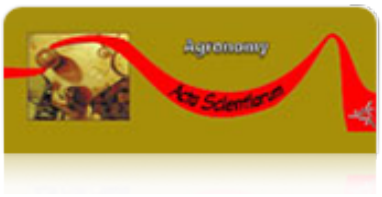

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Survival of pathogens after dormancy in apple tree twigs indicates potential risk as source of inoculum

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ABSTRACT. Several diseases are difficult to control specially in subtropical regions and perennial hosts where the pathogen survive easily within the plant. The aim of this study was to identify which pathogens are surviving after end of one season and at the beginning of the next season to inform which pathogens represent potential risk as primary inoculum to the next season. Survival of pathogens on apple tree twigs was evaluated during dormancy and on beginning of vegetative growth in four orchards located in the two main apple production regions: Palmas and metropolitan region of Curitiba, in Paraná State, Brazil. For this purpose, 10 cm long asymptomatic twigs were collected from 10 randomly selected plants, 24 twigs per plant. Half of the twigs were left directly (without disinfection) in humid chambers for 30 days at 25ºC. The other half of the twigs were disinfected and kept in a freezer for 12 hours at -16ºC by the Over Night Freezing Incubation Technique (Onfit) and then kept in a humid chamber for 30 days at 25ºC. The fungi *Colletotrichum* sp., *Botryosphaeria* sp., *Alternaria* sp., and *Fusarium* sp. were detected in the two sampled dates and methodologies in all evaluated regions. *Neonectria ditissima* was only detected in both orchards from Palmas. The genera found surviving on twigs were confirmed molecularly by BLASTn and were pathogenic in wounded fruits from the cultivar 'Gala'. Our results indicate that pathogens are surviving in orchards after winter treatment and throughout the apple season, being potential sources of inoculum for infections in flowers and fruits, where the pathogens detected cause important diseases as bitter rot, Neonectria fruit rot, white and black rots, and possibly cause Alternaria and Fusarium rots which have not been extensively studied in Brazil.

Keywords: *Malus domestica* Borkh; winter treatments; fungi.

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Introduction

Apple (*Malus domestica* Borkh.) is an important fruit produced in Brazil. Paraná is the third largest apple producing state and the main production areas are in Palmas municipality and Metropolitan Region of Curitiba – MRC. However, fungal diseases are limiting factors, causing indirect and direct losses, and affecting both quality and productivity.

As a temperate fruit tree, the annual season of apple trees comprises two distinct periods: vegetative rest (dormancy) and vegetative growth. At the end of the season after harvest the leaves fall, and the plants limit or cease their growth to survive at low temperatures, beginning the period of vegetative rest or dormancy (Petri, Palladini, & Pola, 2006). In this period, pathogenic microorganisms must endure long periods in the absence of susceptible tissue, so they remain in the orchard in survival phase to perpetuate in this adverse environment. Climatic elements have a strong influence on the occurrence and development of diseases. In temperate climates, where apple is typically grown, inoculum is reduced during winter due to extreme cold, while in tropical climates winters tend to be milder, less affecting the pathogen population in orchards. Especially for perennial crops such as apple trees, the management of diseases in the tropics can be difficult, since favorable conditions for disease development and the presence of susceptible host tissue occurs over long periods (Ploetz, 2007)

As flowers, fruit and leaves are not present, a possible way for the survival of fungi is on woody tissues, as the trunk, branches, or twigs. Most of the pathogens that cause important apple diseases can survive on these parts of the plant, including *Colletotrichum* spp., the causal agent of Glomerella leaf spot and bitter rot (Jones & Sutton, 1996; Crusius, Forcelini, Sanhueza, & Fernandes, 2002; Sutton, Aldwinckle, Agnello, &

Walgenbach, 2014; Hamada & De Mio, 2017); *Botryosphaeria dothidea* causal agent of Botryosphaeria canker and white rot (Jones & Aldwinckle, 1990); and *Neonectria ditissima*, which causes European canker and Neonectria fruit rot (Weber, 2014). To reduce inoculum on woody tissues, winter treatments with eradicant products are performed in the vegetative rest period.

Bud break of apple trees occurs in late winter or early spring, depending on the cultivar, as air temperature increases. The physiological responses of plants, such as dormancy overcoming and bud break, are related to meteorological conditions. After bud break, pathogens that survived the adverse climatic conditions and the winter treatments have the perfect environment to disseminate, infect and colonize the susceptible new plant tissues, as flowers, leaves and fruit.

Despite of winter treatments importance, studies about their efficacy are scarce so it is not known if and which pathogens are surviving after these sprays in the sub-tropical climate as south of Brazil. Post-harvest pathogens, as Alternaria and Fusarium, have been reported causing core rot, probably from early infection on flowers. In addition, little is known about the survival of pathogens in early development of fruit. Producers report frequently that an unusual symptom appears in their orchard, distinct from those caused by the most common pathogens as *Colletotrichum* spp., and the causal agent is often not discovered. The survey of pathogens can help to evaluate the efficiency of winter and vegetative growth treatments, as well as to predict which pathogens have high amount of inoculum with potential to cause epidemics.

While some pathogens might survive superficially on woody tissues, some may have already colonized and be present inside those tissues. Thus, it is important to investigate this with a method that would facilitate the sporulation of pathogens that are more internally and remove those that are on the surface of plant tissues.

In this context, this study evaluated the survival of pathogens at dormancy and after winter treatments (vegetative growth) in 'Gala' apple tree twigs, in Paraná state, confirming pathogenicity in apple fruit. In addition, we investigated which pathogens are surviving internally in twigs, using the Overnight Freezing Incubation Technique (Onfit).

Material and methods

Characterization of the orchards

The collections were carried out in four commercial apple orchards, two of them located in the municipality of Palmas, state of Paraná, called in this work Palmas 01 and Palmas 02, one in Campo Largo and one in Porto Amazonas, those last two from metropolitan region of Curitiba, state of Paraná, Brazil. Twig collections were performed in two periods: beginning of dormancy (before winter treatment) and in vegetative growth (after winter and vegetative growth treatments), in the months of June and September, respectively.

The list of fungicides used and the characterization of climatic conditions of the period between the first and the second collection are presented in Table 1, as well as the characterization of the orchards. Hydrogenated cyanamide (Dormex®) was used in all sampled proprieties for overcoming dormancy, in the following dates: August 22 in Campo Largo; August 20 in Porto Amazonas; September 1st in Palmas 01 and Palmas 02. Mineral oil was used together with Dormex®, being used Agefix® for Campo Largo and Porto Amazonas and Assist® for Palmas 01 and Palmas 02.

Collection of samples and incubation methodologies

To evaluate the presence of pathogens in apple tree twigs, asymptomatic twigs were collected, approximately 10 cm in length, with apical and axillary buds located in the leaf insertion. Twelve twigs of the upper part and twelve twigs of the lower part of the canopy (using half of the canopy height as reference) were collected from ten plants chosen randomly in the orchards, totaling 240 twigs for each of the two collection seasons (dormancy and vegetative growth) in each of the four orchards.

Two methodologies of incubation were used in this work to improve the range of pathogens detected, including those latent in the twigs. Plastic containers, lids and screens were disinfected by soaking in 70% alcohol for at least 1 hour. After drying, the screens were put in the plastic containers with 150 mL of water at the bottom, forming humid chambers.

Six twigs from each plant, three from the upper and three from the lower part of the canopy, were put in an individual humid chamber, so there were two repetitions for each plant for each methodology, totaling 20 repetitions per orchard. From the 24 twigs collected from each plant, half of them were left directly (without

disinfection) in the humid chambers for 30 days at 25ºC, to suit the time required for most of the pathogens to form reproductive structures, such as *N. ditissima*, which needs at least 28 days to form perithecia (personal communication). The other half of the twigs were disinfected by 1 min. soak in 70% alcohol followed by 1 min. soak in 0.5% sodium hypochlorite and 3 rinses in sterile distilled water; then, they were kept in freezer for 12 hours at -16ºC by the Over Night Freezing Incubation Technique (Onfit; Emery, Michailides, & Scherm, 2000) and after that kept in a humid chamber for 30 days at 25ºC.

Table 1. Winter treatments and sprays with fungicides carried out between twig collections periods, and characterization of the climate conditions obtained in the weather stations of Iapar (*Instituto Agronômico do Paraná*), in four orchards of Paraná State in the municipalities of Campo Largo, Porto Amazonas and Palmas, Brazil, in 2016.

Date of pulverization	C. P.[1]	Active Ingredient	Dose[2]	Acc ppt (mm)[3]	Max (°C)[4]	Min (°C)[5]
Campo Largo[6]						
27/07/2016	Ellect	Copper hydroxide	125	0	18.9	7.6
12/09/2016	Bravonil	Chlorothalonil	125	53.1	27.4	14.7
19/09/2016	Dithane	Mancozeb	250	17	21	10.7
19/09/2016	Unix	Cyprodinil	20			
26/09/2016	Dithane	Mancozeb	250	1.1	22.3	9.8
26/09/2016	Prisma	Difenoconazole	12,5			
Porto Amazonas[7]						
08/06/2016	Bordasul	Copper sulphate	300	0	14.5	1.6
25/08/2016	Recop	Copper oxychloride	150	50.2	23.4	11.6
31/08 - 01/09/2016	Previnil	Chlorothalonil	114	13.9	19.5	9.65
08/09/2016	Manzate	Mancozeb	240	39.2	19.6	4.3
08/09/2016	Prisma	Difenoconazole	14			
15/09/2016	Manzate	Mancozeb	240	2.8	21.4	4.3
15/09/2016	Prisma	Difenoconazole	14			
20 - 21/09/2016	Manzate	Mancozeb	300	14.2	19	6
20 - 21/09/2016	Metiltiofan	Methyl thiophanate	70			
27/09/2016	Manzate	Mancozeb	300	1.1	23.3	11.4
27/09/2016	Flint	Trifloxystrobin	10			
Palmas 01[8]						
13/07/2016	Cercobin	Methyl thiophanate	70	0	18.84	10.3
22/07/2016	Bordasul	Copper sulphate	200	66.6	14.83	5.7
05/08/2016	Captan	Captan	250	10.6	22.75	15.2
16/08/2016	Captan	Captan	250	54.3	22.07	9.4
04/09/2016	Bordasul	Copper sulphate	100	3.6	18.13	11.0
09/09/2016	Isatalonil	Chlorothalonil	250	39.4	20.36	7.5
14/09/2016	Isatalonil	Chlorothalonil	250	16.9	14.20	4.73
21/09/2016	Captan	Captan	250	55	18.84	6.7
28/09/2016	Mythos	Pyrimethanil	150	0.2	24.7	9.49
Palmas 02[9]						
13/07/2016	Cercobin	Methyl thiophanate	70	0	18.84	10.3
22/07/2016	Bordasul	Copper sulphate	200	66.6	14.83	5.7
05/08/2016	Captan	Captan	250	10.6	22.75	15.2
16/08/2016	Captan	Captan	250	54.3	22.07	9.4
29/08/2016	Supera	Copper hydroxide	200	130.4	18.82	13.6
06/09/2016	Previnil	Chlorothalonil	150	42.8	11.27	2.91
15/09/2016	Captan	Captan	150	17.1	18.10	2.68
26/09/2016	Mofotil	Methyl thiophanate	100	15.2	22.91	5.44

[1]Commercial product; [2](g or mL of c. p. 100 L^{-1} water); [3]Accumulated precipitation between the sprays; [4]Maximum temperature of the day; [5]Minimum temperature of the day; [6]Cultivar: Imperial Gala; plant spacing (m): 1.20 x 4.0; planting year: 2005; pollinator(s): Imperatriz and Fuji Suprema; [7]Cultivar: Imperial Gala; plant spacing (m): 1.48 x 4.0; planting year: 1998; pollinator(s): Imperatriz and Willi Sharp; [8]Cultivar: Imperial Gala; plant spacing (m): 1.0 x 4.0; planting year: 2004; pollinator(s): Imperatriz; [9]Cultivar: Maxi Gala; plant spacing (m): 1.2 x 4.0; planting year: 2008; pollinator(s): Fuji Suprema.

Identification, isolation, and molecular confirmation of the genera

The identification of the pathogens present on the twigs to the level of genera was made by observation of pathogens reproduction structures (conidia, ascospores, pycnidia or perithecia) after the incubation period using a stereoscopic magnifying glass (Zeiss®) and a light microscope (Olympus®). Morphological attributes of the pathogens were compared to the literature to confirm the genera. The incidence of pathogens in each plant was recorded. If one or more of the twigs in a humid chamber presented a certain fungal genus, the incidence of this pathogen for this repetition was 1. If no signs of a pathogen were found in the humid chamber, the incidence was 0. The results were expressed as mean percentage of incidence on all plants on

each orchard. Direct isolation of the genera *Alternaria, Botryosphaeria, Neonectria, Fusarium* and *Colletotrichum* was also performed, placing the observed reproductive structures in Petri plates containing Potato Dextrose Agar (PDA) medium amended with Lactic Acid. Their genus was confirmed molecularly by extracting the DNA of the isolates obtained from 7-day-old cultures, using the modified CTAB protocol (Pereira et al., 2019). The genomic DNA of the monosporic isolates was amplificated on the ITS1-ITS2 region of the 5.8 ribosomal subunit using primers ITS1 and ITS4, as described by White, Bruns, Lee, and Taylor (1990). The ITS sequence was aligned in the database of the GenBank of the National Centre for Biotechnology Information (NCBI), through the BLAST program (Basic Local Alignment Search Tool; http://www.ncbi.nlm.nih.gov/BLAST/). The preservation of the monosporic cultures was performed in filter paper discs in Eppendorf tubes containing silica gel.

Experimental design

A completely randomized design experiment with 2 treatments (on dormancy and on vegetative growth) and 120 replicates (12 twigs/tree, 10 random trees in each orchard) were used to evaluate the differences in the incidence of each pathogen detected in twigs at different sampling times. After analysis of homogeneity of variances and Anova, a comparison of means between the incidence of each pathogen in the collection of dormancy and the period of vegetative growth was made by the t test ($p < 0.05$). The same design and analyses were made for the comparison between the incidence of pathogens with and without Onfit. Analyses were performed using the R software (R Studio Team, 2015).

Pathogenicity test on fruit

For the pathogenicity test, fruit of the cultivar Gala were used. Their surface was disinfected by immersion in 70% alcohol for one minute, followed by 1% sodium hypochlorite and rinsed three times in distilled water. For drying, the fruits were kept on paper towel for 1 hour at room temperature. They were then individually placed in plastic pots containing moistened filter paper. In the upper part of the fruits, two wounds of 3 mm depth were made with autoclaved toothpicks. Mycelial discs of 5 mm were used to inoculate each pathogen on Gala fruits. The discs were extracted from the edge of seven days old colonies of the previously identified isolates of *N. ditissima* (two isolates from Palmas), *Colletotrichum* sp., *Botryosphaeria* sp., *Alternaria* sp., and *Fusarium* sp. (one isolate/pathogen/orchard). Fruit inoculated with PDA culture medium discs without pathogen structures were used as controls. After the inoculation, the fruits were stored on shelves in a room with a temperature of 25 ± 2ºC and 12 hours photoperiod. Evaluation was made on the 20th day, by observing the presence of symptoms and morphological confirmation of pathogenicity by observing the reproductive structures (signs on fruits) in light microscope. The experimental design was completely randomized, and each isolate from each region had five replicates of one fruit with two wounds, totaling 50 fruits. Data analysis was qualitative, considering the incidence and confirmation of the structures of the inoculated pathogen.

Results and discussion

The mean incidence of pathogens in apple tree twigs collected in different orchards is shown in Table 2. The incidence of pathogens in apple tree twigs maintained under favorable conditions of humidity and temperature resulted in a higher incidence in the dormancy period when compared to vegetative growth for most pathogens. Pathogens *N. ditissima, Colletotrichum* sp. and *Botryosphaeria* sp. presented a reduction in incidence between the dormancy period and on vegetative growth, except for *Botryosphaeria* sp. in the Palmas municipality, where the behavior was inverse, and the frequency on vegetative growth was significantly higher, reaching up to 80% of the twigs evaluated in Palmas 02.

The pathogen *N. ditissima* was not found in the Metropolitan Region of Curitiba, in the two orchards sampled in Campo Largo and Porto Amazonas (Figure 1). In the municipality of Palmas (Palmas 01 and 02 orchards), it was the fungus with the highest incidence on dormancy period, above 70%, and its frequency reduced by one third in vegetative growth (Figure 1).

In Campo Largo, Porto Amazonas and Palmas 02, the genus *Colletotrichum* had a frequency of more than 30% in the dormancy collection, whereas on vegetative growth no pathogen structure was found in the evaluated twigs (Figure 1A, B, and D); in Palmas 01 there was a significant reduction in the collection during dormancy, with frequency less than 10% (Figure 1C).

Table 2. Incidences (%) of fungi genera in 'Gala' apple tree twigs collected during dormancy (June) in the orchards located in Campo Largo, state of Paraná, Porto Amazonas, state of Paraná, Palmas, state of Paraná 01, Palmas, state of Paraná 02, Brazil, with Humid Chamber incubation (25ºC for 30 days) and with Over Night Freezing Incubation Technique (Onfit; 15 hours at -16ºC plus incubation in humid chamber at 25°C for 30 days).

Fungi genera	Incidences (%) of fungi genera at dormancy							
	Campo Largo		Porto Amazonas		Palmas 01		Palmas 02	
	HC[1]	OF[2]	HC	OF	HC	OF	HC	OF
Colletotrichum sp.	65a	20b	55a	10b	35	5	30	10
Fusarium sp.	60	70	60	30	55	45	45	40
Neonectria sp.	0	0	0	0	70a	10b	85a	15b
Alternaria sp.	50	60	45	70	30	45	30	40
Botryosphaeria sp.	60	30	30	20	30	15	20	20

Letters indicate significant difference by t test at 5% between periods of collection, considering twenty replications for each orchard at each time of sampling. [1]Humid Chamber; [2]Overnight Freezing + Humid Chamber.

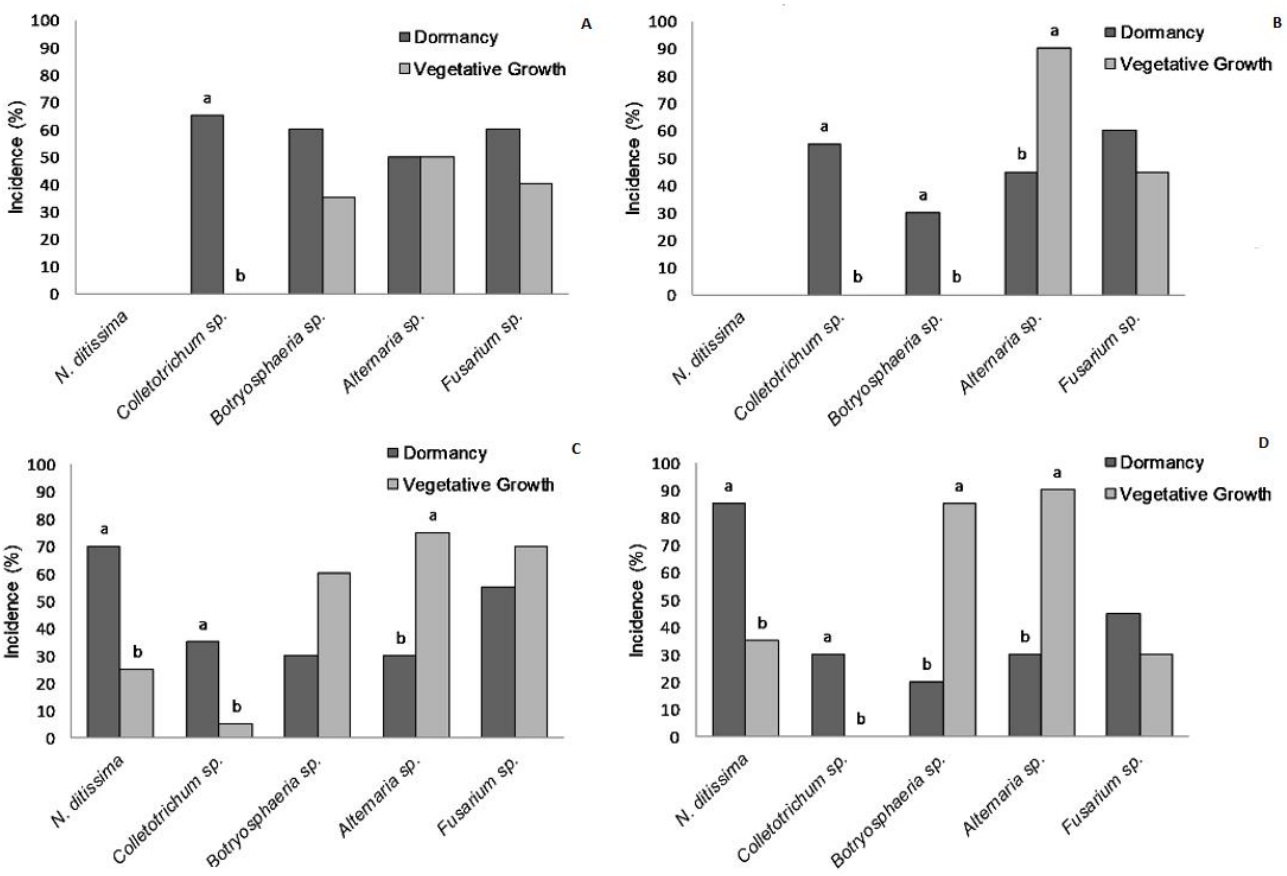

Figure 1. Incidences (%) of fungi genera in 'Gala' apple tree twigs collected during dormancy on June and during vegetative growth on September in the orchards located in Campo Largo, state of Paraná (A), Porto Amazonas, state of Paraná (B), Palmas, state of Paraná 01 (C), Palmas, state of Paraná 02 (D), Brazil, incubated in humid chamber at 25ºC for 30 days. Letters indicate significant difference by t test at 5% between periods of collection, considering twenty replications for each orchard at each time of sampling.

Differently from the previous genera, for *Alternaria* and *Fusarium*, in general, there was no reduction in the incidence of pathogens on vegetative growth. For *Alternaria* sp. the incidence reached 90% in Porto Amazonas, almost two folds in Palmas 01 and Palmas 02, and remained in the same frequency with high incidence (50%) in Campo Largo. The incidence of *Fusarium* sp. remained the same between the two collection seasons, with an average incidence of 50% (Figure 1).

Twigs collected on vegetative growth showed similar behavior to that observed in dormancy after Onfit (Figure 2), maintaining the incidence of *Alternaria* and *Fusarium* genera unchanged by Overnight Freezing treatment. Still regarding vegetative growth, in the orchard of Porto Amazonas, the twigs presented only the incidence of three genera, *Botryosphaeria*, *Alternaria* and *Fusarium* (Figure 2). As observed in dormancy, the pathogen *N. ditissima*, detected in the Palmas 01 and 02 orchards, reduced the incidence after Onfit on vegetative growth (Figure 2). The genus *Colletotrichum* had low incidence with and without Onfit, not being verified in Porto Amazonas and Palmas 02 (Figure 2).

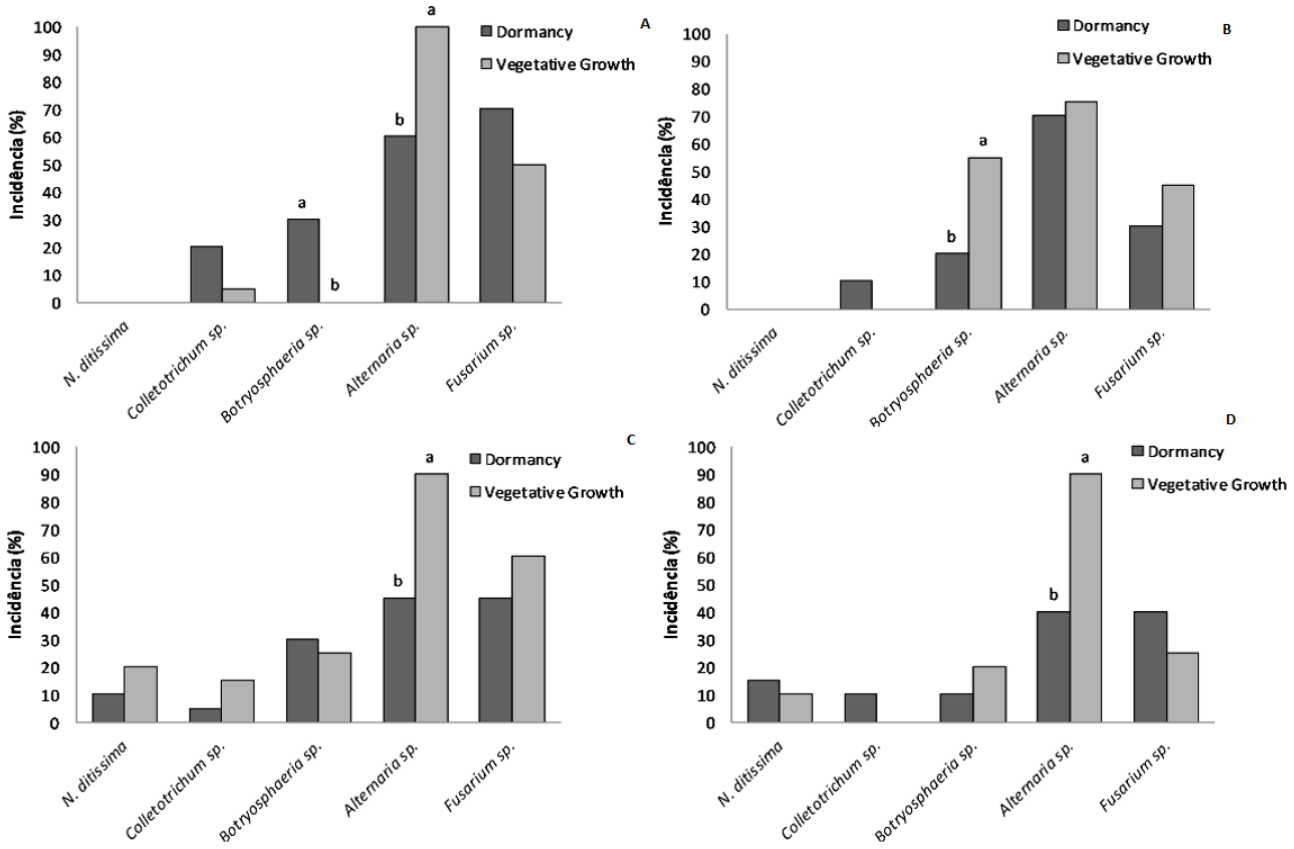

Figure 2. Incidences (%) of fungi genera in 'Gala' apple tree twigs collected during dormancy on June and during vegetative growth on September in the orchards located in Campo Largo, state of Paraná (A), Porto Amazonas, state of Paraná (B), Palmas, state of Paraná 01 (C), Palmas, state of Paraná 02 (D), Brazil, with the Over Night Freezing Incubation Technique (Onfit; 15 hours at -16ºC plus incubation in humid chamber at 25ºC for 30 days). Letters indicate significant difference by t test at 5% between periods of collection, considering twenty replications for each orchard at each time of sampling.

The incidence of the pathogens surviving on twigs collected during dormancy and subjected to Overnight Freezing did not differ from the incidence of pathogens on twigs incubated only in humid chambers, independent of the orchard. The exceptions were in the reduction on the incidence of *Colletotrichum* sp. in Campo Largo and Porto Amazonas and of *N. ditissima* in both orchards of Palmas, after Overnight Freezing (Table 2).

For both collections, during dormancy and vegetative growth, the incidence of the genus *Fusarium* on vegetative growth did not differ with the two techniques used for all orchards (Tables 2 and 3). The genus *Alternaria* also showed no differences in incidence, except in Campo Largo orchard on vegetative growth (Table 3). It also had high incidence despite the incubation technique used at all orchards, always above 50% (Tables 2 and 3). *Botryosphaeria* sp. had significant reduction in incidence after ONFIT in three orchards, but in Porto Amazonas orchard during vegetative growth the behavior was the opposite, presenting significantly higher incidence after Overnight Freezing (Table 3). *Colletotrichum* sp. was not found in twigs of Porto Amazonas and Palmas 02 orchards on vegetative growth even with Overnight Freezing incubation, and in the other two orchards presented a low incidence, below 20%, after this technique (Table 3). *N. ditissima* has not been found even after Onfit in the Campo Largo and Porto Amazonas orchards in both collection dates (Tables 2 and 3). In Palmas 01 orchard the incidence of *N. ditissima* was not different and in Palmas 02 there was a significant reduction in the incidence, but the pathogen could still be found on the twigs submitted to Onfit (Tables 2 and 3).

It was possible to confirm at genus level the previously morphological and cultural identified isolates of *Alternaria* spp., *Colletotrichum* spp., *N. ditissima*, *Fusarium* spp. and *Botryosphaeria* spp. by BLASTn with the sequences from the extraction of fungal colonies isolated from apple tree twigs. Pathogens isolated from twigs and inoculated on wounded fruit were able to cause typical symptoms and signs resulted from the infection, colonization, and reproduction of the fungi (Figure 3). All inoculations resulted in lesions, and the confirmation of the pathogenicity test was made by morphological identification of the structures (signs) formed on the surface of the fruit with a light microscope, re-isolation of the pathogen on PDA medium and confirmation of the colony by its morphological and molecular characteristics.

Table 3. Incidences (%) of fungi genera in 'Gala' apple tree twigs collected during vegetative growth (September) in the orchards located in Campo Largo, state of Paraná, Porto Amazonas, state of Paraná, Palmas, state of Paraná 01, Palmas, state of Paraná 02, Brazil, with Humid Chamber (HC) incubation (25°C for 30 days) and with Over Night Freezing (OF) incubation (15 hours at -16°C plus incubation in humid chamber at 25°C for 30 days).

Fungi genera	Incidences (%) of fungi genera at vegetative growth							
	Campo Largo		Porto Amazonas		Palmas 01		Palmas 02	
	HC[1]	OF[2]	HC	OF	HC	OF	HC	OF
Colletotrichum sp.	0	5	0	0	5	15	0	0
Fusarium sp.	40	50	55	45	70	60	30	25
Neonectria sp.	0	0	0	0	25	20	35a	10b
Alternaria sp.	50b	100a	90	75	75	90	90	90
Botryosphaeria sp.	35a	0b	0b	55a	60a	25b	85a	20b

Letters indicate significant difference by t test at 5% between periods of collection, considering twenty replications for each orchard at each time of sampling. [1]Humid Chamber; [2]Overnight Freezing + Humid Chamber.

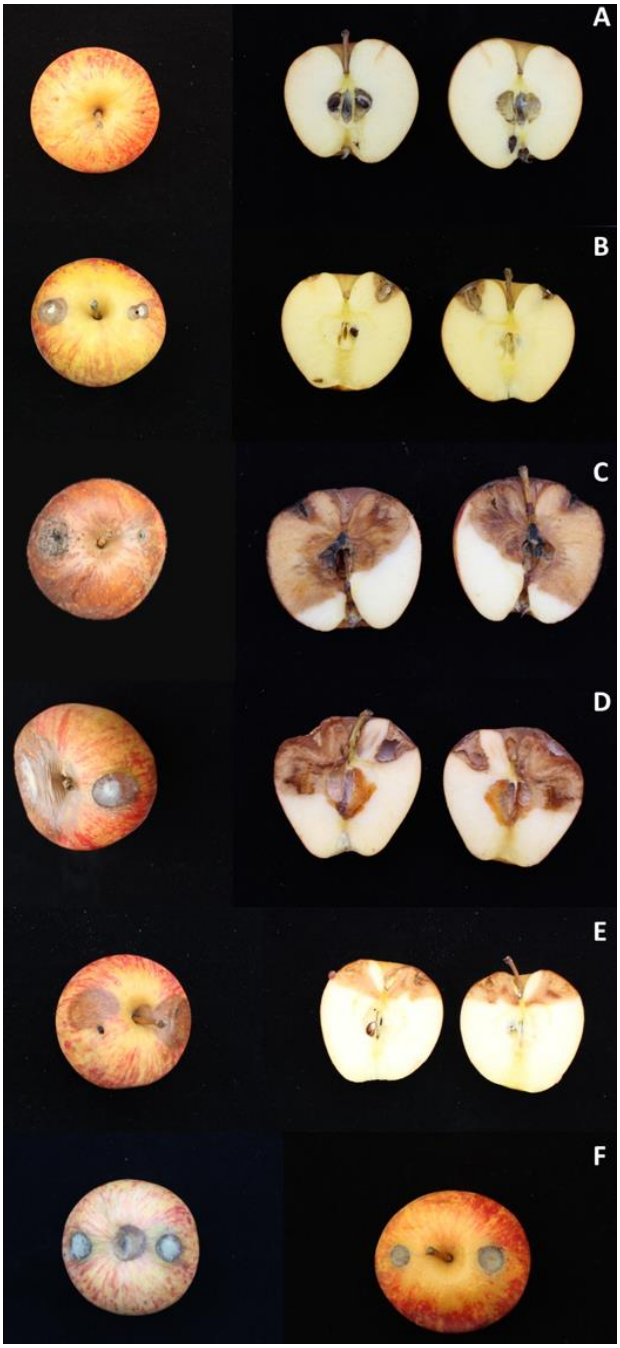

Figure 3. Symptoms of *Neonectria ditissima*, *Colletotrichum* sp., *Botryosphaeria* sp., *Alternaria* sp. and *Fusarium* sp. isolates from twigs inoculated as mycelial plugs on Potato Dextrose Agar (PDA) on detached wounded 'Gala' apple fruit. A = Control (PDA plug without fungi), B = *Neonectria ditissima*, C = *Colletotrichum* sp., D = *Fusarium* sp., E = *Botryosphaeria* sp., and F = *Alternaria* sp.

Several important pathogens for apple production, such as *Neonectria ditissima*, *Colletotrichum* sp., *Botryosphaeria* sp., *Alternaria* sp. and *Fusarium* sp. were detected both at the beginning of the dormancy and after winter treatments (vegetative growth), indicating high potential of being primary inoculum for diseases development in both regions sampled in Paraná. In addition, some of these pathogens (*Alternaria* and *Fusarium*) have not been targeted for control during this period, indicating a risk for a future epidemic. The presence of *N. ditissima* was confirmed on twigs of the two orchards of the municipality of Palmas (Palmas 01 and 02). In the twigs collected in Campo Largo and Porto Amazonas no structure of this fungus was found, indicating its absence in these orchards.

In Brazil, normative instruction No. 12 of the *Ministério da Agricultura, Pecuária e Abastecimento* (Mapa) of May 23 (2014) changed the status of the pathogen *Neonectria ditissima* from A1 (Quarantine Absent Disease) to A2 (Quarantine Present Disease). There have been cases in Brazil in most of the producing regions of Rio Grande do Sul and Santa Catarina States, but in Paraná State the disease is still supposedly restricted to Palmas and Guarapuava regions, which could be confirmed by our study.

In twigs from the two orchards of the municipality of Palmas, only the perfect stage of *N. ditissima* was found. Although the twigs were collected without symptoms, many *N. ditissima* cankers were observed in the other twigs of sampled plants. Signs of *N. ditissima* as perithecia were detected in the evaluated twigs at 30 days. Even if the fungus is latent, this short period of time was enough to produce these structures by the pathogen, which agrees with previous data in which the production of perithecia in detached twigs in only 28 days (personal communication). Although lower incidence of *N. ditissima* was detected during vegetative growth, the results demonstrate the high capacity of the pathogen to produce large amounts of inoculum in a short time, serving as source of inoculum mainly in this stage of the apple tree, when the floral structures are susceptible to pathogen infections leading to pre- or postharvest fruit rot. It may also be a source of inoculum for infections in twigs and apple fruit (Weber, 2014). We were able to find high incidences of the pathogen in tissues that did looked healthy, which shows that control based on visual symptoms is not enough for management of European Canker. It also highlights the importance of strict measures based on the pathogen exclusion in regions where the pathogen has not yet been detected, such as Campo Largo and Porto Amazonas.

The reduction in the incidence of *N. ditissima* in vegetative growth can be explained by winter pruning, which is one of the basis of European Canker control, when the removal of the symptomatic twigs is facilitated by better visualization of cankers in this period (Weber, 2014), along with the use of copper fungicides and other fungicides such as Methyl Thiophanate and Captana, registered at the *Agência de Defesa Agropecuária do Paraná* (Adapar) to control the disease, which contributed to the reduction in the incidence of the pathogen between dormancy until in vegetative growth. The use of these fungicides, however, was not totally effective, since the incidence in the collection in vegetative growth still exceeds 30%, probably due to the high incidence of the disease in the orchards. Therefore, the control measures used were not enough to remove the inoculum of *N. ditissima* in the orchards, and these should be complemented to reduce the losses recorded with this disease in apple orchards and to avoid its spread to orchards nearby.

There was a drastic reduction in the incidence of the genus *Colletotrichum* at all collection orchards between dormancy and vegetative growth collections. The importance of the diseases caused by this genus in apple trees results from its wide distribution (Boneti, Ribeiro, & Katsurayama, 1999; Hamada, Nesi, Alves, & De Mio, 2012; Sutton et al., 2014; Moreira, Peres, & De Mio, 2019) and its caused damage, which can vary from 50 to 75% and the large amount of fungicide sprays required for its control (Ceresine, Leite, & Tsuneta,1992; Hamada et al., 2012). Crusius et al. (2002) reported that the survival of *Colletotrichum* spp. occurred in the dormant apple tree, whereas isolates obtained from fallen leaves in the soil and mummified fruits induced symptoms of bitter rot in the fruits, only isolates from twigs and buds caused Glomerella leaf spot. Winter treatments did not eradicate the primary inoculum of the fungus in an experiment conducted by Crusius et al. (2002). Two sprays of 0.3% copper oxychloride reduced the initial inoculum of the fungus by 65 - 84.6% in buds and 85.6 - 93.7% in twigs. The reduction of the primary inoculum was also found in the present study, even though there was no incidence of the genus in three of the four orchards where it was collected on vegetative growth, which may be the result of winter treatment with cupric fungicides (copper sulphate and copper oxychloride) and other fungicide sprays in all orchards. In spite of the results found, it is important to note that this reduction of the inoculum in twigs does not mean that no disease will occur, since Crusius et al. (2002) in a study conducted in Santa Catarina State found no difference in the initial incidence of the

disease between sprayed and non-sprayed plants, indicating that even minimal amounts of inoculum could lead to Glomerella leaf spot epidemics. In Parana State the survival of *Colletotrichum* in apple orchard was confirmed in twigs, buds and also on fallen leaves, and the isolates were pathogenic on leaves and apple fruit (Hamada & De Mio, 2017).

In the collection orchards in Palmas, the incidence of the genus *Botryosphaeria* increased from dormancy to vegetative growth, probably due to the temperature increase favoring the development and maturation of pycnidia with conidia, corroborating the results found by Copes and Hendrix Jr. (2004) that for *Botryosphaeria dothidea*, the most commonly isolated species of apple trees, temperatures ranging from 6 to 30ºC were favorable for conidia formation, but with an optimum peak of production at 24ºC, a temperature close to those observed during vegetative growth in the orchards.

Alternaria was isolated from leaf spots on Gala apples from an orchard in Paraná State and morphologically identified as *Alternaria mali* (Rollemberg, Fayad, Hamada, & De Mio, 2011). Since the species of Alternaria causing leaf blotch and fruit spot is not certain, with multiple *Alternaria* spp. groups (*Alternaria arborescens, A. tenuissima, and A. alternata, A. mali* and *A. longipes*) being associated with apple diseases worldwide (Harteveld, Akinsanmi, & Drenth, 2013), it is important to carry future studies with the molecular identification of the species. Leaves of the canopy, leaf residue on the soil, twigs and buds are sources of inoculum of *Alternaria* spp. (Harteveld, Akinsanmi, Chandra, & Drenth, 2014). The survival of the inoculum of *Alternaria* spp. (Yousefi & Shahri, 2009) and the survival of *A. alternata* has been reported in buds being a source of inoculum for the infection and death of flowering pear buds (Wenneker, Joosten, Anbergen, Vink, & van Bruggen, 2011). Studies by Harteveld et al. (2013) indicate that, although the leaf residue provides the largest amount of *Alternaria* spp. inoculum in the apple orchard throughout the year, the importance and contribution of other parts of the plant to the inoculum in the orchard should be taken into account because in winter twigs and buds are available. These authors also verified that the relative humidity of the twigs (combination of precipitation and high temperature) was the factor that most influenced the dynamics of the spore production. The production of spores in twigs is reduced with high precipitation, but the increase in temperature after precipitation increases the relative humidity, and, consequently, spore production. Besides sporulation, relative humidity was also indicated as a factor that strongly influences infection of *Alternaria* spp. (Rotem, 1994) and the incidence of Alternaria leaf spot (Yoon, Lee, Park, & Park, 1989; Thakur & Sharma, 2010). Therefore, elevation of temperature in vegetative growth, along with the occurrence of rainfall, may have generated ideal conditions for sporulation of the pathogen in the twigs.

In addition, the winter treatment in all the orchards was not directed to *Alternaria* spp., but to other fungi of greater importance for the culture in the state of Paraná, mainly *Colletotrichum* spp. Another aspect that could be considered is that cellular walls of *Alternaria* spp. conidia are generally thick and formed of several cells, which could also be related to the survival even after winter treatments. This fact was evidenced by Kim, Lee, and Kim (2017), who found *A. alternata* isolates resistant to the fungicide iprodione. The isolates showed visible cellular alterations during sporulation, forming multicellular conidia with thickened double-celled walls and accumulation of lipidic bodies in the cytoplasm, which can inhibit fungicide penetration into conidial cells, reducing fungicide efficiency, and used as energy and nutritional sources by the fungus.

There are more than 70 species of the genus *Fusarium* present in several regions of the world (Leslie & Summerell, 2006), as well as rhizosphere, endophytic or pathogenic colonizers. The fungus *F. avenaceum* was identified as the causal agent of apple core rot (Schroers et al., 2008). This disease represents a potential economic problem for apple producers and a safety issue for consumers due to the potential production of mycotoxins during infections, especially for the production of apple juice and cider, since infected apples hardly have external symptoms, which makes it difficult to remove them in the processing (Sorensen, Stjepanovic, Romarheim, Krekling, & Storebakken, 2009). Studies on the genus in apple trees are scarce and should be performed to better understand the pathosystem, especially since high incidences of *Fusarium* genera were detected in all orchards before and after winter treatments and surviving superficially and internally on twigs.

The twigs disinfected and frozen by Onfit, which deteriorates the superficial microorganisms and degrades the cells of the external twig tissues, in general had fewer incidence of fungus genera or did not differ in relation to the non-use of the technique, both in the dormancy and during vegetative growth. This shows that pathogens, in general, are surviving more superficially in the twigs. The genus *Alternaria*, which had very high incidence rates on vegetative growth without Onfit, also had more than 70% incidence after the use of the

technique in all the localities, being of 90% in Campo Largo orchards, indicating that the pathogen can survive even at extremely low temperatures, probably due to its thick-walled cellular structure, and may be surviving in more internal tissues in the twigs.

Inoculation of the isolates of *N. ditissima, Colletotrichum* sp.*, Botryosphaeria* sp.*, Alternaria* sp. and *Fusarium* sp. resulted in typical symptoms in the fruits, proven by the morphological identification of signs of the pathogens formed on the lesions. It is known the importance of the inoculum, constituted by the epiphytic population of the fruits, in the development of rot, especially those with long latent period (Emery et al., 2000); these populations may come from insufficiently treated twigs during dormancy period. If control measures are not taken until the fruiting period of the apple tree in orchards, losses from fruit rot may occur. The sensitivity of the isolates surviving on twigs to fungicides should be investigated in the future.

Conclusion

Neonectria ditissima, Colletotrichum sp.*, Botryosphaeria* sp.*, Alternaria* sp. and *Fusarium* sp. were detected in 'Gala' apple tree twigs collected in dormancy and in vegetative growth. The twigs are source of inoculum for fruit infections by *N. ditissima*, *Colletotrichum* sp., *Botryosphaeria* sp., *Alternaria* sp. and *Fusarium* sp., which were pathogenic in fruits. All these genera also survive internally in twigs. Winter treatments of apple trees should be reviewed.

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References

Boneti, J. I. S., Ribeiro, L. G., & Katsurayama, Y. (1999). *Manual de identificação de doenças e pragas da macieira*. Florianópolis, SC: Epagri.

Ceresine, P. C., Leite, R. P., & Tsuneta, M. (1992). Efeito de tratamentos químicos no controle de mancha foliar de *Glomerella* em macieira, no estado do Paraná. *Fitopatologia Brasileira, 17*, 258-267.

Copes, W. E., & Hendrix Jr., F. F. (2004). Effect of temperature on sporulation of *Botryosphaeria dothidea, B. obtusa,* and *B. rhodina*. *Plant Disease, 88*(3), 292-296. DOI: https://doi.org/10.1094/PDIS.2004.88.3.292

Crusius, L. U., Forcelini, C. A., Sanhueza, R. M. V., & Fernandes, J. M. C. (2002). Epidemiology of apple leaf spot. *Fitopatologia Brasileira, 27*(1), 65-70. DOI: https://doi.org/10.1590/S0100-41582002000100010

Emery, K. M., Michailides, T. J., & Scherm, H. (2000). Incidence of latent infection of immature peach fruit by *Monilinia fructicola* and relationship to brown rot in Georgia. *Plant Disease, 84*(8), 853-857. DOI: https://doi.org/10.1094/PDIS.2000.84.8.853

Hamada, N. A., & De Mio, L. L. M. (2017). Survival of pathogenic *Colletotrichum* isolates on dormant buds, twigs and fallen leaves of apple trees in commercial orchards. *Fruits, 72*(3), 158-165. DOI: https://doi.org/10.17660/th2017/72.3.5

Hamada, N. A., Nesi, C. N., Alves, G., & De Mio, L. L. M. (2012). Fungicidas associados ou não a fosfitos no controle da mancha foliar de *Glomerela* em macieira. *Revista Brasileira de Ciências Agrárias, 7*(suppl.), 831-837. DOI: https://doi.org/10.5039/agraria.v7isa2394

Harteveld, D. O. C., Akinsanmi, O. A., & Drenth, A. (2013). Multiple *Alternaria* species groups are associated with leaf blotch and fruit spot diseases of apple in Australia. *Plant Pathology, 62*(2), 289-297. DOI: https://doi.org/10.1111/j.1365-3059.2012.02637.x

Harteveld, D. O. C., Akinsanmi, O. A., Chandra, K., & Drenth, A. (2014). Timing of infection and development of *Alternaria* diseases in the canopy of apple trees. *Plant Disease, 98*(3), 401-408. DOI: https://doi.org/10.1094/PDIS-06-13-0676-RE

Jones, A. L., & Aldwinckle, H. S. (1990). *Compendium of apple and pear diseases*. St. Paul, MN: American Phytopathological Society.

Jones, A. L., & Sutton, T. B. (1996). *Diseases of the tree fruits in the east*. East Lansing, MI: Michigan State University Cooperative.

Kim, E., Lee, H. M., & Kim, Y. H. (2017). Morphogenetic alterations of *Alternaria alternata* exposed to dicarboximide fungicide, iprodione. *Plant Pathology, 33*(1), 95-100. DOI: https://doi.org/10.5423/PPJ.NT.06.2016.0145

Leslie, J. F., & Summerell, B. A. (2006). *The Fusarium laboratory manual*. Oxford, GB: Blackwell Publishing.

Moreira, R. R., Peres, N. A., & De Mio, L. L. M. (2019). *Colletotrichum acutatum* and *C. gloeosporioides* species complexes associated with apple in Brazil. *Plant Disease, 103*(2), 268-275. DOI: https://doi.org/10.1094/pdis-07-18-1187-re

Pereira, W. V., Padilha, A. C. N., Kaiser, J. A. O., Nesi, C. N., Fischer, J. M. M., & De Mio, L. L. M. (2019). *Monilinia* spp. from imported stone fruits may represent a risk to Brazilian fruit production. *Tropical Plant Pathology, 44*(2), 120-131. DOI: https://doi.org/10.1007/s40858-018-0243-z

Petri, J. L., Palladini, L. A., & Pola, A. C. (2006). Dormência e indução à brotação em macieira. In Epagri (Ed.), *A cultura da macieira* (p. 261-297). Florianópolis, SC: Epagri.

Ploetz, R. C. (2007). Diseases of tropical perennial crops: challenging problems in diverse environments. *Plant Disease, 91*(6), 644-663. DOI: https://doi.org/10.1094/PDIS-91-6-0644

R Studio Team. (2015). *RStudio*. Boston, MA: R Studio Team. Retrieved from http://www.rstudio.com/

Rollemberg, C. L., Fayad, F., Hamada, N. A., & De Mio, L. L. M. (2011). First report of *Alternaria mali* on apples in Brazil. *Phytopathology, 101*, S155.

Rotem, J. (1994). *The genus Alternaria: biology, epidemiology, and pathogenicity*. St. Paul, MN: American Phytopathological Society.

Schroers, H. J., Sørensen, J. L., Thrane, U., Nielsen, K. F., Jerjav, M., Munda, A., & Frank, J. (2008). High incidence of *Fusarium avenaceum* (Ascomycota, Nectriaceae, Gibberella) and moniliformin in apples showing wet core rot symptoms. *Plant Pathology, 90*(suppl. 2), 326.

Sorensen, M., Stjepanovic, N., Romarheim, O. H., Krekling, T., & Storebakken, T. (2009). Soybean meal improves the physical quality of extruded fish feed. *Animal Feed Science and Technology, 149*(1-2), 149-161. DOI: https://doi.org/10.1016/j.anifeedsci.2008.05.010

Sutton, T. B., Aldwinckle, H. S., Agnello, A. M., & Walgenbach, J. F. (2014). *Compendium of apple and pear diseases and pests* (2nd ed.). St. Paul, MN: American Phytopathological Society.

Thakur, V. S., & Sharma, N. (2010). Epidemic outbreak of apple blotch disease: epidemiology and management in Himachal Pradesh. *Indian Phytopathology, 63*(2), 141-144.

Weber, R. W. S. (2014). Biology and control of the apple canker fungus *Neonectria ditissima* (syn. *N. galligena*) from a Northwestern European perspective. *Erwerbs-Obstbau, 56*(3), 95-107. DOI: https://doi.org/10.1007/s10341-014-0210-x

Wenneker, M., Joosten, N. N., Anbergen, R. H. N., Vink, P., & van Bruggen, A. S. (2011). Epidemiology and effective control of Altenaria altenata, causal agent of dead (dormant) flower bud disease of pear. *Acta Horticulturae, 909*, 485-490. DOI: https://doi.org/10.17660/ActaHortic.2011.909.56

White, T.J., Bruns, T., Lee, S., & Taylor, J. (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In M. A. Innis, D. H. Gelfand, J. J. Sninsky, & T. J. White (Eds.), *PCR protocols. A guide to methods and applications* (p. 315–322). San Diego, CA: Academic Press.

Yoon, J. T., Lee, J. T., Park, S. D., & Park, D. O. (1989). Effects of meteorological factors on the occurrence of alternaria leaf spot caused by *Alternaria alternata* f. sp. *mali*. *Korean Journal of Plant Pathology, 5*(3), 312-316.

Yousefi, A., & Shahri, M. H. (2009). Brown spot disease of peach and apricot trees, pathogenicity and overwinter. *Asian Journal of Plant Pathology, 3*(3), 61-69. DOI: https://doi.org/10.3923/ajppaj.2009.61.69