliar. A suscetibilidade de frutos a infecções por *P. citricarpa* entre outubro a julho foi avaliada em dois pomares de 'Valencia' em SP. Os sintomas da doença e a queda de frutos foram mais intensos em frutos inoculados de outubro a fevereiro, com menos sintomas entre março e julho. As maiores severidades da doença foram de 20, 15 e 10% nos frutos inoculados com 10⁵ esporos/mL mensalmente (total de 10 vezes) de outubro a julho e em frutos inoculados apenas uma vez em novembro ou dezembro, respectivamente. A eficiência da aplicação de cobre ou piraclostrobina para a proteção de frutos após a queda de pétalas foi avaliada em laranja 'Natal' em SP. A incidência da doença foi reduzida em ~50% com aplicações de cobre a partir da queda de pétalas até junho/agosto, enquanto as reduções por aplicações de QoI foram de 80 a 90%. A falta de uma aplicação de QoI durante 38 a 42 dias não resultou em aumento da doença, embora a falta de uma aplicação de cobre durante 26 a 28 dias entre dezembro e março aumentaram a intensidade da doença no fruto. De acordo com os resultados obtidos em diferentes experimentos que avaliaram a quantidade de inóculo, a suscetibilidade dos frutos e eficácia dos fungicidas, o controle da pinta preta não pode ter falhas principalmente de novembro a fevereiro devido à presença de inóculo e condições climáticas mais favoráveis para infecções dos frutos e ocorrência de doença nos pomares paulistas.

Palavras-chave: *Citrus* spp., qPCR, Armadilha de esporos, Inoculação artificial, Proteção de frutos, Fungicidas

ABSTRACT

Inoculum monitoring, fruit susceptibility to infection and fungicide efficacy for citrus black spot control during fruit development in sweet orange orchards

The pathogen *Phyllosticta citricarpa* produces ascospore and pycnidiospore, which play an important role in the epidemiology of citrus black spot (CBS) in Brazilian conditions. However, the detection and quantification of the two types of *P. citricarpa* inoculum during the season are poorly studied. Moreover, the citrus fruit susceptibility to *P. citricarpa* infections as well as the critical period to CBS control have been reported as variable in different CBS-affected areas worldwide. Therefore, this study aimed to: (i) monitor and quantify both ascospores and pycnidiospores in commercial orchards; (ii) determine the susceptibility of sweet orange fruit by artificial inoculation of *P. citricarpa* at different developmental stages in commercial orchards, and (iii) identify the efficacy of copper oxychloride and pyraclostrobin fungicides sprayed at different fruit developmental stages for CBS control in commercial orchard. Monitoring of *P. citricarpa* inoculum was performed in two 'Valencia' sweet orange orchards during two seasons in São Paulo (SP) state, Brazil, by using young citrus trees as spore trap combined with quantitative polymerase chain reaction (qPCR) analyses. Traps kept under the canopy of trees had up to 407 ITS copies/cm², while the peak for traps kept outside the canopy was about 60 ITS copies/cm². *P. citricarpa* ITS copies were mainly detected between October to March, and the peaks were usually found from November to February. Fewer than 20 ITS copies/cm² were detected from March to July. The amount of ITS was related to rainy days (≥ 5 mm) and leaf wetness duration. The susceptibility of fruit to *P. citricarpa* infections by artificial inoculation from October to July was assessed in two 'Valencia' orchards in SP. CBS symptoms and fruit drop were observed in high levels when fruit were inoculated from October to February, while from March to July the symptoms were expressed in low intensities. The highest CBS severities were 20, 15 and 10% reached on fruit inoculated with 10⁵ pycnidiospore/mL 10 times from October to July, only in November or only in December, respectively. The efficacy of copper or pyraclostrobin spray at different times after petal fall was assessed in 'Natal' sweet orange in SP. Both fungicides applied only once consistently reduced CBS symptoms from December to March. CBS incidences were reduced ~50% with copper fungicide from petal fall through June/August, while reductions for QoI fungicide were 80 to 90%. The absence of a single QoI application for 38-to-42 days did not result in CBS increase, whereas trees without copper for a period of 26-to-30 days from December to March had greater CBS intensity on fruit. Taking into account the consistence of the results obtained in different trials that assessed not only the inoculum but also the susceptibility of fruit and efficacy of fungicides, CBS control failures may not occur mainly from November to February due to the presence of conditions highly favorable for fruit infections and CBS occurrence in SP sweet orange orchards.

Keywords: *Citrus* spp., qPCR, Spore trap, Artificial inoculation, Fruit protection, Fungicides

1. GENERAL INTRODUCTION

Citrus black spot (CBS) is caused by the ascomycete *Phyllosticta citricarpa* McAlpine Aa (synonym: *Guignardia citricarpa* Kiely). *P. citricarpa* is known as a pathogen that infects citrus species and cultivars (Baayen *et al.*, 2002) in countries of Africa, Oceania, South America (Cartens *et al.*, 2017), and the USA (Schubert *et al.*, 2012; Er *et al.*, 2014). The disease causes lesions on the rind which leave the fruit unfit for fresh-fruit market, and in more severe cases may lead to premature fruit drop.

The pathogen has as inoculum both asexual and sexual spores (McOnie, 1964a; Kotzé, 1981). In the asexual phase, *P. citricarpa* produces pycnidiospores into pycnidia on lesions formed in fruit, twigs and leaf litter (McOnie, 1964a; Kotzé, 1981). Pycnidiospores are released from mucilage through water and dispersed by splashing or washed off by rain to relatively short distances, infecting susceptible leaves and fruit, mainly within the tree canopy (Kotzé, 2000; Spósito *et al.*, 2011). In sexual stage, ascospores are produced inside pseudothecia in citrus leaf litter, and are disseminated by wind being responsible for primary infections (Kotzé, 1981). Although the two types of inoculum produced by the fungus have been considered as important in disease cycle in Brazil and Ghana, in South Africa only ascospore has been related to the CBS development and in Florida (USA) only pycnidiospore was found in citrus orchards (Kotzé, 1981; Spósito *et al.*, 2011; Wang *et al.*, 2016).

Monitoring and quantifying of *P. citricarpa* inoculum has been performed by volumetric spore traps that capture only ascospores in distinct citrus growing regions (McOnie 1964b; Reis *et al.*, 2006; Fourie *et al.*, 2013; Dummel *et al.*, 2015). In addition, the traditional spore traps have the inconvenient of capturing not only the ascospores of the pathogen *P. citricarpa*, but also the ascospores of other species such as the endophyte *P. capitalensis* (Baayen *et al.*, 2002; Meyer *et al.*, 2006; Truter *et al.*, 2007). Alternative traps need to be developed in order to capture both ascospore and pycnidiospore under citrus canopy tree. The quantification of only *P. citricarpa* inoculum may be performed by using molecular methods, especially quantitative polymerase chain reaction (qPCR) methods with species-specific primers (West *et al.*, 2008). The qPCR procedure has already been used to detect and quantify plant pathogens in air samples (Rogers *et al.*, 2009; Klosterman *et al.*,

2014; Dung *et al.*, 2018; Moyo *et al.*, 2020; Primiano *et al.*, 2021). Moreover, qPCR may be an alternative to microscopic spore counting as it may reduce the errors in visual identification (Cao *et al.*, 2016).

The pathogen *P. citricarpa* affects all commercially grown citrus species and cultivars of oranges, lemons, limes and tangerines, except sour orange (*C. aurantium*) and its hybrids (Kotzé, 1981) as well as Tahiti' acid lime (*C. latifolia*) (Baldassari *et al.*, 2008; Silva Junior *et al.*, 2016a). There is no consensus regarding the fruit susceptibility period to *P. citricarpa* infection in literature. The fruit susceptibility period is reported to start after petal fall stage and may last up to four (Kellerman and Kotzé, 1977), five (Kiely, 1948; Kotzé, 1981), six (Calavan, 1960; Klotz, 1978; Baldassari *et al.*, 2007) or up to 7 months (Brentu *et al.*, 2012). In experiments performed with artificial inoculation in green house, the CBS symptoms were observed in high levels from green fruit at 1.5, 3 and 5 cm in diameter to the color change stage or the beginning of ripening in 7 cm fruit (Frare *et al.*, 2019). The infections of fruit by *P. citricarpa* may be related to ontogenic resistance (young fruit more susceptible than old ones), the local pressure of inoculum and weather conditions, but there is limited information on this subject in the literature.

Management of CBS has been focused on eradicating and/or reducing the two types of *P. citricarpa* inoculum as well as protecting the fruit by fungicide sprays (Silva Junior *et al.*, 2016a,b). In South African and Australian orchards, where CBS has been managed for several decades, the chemical control is restricted to the critical period of *P. citricarpa* infection, from October to January/February (Kotzé 1981; Schutte *et al.*, 2003; Miles *et al.*, 2004). However, the CBS control period adopted in these countries is not enough to control the disease at acceptable levels in Brazil, thus it required to extend applications until March/April (Lanza *et al.*, 2018).

The main fungicides used to manage CBS in Brazil are copper-based (copper hydroxide, copper oxychloride and cuprous oxide) and quinone outside inhibitor, QoI (pyraclostrobin, azoxystrobin and trifloxystrobin), combined or not with mineral or vegetable oil (Silva Junior *et al.*, 2016a). In Brazil, the CBS chemical control is performed with two copper-based fungicide sprays at petal fall stage and 21 to 28 days later, followed by four to five QoI sprays at 35-42-day interval. (Silva Junior *et al.*, 2016a; Lanza *et al.*, 2018). The two first copper sprays have been used to control other citrus diseases such as citrus scab (*Elsinoe* spp.) and melanose

(*Diaporthe citri*) (Silva Junior *et al.*, 2016a). Due to the introduction of CBS in the 1990s, the control program for fungal diseases in SP needed to be extended, and QoI fungicides applications were included in the management after the two copper ones. However, the right time from October to March/April to use each of the fungicide groups, in which they are the most effective, is still undefined.

1.1. Objectives

This study was conducted to better understand three important issues related to the effective method for quantification of both *P. citricarpa* inoculum at the same time in the orchards, the stages in which the fruit is more susceptible to *P. citricarpa* infection as well as the efficacy of fungicides registered for CBS throughout the fruit developmental stage. The specific aims were to:

- (i) Monitor and quantify both ascospores and pycnidiospores of *P. citricarpa* by quantitative PCR analyses of leaves collected from young citrus trees used as traps in commercial sweet orange orchards during the fruit development;
- (ii) Determine CBS incidence, severity and premature drop of sweet orange fruit inoculated in the field with three concentrations of *P. citricarpa* inoculum at ten fruit developmental stages;
- (iii) Identify the efficacy of copper-based and QoI fungicides sprayed for fruit protection at different fruit developmental stages for CBS control in commercial orchard.

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2. MONITORING OF *PHYLLOSTICTA CITRICARPA* INOCULUM IN SWEET ORANGE ORCHARDS USING MOLECULAR ANALYSIS AND YOUNG CITRUS TREES AS SPORE TRAP

ABSTRACT

The quantification of *Phyllosticta citricarpa* ascospores in citrus orchards has long been performed by the use of volumetric spore traps. However, this trap has the disadvantages of not capturing pycnidiospores and the impossibility of visual differentiation of *P. citricarpa* ascospore from other *Phyllosticta* species. Thus, this work aimed to develop an effective method to capture both ascospores and pycnidiospores of *P. citricarpa* by using young citrus trees as spore trap combined with qPCR for inoculum quantification during the fruit developmental stages in commercial orchards. *Phyllosticta citricarpa* inoculum was monitored and quantified during two seasons in two commercial 'Valencia' sweet orange orchards located in Mogi Guaçu and Brotas, São Paulo (SP) state. The traps of young citrus trees were placed under the tree canopy or outside the canopy for different 14-day periods from October to July in all areas. Genomic DNA from young citrus trap leaves was extracted, and the number of copies of *P. citricarpa* ITS was estimated by qPCR. The number of copies of *P. citricarpa* ITS were correlated with weather variables (number of days with ≥ 5 mm of rain, number of hours of leaf wetness duration (LWD) and mean temperature during LWD) and also associated with the number of infection events of ascospores and pycnidiospores predicted by the decision support system developed in South Africa (CRI-PhytRisk). Overall, high quantity of *P. citricarpa* ITS copies were detected in young citrus traps between October to March, regardless of the trap place, season and location. In Mogi Guaçu in season 1, the peaks of ~ 200 and ~ 60 ITS copies/cm² were observed in January in young tree traps kept under and outside the canopy, respectively. Numbers from 50 to 120 ITS copies/cm² were also observed from October to February in traps kept under the canopy. In the following season in this area, a peak of about 400 ITS copies/cm² was observed in late November, and another of ~ 100 in early December in traps kept under the canopy. In the other 14-day periods, the amount of ITS was lower than 10 copies, irrespective of the trap position. In Brotas, the quantities of 40 to 80 ITS copies/cm² were recovered between November to February in traps kept under the canopy, while less than 10 ITS copies were found in other periods. In traps kept outside the canopy, a peak of 35 copies was registered in December/January, lower than 10 copies in other periods from November to February. The numbers of days with rainfall ≥ 5 mm and the hours of LWD during the rain were strongly associated with the amount of ITS detected in each 14-day period. In addition, the number of ITS copies detected from traps kept under the canopy was better fitted with the number of events suitable for infection of ascospores + pycnidiospores, whereas the ITS copies from traps kept outside the canopy were more related to the ascospore infections predicted by CRI-PhytRisk. The results demonstrated that young citrus trees may be used as spore traps to capture both *P. citricarpa* inoculums in the citrus orchard and qPCR was sensitive and effective to quantify the pathogen on leaves from this kind of trap. This study contributes for a better understanding of the relationship between *P. citricarpa* inoculums and weather, which may also be used for adjustment in the CBS control program.

Keywords: *Citrus* spp.; fungal disease; source of inoculum, epidemiology

2.1. Introduction

Citrus black spot (CBS), caused by *Phyllosticta citricarpa*, is one of the main fungal diseases of citrus worldwide, which depreciates the fruit for the fresh market and causes premature fruit drop leading to yield losses (Kotzé, 1981; EFSA, 2014). CBS symptoms are expressed on leaves, twigs and fruit and affects the main citrus species and cultivars. The CBS symptoms are diverse, rind-limited and expressed in six types: hard spot, freckle spot, virulent spot, false melanose, lacy spot, and cracked spot (Goes *et al.*, 2000; Kotzé 1981; Silva Junior *et al.*, 2016). Although *P. citricarpa* is listed as an A1 pathogen in the European Union (EFSA, 2014), the pathogen was isolated from asymptomatic leaf litter collected in *Citrus sinensis* and *C. limon* orchards located in Italy, Malta and Portugal (Guarnaccia *et al.*, 2017).

Phyllosticta citricarpa has both sexual and asexual phase known. In the sexual phase, airborne ascospores are formed in pseudothecia in leaf litter on the orchard ground within 40 to 180 days after leaf drop. Periods driven by wetting and drying of leaves and alternating temperature during the day and at night have been considered as necessary conditions for the maturation of pseudothecia (Fourie *et al.*, 2013; Lee and Huang 1973). Mature ascospores are ejected from water-wetted pseudothecia and dispersed by air currents up to 25 m (McOnie 1964a, Kotzé, 1981; Fourie *et al.*, 2013; Spósito *et al.*, 2007; 2008). *Phyllosticta citricarpa* also reproduces asexually by pycnidiospores (conidia) released from pycnidia formed in diseased fruit or on dead twigs, leaves in the canopy as well as in leaf litter (Balsassari *et al.*, 2006; Kotzé 1981). Pycnidiospores are dispersed by rain splash downward within the tree only to a distance of 80 cm (Spósito *et al.*, 2011).

The two types of spores infect susceptible citrus tissues and both may play a role in CBS epidemiology (Kotze 1981; Spósito *et al.*, 2011), although their importance has been considered as variable in the citrus regions most affected by CBS. Studies conducted in South Africa have reported that ascospore is the main inoculum due to its dispersal over longer distances as opposed to limited pycnidiospore dissemination via rain splash (Fourie *et al.*, 2013; Kotzé, 1981; McOnie 1964b; Korf 1998). However, in other regions such as Brazil and USA, the

pycnidiospores may play a prominent role in CBS epidemiology (Carstens *et al.*, 2017; Spósito *et al.*, 2011; Wang *et al.*, 2016; Hendricks *et al.*, 2017).

Quantification of ascospores in citrus orchards has been performed with spore traps by counting the number of spores in circular trap disc (Fourie *et al.*, 2013; McOnie 1964a; Reis *et al.*, 2006). Ascospores have been trapped during spring, summer and autumn (Kiely, 1948). However, the ascospores of *P. citricarpa* are similar to those produced by other non-pathogenic species in the *Phyllosticta* genus (Baayen *et al.*, 2002; Peres *et al.*, 2007; Meyer *et al.*, 2001, 2006; *et al.*, 2012; Wulandari *et al.*, 2009; Wikee *et al.*, 2013; Glienke *et al.*, 2011). For instance, the endophytic *P. capitalensis* frequently found in citrus orchards that produces ascospores morphologically similar to those of *P. citricarpa* may also be considered in spore trap assessments (Baayen *et al.*, 2002; Glienke *et al.*, 2011; Wikee *et al.*, 2013).

Monitoring and quantifying the ascospores of *P. citricarpa* has long been employed in distinct citrus-growing areas by use of volumetric spore traps (McOnie, 1964a; Kotzé, 1981; Reis *et al.*, 2006; Dummel *et al.*, 2015; Fourie *et al.*, 2013; Tran *et al.*, 2020; Moyo *et al.*, 2020). Spore trap has proved to be an accurate measure for quantifying ascospores in citrus orchards; however, the identification of spores by morphological characteristics is laborious, time consuming, requires knowledge of the fungus and it does not identify the spores at species level (Baayen *et al.*, 2002; Truter *et al.*, 2007; Tran *et al.*, 2020). Furthermore, this kind of spore trap is specific for ascospores and does not capture pycnidiospores of *P. citricarpa*. Due to the difficult in quantifying pycnidiospores, there is no study in the literature regarding the assessment of this inoculum of *P. citricarpa* in citrus growing areas.

Molecular techniques may be useful to simultaneously quantify the two types of *P. citricarpa* inoculum in the field. Quantitative PCR (qPCR or real-time PCR) and its derivations have been used as an alternative to detect and quantify airborne inoculum based on nucleic acids in different pathosystem (Rogers *et al.*, 2009; Klosterman *et al.*, 2014; Dung *et al.*, 2018; Moyo *et al.*, 2020; Primiano *et al.*, 2021). Quantitative PCR has been successful used for detection and quantification of plant pathogens airborne inoculum in other crops such as *Venturia inaequalis* in apple (Meitz-Hopkins *et al.*, 2014; Torfs *et al.*, 2019), *Fusarium circinatum* in pine (Quesada *et al.*, 2018) and *Blumeria graminis* f. sp. *tritici* in wheat (Cao *et al.*, 2016).

Pathogens such as *P. citricarpa* that produce both sexual and asexual spores are difficult not only to monitoring the inoculum but also to control the disease. The spores may require specific environmental conditions to be produced, disseminated and to infect citrus tissues (Perryman *et al.*, 2014; Spósito *et al.*, 2011). A better understanding of *P. citricarpa* inoculum availability in the orchards during the fruit developmental stages may provide useful information to improve CBS management in a more sustainable and cost-effective perspective (Tran *et al.*, 2020). Therefore, in order to monitoring both ascospores and pycnidiospores of *P. citricarpa* in in the field, the aims of the present work were (i) to establish a fast and reliable procedure based on the qPCR for the detection and quantification of *P. citricarpa* by using young citrus trees as spore traps; (ii) to determine the amount of *P. citricarpa* inoculum produced during the fruit developmental stages in different sweet orange orchards in São Paulo citrus belt and (iii) to correlate the amount of *P. citricarpa* inoculum with the environmental conditions.

2.2. Materials and Methods

2.2.1. Experimental area

The study was conducted during the 2018/2019 and 2019/2020 growing seasons in two commercial orchards of 'Valencia' sweet orange (*C. sinensis*) grafted onto Rangpur lime rootstock (*C. limonia*) located in São Paulo citrus belt, where CBS frequently occurs and causes damage. In the first season, *P. citricarpa* inoculum was monitored and quantified in a 16-year-old orchard, with 346 trees per hectare (7.6 m x 3.8 m), located in the municipality of Mogi Guaçu, SP, Brazil (22°10'44.8"S, 47°02'34.7"W, altitude 615 m a.s.l). In the following season, the inoculum was monitored in the same orchard used in the first season, as well as in a 15-year-old orchard with 357 trees/hectare (7.0 m x 4.0 m), located in the municipality of Brotas, SP, Brazil (22°10'02"S, 47°57'16"W, altitude 740 m a.s.l). Both areas received no fungicide sprays for CBS control during the period of experimentation.

Weather data were obtained from iMetos 3.3 automatic stations (Metos, Pessl Instruments, Austria) installed in each location and programmed to record

maximum, minimum and mean temperatures (°C), relative humidity (%), precipitation (mm) and leaf wetness duration (LWD) (h) every 60 min. Leaf wetness was recorded by the sensor and also estimated by the number of hours with relative humidity higher than 90% (NHRH>90%) (Sentelhas *et al.*, 2008).

2.2.2. Monitoring of *Phyllosticta citricarpa* in sweet orange orchards

The *P. citricarpa* inoculum was monitored and quantified in the orchards by using young Valencia trees as traps. All young tree were produced in certified and covered nurseries and presented at least five leaves each. Monitoring of inoculum was performed in five rows ('main row') per orchard and two adjacent rows on either side of each row were used as guard rows, totaling an experimental area of 15 rows. In main row, one young Valencia tree trap was kept under a tree canopy and a second tree trap was kept in an open space where a tree had been removed (approximately 3 m away from tree canopies). A total of 10 young tree traps (5 kept under a tree and 5 kept outside the canopy) were collected from each orchard every 14 days and replaced with other young tree traps from October to July of each season.

Inoculum of *P. citricarpa* deposited on leaves of young tree traps during the different 14-day intervals was quantified by the qPCR procedure described in the following items. Five leaves were collected from each young tree trap, placed in plastic bags and kept in freezer at -4°C. A fragment of approximately 3 x 3 cm (~ 9 cm²) was removed from each leaf and a pooled sample of five leaf fragments (~ 45 cm²) of a specific tree trap were used to quantify the pathogen DNA. Then, the procedure of DNA extraction, qPCR reactions and *P. citricarpa* spore estimation was performed based on the method established below.

2.2.3. DNA extraction

Total DNA was extracted from citrus leaves according to the modified cetyl trimethyl ammonium bromide (CTAB) method from Murray and Thompson (1980). Each sample was placed in an extraction bag and 3 mL of CTAB buffer (2% CTAB, 50 mM Tris-HCl [pH 8], 5% NaCl, 20 mM EDTA, 0.5% PVP, 2% β-Mercaptoethanol)

was added before the material was grinded with a Homex 6 homogenizer (Bio-Rad). The samples extract were transferred to 2 mL microtubes and incubated in a water bath at 65°C for 30 min, in order to rupture the spore cell walls. The samples were centrifuged at 16000 g for 5 min at room temperature (22°C). Supernatant aliquots of 900 µL were transferred to new microtubes where 900 µL of a 24:1 (v/v) CIA solution (chloroform: isoamyl alcohol) was added and tubes inverted for 30 seconds to mix the contents. The microtubes were centrifuged at 16,000 g for 10 min and 800 µL of the supernatant transferred to 1.5 mL microtubes where 480 µL of 96% isopropanol was added to precipitate the DNA. The tubes were then incubated at -20°C for 30 min before centrifuging at 16,000 g (at 4°C) for 10 min and discarding the supernatant. The precipitate was washed with 900 µL of 70 % ethanol and centrifuged at 16,000 g for 10 min. This last step was performed twice to increase purity of the DNA. Then pellet was air dried and finally resuspended in 30 µL water. The DNA was quantified using a Nanodrop 100 spectrophotometer (Thermo Fisher Scientific).

2.2.4. Quantitative PCR

The primers GcF1/GcR1 and the GcP1 probe were used in qPCR assays targeting the ITS1 rRNA region (Van Gent-Pelzer *et al.*, 2007). The qPCR reactions were performed on an ABI StepOne Quantitative PCR thermocycler (Applied Biosystems, Foster City, USA). Reactions for *P. citricarpa* detection were performed in a 12 µL volume containing 250mM of each primer, 100mM of the probe, 1x Master Mix Path ID (Ambicon Corp.) and 100 ng of DNA. The thermocycler was programmed for an initial 95°C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, and primer annealing and extension at 60°C for 60 s. DNA of *P. citricarpa* and DNA of pathogen-free citrus were used as positive and negative controls, respectively. Aliquots (4 µL) of each DNA sample were run in triplicate and reactions were performed in 96-well plates. The Cq values were used to estimate the number of *P. citricarpa* in each sample from the orchards by interpolation with the standard curve of known ITS copy number.

2.2.5. Plasmid and standard curves

The plasmid cloning was performed to develop a standard curve and establish a relationship between quantification cycle (C_q) values and number of *P. citricarpa* ITS-1 copies. The Gc-specific plasmid template was developed by cloning a product of the qPCR using *P. citricarpa* DNA from isolate LRS 42/12 as a template. A 69 bp fragment of the ITS region was amplified using the primers GcF1 and GcR1 as described above. The PCR product was purified using Wizard SV gel and PCR clean-up system (Promega) and ligated to pGEMT_easy vector (Promega). Chemically-competent *Escherichia coli* (JM109) cells were transformed with 10 µL of ligation mixture for 20 min on ice, heat shocked at 42 °C for 2 min and immediately placed on ice for 2 min and 800 µL of LB (Luria-Bertani) medium added. The cells were incubated at 37 °C, with shaking at 150 rpm for 1h and 30 min, before being plated on LB solid medium containing 100 µg/mL ampicillin (amp), 0.5mM IPTG and 80µg/ml X-Gal. The positive clones were confirmed by BLASTN analysis of the ITS fragment from PCR with the primers GcF1 and GcR1. The BLASTN results showed 100% of identity from 70 bp with *Phyllosticta citricarpa* strain (Accession number MT649656.1).

2.2.6. Efficiency of quantitative PCR for detection and quantification of *Phyllosticta citricarpa*

The limit of detection (LOD) of *P. citricarpa* with the quantitative PCR (qPCR) assay developed by Van Gent-Pelzer et al. (2007) was determined in sweet orange leaves before the use of this material as spore traps for quantification of pathogen inoculum in citrus orchards. Leaves of 'Valencia' sweet orange trees generated by tissue culture, to guarantee complete exclusion of the pathogen, were inoculated with different concentrations of a *P. citricarpa* pycnidiospore suspension. Suspensions were prepared with isolate LRS42/12 previously characterized as *P. citricarpa* and grown on potato dextrose agar (PDA) (Difco) at 25°C for 21 days. The spore concentrations were adjusted from 10⁰ to 10⁵ spores/mL. An aliquot of 1 mL of each spore concentration was deposited on fragments of leaves (45 cm²) to be used in a sensitivity qPCR assay. The specificity of the primers (GcF1/GcR1) and

probe (GcP1) (Van Gent-Pelzer *et al.*, 2007) was evaluated by PCR analyses of *P. citricarpa*, *P. capitalensis*, healthy citrus leaf tissues and '*Candidatus Liberibacter asiaticus*', the bacterium associated with huanglongbing (HLB).

2.2.7. Data analyses

The limit of detection (LOD) was determined as the minimum concentration of pycnidiospore at which all replicates tested positive by qPCR (Nutz *et al.*, 2011). A standard curve used to estimate the number of *P. citricarpa* ITS copies from samples from the field were developed with plasmid serial dilutions (10 \times) in healthy citrus DNA at a concentration of 100ng/ μ L ranging from 8.91 \times 10⁸ to 8.91 \times 10¹ copies/ μ L. In order to estimate the amount of ascospores and pycnidiospores in each sample, the C_q values obtained from *P. citricarpa* DNA extracted in the trap leaves were converted in number of ITS copies, using the plasmid standard curve. Then, these estimated ITS copies were transformed in number of spores based on the average quantity of 50 ITS copies present in each nucleus (Hu *et al.*, 2014). The amount of two nuclei per pycnidiospore and four nuclei per ascospore was assumed for the data analysis (Hu *et al.*, 2014). Linear regressions were performed using SigmaPlot software (version 14.0).

A simple linear regression was used to estimate the relationship between the number of estimated ITS copies of *P. citricarpa* from orchard samples and the weather variables (number of days with \geq 5 mm of rain, number of hours of LWD and mean temperature during LWD) of each period in which each trap was kept in the field. The precision of the model was estimated by regression coefficient of determination (R²) and by residual variation (Campbell and Madden, 1990). Analyses were performed using software R version 3.6.1 (R Core Team 2019).

The number of infection periods and inoculum pressure for the two-week trap periods were estimated using CRI-PhytRisk (www.cri-phytrisk.co.za). CRI-PhytRisk is a web-based decision support system that provides information on the risk of CBS infection for each citrus growing region in South Africa on a daily basis (Moyo and Fourie, 2019). Hourly weather data, including temperature, relative humidity, rainfall, wind direction and speed, collected from automatic weather stations during the two-week trap period were converted to 3-hour periods before

being analyzed in CRI-PhytRisk. Simple linear regression of predicted infection events by CRI-PhytRisk and *P. citricarpa* ITS copies quantification from samples collected in the field were conducted.

2.3. Results

2.3.1. Efficiency of quantitative PCR for detection and quantification of *Phyllosticta citricarpa*

Detection of *P. citricarpa* by quantitative PCR was inaccurate after 35 cycles, which corresponded to approximately 100 spores. Samples with *P. citricarpa* DNA quantities above this threshold were considered undetected. Samples with *P. citricarpa* DNA quantities above this threshold were considered undetected. The primers and probe used (GcF1/GcR1 and GcP1) were found to be specific to *P. citricarpa* as no amplification was observed for DNA extracted from other pathogens and citrus leaves (Data not shown). The TaqMan assay for amplification of plasmid ITS containing a single copy of the fragment from *P. citricarpa* did not show primer-dimer formation for the set of primers. Thus, the presence of a single specific product of the correct size in melt-curve analysis at ca. $77.8 \pm 0.5^\circ\text{C}$ confirmed this statement.

2.3.2. Determination of LOD and standard curves

The lowest possible pycnidiospore concentration giving reliable amplification (limit of detection, LOD) of samples in the serial dilution was 100 pycnidiospore/mL using GcF1 and GcR1 primers ($C_q = 35$; $SD = 0.46$), which corresponded to approximately 5 ascospores with four nuclei each. The linear regression of *P. citricarpa* plasmid ITS indicated that an average C_q of 9.72 ± 0.38 and 32.82 ± 0.49 corresponded to 8.9×10^9 and ~ 90 copies of target ITS, respectively (Figure 1A). The relationship between C_q values and the log of number of ITS copies may be explained by linear regression equation: [$C_q = 39.6285 - (3.3525 \times \text{Number of ITS copy})$; $R^2 = 0.99$; $P < 0.001$] with an amplification efficiency of 99%.

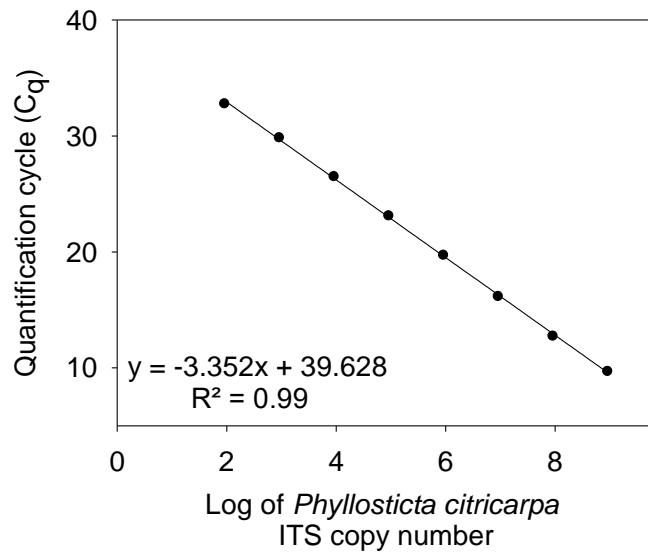


Figure 1 - Relationship between quantification cycle (Cq) values in quantitative PCR assay and number of *Phyllosticta citricarpa* ITS copies. The dots represent the values obtained with dilutions repeated three times, and the lines and equations represent the standard curves estimated by linear regression.

2.3.3. Monitoring of *Phyllosticta citricarpa* in sweet orange orchards

A total of 600 leaf samples were collected from young tree traps: 400 in Mogi Guaçu (200 per season) and 200 in Brotas (season 2). In the quality test control, 87% of these samples had standard curve slope below 0.98 and plate efficiency values between 0.9 and 1.1 and were used in the analyses.

In Mogi Guaçu during the season 1, *P. citricarpa* inoculum was detected by qPCR of leaf samples from tree traps throughout the trapping period, except in two 14-day periods in December and January from traps kept outside the canopy and in two other periods of June and July from traps kept in both places (Figure 2A). The total number of ITS copies recorded reached 198 copies/cm² on young tree traps kept under the tree canopy and 65 ITS copies/cm² on young tree traps placed in open space (Figure 2A). High quantities of ITS copies were obtained from tree traps kept under the tree canopy during two-week periods in late November to early December (100 copies/cm²), January (198) and February (116) (Figure 2A). The peak of 65 ITS copies/cm² was observed in January in trees kept outside the canopy, while fewer than 25 ITS copies/cm² were recovered from leaf traps in the other two-week periods (Figure 2A).

The highest numbers of 3-hourly periods suitable for pycnidiospore infections predicted by CRI-PhytRisk were observed from November to March during the different 14-day periods, with two peaks of seven infection periods estimated from November 22 to December 06 and from January 03 to 17. After March, the number of pycnidiospore infection periods did not surpass three events (Figure 2B). The number of periods for ascospore infection varied from 2 to 4 events from December to March, and none or only one event was predicted from October to November and after March (Figure 2B).

The number of days with rainfall ≥ 5 mm ranged from 1 to 7 from November to early June, with the seven-day peak recorded in the period from December 20 to January 03. No rainy day ≥ 5 mm was recorded from first week of June to July (Figure 2B). The longest accumulated LWD during rainy days from 80 to 90 hours was observed during three periods: November 22 to December 06, January 03 to 17 and February 14 to 27 (Figure 2B). Wetness duration from 15 to 60 hours was observed in the other two-week periods from November to June, in which rainfall ≥ 5 mm was recorded (Figure 2B). The average temperature during LWD ranged from 22 to 26° C from November to March, and decreased from April to June. Temperature from June to July was not shown as LWD and rainfall ≥ 5 mm were not registered in the last four 14-day periods (Figure 2B).

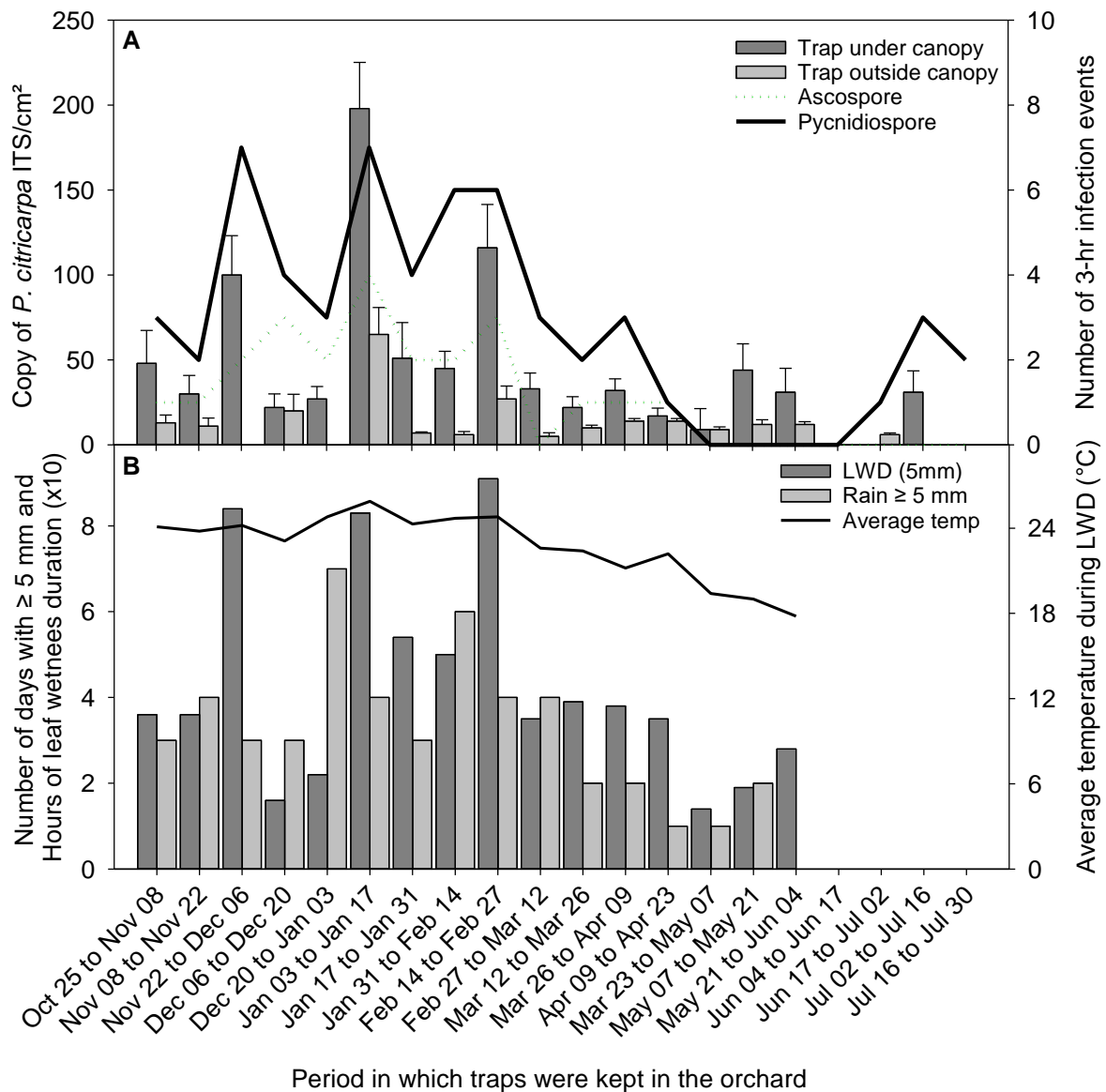


Figure 2 - Number of *Phyllosticta citricarpa* ITS copies per cm² extracted from leaves of young citrus tree traps and detected by qPCR (A). Field samples were collected during different periods of 14 days between late October 2018 and late July 2019. Numbers of 3-hour periods predicted to be suitable for ascospore or pycnidiospore infection by CRI-PhytRisk system (A). Numbers of days with rainfall ≥ 5 mm, leaf wetness duration (LWD) in rainy days, and temperature during the LWD registered for the periods in which the traps were kept in the 'Valencia sweet orange orchard located in the municipality of Mogi Guaçu, São Paulo, Brazil. Trap under canopy: tree traps kept under the tree canopy. Trap outside canopy: tree traps kept in open space where trees were eradicated from the orchard.

In Mogi Guaçu in 2019/2020, although the number of *P. citricarpa* ITS copies reached two peaks of about 400 and 100 in traps kept under the tree canopies from mid-November to early December, fewer than 20 ITS copies were detected in both traps placed under or outside the canopies in the other 2-week

periods from October to July (Figure 3A). In the two-week period, in which 407 ITS copies were amplified, the highest infection events of ascospore (6) and pycnidiospore (9) were estimated by CRI-PhytRisk (Figure 3A). Although fewer than 20 ITS copies were recovered from trap leaves before November or between December and March, the infection events for pycnidiospores varied from 3 to 6 and for ascospores from 1 to 4 in the different 14-day periods (Figure 3A). After March, lower values of both ITS copies and infection periods were observed (Figure 3A).

Rainfall events from 2 to 9 days and LWD from 30 to 110 hours occurred during the periods of 14 days mainly from November to April, and no rainfall or only one rainy day associated with around 20 hours of LWD was recorded in the two-week periods between April to July (Figure 3B). The highest numbers of rainy days (9) as well as hours of LWD (~110) were observed in November (Figure 3B) when the peak of ITS copies was registered. As in the previous season, the average temperature during LWD was around 24° C in the two-week periods from November to March, and the temperature decreased for averages below 18° C after April (Figure 3B).

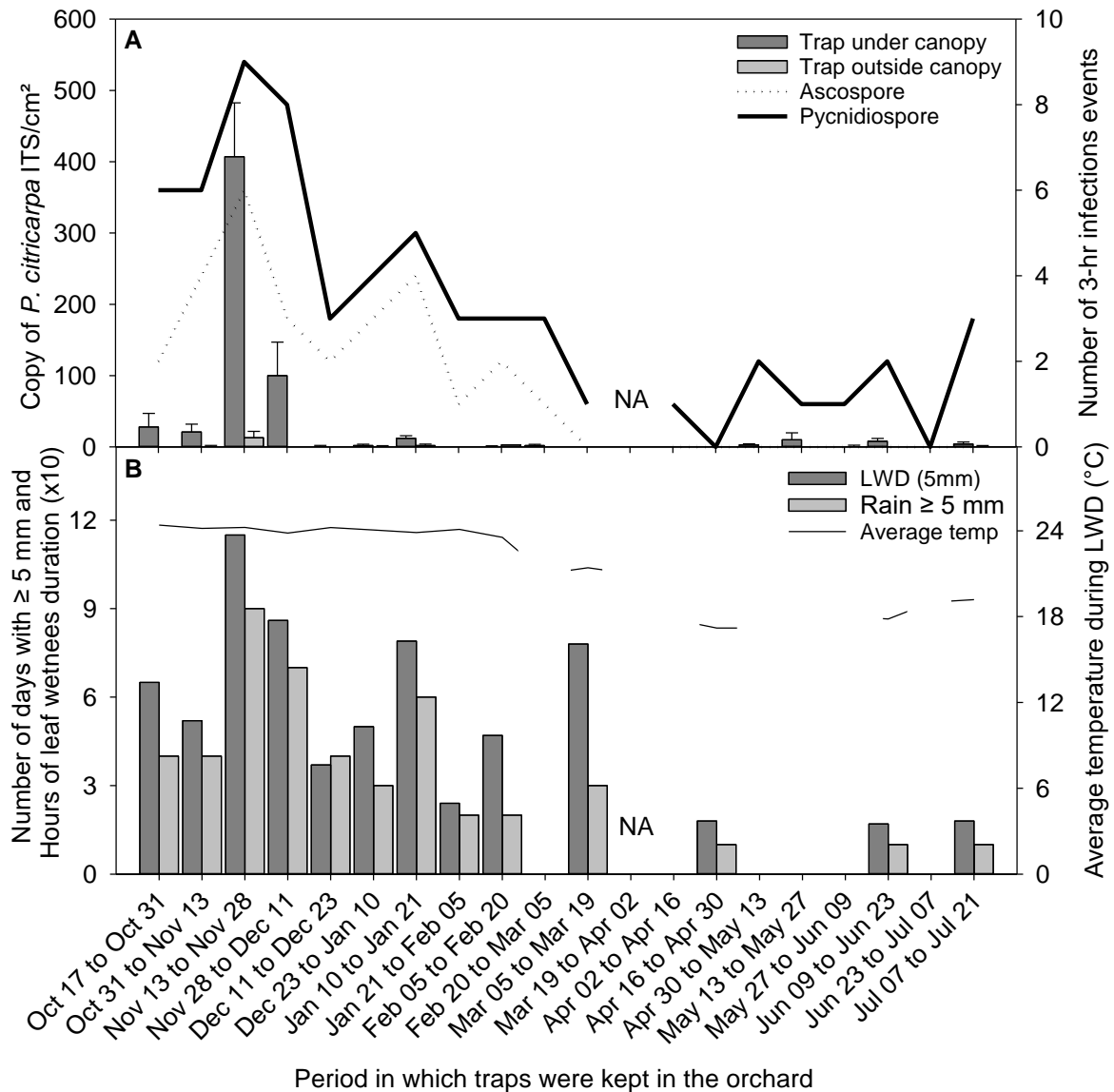


Figure 3 - Number of *Phyllosticta citricarpa* ITS copies per cm² extracted from leaves of young citrus tree traps and detected by qPCR (A). Field samples were collected during different periods of 14 days between late October 2019 and late July 2020. Numbers of 3-hour periods predicted to be suitable for ascospore or pycnidiospore infection by CRI-PhytRisk system (A). Numbers of days with rainfall \geq 5 mm, leaf wetness duration (LWD) in rainy days, and temperature during the LWD registered for the periods in which the traps were kept in the 'Valencia sweet orange orchard located in the municipality of Mogi Guaçu, São Paulo, Brazil. Trap under canopy: tree traps kept under the tree canopy. Trap outside canopy: tree traps kept in open space where trees were eradicated from the orchard. NA: No samples were collected during this period due to government restrictions in Brazil concerning the COVID-19 pandemic.

In Brotas during the 2019/2020 season, ITS of *P. citricarpa* were detected mainly from early November until early March, with numbers of copies ranging from 0 to 76 ITS copies/cm² in young citrus traps under the canopy and from 0 to 34 copies/cm² in traps kept outside the canopy. The highest amount of 76 ITS

copies/cm² was amplified in late January to early February in young tree traps kept under the canopy, followed by 39 to 51 copies observed in the samples collected in two-week periods from November to January (Figure 4A). There were no ITS recovered from tree trap samples from April to July, except for traps kept under a tree canopy between June 10 and 23 (Figure 4A). CRI-PhytRisk predicted up to four 3-hour periods suitable for ascospore infection from November to February, and up to eight 3-hour periods suitable for pycnidiospore infection from November to April. The peaks of 8 and 7 events suitable for pycnidiospore infections were predicted in November and February, while the maximum of 4 events were found for ascospore infections within this period (Figure 4A).

The number of rainy days with ≥ 5 mm ranged from 1 to 7 during the period between November and March. No more than 2 rainfall days were observed in the trapping periods between April and July, and no rain was measured in four of the trapping periods in this interval (Figure 4B). More than 70 hours of leaf wetness were recorded during six of the trapping periods between November and March (Figure 4B). Wetness duration below 30 hours was observed in the trapping periods from April to July, in which no more than 2 rainy days were recorded (Figure 4B). Average temperatures above 22° C were recorded from November to March, and after April the average temperature ranged from 16 to 19° C (Figure 4B).

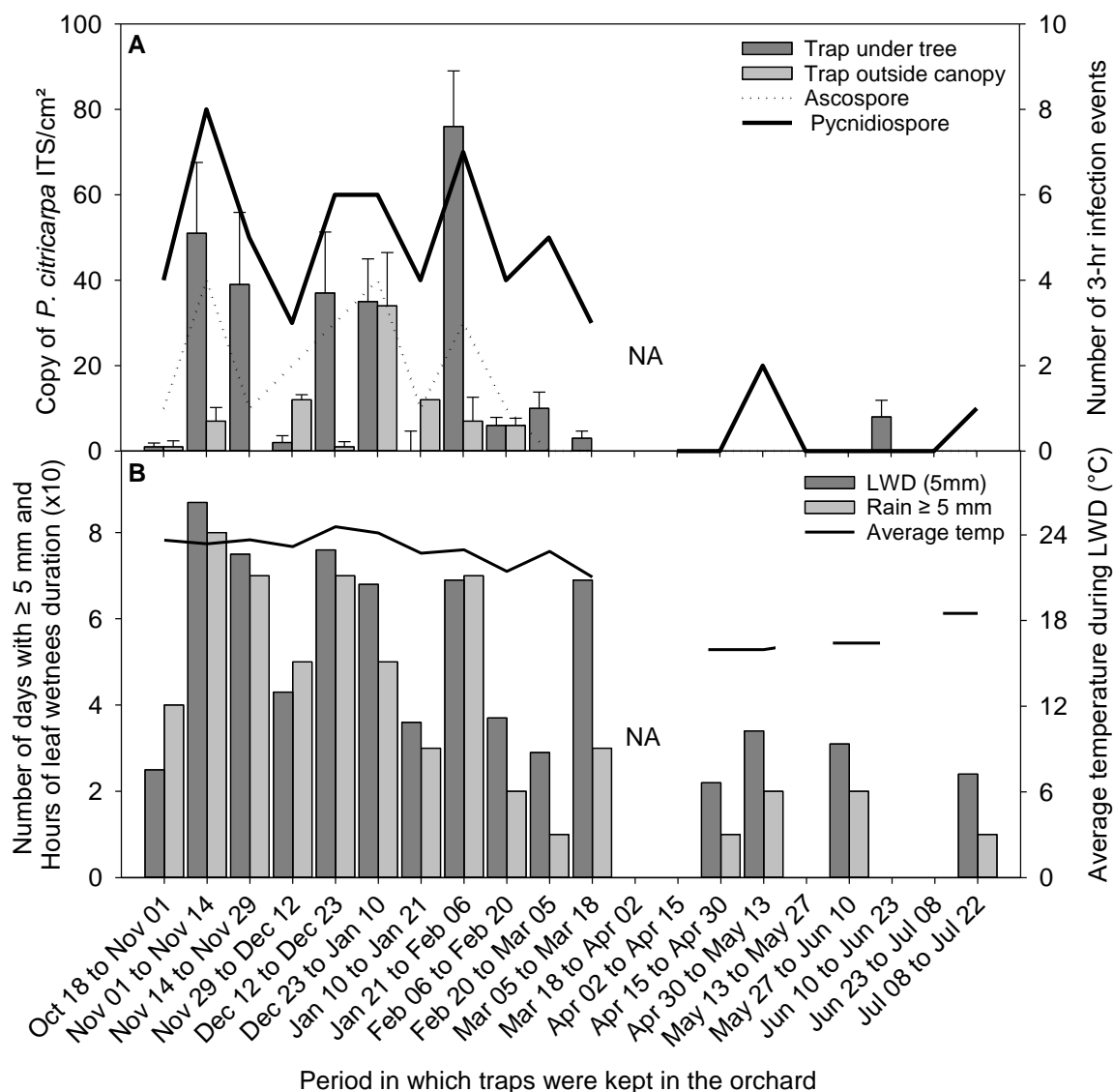


Figure 4 - Number of *Phyllosticta citricarpa* ITS copies per cm² extracted from leaves of young citrus tree traps and detected by qPCR (A). Field samples were collected during different periods of 14 days between late October 2019 and late July 2020. Numbers of 3-hour periods predicted to be suitable for ascospore or pycnidiospore infection by CRI-PhytRisk system (A). Numbers of days with rainfall ≥ 5 mm, leaf wetness duration (LWD) in rainy days, and temperature during the LWD registered for the periods in which the traps were kept in the 'Valencia sweet orange orchard located in the municipality of Brotas, São Paulo, Brazil. Trap under canopy: tree traps kept under the tree canopy. Trap outside canopy: tree traps kept in open space where trees were eradicated from the orchard. NA: No samples were collected during this period due to government restrictions in Brazil concerning the COVID-19 pandemic.

The number of 3-hour periods suitable for infections by ascospores and pycnidiospores as well as the sum of these periods, predicted by CRI-PhytRisk, was significantly associated with the number of ITS copies detected in young tree traps kept under tree canopies for all seasons and locations (Table 1). However,

pycnidiospore infection events were not significantly related to the amount of ITS extracted from young tree traps kept outside tree canopies in the orchards in Mogi Guaçu in 2018/2019 season and Brotas in 2019/2020 (Table 1). Although all relationships among ITS copies extracted from traps kept under the canopy and CRI-PhytRisk outputs were significant for the three datasets as well as pooled data, the coefficients of determination (R^2) were slightly higher when the analyses considered the sum of ascospores and pycnidiospores (Table 1). On the other hand, ITS copies extracted from traps maintained outside the canopy were more correlated with ascospore (R^2 from 0.42 to 0.47) than the sum of inoculum (R^2 from 0.24 to 0.37), while for two datasets the regressions were not significant for pycnidiospore ($R^2 = 0.14$) (Table 1).

Table 1 - Parameters estimated by linear regression model fitted to the relation between CRI-PhytRisk outputs and number of *Phyllosticta citricarpa* ITS copies detected from young citrus traps kept under tree canopies or away from tree canopies during 20 two-week trapping periods from November to July in two orchards of 'Valencia' sweet orange located in Mogi Guaçu and Brotas, São Paulo state, Brazil, during 2018/2019 and 2019/2020 seasons.

| Trap position | CRI-PhytRisk outputs ¹ | Adjusted R ² | a | b | P> t |
|----------------------------------|-----------------------------------|-------------------------|---------|--------|---------------------|
| <i>Mogi Guaçu 2018-19 season</i> | | | | | |
| Under the canopy | Ascospore | 0.53 | 9.907 | 28.602 | <0.001*** |
| | Pycnidiospore | 0.55 | -1.689 | 15.610 | <0.001*** |
| | A+P | 0.59 | -1.219 | 11.005 | <0.001*** |
| Outside the canopy | Ascospore | 0.42 | 2.361 | 7.904 | 0.001** |
| | Pycnidiospore | 0.14 | 3.747 | 2.703 | 0.062 ^{ns} |
| | A+P | 0.24 | 2.253 | 2.299 | 0.015* |
| <i>Mogi Guaçu 2019-20 season</i> | | | | | |
| Under the canopy | Ascospore | 0.42 | -20.179 | 35.05 | 0.001** |
| | Pycnidiospore | 0.43 | -48.228 | 24.825 | 0.001** |
| | A+P | 0.46 | -40.589 | 15.384 | <0.001*** |
| Outside the canopy | Ascospore | 0.44 | -0.512 | 1.133 | 0.001** |
| | Pycnidiospore | 0.29 | -0.980 | 0.666 | 0.010* |
| | A+P | 0.37 | -0.938 | 0.447 | 0.003** |
| <i>Brotas 2019-20 season</i> | | | | | |
| Under the canopy | Ascospore | 0.55 | 3.897 | 11.553 | <0.001*** |
| | Pycnidiospore | 0.64 | -5.235 | 6.673 | <0.001*** |
| | A+P | 0.67 | -3.674 | 4.664 | <0.001*** |
| Outside the canopy | Ascospore | 0.47 | -0.573 | 3.973 | <0.001*** |
| | Pycnidiospore | 0.14 | -0.617 | 1.295 | 0.061 ^{ns} |
| | A+P | 0.26 | -1.277 | 1.140 | 0.012* |
| <i>Pooled data</i> | | | | | |
| Under the canopy | Ascospore | 0.40 | -1.887 | 26.399 | <0.001*** |
| | Pycnidiospore | 0.36 | -16.482 | 15.197 | <0.001*** |
| | A+P | 0.41 | -14.810 | 10.505 | <0.001*** |
| Outside the canopy | Ascospore | 0.20 | 1.415 | 3.317 | <0.001*** |
| | Pycnidiospore | 0.08 | 1.254 | 1.361 | 0.015* |
| | A+P | 0.14 | 0.777 | 1.088 | 0.002** |

¹Number of 3-hour periods suitable for ascospore and pycnidiospore infection, as predicted by CRI-PhytRisk. A+P is the sum of 3-hour periods suitable for ascospore and pycnidiospore infection as predicted by CRI-PhytRisk. The relationship between periods suitable for infection and number of *Phyllosticta citricarpa* ITS copies were estimated by linear regression model ($y = a + bx$), where y is the number of ITS copies, a is the intersection; b is the slope of the line, R^2 is the coefficient of determination, and p is the level of significance (^{ns}not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

The number of *P. citricarpa* ITS copies extracted from the tree traps was positively related to the number of days with rainfall ≥ 5 mm as well as hours of LWD in rainy days of 5 mm, regardless the trap position, season and municipality. The LWD combination with average temperature during rainfall ≥ 5 mm was also strongly

related to the amount of ITS copies. However, the relationships between the average temperature during the LWD periods and ITS copies showed lower adjusted R^2 for all datasets, and were not significant for one dataset (Table 2).

In Mogi Guaçu, the adjusted R^2 from 0.60 to 0.72 were obtained in the first season for the relationships between ITS copies from traps kept under tree canopies and LWD*temperature, LWD and rainfall. Values of adjusted R^2 lower than 0.57 were obtained for these relationships by using numbers of ITS copies recovered from traps kept outside the canopy. A weaker relationship ($R^2 < 0.32$) was estimated between average temperature during LWD and ITS copies extracted from traps kept in both positions (Table 2). In the following season, strong associations with R^2 from 0.37 to 0.52 were observed for ITS copies recovered from traps kept in both places (under and outside the canopy) and LWD, rainfall and LDW*Temperature. The average temperature was not significantly associated with the amount of ITS copies from traps kept in both places (Table 2).

In Brotas, the number of rainy days, LWD and LWD*temperature were strongly correlated with ITS copies extracted from traps kept under tree canopies (R^2 from 0.64 to 0.67) and outside tree canopies (0.39 and 0.43). The temperature during LWD was significantly associated with ITS copies, but with R^2 below 0.27 (Table 2). Linear regression using the pooled data from the orchards in both seasons showed results similar to those obtained for analyses performed for each orchard and season. The variables such as number of rainy days, LWD during rainfall and LWD*temperature were more related to the numbers of ITS copies extracted from trap leaves compared to temperature during LWD. The adjusted R^2 varied from 0.41 to 0.72 for data obtained from traps kept under the canopy and from 0.23 to 0.28 for those maintained outside the canopy (Table 2).

Table 2 - Parameters estimated by linear regression model fitted to the relation between weather variables and number of *Phyllosticta citricarpa* ITS copies extracted from young citrus traps kept under or outside tree canopies during 20 two-week trapping periods from November to July in two orchards of 'Valencia' sweet orange located in Mogi Guaçu and Brotas, São Paulo state, Brazil, during 2018/2019 and 2019/2020 seasons.

| Trap position | Variable | Adjusted R ² | a | b | P> t |
|----------------------------------|-----------------------------------|-------------------------|---------|--------|---------------------|
| <i>Mogi Guaçu 2018-19 season</i> | | | | | |
| Under the canopy | Days with ≥ 5mm rain ¹ | 0.60 | -6.914 | 18.109 | <0.001*** |
| | LWD during rain ² | 0.66 | -6.529 | 1.476 | <0.001*** |
| | Temperature in LWD ³ | 0.32 | -154.9 | 9.022 | 0.006** |
| | LWD*Temperature ⁴ | 0.72 | -3.376 | 0.057 | <0.001*** |
| Outside the canopy | Days with ≥ 5mm rain | 0.46 | -1.025 | 5.112 | <0.001*** |
| | LWD during rain | 0.46 | -0.267 | 0.396 | <0.001*** |
| | Temperature in LWD | 0.19 | -37.78 | 2.314 | 0.03** |
| | LWD*Temperature | 0.57 | 0.365 | 0.0179 | <0.001*** |
| <i>Mogi Guaçu 2019-20 season</i> | | | | | |
| Under the canopy | Days with ≥ 5mm rain | 0.49 | -31.92 | 27.500 | <0.001*** |
| | LWD during rain | 0.37 | -31.037 | 1.787 | 0.004** |
| | Temperature in LWD | 0.02 | -149.99 | 8.436 | 0.259 ^{NS} |
| | LWD*Temperature | 0.44 | -32.116 | 0.081 | 0.081 |
| Outside the canopy | Days with ≥ 5mm rain | 0.52 | -0.754 | 0.912 | <0.001*** |
| | LWD during rain | 0.41 | -0.771 | 0.061 | 0.002** |
| | Temperature in LWD | 0.04 | -5.098 | 0.300 | 0.210 ^{NS} |
| | LWD*Temperature | 0.50 | -0.825 | 0.003 | <0.001*** |
| <i>Brotas 2019-20 season</i> | | | | | |
| Under the canopy | Days with ≥ 5mm rain | 0.65 | -4.577 | 7.285 | <0.001*** |
| | LWD during rain | 0.64 | -7.896 | 0.743 | <0.001*** |
| | Temperature in LWD | 0.27 | -62.129 | 3.776 | 0.013* |
| | LWD*Temperature | 0.67 | 6.945 | 0.031 | <0.001*** |
| Outside the canopy | Days with ≥ 5mm rain | 0.43 | -2.241 | 2.256 | 0.002** |
| | LWD during rain | 0.41 | -3.175 | 0.227 | 0.002** |
| | Temperature in LWD | 0.22 | -21.588 | 1.244 | 0.035* |
| | LWD*Temperature | 0.39 | -2.538 | 0.009 | 0.003** |
| <i>Pooled data</i> | | | | | |
| Under the canopy | Days with ≥ 5mm rain | 0.41 | -15.611 | 17.51 | <0.001*** |
| | LWD during rain | 0.41 | -18.343 | 1.456 | <0.001*** |
| | Temperature in LWD | 0.13 | -127.61 | 7.39 | 0.004** |
| | LWD*Temperature | 0.38 | -43.462 | 0.023 | 0.002** |
| Outside the canopy | Days with ≥ 5mm rain | 0.28 | -0.619 | 2.596 | <0.001*** |
| | LWD during rain | 0.23 | -0.412 | 0.196 | <0.001*** |
| | Temperature in LWD | 0.15 | -24.089 | 1.423 | 0.002** |
| | LWD*Temperature | 0.19 | -4.803 | 0.004 | 0.012* |

¹Number of days with ≥ 5 mm rain; ²Sum of hours with leaf wetness duration (LWD) in the days with rain ≥ 5 mm; ³Average temperature during the LWD, ⁴Average temperature during the LWD combined with hours of LWD. Linear regression between weather variables and number of *Phyllosticta citricarpa* ITS copies estimated by linear regression model ($y = a + bx$), where y is the number of ITS copies, a is the intersection; b is the slope of the line, R^2 is the coefficient of determination, and p is the level of significance (^{NS}not significant; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$).

2.4. Discussion

This study demonstrated for the first time that young citrus trees used as spore traps combined with quantitative PCR analyses may be an effective procedure to capture and quantify together both kinds of *P. citricarpa* inoculums in citrus growing areas. The monitoring of *P. citricarpa* inoculum in sweet orange orchards in São Paulo state, Brazil, by using this sensible methodology showed that the number of *P. citricarpa* ITS copies detected in trap leaves was usually higher during fruit development between October to March than from April to July. ITS copies of *P. citricarpa* were detected in high levels mainly between November and February from young citrus traps kept under sweet orange canopies. The amount of inoculum was positively associated to the number of days with rainfall ≥ 5 mm, the hours of LWD in rainy days as well as the combination of LWD with temperature. In addition, the amount of *P. citricarpa* inoculum captured in traps maintained under the tree canopies was more related to the sum of infection events by ascospore and pycnidiospore, whereas the ITS copies extracted from leaf traps kept outside the canopies were more related to ascospore events.

The inoculum of *P. citricarpa* was estimated based on the number of ITS copies detected in trap leaves since the traps may capture both ascospores (wind-spread from leaf litter) and pycnidiospores (washed down from the canopy) at the same time under tree canopies or mainly ascospores in the place of eradicated trees. This amount of ITS copies may be converted to the *P. citricarpa* spores by using around 50 ITS copies per nuclei, two nuclei per pycnidiospore and four per ascospore, both inoculums presumed to be unicellular (Hu *et al.*, 2014). Therefore, the amount of *P. citricarpa* ITS copies recorded in tree traps kept outside tree canopies may be considered only as ascospores, and the recovered copy of 200 ITS represents one ascospore. For instance, the peak of around 70 ITS copies/cm² in a 14-day period in January (Mogi Guaçu, season 1) may be converted in ~3150 copies or ~15 ascospore per leaf sample of 45 cm². In citrus traps maintained under the tree canopy, both ascospore and pycnidiospore may have been deposited on the leaves and the peak of about 400 ITS copies/cm² (~18,000 copies per 45 cm²) found in the same orchard and in a two-week period may be considered as ~180 pycnidiospores or ~90 ascospores per sample. The findings obtained in our study

corroborate with Reis et al. (2006) that reported low to moderate numbers of ascospores from October to March with peak of capture by volumetric spore traps in January and February in orchards from São Paulo citrus belt, and Bellotte et al. (2013) that observed fluctuations of *P. citricarpa* throughout the season in orchard also in São Paulo, with ascospore peaks between November and January. These studies reported the amount of ascospores varying from ~50 to up to 1,200 per week in January volumetric traps. However, comparisons between the two methods for inoculum quantification may not be performed as the disk area of conventional traps for ascospore capture is larger than the leaf trap surface used in our work, and PCR analyses consider only *P. citricarpa* spores, while the visual assessments include *P. citricarpa* and *P. capitalensis* ascospores.

In general, young citrus traps placed at a minimum distance of 3 m from the sweet orange canopies had peaks of capture 2- to 20-fold lower than the amounts recovered by traps installed under the tree canopies. This difference may be associated to the capture of pycnidiospores washed down from fruit, dead twigs and other tissues to the leaf trap. Conidia are dispersed over short distances of less than 1 m by splashing or washed by rain to nearby susceptible tissues located mainly below the inoculum source (Spósito et al., 2011), while ascospores may be spread by the wind over distances estimated at around 25 m (Spósito et al., 2007). Our findings are in accordance with Tran et al. (2020) in Australia, which monitored *P. citricarpa* inoculum in leaf litter and in volumetric spore trap and concluded that even in leaf litter pycnidia and pycnidiospores were more abundant than pseudothecia with ascospores. In addition, the difference among the amounts of inoculum from the two trap positions reinforced Spósito et al (2010) assertion that weather conditions in São Paulo citrus belt are favorable for production of both *P. citricarpa* inoculum in the orchards.

Biweekly observations of reproductive structures of *P. citricarpa* collected from this two-season study showed that the inoculum fluctuated throughout the fruit development period in both areas and seasons, with the peaks always from November to February. The inoculum discharged that generated these peaks was associated with LWD during rainy periods. The total LWD during rainfall ≥ 5 mm from October to July in the three experiments varied from 3573 (Brotas) to 3852 hours (Mogi Guaçu, season 1) and may explain the differences in the inoculum recovered

from the leaf traps in the different areas. LWD has been reported as a weather variable that may influence not only spore production, but also maturation and discharge (James and Sutton, 1982). The numbers of days with rainfall were another important variable correlated with the amount of inoculum. Although the numbers of rainy days with ≥ 1 mm or total volume of rain also showed positive correlations ($R^2_{adj} < 0.30$) with inoculum in the traps, the rainfall ≥ 5 mm was better correlated with the amount of *P. citricarpa* quantified in the orchards ($R^2_{adj} = 0.41$). This positive relationship between pathogen ITS copies detection and precipitation indicated that rainfall events potentially triggered the release of ascospore, which requires dry and wet alternating periods for spore maturation and discharge (Kotzé, 1981; 2000), and facilitates the washing down of pycnidiospore from twigs and fruit (Spósito *et al.*, 2008; 2011; Perryman *et al.*, 2014). In our study, the peaks in inoculum quantification were usually registered in 14-day periods of 7 to 9 days with rainfall ≥ 5 mm. McOnie (1964a) reported that rainy days of ≥ 5 mm after September 1 in South Africa were associated to the onset of ascospore release. However, other studies suggested that ascospore release did not always coincide with rainfall periods, and other sources of moisture such as irrigation, dew, and relative humidity may be playing a role in the ascospore discharge (Rossi *et al.*, 2009; Bellotte *et al.*, 2013; Fourie *et al.*, 2013; Moyo *et al.*, 2020).

In our study, there were no considerable fluctuations on the average temperature during LWD in the two-week periods during summer and autumn. However, the temperature decreased in the periods from autumn to winter. This reduction in the average temperature coincided with the end of the rainy season. A reduction of approximately 90% in *P. citricarpa* ITS detection in all orchards was observed during the winter. Our study only included weather data from different areas of São Paulo citrus belt that have Cwa (Mogi Guaçu) and Cfa (Brotas) climates with warm temperatures and rainy summers (Alvares *et al.*, 2013). As the temperature is not very variable among these areas and seasons, additional studies may be conducted in other citrus growing areas worldwide to better understand the direct effect of temperature in the inoculum production. Fourie *et al.* (2013) modeling the effect of temperature and wetness on *P. citricarpa* pseudothecium maturation and ascospore release reported that the majority (>95%) of ascospore release events occurred at temperatures $\geq 18^\circ\text{C}$. Mean temperatures closer to the reported

optimum of 21 to 28°C from September 1 plays an active role in the start of the ascospore release (Lee and Huang, 1973). Moyo et al. (2020) developed a new pseudothecium maturation and ascospore release model for *P. citricarpa*, and considered both wetness and temperature as the two main weather factors that influence the maturation of pseudothecia of *Phyllosticta* spp.

The amount of inoculum quantified from traps was also associated with the prediction of CRI-PhytRisk system developed in South Africa, which considers temperature, dew point, relative humidity, rainfall, wind direction and speed for estimating the events favorable for inoculum release and infection. Ascospore infections predicted by the system were strongly related to the capture of inoculum outside the sweet orange canopies, while the sum of ascospore and pycnidiospores was associated to the amount of *P. citricarpa* ITS quantified under the tree canopy. Overall, the peaks of inoculum captured in traps kept under the canopy were always associated with more than seven periods of 3-hour events for pycnidiospores and three for ascospores during 14-day intervals.

The molecular method proposed here has been an alternative to quantify pathogen inoculum in the field in different crops (Rogers et al., 2009; Klosterman et al., 2014; Dung et al., 2018; Moyo et al., 2020; Primiano et al., 2021). Most of these studies were focused on quantification of airborne spores in volumetric spore traps and replacement of morphological assessments. Quantification of inoculum in plant tissue was performed by Primiano et al. (2021), which developed a qPCR protocol to quantify urediniospores of *Neophytophthora tropicalis*, causal agent of Asian grapevine leaf rust, both in adhesive tapes and in grapevine leaves. Although this procedure has been effective to detect and quantify pathogen inoculum, the steps from DNA extraction to calibration quantification by qPCR are very laborious and sensible. The quality of the nucleic acids during the extraction depends on several factors including the storage of samples, type of propagule, collection matrix, and the presence of non-target particles. The estimated cost of the quantitative PCR is another subject to be explored to assess the feasibility of employing the method. Thus, this method has been indicated to generate information related to the pathogen, host and climate association useful for improving disease control as well as developing decision support systems for routine quantification of inoculum in the field by growers.

Some trials related to the correlations between *P. citricarpa* inoculum and CBS symptoms may be performed as the next steps, since the disease intensity as well as CBS incubation period depends on the period of infection and concentration of spores deposited on the fruit surface (Frare *et al.*, 2019). The identification of the critical period for inoculum production is an important information that needs to be associated to the susceptibility of fruit and the efficacy of control measures in order to better understand CBS occurrence in different intensities. Further studies may also quantify the inoculum in tree traps placed in different positions inside the canopy, during different periods shorter than 14 days, and in more CBS-affected areas worldwide in order to more accurately clarify relationships between inoculum and weather variables. In addition, the quantification of *P. citricarpa* and *P. capitalensis* ascospores by visual assessments and qPCR analyses may be performed to estimate the proportion of each species in the orchard along the season.

Taking into account the consistent results obtained in two different areas and seasons, our data showed that young citrus tree traps associated with qPCR analyses are viable to capture and quantify the two kinds of *P. citricarpa* inoculums. Thus, the very sensitive qPCR method developed here to detect low amounts of spores is promising to monitor the inoculum in citrus growing areas as an alternative for replacing conventional traps that use visual counting only of ascospores produced by different *Phyllosticta* species. The establishment of the main weather variables associated with the discharge and spread of inoculum in citrus orchards may provide useful information to improve CBS control by using rainfall and wetness data to anticipate or postpone a given CBS control measure based on more or less favorable conditions for the *P. citricarpa* inoculum production.

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3. CITRUS BLACK SPOT INTENSITY ON SWEET ORANGE IS AFFECTED BY *PHYLLOSTICTA CITRICARPA* INOCULUM CONCENTRATION AND FRUIT DEVELOPMENTAL STAGE

ABSTRACT

Symptoms of citrus black spot (CBS), caused by *Phyllosticta citricarpa*, may be observed in different species and cultivars. The expression of CBS symptoms on sweet orange fruit inoculated in greenhouse has been affected by the developmental stage and inoculum concentration. However, there is little information in the literature concerning the period in which the fruit are susceptible to *P. citricarpa* infections under field conditions. This study aimed to assess the influence of fruit inoculation month and concentration of *P. citricarpa* pycnidiospore on CBS symptom intensity and fruit drop. The experiment was conducted in two commercial 'Valencia' orchards located in Boa Esperança and Itapólis, São Paulo state, Brazil. Fruit were inoculated only once from October to July, with three concentrations (10^1 , 10^3 and 10^5 pycnidiospores/mL) of *P. citricarpa* suspension. Fruit inoculated every 30 days from October to July (totalling 10 inoculations in the same fruit) and non-inoculated were used as controls. CBS incidence and severity on fruit as well as premature fruit drop were monthly assessed from March to December in both areas. CBS symptoms and fruit drop were observed in high levels for fruit inoculated from October, at petal fall stage, to March, when the fruit had around 5.5 cm in diameter and was unripe, regardless of the areas. Conversely, from April to July usually less CBS symptoms were expressed on fruit by artificial inoculations in both areas, and in some months and concentrations did not differ from the values observed on non-inoculated fruit. In season 1, the highest CBS severities were around 16.8, 9.5 and 5.3% on fruit inoculated 10 times with 10^5 pycnidiospores/mL, only in December or in January, respectively. In season 2, CBS severities of 22.3, 16.5 and 11.2% were observed on fruit inoculated 10 times or only in November or in December, respectively. In the two areas, the lowest proportions of fruit attached to the tree of 42% were observed in plots with fruit monthly inoculated (season 1), followed by values of 56% and 63% in plots with fruit inoculated only in December (season 1) or November (season 2), respectively. Overall, CBS incidence and severity and fruit drop were greater on fruit inoculated with 10^5 pycnidiospores/mL compared to the lower concentrations. Thus, our results showed that sweet orange fruit may be usually infected from October to February in São Paulo citrus belt, with a most severe period from November and December. These findings may be used by citrus growers to make decisions on the timing and interval of fungicide sprays to control CBS during fruit developmental stage in sweet orange orchards.

Keyword: *Citrus* spp., artificial inoculation, CBS, Susceptibility period, fruit drop

3.1. Introduction

Phyllosticta citricarpa (McAlpine) Van der Aa (synonym *Guignardia citricarpa* Kiely) is the causal agent of citrus black spot (CBS) disease. Even though the disease symptoms do not reduce the quality of orange juice, CBS lesions have been associated with premature fruit drop that leads to significant yield losses under high disease intensity conditions (Kotzé, 1981). CBS disease was first reported in Australia (Benson, 1895), and is currently spread in tropical and subtropical citrus growing regions of Africa, Asia, Oceania, South America and North America (Kotzé 1981; Schubert *et al.*, 2012; Yonow *et al.*, 2013; Wang *et al.*, 2016; EPPO, 2017). Although *P. citricarpa* was detected in citrus orchards in Europe in the absence of any CBS disease symptoms (Guarnaccia *et al.*, 2017), the pathogen is considered an A1 quarantine pest in this region (EU; Annex1/A1) (EFSA, 2014).

Symptoms of CBS are expressed in six different types, i.e., hard spot, freckle spot, virulent spot, false melanose, lacy spot and cracked spot (Kiely 1948; Goes *et al.*, 2000; Silva Junior *et al.*, 2016a). The expression of different types of lesions may be influenced by the fruit age at infection time and the inoculum concentration (Frare *et al.*, 2019). CBS symptoms have been observed on sweet oranges (*Citrus sinensis*), lemons (*C. limon*), mandarins, tangerines, clementines (*C. deliciosa*, *C. reticulata*, and *C. clementina*), and some limes (*C. aurantiifolia* and *C. limettioides*) (Kotze, 1981, Baldassari *et al.*, 2008, Silva Junior *et al.*, 2016a). CBS symptoms have never been observed on Tahiti lime (*C. latifolia*) fruit, but *P. citricarpa* was isolated from leaf and fruit tissues of this citrus species (Baldassari *et al.*, 2008). In spite of sour orange (*C. aurantium*) being considered as resistant worldwide, CBS symptoms have been observed in Brazil at very low severity levels (Silva Junior *et al.*, 2016a). Sweet orange cultivars show the same susceptibility to *P. citricarpa* infections; however, the most severe losses have been observed in late maturing cultivars, such as 'Valencia' (Spósito *et al.*, 2004b).

Phyllosticta citricarpa produces pycnidiospores (conidia) by asexual reproduction and ascospores as sexual spores. Both pycnidiospores and ascospores play an important role in the CBS epidemiology, with their relative importance depending on different factors, such as environmental conditions, citrus species and cultivars (Spósito *et al.*, 2011; Tran *et al.*, 2017). Pycnidiospores are

produced in pycnidia on lesions of fruit, leaves, dead twigs in the tree canopy, as well as on leaf litter on the orchard ground. Ascospores are produced in pseudothecia only on leaf litter (Kotzé, 2000; Baldassari *et al.*, 2008; Spósito *et al.*, 2011). The dispersion of pycnidiospores occurs by washing down with rainwater from the surface of pycnidia on CBS lesions to nearby susceptible tissues of fruit, twigs and leaves (Brentu *et al.*, 2012; Spósito *et al.*, 2011). As pycnidiospores are transported over short distances, they are associated to the pathogen spread within the tree canopy (Reis *et al.*, 2006; Spósito *et al.*, 2011). Mature ascospores are mainly spread by air currents (McOnie, 1964a) and have been related to spread among trees (Spósito *et al.*, 2011). Infected propagation materials (budwood or nursery trees) have been suggested as responsible for the introduction of CBS into new areas (Marchionatto, 1926; Kiely, 1948; Wager, 1953; McOnie, 1964ab; Kotzé, 1981; Gottwald *et al.*, 2021).

In citrus growing areas of Australia and South Africa, ascospores are the main source of inoculum in CBS epidemics (McOnie, 1964a; Kotzé, 1981; Tran *et al.*, 2017). However, Tran *et al.* (2018) found similarities in CBS incidence and severity caused by ascospores and pycnidiospores in greenhouse conditions in Australia. Pycnidiospores may play a more significant role in CBS epidemics in regions such as Brazil and Ghana, where rainfall is more frequent or occurs in higher volumes for a longer period and young and ripe fruit overlap in the tree canopy (Baldassari *et al.* 2006; Spósito *et al.* 2008, 2011; Carstens *et al.*, 2017). *P. citricarpa* spores infect susceptible tissues of leaves and fruit in the presence of adequate wetness and temperature, but there is not quantitative information about environmental requirements for infection (Martínez-Minaya, *et al.*, 2015).

Control of CBS currently relies on calendar-scheduled preventive fungicide sprays during the fruit susceptibility period (Schutte *et al.*, 2003; Miles *et al.*, 2004; Silva Junior *et al.*, 2016b; Lanza *et al.*, 2018). However, there is no unanimity in the literature concerning the period in which fruit are susceptible to *P. citricarpa* infections as well as the critical period for CBS control in commercial orchards worldwide (Kotzé 1981; Schutte *et al.*, 2003; Miles *et al.*, 2004; Lanza *et al.*, 2018; Frare *et al.*, 2019). In South Africa, the fruit susceptibility period extends from fruit set until 4 to 5 months later (Kotzé, 1981; Schutte *et al.*, 2003), whereas in Brazil and Ghana the susceptibility has been reported to be up to 6-7 months after petal

fall (Baldassari *et al.*, 2009; Brentu *et al.*, 2012; Lanza *et al.*, 2018). This susceptibility period ranged from 40 to 340 days under controlled conditions, depending on the type of symptoms and fruit development stage at inoculation time (Aguiar *et al.*, 2012; Frare *et al.*, 2019). Frare *et al.* (2019) demonstrated that sweet orange cultivars Hamlin, Pera and Valencia are similarly susceptible to *P. citricarpa* infections. However, the incubation periods are shorter and incidences of CBS symptoms are higher following inoculation with higher inoculum concentration (10^5) and smaller fruit diameter (1.5 cm).

Studies conducted in greenhouse have provided valuable data on how CBS symptoms are expressed in sweet orange fruit artificially inoculated with *P. citricarpa*. However, the association of CBS severity with fruit drop in commercial orchards remains unclear. Field investigations related to fruit susceptibility during its developmental phases may provide further information for establishing a more effective and sustainable CBS control program. Therefore, this study aimed to determine the CBS intensity and losses due to premature drop of sweet orange fruit inoculated with different concentrations of *P. citricarpa* pycnidiospores at various fruit developmental stages in São Paulo state, Brazil.

3.2. Materials and Methods

3.2.1. Inoculum material

Phyllosticta citricarpa was isolated from naturally infected fruit of Valencia sweet orange in an orchard located in the central region of São Paulo citrus belt, in which the field trials were conducted. The isolate derived from single spore culture was grown on potato dextrose agar (PDA) at 24°C under continuous light for 20 days. The molecular identification was performed by conventional PCR using species-specific primers, GCP1 and GCP2, for *P. citricarpa* (Stringari *et al.*, 2009). Spore suspensions were prepared by flooding 20-day-old culture plates with 10 mL of sterile distilled water, gently scraping the colony surfaces with a scalpel, and then filtering the suspension through a sterile gauze pad. The inoculum was adjusted to 10^1 , 10^3 and 10^5 pycnidiospores/mL using a hemocytometer. A liter of suspension

at 10^5 pycnidiospores/mL was obtained with about five colonies of *P. citricarpa* grown on PDA during 20 days.

3.2.2. Experimental design and inoculation method

Field trials were performed during the 2017/2018 and 2018/2019 seasons in two commercial orchards of late maturing 'Valencia' sweet orange (*Citrus sinensis*) trees grafted onto Rangpur lime (*Citrus limonia*). In 2017/2018, the field trial was conducted in a 15-yr-old orchard located in the municipality of Boa Esperança do Sul (latitude 21°54'10.2"S, longitude 48°25'59.8"W, altitude 490 m a.s.l.). In the following season, the trial was performed in an 8-yr-old orchard located in Itápolis (21°25'45.49"S, 48°44'43.9"W, 540 m a.s.l.). The two municipalities are located in the central region of São Paulo state, Brazil, where the Koppen climate classification is Aw - tropical with dry winter (Alvares *et al.*, 2013), had a low incidence of CBS in previous seasons before the trial.

In both locations, treatments were arranged in a randomized complete block design and five replicates (rows of ~100 trees). At the beginning and end of each row, five trees were kept as buffer trees. Two guard rows were used above and below the field trial rows to avoid spray effects. Inoculations of fruit were monthly performed from October to July (10 months). At each month of inoculation, three concentrations (10^1 , 10^3 and 10^5 pycnidiospores/mL) were used to inoculate 10 fruit attached in a given tree per concentration in each block row (total of 50 fruit per concentration per month). The inoculations were performed in the three innermost trees of the plot. Fruit monthly inoculated (performed every ~30 days totalling 10 inoculations in the same fruit) from October to July with the three concentrations were used as control. In each block row, ten non-inoculated fruit were used as control, totaling 50 fruit samples. Fruit were always inoculated during the late afternoon, with 10 mL of spore suspension, using a hand sprayer with 500-mL capacity and thereafter individually bagged using a plastic bag (25 x 35 cm) for 48 hours to simulate a humid chamber.

3.2.3. Weather data collection

Meteorological data were collected using automatic meteorological stations iMetos 3.3 model (Pessl Instruments, Austria) installed at distances of 1 and 2 km from Boa Esperança do Sul and Itápolis field trials, respectively.

3.2.4. Assessments and data analyses

Assessment of incidence and severity of CBS symptoms on fruit attached to the tree were monthly performed in both seasons from the beginning of CBS symptom expression (March) until harvest (November). Fruit were harvested and immersed for one minute in a solution composed of ethephon at 2.10 g/L (Ethrel[®], Bayer CropScience) and imazalil at 0.25 g/L (Magnate 500 EC[®], Adama) to induce CBS symptom expression from latent infections. Fruit samples were kept in Fundecitrus laboratory at room temperature (24°C) for 20 days with constant light (Baldassari *et al.*, 2007). The last CBS assessment was performed in December on detached fruit after the post-harvest treatment above.

The assessments involved recording CBS incidence (percentage of inoculated fruit with at least one CBS lesion) and CBS severity (percentage of fruit area affected by the disease). CBS severity was estimated using a 6-level scale, considering all types of CBS symptoms on the outer canopy-facing portion of the fruit, adapted by Silva Junior *et al.* (2016a) from Spósito *et al.* (2004a). The area under incidence progress curve (AUIPC) and area under severity progress curve (AUSPC) were calculated for each season by using data from March to December (Madden *et al.* 2007). The data were tested for normality and equality of variances. Where required, data were transformed using the square root transformation but actual means are presented. The least significant difference was determined at $p < 0.05$.

The design was a randomized block with 34 treatments (11 inoculation time with three concentrations, and the non-inoculated control). The means of AUIPC and AUSPC for both seasons were compared using software R version 3.6.1 (R Core Team 2019) with the add-on package 'ExpDes'. When differences by the F

test were significant ($p < 0.05$), the averages were compared by the Scott-Knott test at 5% probability (www.r-project.org).

Premature fruit drop associated to CBS of each period of inoculation and spore concentration were analyzed using nonparametric Kaplan-Meier survival analysis (Kaplan and Meier, 1958) to compare the survival probabilities (%) and the survival time means (average time to fruit drop). Data from the two seasons were analyzed separately, and each fruit was considered a replication. The effects of three spore concentrations on the CBS fruit drop were compared to the non-inoculated fruit drop by using the pooled data of different inoculation times for each concentration. The effects of monthly inoculation as well as a single inoculation in a given month from October to July on the fruit drop caused by CBS were compared to the non-inoculated fruit drop using the pooled data of the three concentrations for the different inoculation times. This analysis was performed using SigmaPlot 14.0 (Systat Software Inc., San Jose, CA, USA). The influence of these effects on survival functions was evaluated with the log-rank test at the 5% probability level. Within significant effects, individual survival curves were separated with multiple log-rank comparisons that were pairwise compared using the Holm-Sidak test at the 5% probability level. The time to fruit drop were compared using 95% confidence intervals (Copes and Thompson 2008).

Diameter and color index (CI) of fruit were monthly assessed on samples of 25 fruit randomly collected (five per block) from the first inoculation (October) until post-harvest treatment (December of the following year). Due to the color index having to be assessed in the laboratory, the fruit were not the same ones used for inoculation. The fruit diameter was measured at equatorial region of each fruit with a caliper (Eccofer, 150 mm, Curitiba, Paraná) and CI of the fruit peel was measured using a portable Chromometer CR-400 (Konica Minolta Sensing Inc, Tokyo, Japan) at two points (one per side) on the equatorial region of each fruit (Frare et al. 2019). The CI was expressed using the transformation: $CI = 1000a/Lb$, in which a is the variation in green and red (red-greenness), b = variation in blue and yellow (blue-yellowness), and L is variation in light and darkness (lightness). Negative and positive values indicate fruit with green and yellow/orange peel, respectively. A value of zero indicates the midpoint of the colour break period (Jiménez-Cuesta *et al.*, 1981, Frare *et al.*, 2019).

As additional analysis, the average temperatures recorded during the 48-hour periods of humid chamber from October to July inoculations were used to estimate the percentages of *P. citricarpa* appressorium formation by using the surface response model described by Noronha (2002). The model is expressed by:

$$Y = (0.15)*((T-(9.8))^{(0.37)}(((43.34)-T)^{(0.73)})*(((20.42)/(1+(10.36)\exp(-(0.14)WD))))),$$

where Y is the relative percentage of appressoria, T is the temperature in °C and WD is the wetness duration in hours. Through this model, the minimum, average and maximum temperature for the appressorium formation was estimated as 9.8, 20.4 and 43.34°C, respectively.

3.3. Results

3.3.1. Progress of CBS symptoms on fruit

The average temperature ranged from 17.3 to 25.9 °C in season 1 and 19.4 to 27.2 °C in season 2 during the 48 hours in which inoculated fruit were left in a humid chamber of inoculations performed from October to July. In the first season, average temperatures recorded during 48 hours were 25.0, 24.2, 23.5, 25.8, 23.5, 25.9 and 23.8°C from October to April. This slightly variation of the temperature did not consistently affect the percentages of *P. citricarpa* appressorium formation, which varied from 68.2 to 70.6% based on model of Noronha (2002). The lowest temperatures of 17.3, 18.8 and 17.6°C were recorded from May to July. In the following season, averages temperature above 23°C were also recorded from October to April, and the values were: 23.0, 23.3, 27.2, 26.5, 24.1, 25.3 and 24.1°C. The percentage of appressorium formation for these months ranged from 66.3 to 70.8%. Temperatures of 20.6, 20.8 and 19.5 were registered during the humid chamber of May, June and July, which represented an estimation of around 71% of appressorium.

Symptoms of CBS were observed in 100% of the 'Valencia' fruit monthly inoculated from October to July, regardless of the inoculum concentration and season (Figures 1A-F). In the first season, the incidence of CBS symptoms, as

assessed from May to December, on fruit inoculated with 10^1 pycnidiospores/mL was lower than 63.1% for fruit inoculated only in a given month (Figure 1A). CBS incidences above 32% were evaluated in the last assessment when fruit was inoculated from October to February (Figure 1A). CBS incidences on fruit inoculated with 10^3 pycnidiospores/mL only in December, January or February varied from 70% to 80% diseased fruit (Figure 1B). Inoculations with 10^5 pycnidiospores/mL resulted in CBS incidence of more than 90% on fruit inoculated from October to February (Figure 1C). CBS incidences were lower than 60% when fruit were inoculated in a given month from March to July in the first season, regardless of the inoculum concentration (Figures 1A-C). The average CBS incidence of non-inoculated fruit used as control was 42.9% (Figure 1A-C). In the 2018/2019 season, the CBS incidences were high and more than 77% of diseased fruit was observed, regardless of the inoculation month and inoculum concentration (Figures 1D-F). The percentage of non-inoculated fruit with at least one CBS lesion was lower than 40% (Figures 1D-F).

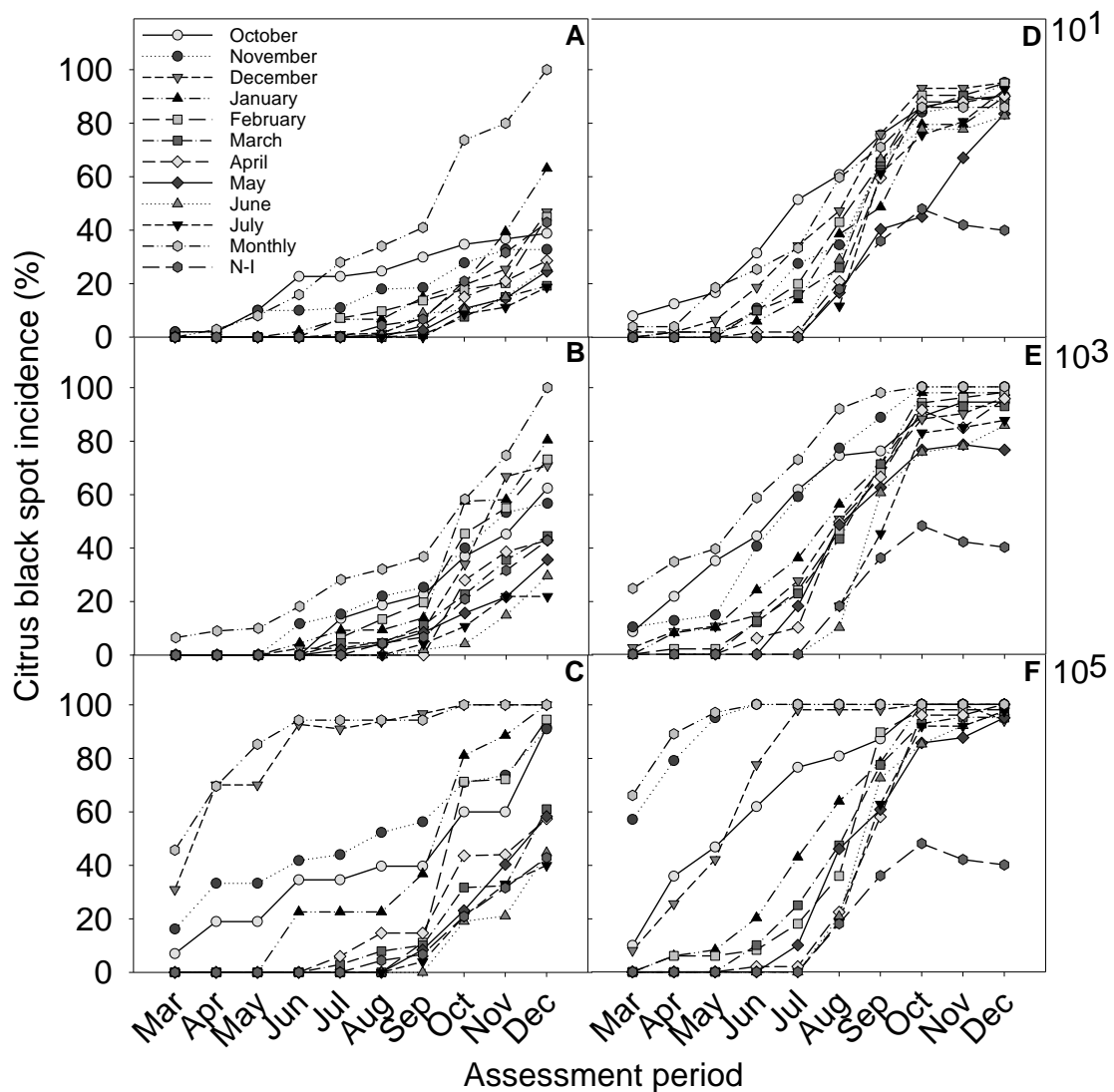


Figure 1 - Progress of citrus black spot incidence (% diseased fruit) assessed from March to December in commercial orchards of 'Valencia' sweet orange in the municipalities of Boa Esperança do Sul, season 1 (A-C) and Itapópolis, season 2 (D-F), São Paulo state, Brazil. Fruit were inoculated with 10^1 (A and D), 10^3 (B and E) or 10^5 (C and F) pycnidiospores/mL of *Phyllosticta citricarpa* once from October 2017 to July 2018 (A-C) and from October 2018 to July 2019 (D-F). Fruit monthly inoculated from October to July and non-inoculated fruit (N-I) were used as controls.

Severity of CBS on fruit inoculated with 10^1 or 10^3 pycnidiospores/mL was low during the assessment period and ranged from 0.2 to 3.1% in season 1 for the last assessment (Figures 2A-B). The highest CBS severities were found with 10^5 pycnidiospores/mL on fruit inoculated 10 times from October to July (16.9%), followed by fruit inoculated only in December (9.5%), January (5.3%), February (3.1%), October (2.9%) and November (2.3%) (Figure 2C). CBS severity on fruit inoculated from March to July varied from 0.4 to 1.5%, and on non-inoculated fruit

was 0.4% (Figure 1A-C). Similar to season 1, fruit inoculated at different periods, with 10^1 and 10^3 pycnidiospores/mL in season 2, had CBS severities varying from 2.0 to 5.9% during the assessment period (Figures 2D-E). The highest CBS severity in the last assessment on fruit inoculated 10 times with 10^5 pycnidiospores/mL was 22.3%, followed by inoculation only in November (16.5%), December and February (9.8%), January (8.8%) and October (7.1%) (Figure 2F). The fruit inoculated with 10^5 pycnidiospores/mL from March to July had average severities between 3.8% and 5.8%, while non-inoculated fruit had 1.1% of diseased area (Figure 2F).

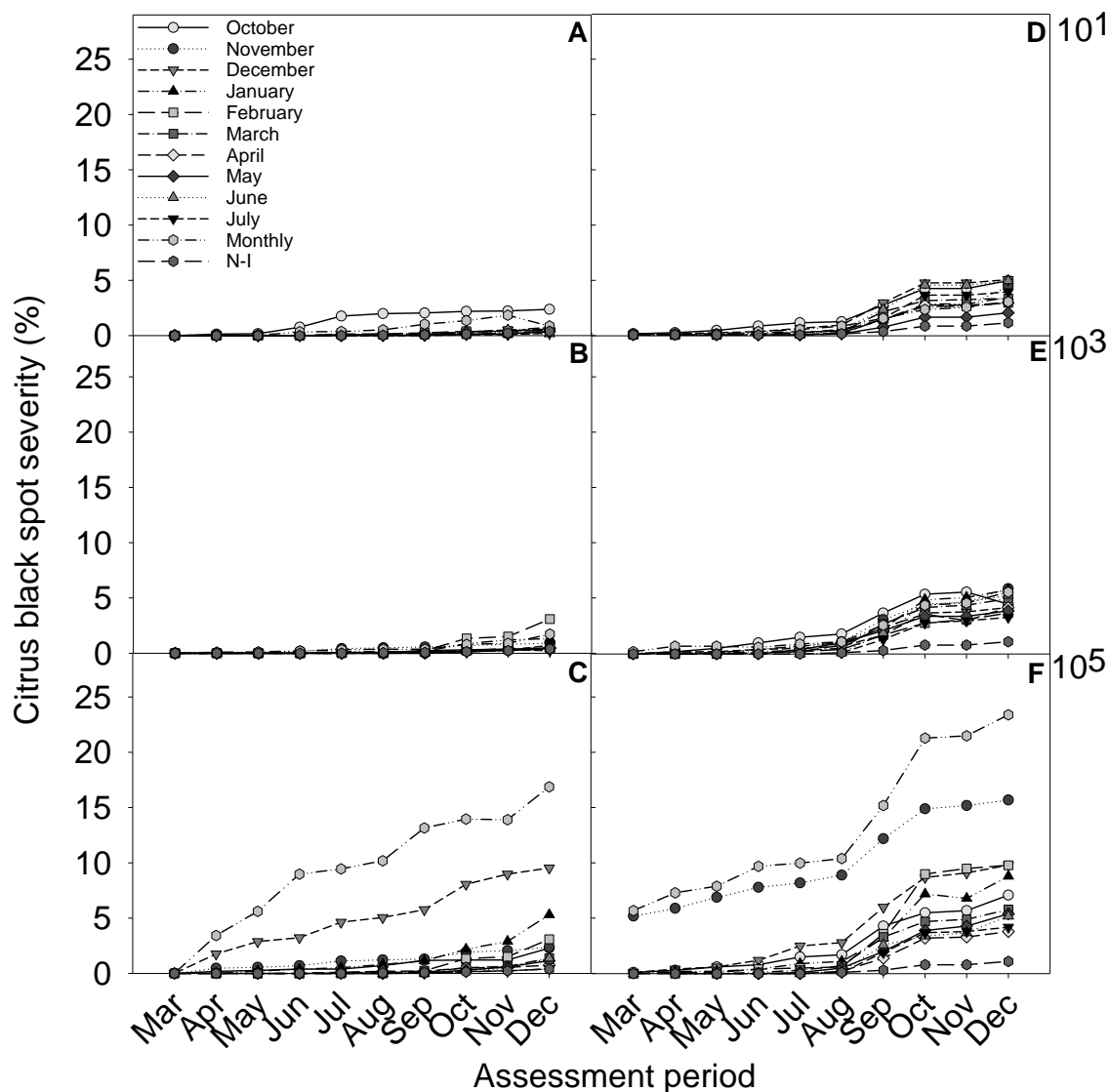


Figure 2 - Progress of citrus black spot severity (% diseased fruit peel area) on fruit assessed from March to December in commercial orchards of 'Valencia' sweet orange in the municipalities of Boa Esperança do Sul, season 1 (A-C) and Itapópolis, season 2 (D-F), São Paulo state, Brazil. Fruit were inoculated with 10^1 (A and D), 10^3 (B and E) or 10^5 (C and F) pycnidiospores/mL of *Phyllosticta citricarpa* once from October 2017 to July 2018, season 1 (A-C) and from October 2018 to July 2019, season 2 (D-F). Fruit monthly inoculated from October to July and non-inoculated fruit (N-I) were used as controls.

3.3.2. Effect of inoculation time and *Phyllosticta citricarpa* spore concentration on the expression of CBS symptoms on fruit

In the first season, the highest averages of AUIPC of 24,304, 22,990 and 13,239 were observed by fruit inoculated 10 times, only in December or in November with 10^5 pycnidiospores/mL, respectively (Figure 3A). These averages were

significantly higher ($P < 0.05$) than AUIPC from 4,588 to 10,080 obtained for fruit inoculated 10 times with 10^1 and 10^3 pycnidiospores/mL, or once in a given month from October to February (10^3), or in October, January or February (10^5) (Figure 3A). Fruit inoculated once from November to February (10^1), in March or April (10^3), and performed in March, April, May or July (10^5) resulted in AUIPC from 2,201 to 3,805, which differed from the average of 2,127 obtained for non-inoculated fruit ($P < 0.05$). However, AUIPC from 686 to 1,584 obtained for fruit inoculated in October or from March to July (10^1), from May to July (10^3), or in June (10^5) did not differ significantly from the AUIPC observed for non-inoculated fruit (Figure 3A).

In the following season, the effects of inoculation month and spore concentration on the AUIPC were similar to season 1. The highest AUIPC averages around 26,000 were obtained when the fruit was monthly inoculated with 10^5 pycnidiospores/mL or only in November (Figure 3B). Inoculations only in October or December with this high concentration or fruit inoculated 10 times with 10^3 pycnidiospores/mL were associated with high AUIPC averages between 17,991 and 19,481, which differed ($P < 0.05$) from the highest values, as well as the lowest averages of 13,829 and 15,052 observed, respectively, for fruit inoculated in October or November with 10^3 pycnidiospores/mL (Figure 3B). AUIPC values from 9,749 to 12,625 were observed for fruit monthly inoculated (10^1) or only in October, November or February (10^1), from December to March (10^3) and from January to March (10^5) (Figure 3B). Most of fruit inoculated from April to July, regardless of spore concentration, showed AUIPC between 6,488 and 9,336 significantly higher than the average of 4,717 observed for non-inoculated fruit, except for fruit inoculated in May with the lowest concentration that was 5,281 (Figure 3B).

The CBS severity progress represented by AUSPC was also affected by the inoculation month and spore concentration. In the first season, inoculations with 10^5 pycnidiospores/mL monthly performed or only in December resulted in the highest AUSPC of 1,511 and 1,105, respectively (Figure 3C). AUIPC from 117 to 293 were obtained in the other months from October to February, in which the fruit received 10^5 pycnidiospores/mL, and for fruit inoculated with 10^3 pycnidiospores/mL only from November to January or for fruit monthly inoculated (Figure 3C). AUSPC for fruit inoculated from March to July were very low and ranged from 7 to 54, regardless of the spore concentration, and the averages did not differ from the AUSPC of 10

obtained for non-inoculated fruit, except for fruit inoculated with 10^5 pycnidiospores/mL in March (54), April (37) or June (37) (Figure 3C).

In the second season, the highest AUSPC averages of 3,221 and 2,506 were obtained for fruit monthly inoculated or only in December with 10^5 pycnidiospores/mL, respectively (Figure 3D). Inoculations from December to February with 10^5 pycnidiospores/mL as well as monthly or only in November with 10^5 pycnidiospores/mL resulted in AUSPC from 577 to 937 (Figure 3D). These values differed from the two highest AUSPC and from the values from 298 to 509 observed for fruit inoculated in October, March, May or June (10^5), from December to February (10^3) or from November to January or monthly (10^1). All the AUSPC averages differed from the value of 57 observed for non-inoculated fruit (Figure 3D).

In general, the increase in *P. citricarpa* spore concentration resulted in increases in both AUIPC and AUSPC, mainly when fruit was inoculated once from October to March or monthly with 10^5 compared to 10^1 pycnidiospores/mL (Figure 3A-D). The CBS progress was not affected by *P. citricarpa* spore concentration only for AUIPC in February, April and July (season 1) and in June (both seasons), as well as for AUSPC in May and July (season 1) and April and June (season 2) (Figure 3A-D). Peaks of AUIPC and AUSPC were observed when the fruit was inoculated with the highest spore concentration (10^5 pycnidiospores/mL) monthly in both seasons or only in December in season 1 or November in season 2 (Figure 3A-D).

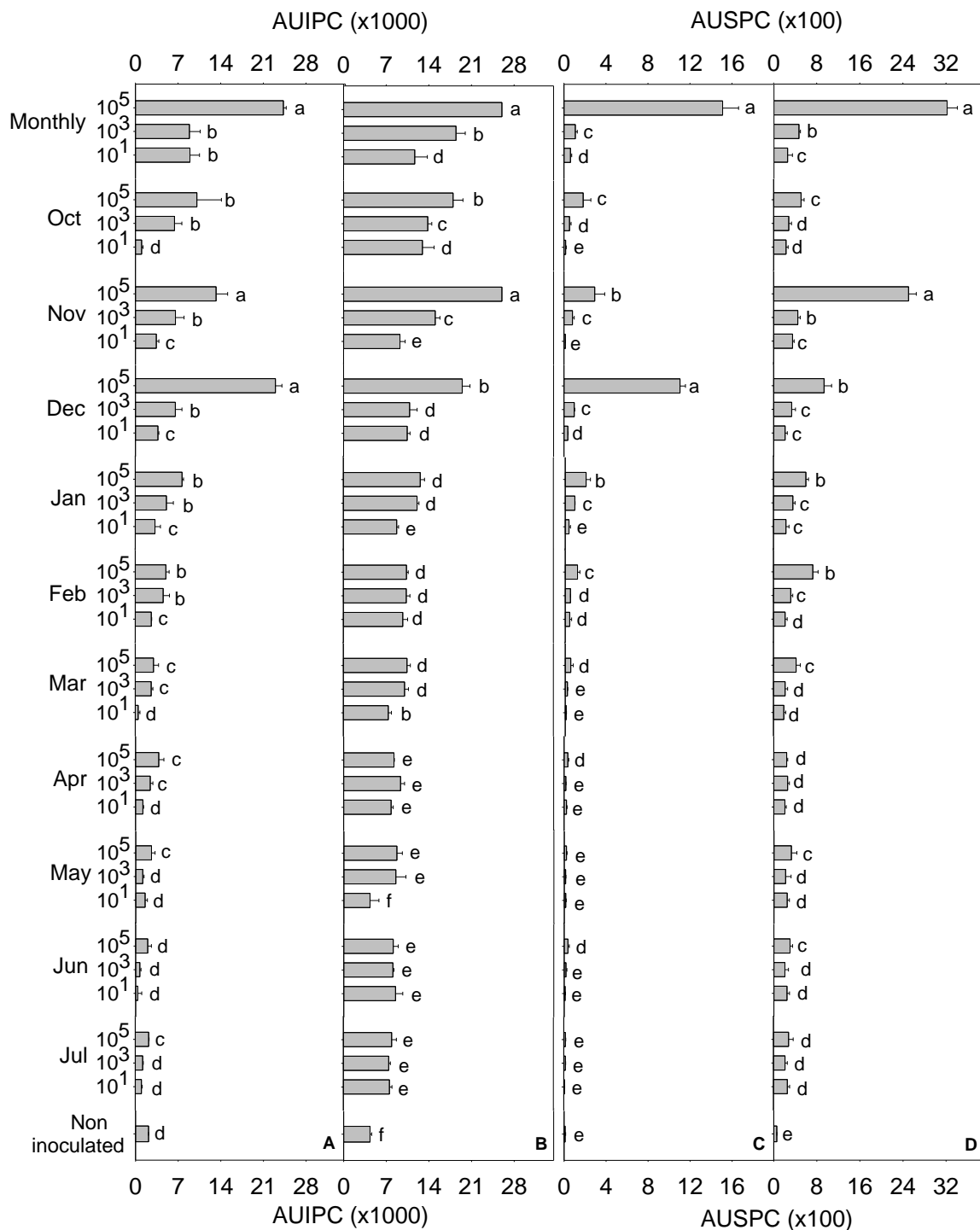


Figure 3 - Area under incidence progress curve, AUIPC (A-B) and severity progress curve, AUSPC (C-D) of citrus black spot on fruit assessed from March to December in Valencia' sweet orange commercial orchards 'in the municipalities of Boa Esperança do Sul in 2017/2018, season 1 (A and C), and Itapólis in 2018/2019, season 2 (B and D), São Paulo state, Brazil. Fruit were inoculated with 10¹, 10³ or 10⁵ pycnidiospores/mL of *Phyllosticta citricarpa* once from October 2017 to July 2018 (season 1) and from October 2018 to July 2019 (season 2). Fruit monthly inoculated from October to July and non-inoculated fruit (N-I) were used as controls. At each variable assessed and season, columns with the same letter do not differ by Scott-Knott's test ($P < 0.05$). Bars represent the standard error of the mean.

3.3.3. Effect of inoculation time and inoculum concentration on fruit drop

Survival analysis showed that proportions of fruit that remained on the tree canopy throughout the assessments varied with the inoculation months and spore concentrations (Figure 4). The lowest proportions of the remained fruit on the canopy were 0.48 in season 1 and 0.59 in season 2 when they were monthly inoculated in a total of 10 inoculations on same tree from October to July. These proportions did not differ from those obtained for fruit inoculated in the two seasons only in November (0.68 and 0.63) or December (0.56 and 0.77) (Figure 4A-C). In the first season, the proportions from 0.83 to 0.95 of fruit remained on the tree obtained by inoculations in other months did not differ from the 0.88 observed for non-inoculated fruit, except for October (0.69) and May (0.81). In the following season, the proportion of 0.91 non-inoculated fruit remained on the tree was significantly similar to the proportions from 0.77 to 0.89 obtained for fruit inoculated from December to July. Monthly inoculations or inoculations performed only in November were associated with lower retention of the fruit on the canopy compared to non-inoculated control (Figure 4C).

In both seasons, the proportions of fruit remaining on the tree of 0.88 and 0.91 greatly higher in non-inoculated fruit were not significantly different from 0.84 and 0.86 estimated for fruit inoculated with 10^1 pycnidiospore/mL, and 0.83 found on the inoculation of 10^3 pycnidiospore/mL in the first season ($P < 0.05$). The inoculation of fruit with 10^5 pycnidiospore/mL significantly increased the proportion of fruit drop compared to non-inoculated fruit and fruit inoculated with lower concentrations, as only 0.71 and 0.74 fruit remained attached to the tree (Figures 4A-B).

In season 1, the monthly inoculated fruit or only in November or December remained attached to the tree canopy for an average period of 321, 331 and 334 days, while the periods from 365 (January) to 379 days (June and July) were significantly similar to 370 days found for non-inoculated fruit. In the following season, the lowest survival times were observed for monthly inoculated fruit (342 days) or only in November (344 days), and the highest periods from 374 to 380 days were observed from February to July that were significantly similar to 374 days found for non-inoculated fruit. Survival time of 351 days estimated for fruit inoculated with

10^5 pycnidiospores/mL in the first season was lower than values from 363 to 370 days observed for fruit inoculated with lower concentration and non-inoculated, while in the second season, a period of 364 days estimated for the highest concentration was lower than 374 days obtained for non-inoculated fruit.

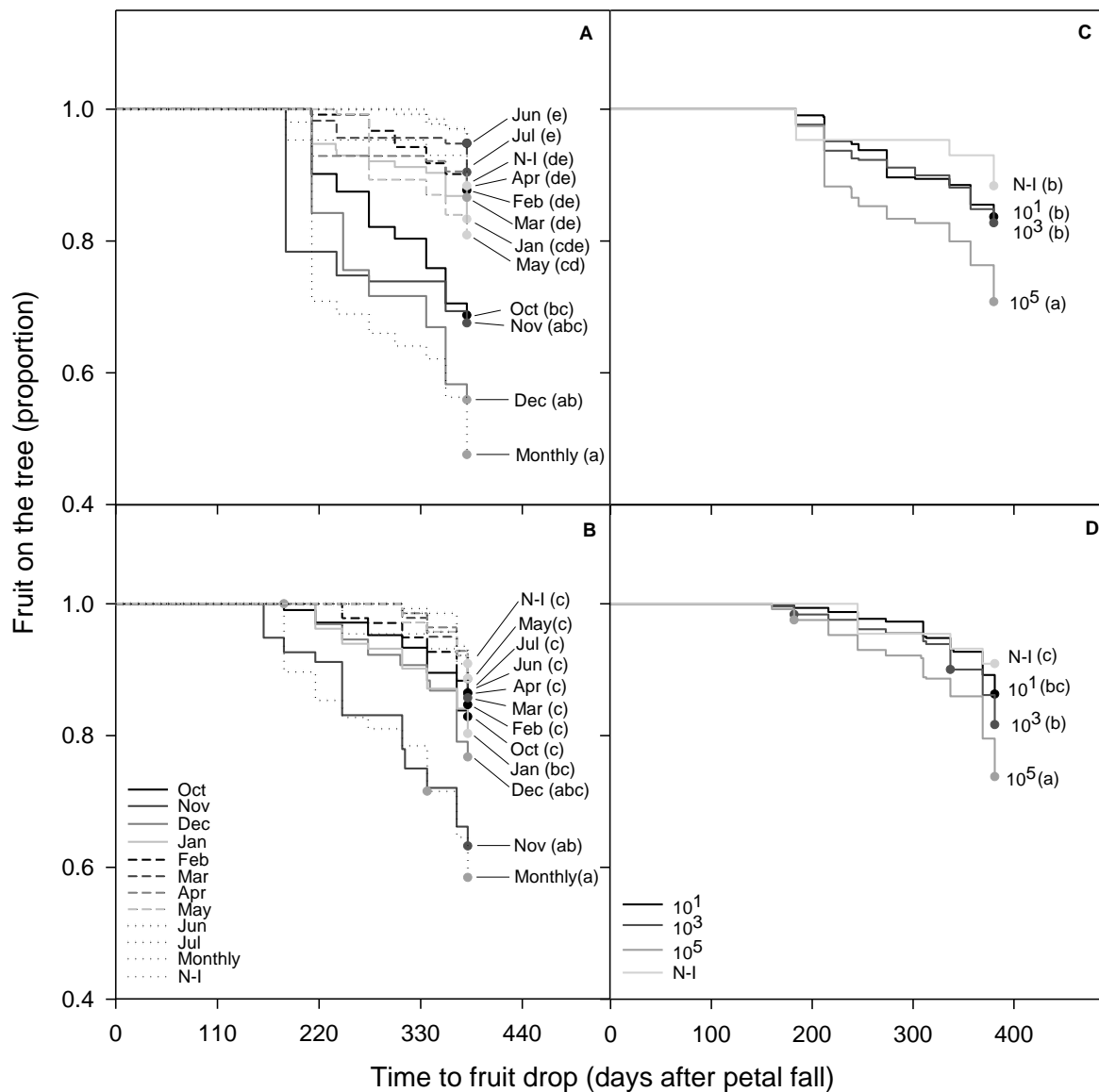


Figure 4 - Estimation of the Kaplan-Meier curves by survival analysis that describes the probability over time of fruit to remain attached to the tree in commercial orchards of 'Valencia' sweet orange in the municipalities of Boa Esperança do Sul in 2017/2018, season 1 (A-B), and Itapópolis in 2018/2019, season 2 (C-D), São Paulo state, Brazil. The fruit was inoculated once from October 2017 to July 2018 (A) and from October 2018 to July 2019 (B) with 10^1 , 10^3 or 10^5 pycnidiospores/mL of *Phyllosticta citricarpa*. Fruit monthly inoculated from October to July and non-inoculated fruit (N-I) were used as controls. The steps indicate the time in which fruit drop events occurred. Survival curves related to inoculation months (A and C) or spore concentrations (B and D) followed by the same letter did not differ significantly according to the log-rank test that were pairwise compared using the Holm-Sidak test ($P = 0.05$).

3.3.4. Diameter and color index of fruit

The first inoculations were performed in October when fruit diameter was around 0.3 and 0.4 cm for seasons 1 and 2, respectively. Fruit size increased until the last inoculation in July when diameter reached approximately 7.2 cm in both seasons (Figures 5 A-B). Due to reduced diameter of fruit in October, color index was not possible to be measured. The color index of fruit changed from dark green in November (CI = -18.1 in season 1 and -17.5 in season 2) to light green in July (CI = -4.7 in season 1 and -3.3 in season 2). In the first season, CBS assessments commenced in March when fruit were 6.0 cm in diameter and -15.8 color index and continued until December when fruit reached approximately 7.0 cm diameter and 2.6 color index (Figure 5A). In the following season, CBS symptoms were also assessed from March to December on fruit with diameters ranging from 5.8 cm to 7.3 cm and color index ranging from -15.4 to 1.5 (Figure 5B).

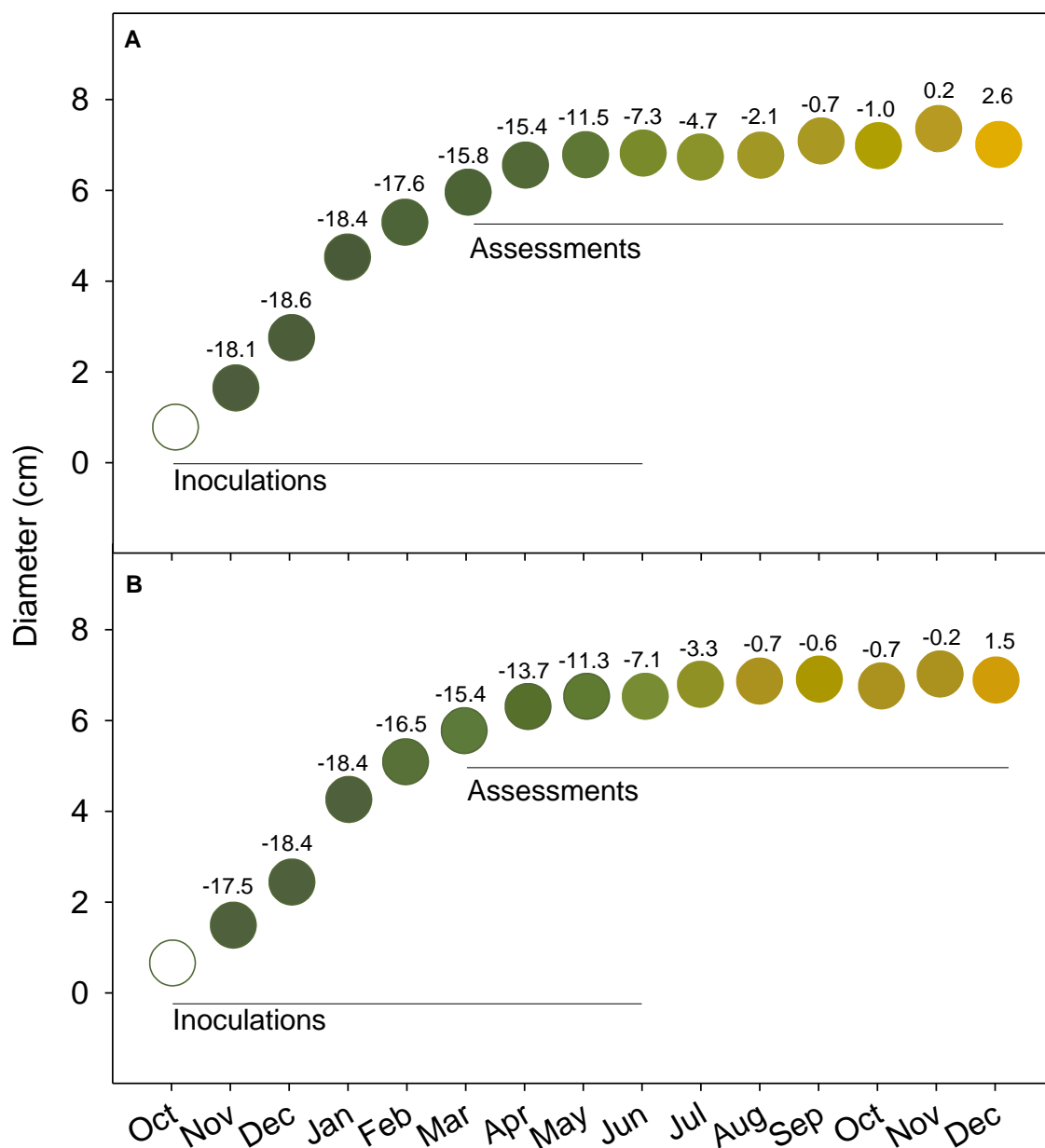


Figure 5 - Diameters (in cm) and color indexes of 'Valencia' sweet orange fruit at different developmental stages. Fruit were inoculated with different concentration of *Phyllosticta citricarpa* pycnidiospores once a month in commercial orchards in the municipalities of Boa Esperança do Sul in 2017/2018, season 1 (A), and Itapópolis in 2018/2019, season 2 (B), São Paulo state, Brazil. Symptoms of citrus black spot were assessed on inoculated fruit from March to December 2018 (A) and 2019 (B). Numbers above the circles represent the color indexes. The color index was not possible to be measured in October due to the reduced diameter of the fruit.

3.4. Discussion

Symptoms of CBS were successfully produced by artificial inoculation of *P. citricarpa* on fruit of Valencia sweet orange in field conditions using different

concentrations of pycnidiospores. Overall, the intensity of CBS symptoms was significantly high on fruit by artificial inoculation from the petal fall stage in October to February when diameter of the unripe fruit was about 5.5 cm. It is noteworthy that within this period both progress of CBS incidence and severity were the highest when fruit were inoculated only in November or December. Overall, fruit inoculations even performed with the highest concentration of inoculum (10^5 pycnidiospore/mL) after March did not result in a significantly increase in the progress of CBS symptoms compared to that of non-inoculated fruit. CBS-related fruit drop was also prominent when the fruit was inoculated only in November or December with the highest *P. citricarpa* spore concentration. Therefore, our findings indicate that CBS intensity gets lower as the fruit ripe, and even the inoculum is available at high levels usually after March, which does not mean significant CBS symptoms expression as well as premature fruit drop.

High CBS severity and premature fruit drop were mainly observed on fruit inoculated from October (petal fall stage) to February (fruit with ~120 days and 5.3 cm diameter), with peaks of symptoms in November when the fruit were ~30 days and 1.5 cm and in December (fruit with ~60 days and 3 cm). A consistent decrease in CBS intensity was observed mainly after March/April (fruit with ~150 to 180 days) as the fruit ripened and had ~6 cm diameter. These findings corroborate results of a recent study conducted in 'Valencia' sweet orange orchard, which demonstrated that spray programs with ~180 days of fruit protection (September to March) were cost-effective as they reduced CBS incidence and severity as well as fruit drop (Lanza *et al.*, 2018). Therefore, our results showed not only the critical period in which the fruit may be infected by *P. citricarpa*, but also that the most severe moment for the fruit infection within this critical period was between November and December, when the fruit were dark green with diameters between 1.5 and 3.0 cm.

Reduced CBS intensities following infection after February/March have been associated with low inoculum production and unfavorable weather conditions (Baldassari *et al.*, 2006; Brentu *et al.*, 2012, Schutte *et al.*, 2003; Miles *et al.*, 2004; Lanza *et al.*, 2018). However, the protocol of artificial inoculations used in this study provided sufficient amount of inoculum and favorable conditions for *P. citricarpa* infection, including during the dry autumn-winter period (May to July) with scarce rains in São Paulo state. In both seasons, the temperatures during the 48-hour

inoculations in the 10 different months (October to July) did not represent a substantial variation in the appressorium formation that varied from 66.3% to 71.4% by Noronha (2002) estimation. Therefore, both temperature and wetness duration along the 10 inoculation months in both seasons were conducive for appressorium formation and consequently for fruit infection by *P. citricarpa*.

The lower CBS severities on fruit inoculated when they were more than 5.0 cm in diameter are indicative of ontogenic (age-related) resistance. This resistance describes the ability of a plant or parts of a plant to resist or tolerate disease as a function of age or maturity. Ontogenic resistance does not necessarily indicate complete resistance, but the resistance level acquired as the plant tissue ages may affect both infection and disease progress (Ficke *et al.*, 2002). The knowledge related not only to the susceptibility of fruit to infection by *P. citricarpa* but also the ontogenic resistance may be used to determine the critical period of sprays to manage CBS in the citrus orchards. In addition, the reduced CBS intensity on fruit inoculated from May to July may also be associated with the shorter period for expression of CBS symptoms from the inoculation to harvest. Most orchards of late-maturing cultivars are usually harvested by December in the São Paulo citrus belt, and the period between inoculations in May and the harvest is less than 210 days. On the other hand, fruit inoculated from October to December had more than 360 days for expression of CBS symptoms, which usually occurs from 45 to 360 days (Frare *et al.*, 2019).

Fruit inoculated with the highest spore concentration, between October and February when diameter ranged from 0.4 to 5.3 cm, were more susceptible to *P. citricarpa* infections than the following months until July. This corroborates results obtained by Frare *et al.* (2019) on fruit of potted sweet orange trees inoculated with *P. citricarpa* pycnidiospore. These authors reported CBS symptoms on fruit with different diameters until 7.0 cm, but the highest incidences of diseased fruit were observed on fruit with 1.5, 3.0 or 5 cm. In our study, even when the fruit were inoculated from April to July with the highest inoculum concentration, the ontogenic resistance was not overcome. In addition, low levels of CBS severity below 4.0% during harvest of fruit that were inoculated after March was not associated with significant premature fruit drop. These findings may be explained by the fact that around 90% of the dropped fruit found in orchards of 'Pera' and 'Valencia' located

in São Paulo citrus belt had CBS severity higher than 4.2 ± 1.0 % (Machado *et al.*, 2021).

The increase in CBS severity was associated with an increase in the concentration of spores used to inoculate the fruit. Fruit inoculated with 10^5 pycnidiospore/mL showed the greatest CBS intensity. This corroborates results obtained by previous studies conducted by Tran *et al.* (2018) and Frare *et al.* (2019). Leaves of 'Troyer' citrange and fruit of 'Murcott' tangor inoculated with *P. citricarpa* ascospores and pycnidiospore at concentration of 10^5 spores/mL had more CBS symptoms compared to the inoculations performed with 10^4 spores/mL (Tran *et al.*, 2018). The percentage of CBS-affected fruit of 'Hamlin', 'Pera' and 'Valencia' sweet orange inoculated with 10^5 pycnidiospore/mL was higher than that observed when fruit were inoculated with 10^3 pycnidiospore/mL, and the average incubation period was 15% shorter when 10^5 pycnidiospore/mL was used (Frare *et al.*, 2019).

In our study, CBS symptoms were mainly observed on fruit inoculated from October until March, period considered by Lanza *et al.* (2018) as the critical for control of the disease in Sao Paulo citrus belt. However, the peak of *P. citricarpa* infections and symptoms expression occurred between November and December. These results suggest that the fruit protection against CBS needs to be performed more carefully within this period. As quinone outside inhibitor (QoI) fungicides have shown efficacy of around 95%, and the copper-based compounds have resulted in an efficacy of ~70% (Silva Junior *et al.*, 2016b), the growers may save QoI sprays for this moment. Moreover, the 42-day interval between QoI sprays, practiced by calendar spray programs may be reduced during this period as a strategy to improve the efficacy of CBS control.

This study conducted in commercial orchards represents an additional step to better understand the susceptibility of sweet orange fruit along the different developmental stages in São Paulo citrus belt. The period between October and March may still be considered as the period to protect fruit against CBS in São Paulo citrus belt, but the citrus growers need to be more concerned in November and December, since the fruit infections within this severe moment is associated with the most CBS-yield losses. This information may also be useful for further investigations in order to develop a disease support system that may determine the timing and interval of fungicide sprays to control CBS during the fruit susceptibility period.

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4. COPPER AND QOI FUNGICIDE EFFICACY ON CITRUS BLACK SPOT CONTROL DURING SWEET ORANGE FRUIT DEVELOPMENT

ABSTRACT

The control of citrus black spot (CBS) is mainly performed in Brazil with applications of copper-based and quinone outside inhibitors (QoI) fungicides. In general, the two first sprays have been performed with copper and the following three to four applications with QoI-fungicides. However, there is little information regarding the effect of these two fungicide groups used in other alternative arrangements. Thus, this study aimed to identify the efficacy of copper oxychloride and pyraclostrobin (QoI) fungicides sprayed at different fruit developmental stages for CBS control in a commercial orchard. Four field trials were conducted during the two seasons in a late-maturing 'Natal sweet orange orchard located in the eastern region of São Paulo state. Copper and pyraclostrobin were sprayed at different times after petal fall until June/August (~280 days later). In field trial 1, different trees received only one copper spray at 10 different times every 26-30 days. In field trial 2, the same trees received 9 sprays every 26-to-30 days, and in one given period of 26-to-30 days, the trees were kept unsprayed. In field trials 3 and 4, the QoI was used in the same way, but the interval was 38-to-42 days and the total of spray periods tested were seven from petal fall to ~290 days later. Trees sprayed 10 times with copper (trials 1 and 2) or seven times with QoI (trials 3 and 4) during the whole CBS control period and non-treated trees (NTC) were used as controls. CBS incidence and severity assessments were monthly performed from May to December in both seasons. Both fungicides applied only once consistently reduced CBS symptoms from December to March. CBS incidences were reduced by approximately 50% compared to NTC by the use of copper fungicide from petal fall through July/August, while reductions for QoI fungicide were 80 to 90%. During CBS spray program, the absence of a single QoI application for 38-to-42 days did not result in CBS increase, whereas trees without copper for a period of 26-to-30 days from December to March had greater CBS intensity on fruit. The volume and frequency of rainfall and the duration of wetness period usually higher in periods from October to February were positively correlated with CBS intensity in the absence of sprays in these periods. This study demonstrated that the calendar-based CBS spray program used in São Paulo citrus belt may be customized based on weather conditions in order to protect the fruit with QoI fungicides during rainy periods, as this group is more effective than copper-based fungicides.

Keywords: *Citrus sinensis*, *Phyllosticta citricarpa*, chemical control

4.1. Introduction

Citrus black spot (CBS) is caused by *Phyllosticta citricarpa* (McAlpine) van der Aa (*Guignardia citricarpa* Kiely), which infects almost all commercial citrus

species and cultivars, especially sweet oranges (Kotzé, 2000). CBS symptoms are associated with external blemishes on fruit rind (Kotzé, 1981). In addition, fruit severely affected by the disease may fall prematurely (Kotzé, 2000; Spósito *et al.*, 2007), leading to considerable yield losses mainly in late-maturing cultivars (Schubert *et al.*, 2012; Araújo *et al.*, 2013).

The CBS management starts with planting disease-free citrus trees, considering that infected propagation material is an important source of inoculum for introduction of the pathogen to new areas (Kotze, 1981; Silva Junior *et al.*, 2016a). The removal of infected leaves from machines used in the citrus farms during routine orchard maintenance is also important to prevent pathogen introduction (Dewdney *et al.*, 2018; Silva Junior *et al.*, 2016a).

The orchard ground may be covered with grass cuttings or mulch to prevent the ascospore discharge from citrus leaf litter (Scaloppi *et al.*, 2012). Compounds such as urea, ammonium sulphate, sugarcane bagasse may be used to increase the decomposition of leaf litter or removing them with machines to reduce ascospore production (Bellotte *et al.*, 2009; Bellotte *et al.*, 2013; Silva Junior *et al.*, 2016a). Removal of mature fruit with CBS symptoms from the orchard before the new flowering may avoid the overlapping of crops and reduce source of pycnidiospore production and dispersion to young fruit (Kotzé, 1996). Pruning of dead twigs (Silva *et al.*, 2017; Silva Junior *et al.*, 2016a), irrigation and balanced nutrition (Kotzé, 1981; Dewdney *et al.*, 2018) have also been used as strategies for CBS management.

Biological control of CBS with agents such as *Trichoderma* spp. and *Bacillus* spp. has been shown to be effective in suppressing *P. citricarpa* growth *in vitro* (Kupper *et al.*, 2011; Tonial *et al.*, 2017; Kupper *et al.*, 2020). Endophytes from medicinal plants or isolated from citrus leaves have also shown inhibitory effect against *P. citricarpa* (Er *et al.*, 2014; Hokama *et al.*, 2016; Peña *et al.*, 2016; Tran *et al.*, 2019).

Although there are several management alternatives for the disease control, CBS management mainly relies on the application of protective and curative fungicides during the critical period to *P. citricarpa* infection, which lasts 4 to 5 months after petal fall stage (Kotzé, 1981; Schutte *et al.*, 1997; Silva Junior *et al.*, 2016b; Lanza *et al.*, 2018). CBS control is based on fungicides with different efficacies from different modes of action groups such as quinone outside inhibitors

(QoI, strobilurins), methyl benzimidazol carbamates (MBC), dithiocarbamates and fixed copper (multisite activity), applied single or mixed with mineral oil (Miles *et al.*, 2004; Schutte *et al.*, 2003; Silva-Junior *et al.*, 2016a; Kellerman and Kotze, 1977). In Southern Africa, the disease was managed with the MBC benomyl until 1981, when the resistance of *P. citricarpa* to this fungicide group was reported (De Wet, 1987; Schutte *et al.*, 2003). The MBC carbendazim as well as the ditiocarbamate mancozeb were used until 2012 in Brazil when these fungicides were removed from the Protecitrust list (Silva Junior *et al.*, 2016a).

In South Africa, Australia and Argentina, sprays of mancozeb and copper-based alone or in mixture with QoI plus oil at 28–35-day spray intervals are recommended during the first 30 weeks from fruit set (from October to January/February), after which the fruit become resistant (Kotze 1981; Grout, 2015; Schutte *et al.*, 1997, 2003, 2012; Fogliata *et al.*, 2011). In Florida, the only fungicide groups registered for CBS control are copper and QoI. The recommendation for these fungicides has been based on monthly applications from early May to mid-September (Hincapie *et al.*, 2014). In the USA, copper products are a mainstay of citrus disease management such as citrus canker (*Xanthomonas citri* subsp. *Citri*) and melanose (*Diaporthe citri*) (Hendricks *et al.*, 2013).

In São Paulo (SP), Brazil, CBS is mainly controlled with the use of copper-based and QoI fungicides. Two copper sprays are commonly applied after petal fall, between September and November with 28-day intervals, followed by three to four strobilurin sprays at 35-day to 42-day intervals (Scaloppi *et al.*, 2012; Silva Junior *et al.*, 2016b). This program protects the fruit for 180–220 days until March or up to May (Lanza *et al.*, 2018). Despite all efforts, chemical control may fail to reduce CBS to acceptable levels depending on the problems related to the choice of products, time and interval of application, weather conditions after sprays, and sprayer setup (Silva Junior *et al.*, 2016a). Moreover, the incorrect use of fungicides may increase production costs and result in the selection of resistant pathogens, including fungicide-resistant *P. citricarpa* (Makowski *et al.*, 2014; Lanza *et al.*, 2018; Savi *et al.*, 2019). The availability of only two fungicide groups (copper and QoI) to control CBS in São Paulo citrus belt requires investigations in order to determine at which times each of the groups has better efficacy in controlling CBS. Thus, this study aimed to determine the efficacy of copper and QoI fungicides for CBS control during

different periods along with the fruit developmental stage of late-maturing sweet orange orchard in São Paulo conditions.

4.2. Materials and Methods

4.2.1. Experimental areas

Four field trials were conducted during the seasons 2016/2017 (season 1) and 2017/2018 (season 2) in a commercial sweet orange orchard located in the municipality of Casa Branca, São Paulo, Brazil (latitude 21°43'35.7"S, longitude 47°06'33.4"W, altitude 684 m a.s.l.). This municipality is positioned in Eastern SP region, where CBS was first reported in the state and frequently occurs. The Koppen-Geiger climate type is Aw, with tropical zone with dry winter (Alvares *et al.*, 2013). The orchard was planted in 1993 and it is composed of late-maturing Natal sweet orange [*Citrus sinensis* (L.) Osbeck] trees grafted onto Sunki mandarin (*Citrus sunki* Hort. ex Tan.), with 408 trees per hectare (spacing 7.0 m x 3.5 m).

The tree-row-volume (TRV) methodology was used to determine the spray volumes applied on the orchard. In season 1, the average tree height and width were 5.0 and 5.3 m, respectively. In season 2, these measures were 5.3 and 5.6 m. Thus, based on the row distance of 7.0 m, the estimated TRVs in season 1 and 2 were 37,500 and 42,300 m³/ha, i.e., 92 and 104 m³/tree, respectively. In season 1 and 2, the spray volumes used were 2,816 and 3,169 L/ha, respectively, which corresponded to 75 mL/m³ of tree canopy, based on the TRV concept (Scapin *et al.*, 2015; Silva Junior *et al.*, 2016).

4.2.2. Fungicides and spray setup

The fungicides used were copper oxychloride (Recop 840 WP, 50% metallic copper, Atar Company, Brazil), at a rate of 70 g of metallic copper/100 L of water and QoI fungicide (Comet EC, 25% of pyraclostrobin, Basf SA) at a standard rate of 3.8 g of pyraclostrobin/100 L of water. The rates of copper and QoI fungicide used were also based on the TRV methodology, which corresponded to 50 mg/m³ and

2.8 mg/m³ of tree canopy, respectively. Mineral oil (Agefix, Packblend) at 0.25% (v/v) was added to the QoI fungicide tank mixture.

Applications were performed with a 4000 L capacity high profile sprayer (Guliver 4000 Bi-lateral, FM Copling, Araraquara, Brazil) with working speed of 2.7 km/h and a tractor power rotation of 540 rpm. Forty-five nozzles per side, model AD3/AC25 Disc & Core (Albuz, France), were used with varying the pressure from 100 to 200 psi and nozzle flow of 0.96 (season 1) and 1.1 L/min (season 2).

4.2.3. Spray program and experimental design

The four field trials were conducted side by side in the same orchard. The treatments of each field trial were arranged in a randomized complete block design, with four replicates. The plots consisted of 30 trees divided into three rows of 10 trees. A guard row between treated rows was left non-sprayed (Silva Junior *et al.*, 2016b; Lanza *et al.*, 2018).

Copper-based and QoI fungicide applications were performed for CBS control at different times after petal fall (Tables 1 and 2), when the fruit had about 0.5 cm in diameter. Trial 1 consisted of 12 treatments, in which trees were sprayed with copper only once at 26-30-day intervals, totaling 10 spray dates, from 70% petal fall to about 280 days later. In the first season, sprays started on October 17 and ended on June 27, while in the following season the period was from December 06 to August 14. Trees that received one copper spray at intervals of 26 to 30 days (totaling 10 spray applications) during almost 280 days of protection as well as non-treated trees (NTC) were used as controls (Table 1). In trial 2, copper was sprayed every 26-30 days, from October to June (season 1) and December to August (season 2), except in just one period of 28 days (unprotected period). Trees once sprayed at a 28-day interval during the period of about 280 days and NTC trees were used as controls (Table 1).

Table 1 - Spray programs with copper (Cu) applied at different periods of 26 to 30-day intervals for control of citrus black spot in a Natal sweet orange orchard, during 2016/2017 and 2017/2018 seasons, in Casa Branca, SP, Brazil.

| Treatments | Spray dates | | | | | | | | | | |
|--|---------------------------|--------|--------|--------|--------|--------|--------|--------|--------|--------|----|
| | Out 17 ^a | Nov 16 | Dec 14 | Jan 10 | Feb 08 | Mar 08 | Apr 04 | May 03 | Jun 01 | Jun 27 | |
| | Dec 06 ^b | Jan 05 | Feb 02 | Feb 28 | Mar 28 | Apr 24 | May 21 | Jun 18 | Jul 17 | Aug 14 | |
| <i>Trial 1 - One period with copper application</i> | | | | | | | | | | | |
| 1 | Cu ^c | - | - | - | - | - | - | - | - | - | - |
| 2 | - | Cu | - | - | - | - | - | - | - | - | - |
| 3 | - | - | Cu | - | - | - | - | - | - | - | - |
| 4 | - | - | - | Cu | - | - | - | - | - | - | - |
| 5 | - | - | - | - | Cu | - | - | - | - | - | - |
| 6 | - | - | - | - | - | Cu | - | - | - | - | - |
| 7 | - | - | - | - | - | - | Cu | - | - | - | - |
| 8 | - | - | - | - | - | - | - | Cu | - | - | - |
| 9 | - | - | - | - | - | - | - | - | Cu | - | - |
| 10 | - | - | - | - | - | - | - | - | - | Cu | - |
| 11 | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 12 | Non-treated control trees | | | | | | | | | | |
| <i>Trial 2 - One period without copper application</i> | | | | | | | | | | | |
| 1 | - | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 2 | Cu | - | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 3 | Cu | Cu | - | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 4 | Cu | Cu | Cu | - | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 5 | Cu | Cu | Cu | Cu | - | Cu | Cu | Cu | Cu | Cu | Cu |
| 6 | Cu | Cu | Cu | Cu | Cu | - | Cu | Cu | Cu | Cu | Cu |
| 7 | Cu | Cu | Cu | Cu | Cu | Cu | - | Cu | Cu | Cu | Cu |
| 8 | Cu | Cu | Cu | Cu | Cu | Cu | Cu | - | Cu | Cu | Cu |
| 9 | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | - | Cu | Cu |
| 10 | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | - | Cu |
| 11 | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu | Cu |
| 12 | Non-treated control trees | | | | | | | | | | |

^aDate of the first spray at the stage of 70% petal fall in the 2016/2017 season. ^bFirst spray at petal fall stage in the 2017/2018 season. ^cCu, spray of copper oxychloride (Recop 840 WP, 50% metallic copper) at 50 mg of metallic copper/m³ of tree canopy.

Field trial 3 was conducted only with QoI applications in a similar way as trial 1. The interval among QoI sprays varied from 38 to 42 days, totaling seven different treatments with applications at a specific time from October to June in season 1, and from December to August in the following season (Table 2). Trial 4 was performed by using QoI fungicide in a similar way as trial 2 conducted with copper. The interval among applications was 38 to 42 days. Trees sprayed seven times during CBS control period of 290 days and NTC trees were used as controls for both QoI trials (Table 2).

Table 2 - Spray programs with quinone outside inhibition (QoI) fungicide applied at different periods of 38-42-day interval for control citrus black spot in a Natal sweet orange orchard, during 2016/2017 and 2017/2018 seasons, in Casa Branca, SP, Brazil.

| Treatments | Spray dates | | | | | | |
|---|---------------------------|--------|--------|--------|--------|--------|--------|
| | Oct 18 ^a | Nov 29 | Jan 10 | Feb 21 | Apr 4 | May 16 | Jun 27 |
| | Dec 06 ^b | Jan 17 | Feb 28 | Apr 11 | May 21 | Jul 3 | Aug 14 |
| <i>Trial 3 - One period with QoI application</i> | | | | | | | |
| 1 | QoI ^c | - | - | - | - | - | - |
| 2 | - | QoI | - | - | - | - | - |
| 3 | - | - | QoI | - | - | - | - |
| 4 | - | - | - | QoI | - | - | - |
| 5 | - | - | - | - | QoI | - | - |
| 6 | - | - | - | - | - | QoI | - |
| 7 | - | - | - | - | - | - | QoI |
| 8 | QoI | QoI | QoI | QoI | QoI | QoI | QoI |
| 9 | Non treated control trees | | | | | | |
| <i>Trial 4 - One period without QoI application</i> | | | | | | | |
| 1 | - | QoI | QoI | QoI | QoI | QoI | QoI |
| 2 | QoI | - | QoI | QoI | QoI | QoI | QoI |
| 3 | QoI | QoI | - | QoI | QoI | QoI | QoI |
| 4 | QoI | QoI | QoI | - | QoI | QoI | QoI |
| 5 | QoI | QoI | QoI | QoI | - | QoI | QoI |
| 6 | QoI | QoI | QoI | QoI | QoI | - | QoI |
| 7 | QoI | QoI | QoI | QoI | QoI | QoI | - |
| 8 | QoI | QoI | QoI | QoI | QoI | QoI | QoI |
| 9 | Non treated control trees | | | | | | |

^aDate of the first spray at the stage of 70% petal fall in the 2016/2017 season. ^bFirst spray at petal fall stage in the 2017/2018 season. ^cQoI, pyraclostrobin (Comet EC, 25% active ingredient) at 2.8 mg of pyraclostrobin/m³ of tree canopy.

4.2.4. Weather data measurement

Hourly weather data as temperature and relative humidity were recorded by an automatic weather station Davis Vantage Pro 2 Wireless Weather Station (Davis Instruments Corp.) installed closed to the experimental areas for the two seasons. Rainfall was daily measured in millimeters with standard rain gauges for two seasons. Rainfall information was used to obtain the accumulated rain (> 0.2 mm) and number of rainy days during different spray periods of around 28 or 42 days, respectively, for copper or QoI sprays, from petal fall stage to approximately 290 days. The relative humidity data were used to calculate the days in which the number of hours with relative humidity was equal or greater than 90% (NHRH \geq 90%). This is considered a model used to estimate the leaf wetness duration (LWD) (Sentelhas *et al.*, 2008; Montone *et al.*, 2016). The numbers of days with up to 8, 12 e 16 hours of NHRH \geq 90% were calculated for the different periods with or without Copper or QoI sprays.

4.2.5. Statistical analyses

Assessments of CBS symptoms were monthly performed from May to December (from 219 to 428 days after petal fall in season 1 and from 154 to 376 days after petal fall in season 2). CBS incidence and severity were assessed on 200 fruit of the four innermost trees of each plot (50 fruits per tree). CBS incidence was measured as the percentage of fruit with CBS symptoms at each assessment. CBS severity was measured as the percentage of diseased area on the outer canopy-facing portion of the fruit. The severity was estimated by using a six-level scale taking into account all types of CBS symptoms on the assessed portion of the fruit (Silva Junior *et al.*, 2016a; Spósito *et al.*, 2004). The standardized area under incidence progress curves (AUIPC*) and area under severity progress curves (AUSPC*) were calculated using data from all assessments in each season (Madden *et al.*, 2007).

The variables AUIPC* and AUSPC* were subjected to analysis of variance (ANOVA) using software R version 3.6.1 (Team R Core, 2019) with the add-on

package 'ExpDes'. The AUIPC* and AUSPC* for each period with or without applications were compared by Scott-Knott test ($P \leq 0.05$).

The influence of weather variables on the progress of CBS incidence and severity was estimated by regression analyses. AUIPC* and AUSPC* estimated for each non-sprayed period were correlated with the accumulated rainfall, number of rainy days, number of days with LWD of 8h, 12h or 16h, and average temperature during the LWD. The relationships between AUIPC* or AUSPC* (y) and weather variables (x) were determined by linear regression analyses, $y = a + bx$. The accuracy of the model was determined by the t test applied to the parameter estimates: linear regression intersection (a), to verify the hypothesis $H_0: a = 0$ and slope of the regression line (b) to test the hypothesis $H_0: b = 1$, at the probability level $p = 0.05$. The precision of the model was estimated by regression coefficient (R^2) and by residual variation (Madden *et al.*, 2007). Moreover, relationships between AUIPC* or AUSPC* and the different weather variables were determined by multiple linear regression. Normality of residuals was visually assessed by using a normal Q-Q (quantile-quantile) Plot. Analyses were performed using software R version 3.6.1 (Team R Core, 2019).

4.3. Results

4.3.1. CBS incidence and severity in trials performed with copper

4.3.1.1. Copper sprayed once during fruit development

In season 1, CBS symptoms began to be expressed in May. The incidence and severity consistently increased until harvest in December in all copper spray 28-day periods (Figures 1A-B). Regardless of the time to copper application, CBS incidence reached almost 100% in the last assessment in all the trees treated with a single spray (Figure 1A). Trees once sprayed with copper at 28-day intervals (totaling 10 applications sprays) showed around 70% of incidence of fruit with CBS in the last assessment, while non-sprayed trees had 100% diseased fruit (Figure 1A). CBS severity on fruit of NTC trees as well as on fruit sprayed with copper only on October 17 peaked at approximately 3.5% at harvest (Figure 1B). Severity of CBS on fruit sprayed only once with copper from November 16 to June 27 ranged

from 2 to 3%. CBS severity lower than 1% was observed only on fruit once treated at 28-day intervals with copper during the whole period of ~280 days (Figure 1B).

Both AUIPC* and AUSPC* were higher for fruit of NTC trees and for fruit sprayed only once on October 17 than the averages obtained for fruit treated only once in other periods from November 16 to June 27 (Figures 1C-D). A single copper application performed from November to June significantly reduced the AUIPC* and AUSPC* compared to NTC tree values, but did not differ from each other. Copper monthly sprayed at around 28-day intervals reduced the AUIPC* and AUSPC*, respectively, by 44% and 71% compared to the values obtained for NTC fruit (Figures 1C-D).

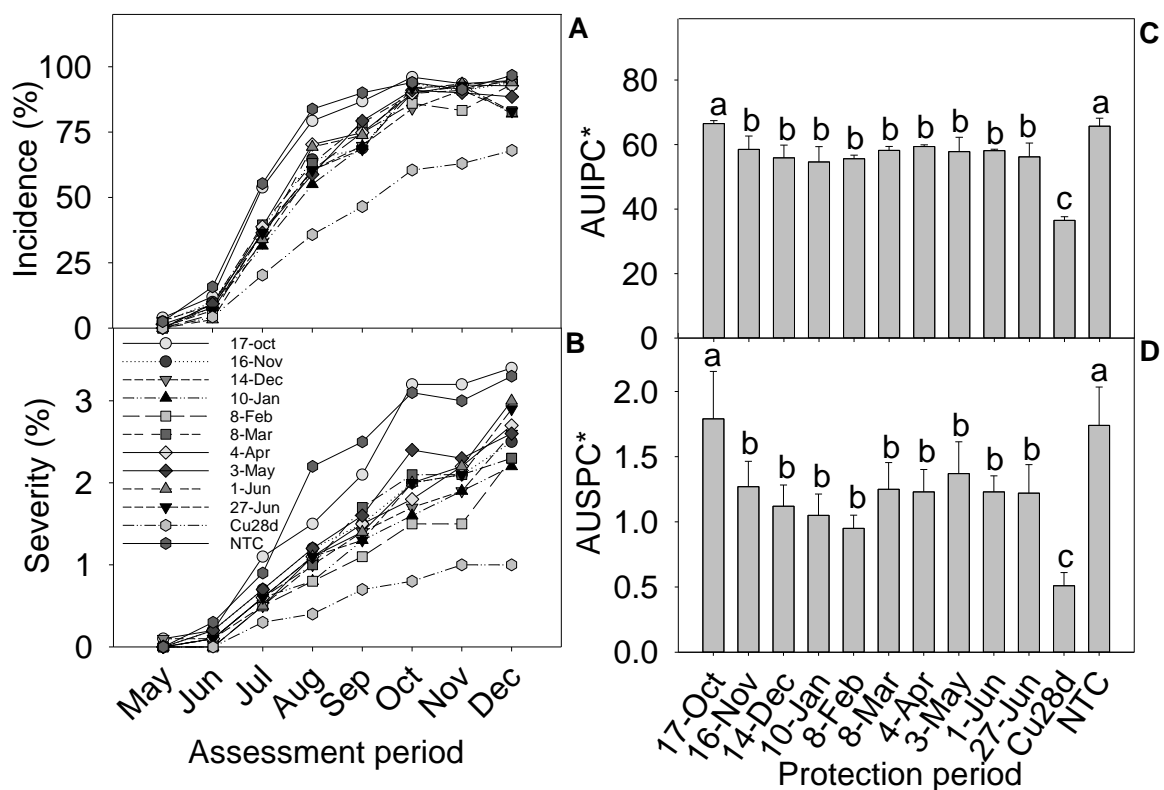


Figure 1 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees treated only once with copper at different times (field trial 1, season 1). Standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); Cu28d, trees once sprayed at 28-day intervals with copper; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

In season 2, the amount of disease symptoms on fruit sprayed once with copper was lower when compared to season 1, regardless of the protection period

(Figures 2A-B). NTC trees reached a maximum of 60% incidence of fruit with CBS and 2% of severity on fruit in the last assessment (Figures 2A-B). The lowest incidence of 22.5% fruit with CBS was found in trees that received monthly sprays every 28-days, followed by 28.3% incidence of fruit observed in protected trees in January (Figure 2A). CBS severity below 0.6% was observed on fruit monthly sprayed at 28-day intervals and only on January 5, followed by fruit sprayed only on February 2 or February 28 with 1.1% (Figure 2B). A single copper application in trees from December 6 to February 28 and on August 14 significantly reduced ($P < 0.05$) the AUIPC* in comparison with NTC, and significantly differed ($P < 0.05$) from other fruit that received a single copper spray from March 28 to July 17 (Figures 2C-D). Copper sprayed on January 5 significantly reduced ($P < 0.05$) the AUIPC* and AUSPC* at 44 and 68%, respectively, compared to NTC trees and did not significantly differ ($P < 0.05$) from fruit sprayed at 28 days in other months (Figures 2C-D).

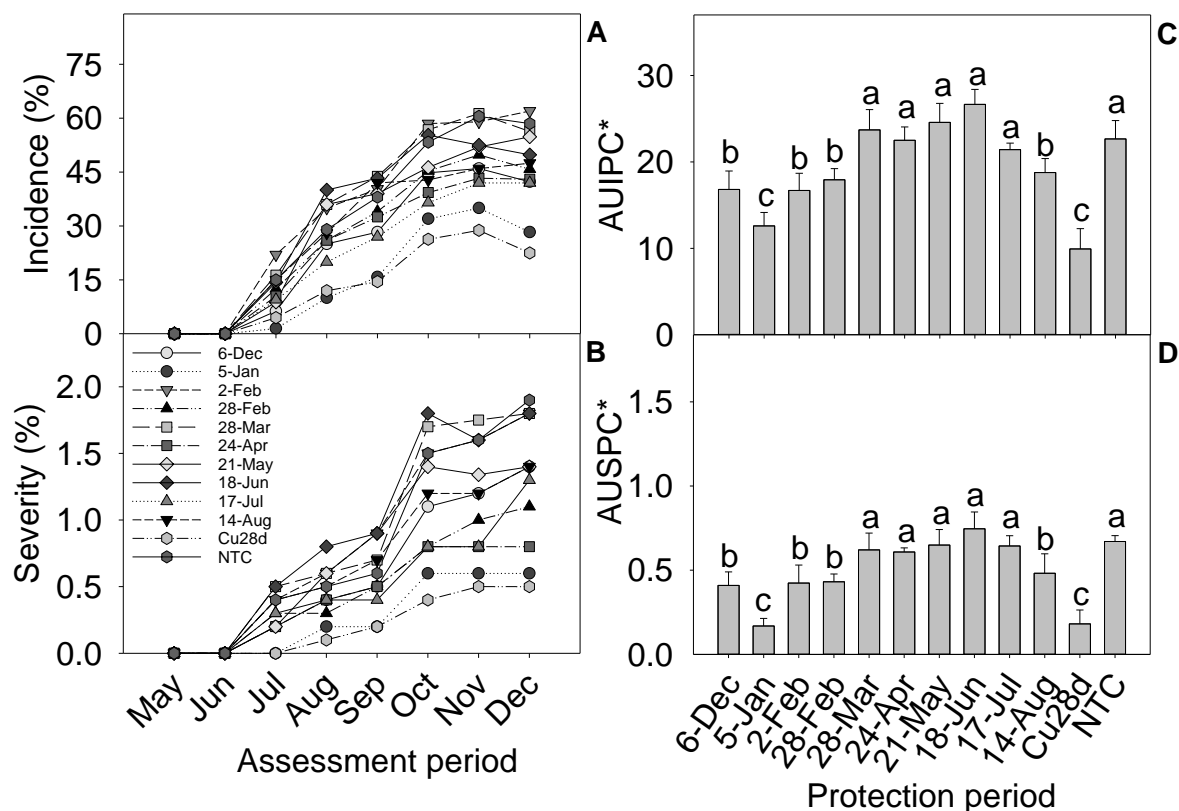


Figure 2 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees treated only once with copper at different times (field trial 1, season 2). Standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); Cu28d, trees once sprayed at 28-day intervals with copper; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

4.3.1.2. Lack of one copper spray during fruit developmental stage

In season 1, the lowest CBS incidence of 68% was observed when copper was monthly sprayed at 28-day intervals. The absence of copper only in May and June did not result in an increment in CBS incidence, while the peak of 86.3% CBS incidence in the last assessment was observed when copper was not sprayed only on February 8 (Figure 3A). CBS severity on fruit from NTC trees reached 3.3%, while the average on fruit monthly treated were about 1%. Fruit without protection on December 14, February 8 or June 27 had CBS severities from 1.4 to 1.7% (Figure 3B), AUIPC* from 47.2 to 51.7 (Figure 3C), and AUSPC* around 1.0% (Figure 3D). Both AUIPC* and AUSPC* for these three treatments did not differ from each other,

but were significantly higher ($P < 0.05$) than averages obtained for other treated fruit and lower compared to non-treated fruit (Figure 3C-D).

In season 2, the peak of CBS incidence and severity on fruit in NTC trees was 71.5% and 2.1%, respectively (Figure 4A-B). Trees without one copper spray on January 5 and March 28 showed the highest CBS incidence in the last assessment with averages of 42 and 46.8%, respectively. The highest CBS severities on sprayed fruit were about 1% observed for fruit without one application from December to March (Figure 4A-B). AUIPC* for fruit without one copper spray from January to March was significantly reduced ($P < 0.05$) compared to NTC trees, but higher than averages obtained for other treatments with sprays (Figure 4C). Fruit without one copper application on January 5, February 2 or March 28 had significantly higher AUSPC* ($P < 0.05$) than fruit without one copper application on other periods (Figure 4D).

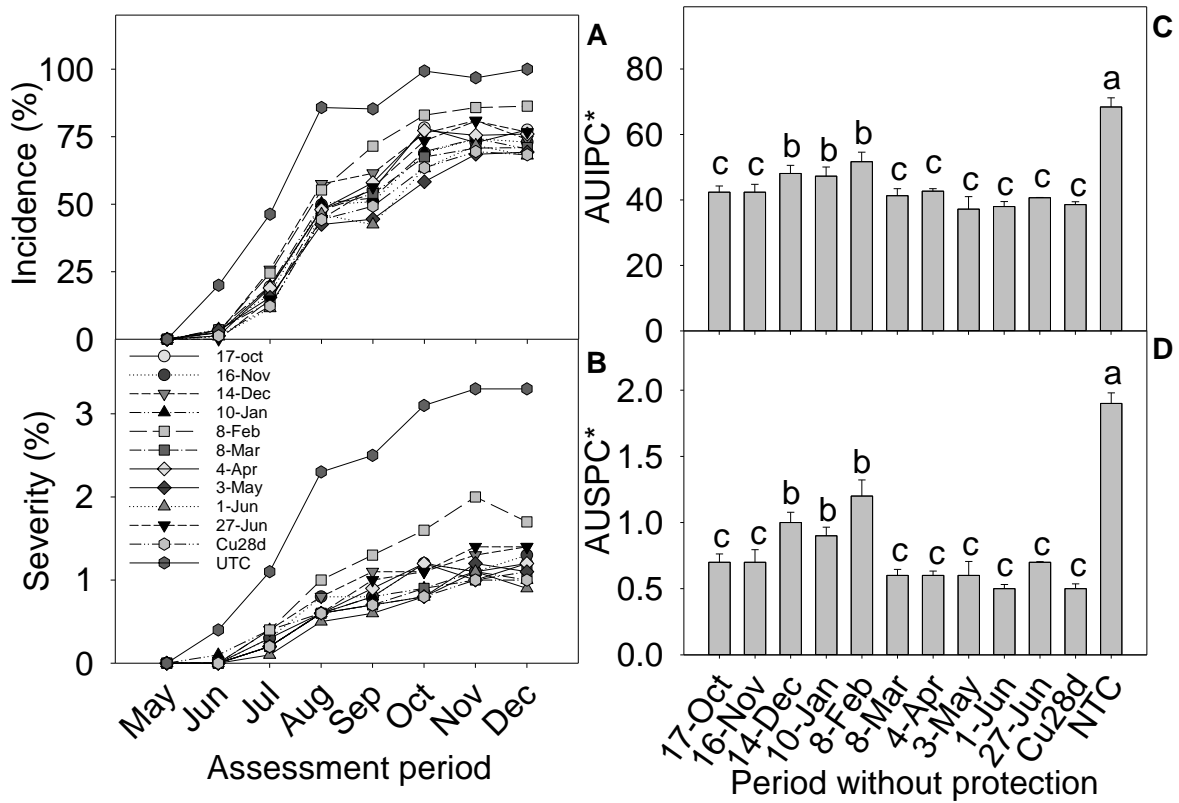


Figure 3 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees under different periods without one copper protection (field trial 2, seasons 1); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); Cu28d, trees once sprayed at 28-day copper intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

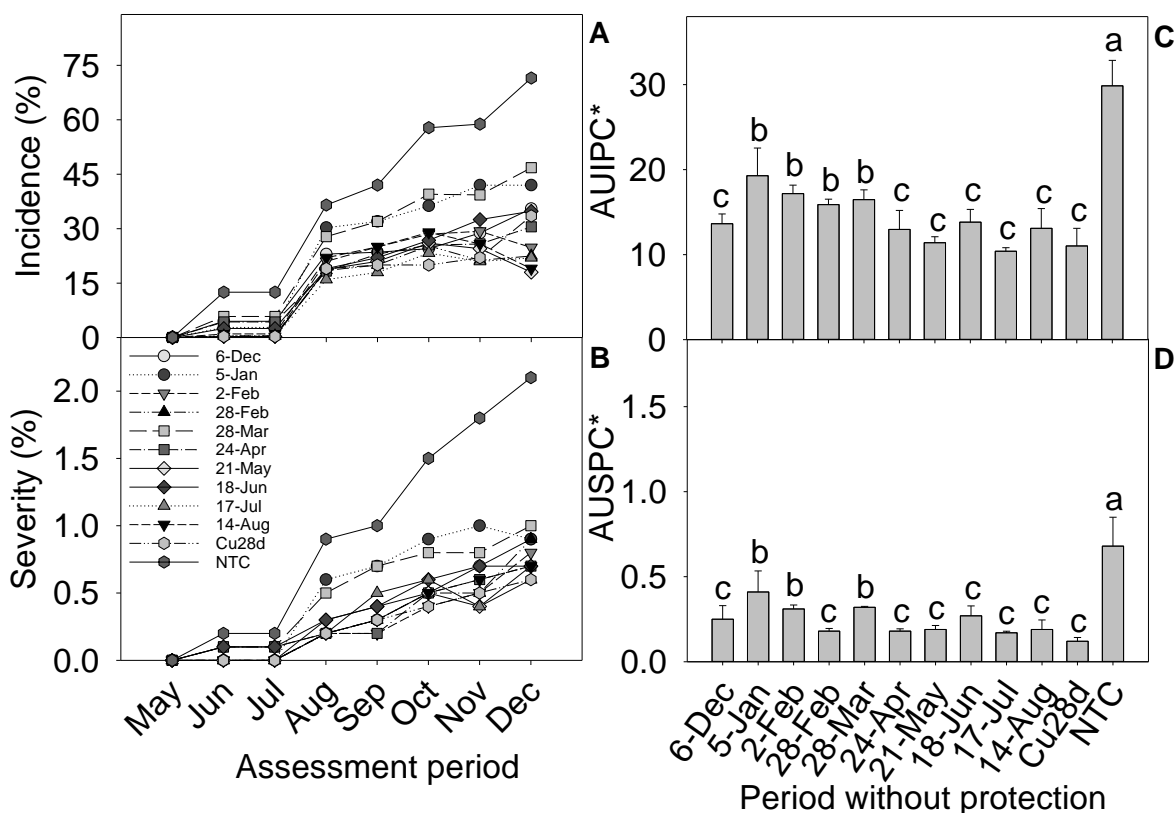


Figure 4 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees under different periods without one copper protection (field trial 2, seasons 2); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); Cu28d, trees once sprayed at 28-day copper intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

4.3.2. CBS incidence and severity in trials performed with Qol

4.3.2.1. Qol sprayed once during fruit developmental stage

In season 1, CBS incidence of fruit that received only one Qol application, regardless of the moment, was above 85%. The incidence of non-treated fruit with CBS was 94%, while only 28.8% of fruit sprayed once at 42-Qol intervals (totaling seven spray applications) had CBS symptoms (Figure 5A). The lowest CBS severity on fruit in the last assessment was 0.4% observed in trees treated once every 42 days, and the highest severity was 3.5% on non-treated fruit (Figure 5B). Fruit that received only one Qol spray on May 16 or June 27 had 2.6% and 2.8% CBS severity, respectively, while the fruit protected with only one spray in other five periods from

October to April presented about 2.0% CBS severity (Figure 5B). The highest reductions in AUIPC* (76%) and AUSPC* (88%) compared to NTC were observed for fruit protected during the whole period with seven sprays (Figures C-D). Among trees that received only one spray, the reductions of both AUIPC* (33% and 26%) and AUSPC* (62% and 58%) were higher than NTC ($P < 0.05$) when the trees were protected, respectively, on January 10 or February 21 (Figure 5C-D).

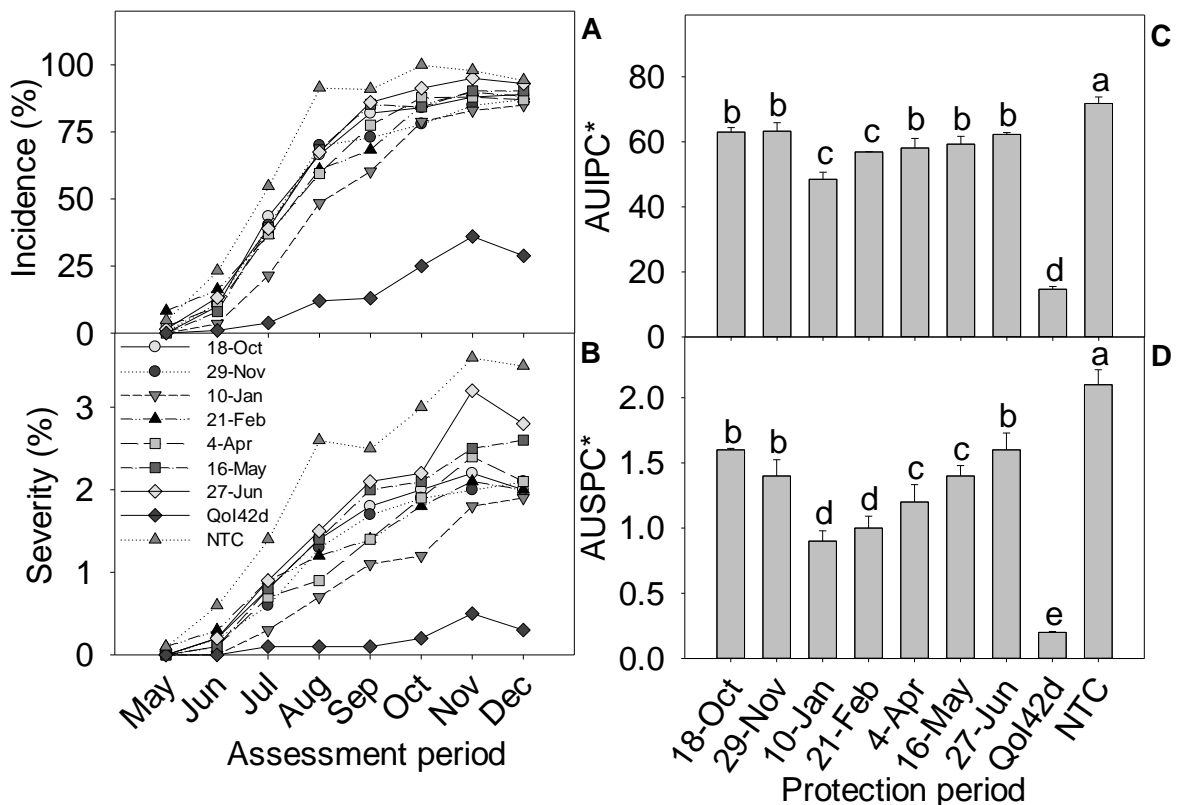


Figure 5 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees treated only once with strobilurin at different times (field trial 3, season 1); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); QoI42d, trees sprayed once at 42-day strobilurin intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

In season 2, fruit that were sprayed with QoI only on April 11 showed in the last assessment a CBS incidence of 76%, severity of 2.3% and did not significantly reduce ($P < 0.05$) the AUIPC* and AUSPC* compared to NTC that had final incidence of 85% and severity of 3.3% (Figures 6A-D). The trees protected only once in other periods as well as the trees sprayed once at 42-day intervals had CBS

intensity and progress reduced compared to NTC, with the highest reductions of AUIPC* (81%) and AUSPC* (98%) observed for trees sprayed during the whole period (Figure 6A-D).

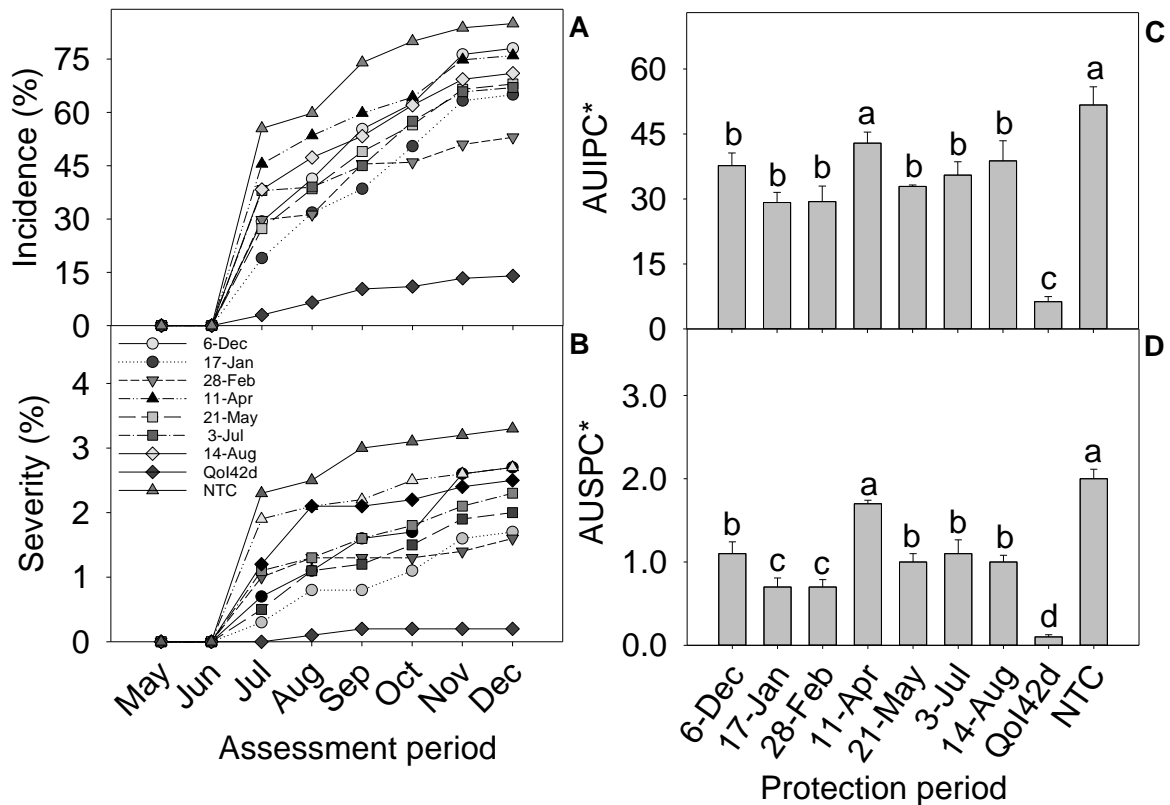


Figure 6 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees treated only once with strobilurin at different times (field trial 3, season 2); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); Qo142d, trees sprayed once at 42-day strobilurin intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

4.3.2.2. Lack of one QoI spray during fruit developmental stage

In field trial 4 during season 1, in which fruit did not receive one QoI application at different times, the progress of CBS incidence and severity was reduced in all treatments with sprays compared to NTC trees. The absence of one QoI spray, irrespective of the period, did not increase CBS intensity compared to trees sprayed once at about 42-day intervals (Figures 7A-D).

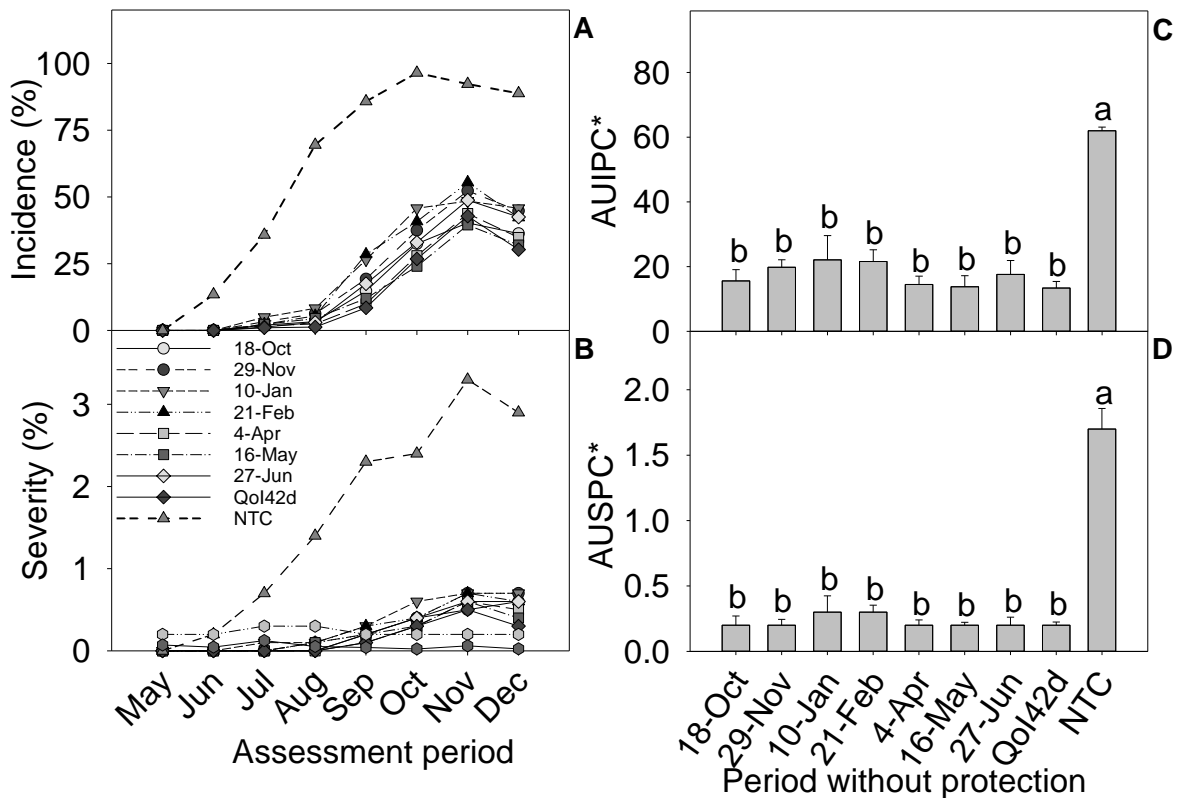


Figure 7 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees under different periods without one strobilurin protection (field trial 4, season 1); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); QoI42d, trees once sprayed once at 42-day strobilurin intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

In the following season, the final CBS incidence and severity of fruit in NTC trees reached 79% and 1.8%, respectively, while the averages of diseased fruit with CBS were lower than 15% and severity below 0.3% when QoI was sprayed six or seven times (Figure 8A-B). Similar to season 1, the lack of one QoI application did not significantly increase ($P < 0.05$) both AUIPC* or AUSPC* compared to trees sprayed once at 42-day intervals (Figure 8C-D).

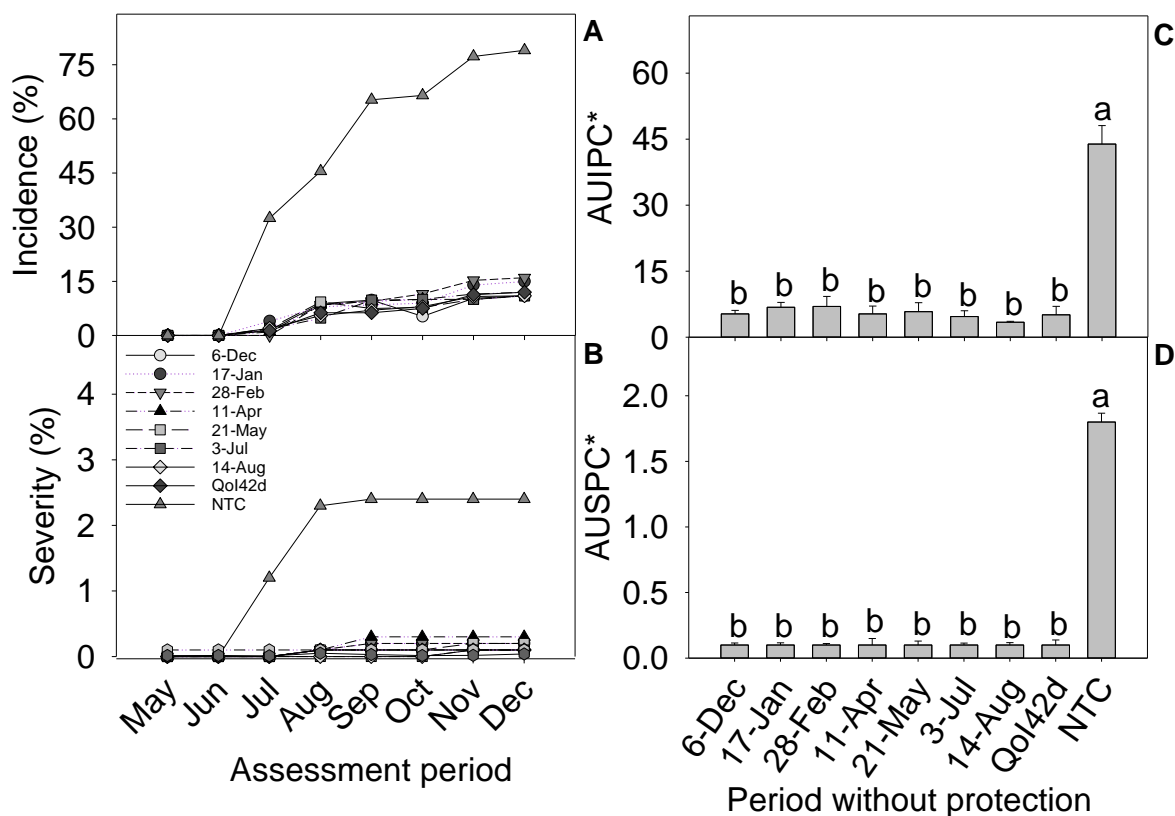


Figure 8 - Progress curves of incidence (A) and severity (B) of citrus black spot from May to December in 'Natal' sweet orange fruit from trees under different periods without one strobilurin protection (field trial 4, season 2); standardized area under incidence progress curves (AUIPC*) and standardized area under severity progress curves (AUSPC*) (C and D); QoI42d, trees once sprayed once at 42-day strobilurin intervals; NTC, Non treated tree control. Columns followed by the same letters do not differ statistically by Scott-Knott test ($P < 0.05$). Bars indicate the standard error of the mean.

4.3.3. Weather measurements

The weather conditions were favorable for CBS development over the two seasons in the experimental area (Tables 3-4). In season 1, rainfall was recorded during each 28-day copper periods and each 42-day QoI periods from October to July, except in the last period of all trials from June to August, in which no rain occurred (Table 3). The total volume of accumulated rain was 1293 mm in the period of 280 days treated with copper and 290 days with QoI. The highest volume registered was 307 mm in the first period of copper application from October 17 to November 15 as well as in the first QoI spray from October 18 to November 28 (Table 3). Rainfall was recorded in 91 days of the period treated with both copper or QoI sprays. The highest numbers of rainy days were registered from January 10 to

February 7 for periods treated with copper and from January 10 to February 20 for periods treated with Qol, in which 20 and 23 days of rain were registered, respectively (Table 3). The average temperature in LWD ranged from 11°C to 22°C in different copper or Qol spray periods in the first season. During the 282 days of copper spray periods, 154, 94 and 41 days with LWD of 8, 12 and 16 hours were registered, respectively, with the highest numbers of days from January 10 and February 20 (Table 3). Based on Qol spray periods, the highest numbers of days with LWD of 8, 12 and 16 hours, were registered in the periods from January 10 to February 20 with 31, 23 and 12 days, respectively.

In season 2, weather data were collected from December 6 to September 25 (Table 4). Rainfall during this period was lower when compared to season 1. The total volume of accumulated rain in 280-day periods of copper protection was 850 mm and in 294 days of Qol protection was 915 mm. The highest volume recorded was 278 mm from January 5 to February 1 considering the ten copper periods, and 382 mm from December 6 to January 16 considering the seven Qol spray periods. The highest numbers of rainy days were 20 days recorded in the copper spray periods from January 5 to February 1 and 18 days in Qol spray periods from February 29 to April 10 (Table 4). In season 2, the average temperature in LWD ranged from 12°C (winter) and 21.2°C (summer) during all 28-day (copper) and 42-day (Qol) periods from December to September. Based on the 28-day copper periods, the highest numbers of days with LWD of 8, 12 and 16 hours were registered in the periods from January 5 to February 1 with 22, 16 and 8 days, respectively. During 42-day Qol periods of fruit protection, the highest numbers of days with LWD of 8, 12 and 16 hours, with 32, 20 and 15 days, respectively, were observed from December 6 to January 16 (Table 4).

Table 1 - Weather variables recorded during periods with or without copper and quinone outside inhibitor (QoI) fungicide applications in a Natal sweet orange orchard during 2016/2017 season, in Casa Branca, SP, Brazil.

| Periods with or without sprays | Accumulated rainfall (mm) ^a | Number of rainy days ^b | Temperature during the LWD (°C) ^c | 8 hours LWD ^d | 12 hours LWD ^d | 16 hours LWD ^d |
|--------------------------------|--|-----------------------------------|--|--------------------------|---------------------------|---------------------------|
| <i>Copper field trials</i> | | | | | | |
| Oct 17 to Nov 15 | 307 | 16 | 19.2 | 19 | 14 | 7 |
| Nov 16 to Dec 13 | 211 | 13 | 20.4 | 14 | 12 | 3 |
| Dec 14 to Jan 9 | 87 | 10 | 21.7 | 9 | 5 | 1 |
| Jan 10 to Feb 7 | 281 | 20 | 20.7 | 27 | 19 | 10 |
| Feb 8 to Mar 7 | 113 | 13 | 20.8 | 14 | 8 | 4 |
| Mar 8 to Apr 3 | 50 | 4 | 18.0 | 20 | 5 | 1 |
| Apr 4 to May 2 | 109 | 5 | 18.2 | 19 | 13 | 5 |
| May 3 to May 31 | 120 | 7 | 17.0 | 19 | 12 | 6 |
| Jun 1 to Jun 26 | 15 | 3 | 15.0 | 10 | 6 | 4 |
| Jun 27 to Jul 25 | 0 | 0 | 11.4 | 3 | 0 | 0 |
| Oct 17 to Jul 25 | 1293 | 91 | 18.2 | 154 | 94 | 41 |
| <i>QoI field trials</i> | | | | | | |
| Oct 18 to Nov 28 | 352 | 20 | 19.1 | 24 | 19 | 7 |
| Nov 29 to Jan 9 | 253 | 19 | 21.3 | 18 | 13 | 4 |
| Jan 10 to Feb 20 | 303 | 23 | 20.6 | 31 | 23 | 12 |
| Feb 21 to Apr 3 | 141 | 14 | 19.1 | 29 | 12 | 4 |
| Apr 4 to May 15 | 133 | 8 | 17.6 | 27 | 15 | 7 |
| May 16 to Jun 26 | 111 | 7 | 16.3 | 25 | 13 | 8 |
| Jun 27 to Aug 8 | 0 | 0 | 11.0 | 4 | 0 | 0 |
| Oct 18 to Aug 8 | 1293 | 91 | 17.9 | 158 | 95 | 42 |

^aAccumulated rainfall (mm) during the 28-day or 42-day periods, in which trees were sprayed or not with copper or QoI, respectively; ^bSum of numbers of rainy days (rain > 0.2 mm) during different spray periods; ^cAverage temperatures (°C) throughout hours of leaf wetness duration (LWD) in different spray periods; ^dNumbers of days with more than 8, 12 or 16 hours of LWD.

Table 2 - Weather variables recorded during periods with or without copper and quinone outside inhibitor (QoI) fungicide applications in a Natal sweet orange orchard during 2017/2018 season, in Casa Branca, SP, Brazil.

| Periods with or without sprays | Accumulated rainfall (mm) ^a | Number of rainy days ^b | Temperature in LWD (°C) ^c | 8 hours LWD ^d | 12 hours LWD ^d | 16 hours LWD ^d |
|--------------------------------|--|-----------------------------------|--------------------------------------|--------------------------|---------------------------|---------------------------|
| <i>Copper field trial</i> | | | | | | |
| Dec 6 to Jan 4 | 167 | 14 | 20.6 | 21 | 12 | 8 |
| Jan 5 to Feb 1 | 278 | 16 | 20.4 | 22 | 16 | 8 |
| Feb 2 to Feb 27 | 155 | 10 | 20.0 | 21 | 9 | 7 |
| Feb 28 to Mar 27 | 83 | 12 | 21.2 | 22 | 11 | 3 |
| Mar 28 to Apr 23 | 68 | 7 | 18.5 | 10 | 7 | 3 |
| Apr 24 to May 20 | 25 | 2 | 16.4 | 10 | 1 | 0 |
| May 21 to Jun 17 | 1 | 1 | 14.7 | 9 | 5 | 0 |
| Jun 18 to Jul 16 | 3 | 1 | 12.0 | 6 | 2 | 1 |
| Jul 17 to Aug 13 | 64 | 6 | 14.6 | 11 | 8 | 6 |
| Aug 14 to Sep 11 | 6 | 3 | 13.3 | 9 | 3 | 0 |
| Dec 6 to Sep 11 | 850 | 72 | 17.2 | 141 | 74 | 36 |
| <i>Strobilurin field trial</i> | | | | | | |
| Dec 6 to Jan 16 | 382 | 23 | 20.4 | 32 | 20 | 13 |
| Jan 17 to Feb 27 | 218 | 17 | 20.4 | 32 | 15 | 9 |
| Feb 28 to Apr 10 | 136 | 18 | 20.5 | 28 | 17 | 6 |
| Apr 11 to May 20 | 40 | 3 | 16.6 | 12 | 4 | 1 |
| May 21 to Jul 2 | 1 | 1 | 14.6 | 11 | 5 | 0 |
| Jul 3 to Aug 13 | 67 | 7 | 13.8 | 15 | 9 | 7 |
| Aug 14 to Sep 25 | 71 | 8 | 15.5 | 16 | 9 | 6 |
| Dec 6 to Sep 25 | 915 | 77 | 17.4 | 146 | 79 | 42 |

^aAccumulated rainfall (mm) during the 28-day or 42-day periods, in which trees were sprayed or not with copper or QoI, respectively; ^bSum of numbers of rainy days (rain > 0.2 mm) during different spray periods; ^cAverage temperatures (°C) throughout hours of leaf wetness duration (LWD) in different spray periods; ^dNumbers of days with more than 8, 12 or 16 hours of LWD.

4.3.4. Relationship between disease intensity and weather variables

The relationships between the different weather variables and CBS intensity for two seasons were explained by linear regression models. The weather variables were more related to AUIPC* than AUSPC* (Figure 9-10). The highest R^2 adj around 0.70 was obtained for the association of AUIPC* with LWD, regardless of the numbers of hours from 8 to 16h and the numbers of rainy days. The relationship between AUIPC* and rainfall volume also had a high R^2 adj = 0.55. Although the average temperature during LWD showed significant association with the AUIPC* ($P < 0.05$), the R^2 adj = 0.12 was very low (Figure 9).

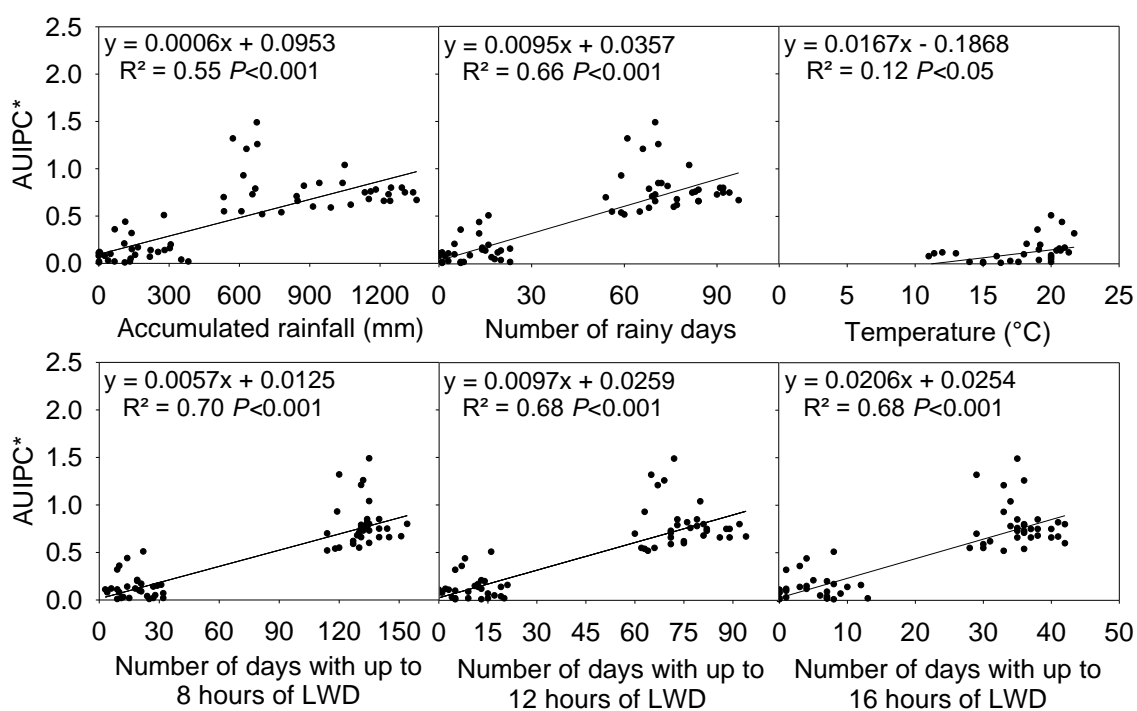


Figure 9 - Relationship between citrus black spot (CBS) standardized area under incidence progress curve (AUIPC*) on Natal sweet orange trees and accumulated rainfall (mm), number of rainy days, average temperature ($^{\circ}\text{C}$), number of days with up to 8 hours of leaf wetness duration (LWD), number of days with up to 12 hours of leaf wetness duration and number of days with up to 16 hours of leaf wetness duration during two seasons. Black circles represent the grouped data recorded in seasons 1 and 2.

The number of rainy days and the number of days with up to 12, 16 and 8 hours of leaf wetness duration were the more related variables to AUSPC* ($P < 0.001$), respectively, with R^2 adj of 0.44, 0.46, 0.47 and 0.5 (Figure 10). The accumulated rainfall was significantly related to AUSPC* with R^2 adj = 0.30. The

average temperature during LWD was not significantly related to AUSPC* (Figure 10).

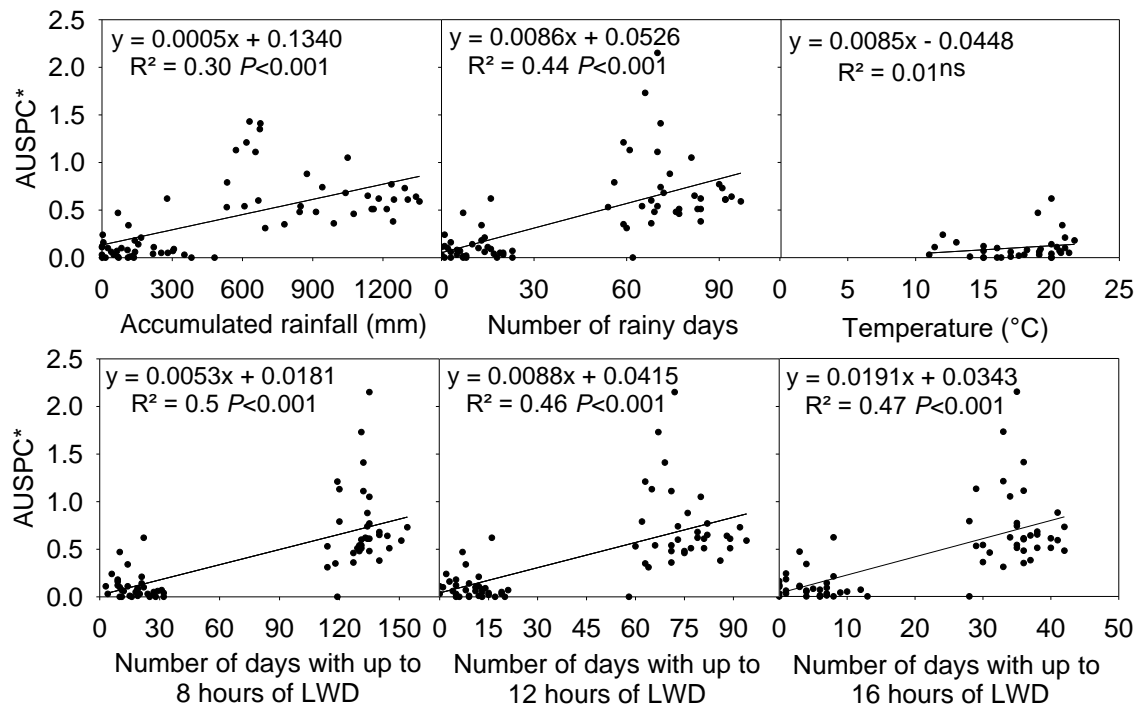


Figure 10 - Relationship between citrus black spot (CBS) standardized area under incidence progress curve (AUSPC*) on Natal sweet orange trees and accumulated rainfall (mm), number of rainy days, average temperature (°C), number of days with up to 8 hours of leaf wetness duration (LWD), number of days with up to 12 hours of leaf wetness duration and number of days with up to 16 hours of leaf wetness duration during two seasons. Black circles represent the grouped data recorded in seasons 1 and 2.

4.4. Discussion

This study focused on fungicide applications during the sweet orange fruit developmental stages confirmed that copper and QoI fungicides are more effective against CBS when sprayed within the rainy periods in São Paulo state, Brazil. Taking into account all field trials and seasons, both fungicides consistently reduced CBS symptoms from petal fall to the end of rainy periods, usually in March. CBS control performed with QoI sprays was more effective than fruit protection with copper. The lack of one QoI spray for a period of 42 days, regardless of the period that lacked this application, did not result in CBS increase, while trees without copper for a period of 28 days from December to March had greater CBS intensity on fruit. In addition, the volume and frequency of rain and the duration of wetness

period were positively correlated with progress of CBS symptoms. Overall, the lack of fruit protection from April to June, regardless of the fungicide, had basically no effect on increasing CBS intensity. Based on these findings, it is suggested that the fruit needs to be protected with QoI, which is more effective than copper, mainly from December to March during CBS control in sweet orange orchards intended for juice production in São Paulo citrus belt.

In the first growing season, the petal fall stage was in mid-October, while in the following season due to the later flowering, the spray program started in early December. Although in both seasons the weather conditions were favorable to CBS occurrence during fruit developmental stages, higher rainfall volumes of around 100 mm or above were observed until May in season 1 and only until March in the following season. As reported by Lanza et al. (2018), CBS spray programs in Brazil need be longer than that applied in South Africa and Australia probably because the weather conditions are more favorable to *P. citricarpa* infection not only until January/February but also until March/April. In the spray programs tested here from petal fall stage to June or August, the absence of a spray from April onwards did not result in increase of CBS symptoms. However, sometimes trees protected only once from April to August had fewer CBS symptoms compared to non-treated trees. These results suggest that CBS spray programs in orchards aimed mainly at the production of fresh fruit need to consider this occurrence of rain in autumn-winter, which may favor the infection of fruit by *P. citricarpa* and the expression of CBS symptoms even at low levels.

In general, the progress of CBS incidences from the beginning of symptom expression to harvest was reduced in almost 50% compared to non-treated trees by using copper fungicide to protect fruit from petal fall to June or August, while the reductions obtained for QoI fungicide were from 80 to 90% considering all the four field trials. Higher reductions for QoI (~90%) than copper sprays (~70%) were also observed for CBS severity progress in both seasons and field trials. These results corroborate other studies that observed higher efficiency of QoI compared to copper-based fungicides (Miles *et al.*, 2004; Silva Junior *et al.*, 2016b). Fixed copper compounds (Frac group M1) act non-specifically at the cell membrane level and once inside the cells, they interfere with numerous enzymatic reactions, with consequent inhibition of spore germination (La Torre *et al.*, 2018) and thereby

prevent the fungal pre-penetration steps and disease progress (Fonseca *et al.*, 2019). QoI fungicides or strobilurins act as respiration inhibitors by binding to mitochondrial cytochrome b (complex III), disrupting ATP production (Olaya *et al.*, 1998). The pyraclostrobin used here is considered locally systemic and may act in both fungal infection and colonization process, due to its high activity against spore germination and mycelial growth (Hincapie *et al.*, 2014; Gao *et al.*, 2017). Pyraclostrobin has been effective against *P. citricarpa* in field as well as *in vitro* assays (Tollig *et al.*, 1996; Schutte *et al.*, 2003; Miles *et al.*, 2004; Rodríguez *et al.*, 2010; Fogliata *et al.*, 2011; Silva Junior *et al.*, 2016b).

In both seasons, spray programs with QoI fungicide every 42 days from October or December (petal fall) to June or August, in which only one application was lacking, regardless of time, did not result in CBS symptom increases compared with the program with all sprays. Nevertheless, programs lacking one copper application, mainly from December to March, contributed to increase CBS intensity compared to the program with copper once sprayed every 28 days from October to December to June or August. These results may be explained by the pyraclostrobin effect after the interval of 42 days used in this study (Gold and Leinhos, 1995) as well as a possible post-infection activity of pyraclostrobin against *P. citricarpa* provided by the following application. The post-infection activity of QoI fungicides has been demonstrated for different pathogens, such as *Elsinoë* spp. causal agent of citrus scab (Bushong and Timmer, 2000) and *Colletotrichum* spp. that causes anthracnose fruit rot in strawberry (Turechek *et al.*, 2006). On the other hand, this effect was not observed for copper as this fungicide group has no curative action on pathogen control.

Although conducive weather conditions for CBS epidemics occurred in both seasons, the amount of rain as well as the number of rainy days were reduced by almost 30 and 20%, respectively, from the first to the second season of this study. This difference implied progress in incidence (AUIPC*) and severity (AUSPC*) around 44% and 27%, respectively, higher in the first season than in the following season that started only later in December. Considering the pooled data from all the four-field trials and both seasons of our study, CBS intensity was strongly related to rainfall and wetness periods. In the period from October to February, which is considered critical for fruit infection by *P. citricarpa* in different citrus growing regions

(Kotzé 1981; Schutte *et al.*, 1997; Milles *et al.*, 2004; Lanza *et al.*, 2018), the highest volume and frequency of rainfall are usually registered. During the two seasons of this study, the volumes of 978 and 624 mm of rain were accumulated in 141 and 85 days in this critical period in season 1 and 2, respectively. The positive association between disease symptoms and rainfall or wetness period may be explained by the water to trigger inoculum release, dispersal and infection (Kotzé 1981; Reis *et al.*, 2006; Spósito *et al.*, 2008, 2011; Hendricks *et al.*, 2020).

In our study, the number of rainy days and the number of days with prolonged wetness for more than 8h also positively correlated to the progress of CBS incidence and severity. Leaf wetness is among the major factors in the development of the infection process of some plant pathogens (Agrios, 2005; Gillespie and Sentelhas, 2008). Under long periods of free water, *P. citricarpa* ascospores and pycnidiospores initiate the infection process by germinating, forming appressoria, and directly penetrating the cuticle layer following successful attachment onto host tissue (Kiely, 1948; McOnie 1967; Kotzé 1981; Magarey *et al.*, 2015). Other studies showed that *Guignardia psidii* which causes black spot on guava required at least 6 hours of wetness period for spore germination and appressorium formation (Escanferla *et al.*, 2009; Soares-Colletti *et al.*, 2015).

Effective chemical control of CBS in commercial orchards has been reported in different citrus growing areas worldwide by using mainly copper, QoI and dithiocarbamates (Miles *et al.*, 2004; Schutter *et al.*, 2003, 2007; Silva Junior *et al.*, 2016b; Lanza *et al.*, 2018). However, in Brazil only copper-based and QoI fungicides may be used for CBS control in orchards intended for juice processing (Silva Junior *et al.*, 2016a). As demonstrated by Lanza *et al.* (2018) and confirmed here, the critical period for CBS control in São Paulo state starts at petal fall stage and usually ends in March/April. However, the rainfall and wetness period, which were the main weather variables related to the CBS intensity, are registered in higher amounts between November and February. Therefore, the program with two copper sprays followed by three or four QoI fungicides may be used only in seasons with petal fall stage until October, since QoI fungicides need to be included in the schedule from November in order to reach higher CBS control levels. Under conditions similar to season 2 of this work, which petal fall stage occurring in December, the program may start with QoI sprays and the copper included in the control program after the

rainy period that commonly takes place until February. Taking into account the consistently results obtained during the two seasons in an orchard of late-maturing cultivar, the application of copper-based and QoI fungicides for fruit protection may be adjusted based on the petal fall stage, the length of rainy periods and the destination of the fruit. These findings added to those obtained in the other studies presented here may provide citrus growers robust information to be used for better understanding the factors that affect CBS control as well as to spray the fungicides available for CBS control at right time.

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5. CONCLUSIONS

Quantitative PCR analyses associated with young citrus tree traps have the potential to be used as a tool for quantifying together the two kinds of *P. citricarpa* inoculums in the orchards. The numbers of *P. citricarpa* ITS copies detected were higher during October to March than from April to July, although peaks of amplification occurred from November to February. The high level of *P. citricarpa* inoculum in the commercial sweet orange orchards is directly influenced by the number of rainy days and hours of leaf wetness.

Artificial inoculation in the field successfully produced CBS typical symptoms on sweet orange fruit mainly from October to February. Fruit inoculated only in November or December had the highest CBS intensity and premature drop. Inoculation after April did not consistently increase CBS symptoms and fruit drop. The increase in concentration of pycnidiospores from 10^1 to 10^5 pycnidiospores/mL inoculated on fruit resulted in an increment of CBS incidence and severity.

Copper and QoI fungicides applications during the sweet orange fruit developmental stages consistently reduced CBS symptoms mainly from December to March. The absence of QoI spray for a period of 42 days did not increase CBS symptom development, while trees without copper for one period of 28 days from December to March resulted in an increase of CBS intensity on fruit. Moreover, fruit protection with both groups of fungicides from April to July had no effect in increasing CBS intensity.

Based on all these findings, the period between October and March needs to be considered as the period to protect fruit against CBS in São Paulo citrus belt. However, applications with QoI during the critical period of rain, usually between November and February, are the most important for the success of CBS control in the sweet orange orchards.