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Original article

Survival of pathogenic *Colletotrichum* isolates on dormant buds, twigs and fallen leaves of apple trees in commercial orchards

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Summary

Introduction – **Glomerella leaf spot on apple (*Malus* × *domestica* Borkh.), due to a complex of *Colletotrichum* species, causes severe leaf spot symptoms leading to early leaf fall, and eventual symptoms on fruit before and after harvest. Under the Brazilian conditions, it is the main apple disease responsible for severe damage in all production areas. This study aimed 1) to verify the survival of *Colletotrichum* spp. in dormant organs, fallen leaves and soil samples from fungicide-sprayed commercial orchards during winter; 2) to verify the survival of *Colletotrichum* spp. on asymptomatic leaves during the vegetative period; and 3) to identify the species complex and to confirm the pathogenicity of the isolates obtained from different parts of the plant (on fruit and leaves).** *Materials and methods* – **The study was conducted in a commercial orchard during the winters of 2010 and 2011, assessing the pathogen survival on buds, twigs, asymptomatic leaves, fallen leaves and soil samples. Fungal isolates from different substrates were inoculated on fruit (with and without wound) and on leaves of apple cv. Gala to prove their pathogenicity.** *Results and discussion* – **This is the first investigation on the survival of the *Colletotrichum* complex in apple under the conditions of Brazilian commercial orchards. All isolates (16) from dormant twigs and fallen leaves were identified as *C. acutatum* species complex. Five (5) isolates from dormant buds were identified as *C. gloeosporioides* species complex and three (3) as *C. acutatum* species complex. According to the data collected, *Colletotrichum* spp. are able to survive during winter in dormant buds, on dormant twigs and fallen leaves, but are most frequent on fallen leaves. The isolates obtained from buds, twigs and fallen leaves were pathogenic on leaves and fruit of apple. Copper sprays during the dormant stage did not completely eliminate the inoculum. The pathogen was not recovered from soil or from asymptomatic leaves with the methodology used.** *Conclusion* – **Fallen leaves on the ground can be a source of inoculum from one season to the next, so they must be considered in disease management programs to avoid the spread of primary inoculum.**

Significance of this study

What is already known on this subject?

- Glomerella leaf spot is the main disease of apple and causes lesions on leaves and fruit, resulting in early defoliation. The pathogen can survive asymptomatically in dormant buds and twigs in the coldest region of Brazil.

What are the new findings?

- The pathogen survives in dormant buds, twigs and on fallen leaves in subtropical region of Paraná State.

What is the expected impact on horticulture?

- Based on these findings, it is recommended to eliminate leaves on the ground to avoid survival of the pathogen on fallen leaves.

Keywords
Brazil, apple, *Malus domestica*, fungal disease, plant pathology, epidemiology

Résumé

Survie des isolats pathogènes de *Colletotrichum* sur les bourgeons dormants, les brindilles et les feuilles mortes de pommiers en verger commercial.

Introduction – **La pourriture amère du pommier (*Malus* × *domestica* Borkh.), due à un complexe d'espèces de *Colletotrichum*, provoque des symptômes sévères de taches foliaires conduisant à une chute rapide des feuilles et des symptômes éventuels sur fruits avant et après récolte. Dans les conditions brésiliennes, c'est la principale maladie de la pomme, responsable de graves dommages dans toutes les zones de production. Cette étude a visé 1) à vérifier la survie de *Colletotrichum* spp. sur les organes dormants, les feuilles mortes et les échantillons de sol des vergers commerciaux traités aux fongicides pendant l'hiver; 2) à vérifier la survie de *Colletotrichum* spp. sur des feuilles asymptomatiques pendant la période végétative; 3) à identifier le complexe d'espèces et à confirmer la pathogénicité des isolats obtenus à partir de différentes parties de la plante (fruits et feuilles).**

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Matériel et méthodes – **L'étude a été menée dans un verger commercial pendant les hivers 2010 et 2011, en évaluant la survie des pathogènes sur les bourgeons, les rameaux, les feuilles asymptomatiques, les feuilles tombées à terre et les échantillons de sol. Des isolats fongiques de différents substrats ont été inoculés à des fruits (avec ou sans plaie) et des feuilles de pommier cv. Gala pour prouver leur pathogénicité.** *Résultats et discussion* – **Cette étude constitue la première enquête sur la survie du complexe *Colletotrichum* sur pommiers dans les conditions des vergers commerciaux du Brésil. Tous les isolats (16) provenant des rameaux dormants et des feuilles tombées ont été identifiés comme étant des espèces de *C. acutatum*. Cinq (5) isolats de bourgeons dormants ont été identifiés en tant que complexe d'espèces de *C. gloeosporioides* et trois (3) comme complexe d'espèces de *C. acutatum*. Selon les données recueillies, les *Colletotrichum* spp. sont capables de survivre pendant l'hiver dans les bourgeons dormants, sur les rameaux dormants et les feuilles tombées, et sont les plus fréquentes sur les feuilles au sol. Les isolats obtenus à partir des bourgeons, des brindilles et des feuilles tombées se sont révélés pathogènes sur les feuilles et les fruits. Les pulvérisations de cuivre au stade de dormance n'ont pas complètement éliminé l'inoculum. Toutefois, aucun pathogène n'a été récupéré à partir du sol ou des feuilles asymptomatiques avec la méthodologie utilisée.** *Conclusion* – **Les feuilles mortes au sol peuvent être une source d'inoculum d'une saison à l'autre, donc elles doivent être prises en compte dans les programmes de gestion des maladies pour éviter la propagation de l'inoculum primaire.**

Mots-clés
Brésil, pommier, *Malus domestica*, maladie fongique, pathologie végétale, épidémiologie

Introduction

Glomerella leaf spot (GLS) is the main foliar disease of apple in Brazil and is caused by several *Colletotrichum* spp., mainly of *C. gloeosporioides* and *C. acutatum* species complex. The disease causes lesions on leaves and fruit, resulting in early defoliation. Favorable conditions for disease development include temperatures above 20 °C and continued leaf wetness of at least for 10 h (Katsurayama *et al*., 2000a).

The disease control is carried out primarily preventively. This approach requires an average of 16 protectant fungicide sprays from the end of flowering to the onset of natural defoliation (Katsurayama *et al*., 2000a), which lasts from September to April depending on the region of Brazil.

Fungicides are used as preventive to control GLS due in part to the short incubation period of the pathogen, only 45 h at 20 °C and above, which thus circumvents the effectiveness of such treatments (Katsurayama and Boneti, 2012). A strategy for improvement of GLS management is to predict when the disease will occur (Katsurayama and Boneti, 2012; Crusius *et al*., 2002). This includes understanding the origin of the primary inoculum (Copes and Thomson, 2008). Primary inoculum is produced following the survival period of the pathogen, and for deciduous plants including apple, coincides with budding (Stadnik *et al*., 2009).

Although the survival of *Colletotrichum* spp. is well documented on several crops (Crusius *et al*., 2002; Copes and Thomson, 2008; Freeman *et al*., 2002), little information can be found about survival strategies of *Colletotrichum* spp. during apple tree dormancy or about differences between members of the species complex. In the conditions of Rio Grande do Sul State (*i.e.*, a low pressure of inoculum) it is reported that the GLS pathogen can survive asymptomatically in dormant buds and twigs but not on fallen leaves (Crusius *et al*., 2002). The present study did not address the ability of the pathogen to survive winter treatments administered by the growers. Our hypothesis was that under high pressure of inoculum and under winter treatments the pathogen could survive on buds, twigs and also leaves on the ground.

This study aimed 1) to verify the survival of *Colletotrichum* spp. in dormant buds, dormant twigs, fallen leaves on soil from commercial orchards (with fungicides sprays) during winter; 2) to verify the survival of *Colletotrichum* on asymptomatic leaves during vegetative period; and 3) to identify species complex and to confirm the pathogenicity on fruit and leaves of the isolates obtained from different parts of the plant.

Materials and methods

This study was conducted in a commercial orchard in Campo Largo, Paraná State, Brazil, during two consecutive years (2010 and 2011). The apple trees were 13-year old cultivar Imperial Gala grafted on rootstock 'M9' and trained to the central leader system. The climate in the region according to Koppen's classification is humid subtropical, with cool summers and no dry seasons (Cfb).

Plants were subjected to 25 fungicide sprays (on average) in the 2009–10 cropping season and to 26 sprays (on average) in the 2010–11 season. The number of sprays (Table 1) varied according to the environmental conditions, specifically with rainfall. The fungicides used were classified as protectants (mancozeb and chlorotalonil, copper sulphate and copper hydroxide) or systemics (methyl tiophanate and piraclostrobin) + protectant fungicide (metiram). In June, winter treatments involved the application of Bordeaux mixture (Bordasul®) on the trees, at a concentration of 0.38% in the middle of the month.

Survival of *Colletotrichum* spp. during dormancy

Assessment of pathogen survival was carried out during the dormant period by monitoring the presence of inoculum over time on plant material including buds, twigs and fallen leaves, and in the soil at 1 cm depth at the edge of the canopy of apple tree.

Samples were collected arbitrarily in two sectors of 0.5 and 0.7 ha (replications) in the same orchard from June to August in 2010 and 2011. Five samples per sector and per year were collected and a composite sample was formed for each plant material (dormant buds, dormant twigs, soil, and fallen leaves).

Isolations from plant materials collected of each sector and per year were performed from 50 g of dormant twigs (approximately 120 pieces of 5 cm), 5 g of dormant buds (approximately 750 buds) and 20 g of fallen leaves (approximately 150 leaves), which were suspended in 100, 50 and 200 mL sterilized water, respectively, containing 20 µL L^{-1} Tween 20 (Biotec®), and incubated on an orbital shaker (Labstore® model 109/TCM, Brazil) for 60 min at 60 rpm. The resulting suspensions were streaked onto five Petri dishes (100 µL $dish^{-1}$) containing *Colletotrichum* spp. semi-selective medium

(Ureña-Padilla *et al*., 2001), with 39 g potato-dextrose-agar media (PDA), 250 mg ampicillin, 150 mg streptomycin sulfate, 5 mg iprodione, 100 µL tergitol and 1 L distilled water.

Isolations from soil were performed from 100 g sifted, air-dried soil samples collected at the edge of the canopy of apple tree at a depth of 1 cm. To each sample, 200 mL sterile water with 20 µL L^{-1} Tween 20 were added. Each sample was subjected to shaking for 60 min at 60 rpm in an orbital shaker, after which it was left on the laboratory bench for 1 h. The resulting suspension was transferred to Petri dishes (100 µL $dish^{-1}$) containing the semi-selective medium (Ekefan *et al*., 2000) for the isolation of *C. gloeosporioides* from soil, consisting of potato-dextrose-agar amended with pencycuron (50 mg L^{-1}), tolclofosmethyl (10 mg L^{-1}), and chlortetracycline (100 mg L^{-1}) in 1 L distilled water. The presence of *Colletotrichum* spp. conidia was observed with a Neubauer Chamber for all suspensions but conidia were not counted due to the small number observed.

Petri dishes were maintained in B.O.D. (Eletrolab®, model 122 FC) at 25 ± 1 °C until the appearance of the first colonies, which were then transferred to Petri dishes, each containing 15 mL PDA (Himedia®, Biosystems Com. Imp. Exp. Produtos Lab. Ltda., Curitiba, Brazil) at 39 g L^{-1}. After 7 days, *Colletotrichum* spp. colonies were identified according to their cultural and morphological characteristics (Boneti *et al*., 1999). Colonies on semi-selective medium were considered to have originated from single conidia. The total number of colonies obtained from a collection represented inoculum density of the original substrate.

Table 1. Phytosanitary treatments for the control of Glomerella leaf spot (GLS) conducted in two sectors sampled of a commercial orchard in Campo Largo, PR, according to the type and number of fungicide applications.

Months	Fungicides[a]	Number of fungicide applications	
		2009–10	2010–11
Sep	Protectant	3	3
	Systemic	3	0
Oct	Protectant	3	2
	Systemic	1	3
Nov	Protectant	1	3
	Systemic	1	0
Dec	Protectant	3–4	4
	Systemic	1	2–3
Jan	Protectant	3–6	4
	Systemic	3–5	1–3
Feb	Protectant	0–4	0–3
	Systemic	0	0
Mar	Protectant	0	0–1
	Systemic	0	0
Apr	Protectant	0	0–1
	Systemic	0	0

[a] Protectant fungicides: mancozeb, chlorotalonil and copper sulphate (concentration 0.38%) and copper hydroxide (concentration 0.35%); systemic + protectant fungicides: tiophanate methyl, pyraclostrobin + metiram. The fungicides were used according to Brazilian rules at MAPA (http://www.agricultura.gov.br/vegetal/registros-autorizacoes/registro).

Survival of *Colletotrichum* spp. on asymptomatic leaves during the vegetative period

Symptomless leaves of apple cv. Imperial Gala were collected monthly from September to March during the two cropping seasons (2009–10 and 2010–11). Approximately 50 leaves (20 g) were placed in Erlenmeyer flasks with 200 mL sterile water plus 20 µL L^{-1} Tween 20 (Biotec®), and agitated for 60 min at 60 rpm on an orbital shaker (Labstore®, model 109/TCM). The resulting suspension was observed in a microscope to check the presence of conidia, and after that aliquots were placed on *Colletotrichum* spp. semi-selective medium (100 µL $dish^{-1}$) (Ureña-Padilla *et al*., 2001), using 5 Petri dishes per sample. Estimates of inoculum levels were based on the number of *Colletotrichum* spp. isolates obtained for each collection, each colony observed on semi-selective medium was considered to represent a single conidium or a mycelial fragment.

Identification of *Colletotrichum* spp. colonies

A sample of 24 isolates obtained were identified according to their cultural and morphological characteristics in culture medium (BDA), which are: mycelial diameter at 7 days after inoculation, size of conidia, colony color (verse and reverse), presence of sectors (specific areas of mycelial growth) and the formation of perithecia.

To determine mycelial growth, mycelial discs (3 mm) were placed in the centers of Petri dishes containing 20 mL of PDA (39 g L^{-1}) and the dishes were incubated in BOD at 25 ± 1 °C, in the dark, using 3 replications per isolate. After 168 h of incubation the colony diameter in orthogonal directions was measured.

The characteristics of the conidia were evaluated through the use of conidial suspensions from monosporic cultures grown on PDA medium maintained for 15 days at 25 ± 1 °C in the dark. The conidial suspension was obtained by scraping the surface of the Petri dishes flooded with distilled water and filtering it in sterile gauze. The characteristics evaluated for conidia were width and length. We measured 25 conidia per isolate in an optical microscope at ×40 magnification.

The evaluation of colony color and sector formation was performed by culturing the isolates under the same conditions described above, using mycelial discs (3 mm) which were placed on dishes containing PDA medium, which were incubated in BOD at 25 ± 1 °C for 7 days, in the dark (in triplicate per isolate) and evaluations were performed 7 days after inoculation. These same Petri dishes were kept for 30 days to observe whether perithecia formed or not. Perithecia were collected and crushed onto a slide for observation by an optical microscope.

For molecular identification, the DNA was extracted from 24 isolates using the extraction kit UltraClean® Microbial DNA Kit (Bio MO) following the manufacturer's instructions. The DNA concentration was quantitated by 0.8% agarose gel stained with GelRed (Uniscience®) and documented System photodocumentation L EX-Pix (Loccus®) visually comparing the intensity of the bands formed with a known weight of standard ("High mass DNA ladder" Invitrogen®). The samples were stored at a temperature of -20 °C. After DNA extraction and for identification by PCR, we used specific primers for *C. acutatum* and *C. gloeosporioides*. For the identification of *C. acutatum* the oligonucleotides CaInt2 (5'-GGG CGC GAA GCC TCT GG-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC - 3') were used (Afanador-Kafuri *et al*., 2003). For the identification of *C. gloeosporioides* the oligonucleotide CgInt (5'- CTC GGC GGC CCG CCG CCT GG- 3") was used as described by Mills *et al.* (1992) together with the ITS4.

The reaction was performed with 12.5 µL of the solution in ultrapure water with 1 µL of the DNA extracted quantified to a concentration of 10–100 ng mL^{-1}, 1.25 µL of PCR buffer 10X, 0.375 mM $MgCl_2$, 1.0 mM dNTP mixture, 0.125 mM of each of the primers and 0.2 U Taq polymerase (all reagents from Invitrogen®). Amplification was performed in a thermocycler with an initial denaturation for a period of 5 min at 95 °C followed by 35 cycles of 30 s at 95 °C, 30 s at 65 °C and 90 s at 72 °C with a final extension by a period of 3 min at 72 °C. After amplification, the PCR products were applied in an agarose gel at 1%. The electrophoretic run was performed at constant current of 5 V cm^{-1} for 1 h. The DNA bands were visualized under ultraviolet transilluminator and photographed. All analyzes were performed comparing the isolates of this study with DNA of *C. acutatum* (TUT-137) and *C. gloeosporioides* (AVO-33- 4B) (Freeman *et al*., 2001).

Pathogenicity of isolates

All 68 isolates obtained in this study were used in the pathogenicity assays on fruit and a sample of 30 isolates on leaves. Isolates were selected from each plant part and according to sporulation ability, including seven isolates from dormant buds at 2010 and 2011, respectively, five isolates from dormant branches at 2011, and 11 isolates from fallen leaves at 2011.

Thirty isolates were inoculated on apple cv. Gala leaves and 68 isolates on fruit to evaluate their pathogenicity. For fruit inoculation, agar discs with mycelium of 7 day old cultures, grown on PDA medium, were placed on the fruit surface with and without wound. One disc was placed on each fruit; there were four fruit (replications) for each evaluated condition (with and without wound) for each isolate. Entomological pins were used to make 1 mm-deep wounds, on which mycelia discs were placed. Fruit were evaluated for appearance of lesions until 16 days after inoculation (DAI).

We used 4 month-old apple seedlings cv. Gala for leaf inoculations, grown in plastic pots filled with substrate (Tropstrato – HT Hortaliças) and maintained in a greenhouse. Fungal isolates were grown on PDA medium (Himedia®) and incubated in BOD (Eletrolab®, model 122 FC) at 25 ± 1 °C for 7 days. Conidia suspensions were prepared by scraping the surface of the mycelium in presence of sterile water.

Concentrations of conidia suspensions were determined with a Neubauer Chamber and adjusted to 1.0×10^4 conidia mL^{-1} with sterile water. Asymptomatic leaves of four plants per isolate were inoculated by spraying the conidia suspension with Wilbes sprayers (capacity: 340 mL; approximate flow rate: 10 mL min^{-1}), until complete coverage of leaf surfaces. Inoculated plants were maintained in a humid chamber (25 °C and 100% RH) for 48 h, and plants were observed for appearance of typical symptoms up to 14 days after inoculation.

Weather data

During the experiment, temperature and relative humidity (RH) were monitored at the weather station of the Technological Institute SIMEPAR, Curitiba, PR (station number 25.264.916). The weather station is located 935 m a.s.l., 30 km from the experimental area. Rainfall was measured daily in the orchard, at the beginning of the centerline of the area using rain gauges (Jprolab Label, Brazil, model: wedge).

Results and discussion

Survival of *Colletotrichum* spp. during dormancy

Although very small numbers of *Colletotrichum* spp. conidia were observed in the suspensions that we prepared from various substrates, they were present since *Colletotrichum* spp. colonies were obtained when conidia or mycelial suspensions were plated on a semi-selective medium. In future studies, in order to increase the number of colonies forming units, we recommend that the protocols we used be modified by increasing the amount of sampled materials and by incorporating a light centrifugation step following incubation.

The presence of *Colletotrichum* spp. conidia in dormant twigs and buds during the resting period as observed in this study was previously reported in apple in Brazil (Crusius *et al*., 2002) and in Norway (Borve and Stensvand, 2007). They were also found in the United States with *C. gloeosporioides* on camellia branches (*Camellia japonica* L.) (Copes and Thomson, 2008), and in Portugal with *C. acutatum* and *C. gloeosporioides* in buds and branches of olive (*Olea europaea* L.) (Talhinhas *et al*., 2011).

Our results showed that dormant twigs and buds infested with *Colletotrichum acutatum* and *C. gloeosporioides* species complex could be an important source of inoculum even when the fungi are present at low levels. For this pathosystem we have observed that minimal amounts of inoculum could trigger a GLS epidemic. In south of Brazil Crusius *et al.* (2002) found no difference in the amount of primary inoculum between fungicide-sprayed areas (calcium polysulfide and copper oxychloride) and untreated areas (control), and they have noticed the same intensity of disease at the end of season.

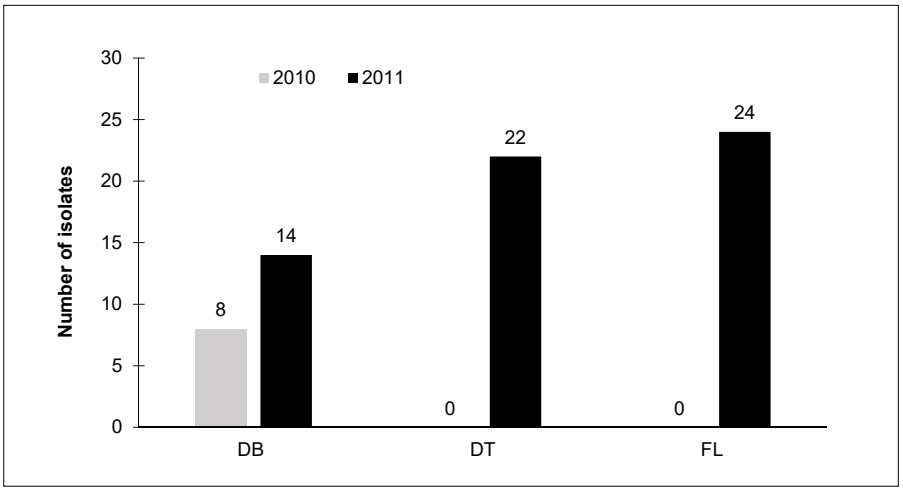

Figure 1. Number of isolates of *Colletotrichum* spp. obtained from dormant buds (DB), dormant twigs (DT) and fallen leaves (FL) of apple trees cv. Gala during winter (June to August) of 2010 and 2011 cropping seasons. Commercial orchard in Campo Largo Municipality, Paraná State, Brazil.

Survival of *Colletotrichum* spp. on asymptomatic leaves during the growing season

No *Colletotrichum* spp. colonies were obtained from asymptomatic leaves during the growing season (September to March). The fact that *Colletotrichum* spp. conidia were absent from asymptomatic leaves, show that *Colletotrichum* spp. did not survive epiphytically in orchards with fungicides sprays. This is in contradiction with the observations of Agrios (2005) with *C. acutatum* on pepper, eggplant and tomato, where the pathogen survived epiphytically without invading the plant for more than 12 months.

Identification of *Colletotrichum* spp. colonies

Colletotrichum spp. conidia were observed in very low frequency under the microscope in all of the suspensions during the 2 years of study. When suspension aliquots were streaked out on semi-selective medium, *Colletotrichum* spp. colonies were observed 7 days after inoculation. The number of colonies depended on the season and substrate.

Eight isolates were obtained in 2010 and 60 in 2011. The isolates recovered in 2010 were obtained in August from dormant buds, and in 2011 all isolates were obtained in June. Fallen leaves yielded the largest number of isolates (Freeman *et al*., 2002), followed by isolates from twigs (Hamada and May-De-Mio, 2013) and from dormant buds (Katsurayama *et al*., 2000b) (Figure 1). No *Colletotrichum* isolates were recovered from soil samples.

The cultural and morphological characteristics of isolates were variable and the identification was confirmed by molecular analysis (Table 2). Isolates were identified as *C. gloeosporioides* and *C. acutatum* species complex. Only isolates of *C. gloeosporioides* complex were detected in dormant buds, while isolates of *C. acutatum* complex were detected in dormant buds, dormant twigs and fallen leaves.

Pathogenicity of isolates

Colletotrichum spp. isolates from dormant buds were pathogenic on fruit and leaves of apple cv. Gala, regardless of year sampled. The isolates collected in 2010 did not induce symptoms on fruit without wound; 75% of isolates caused symptoms of bitter rot (BR) when inoculated on injured fruit. The isolates recovered in 2011 showed a higher frequency of pathogenic isolates, as 92.8% of the isolates induced symptoms of BR on injured fruit; some of the 2011 isolates

Table 2. Morpho-physiological characterization and molecular identification of isolates of *Colletotrichum* spp. obtained during the dormant period of plants (June to August) from dormant buds, dormant twigs and fallen leaves on the projection of plant canopies. Winter of 2010 and 2011, Campo Largo municipality, Paraná, Brazil.

Description		Morpho-physiological characterization							
Years	Isolates	Conidia		Perithecium formation[a]	Colony color[b]		Sectors	Mycelium growth[b]	Molecular identification[c]
		Width (µm)	Leigh (µm)		Verse	Reverse			
Dormant buds									
2010	48	8.9 (7.5–12.5)	4.8 (2.5–7.5)	No	White	White	No	2.85	C.g.
2010	50	8.7 (5.0–12.5)	4.0 (2.5–7.5)	No	White	White	No	5.25	C.g.
2010	51	8.8 (7.5–10.0)	5.5 (2.5–7.5)	No	White	White	No	5.23	C.g.
2010	55	11.0 (7.5–15.0)	6.7 (2.5–10.0)	No	White	Grey	No	4.95	C.g.
2011	128	11.1 (10.0–12.5)	4.7 (2.5–5.0)	Yes	White	Orange	No	2.50	C.g.
2011	129	14.2 (10.0–17.5)	6.5 (2.5–10.0)	No	White	Orange	No	5.70	C.a.
2011	131	9.2 (7.5–12.5)	4.6 (2.5–5.0)	Yes	White	Orange	No	3.32	C.a.
2011	132	8.8 (7.5–12.5)	4.2 (2.5–5.0)	Yes	White	Orange	Yes	6.05	C.a.
Dormant twigs									
2011	156	11.7 (7.5–20.0)	6.8 (2.5–12.5)	Yes	White	Orange	No	2.76	C.a.
2011	157	10.3 (5.0–17.5)	6.3 (2.5–10.0)	Yes	White	Orange	No	3.42	C.a.
2011	161	14.3 (12.5–17.5)	8.2 (5.0–12.5)	Yes	White	Green	No	2.75	C.a.
2011	162	14.4 (10.0–22.5)	8.7 (5.0–12.5)	Yes	White	Grey	No	2.95	C.a.
2011	164	14.0 (10.0–20.0)	7.1 (5.0–10.0)	Yes	White	Grey	No	3.95	C.a.
2011	165	7.1 (5.0–10.0)	4.2 (2.5–5.0)	Yes	White	Grey	No	3.43	C.a.
2011	166	9.7 (7.5–12.5)	4.7 (2.5–7.5)	Yes	White	Grey	No	3.50	C.a.
2011	167	12.7 (7.5–17.5)	5.2 (5.0–10.0)	Yes	White	Grey	No	3.27	C.a.
2011	168	10.0 (5.0–12.5)	5.1 (2.5–7.5)	Yes	White	Orange	No	3.65	C.a.
2011	169	9.5 (7.5–12.5)	5.5 (5.0–7.5)	Yes	White	White	No	4.57	C.a.
2011	171	9.9 (7.5–12.5)	5.9 (5.0–7.5)	Yes	White	White	No	3.65	C.a.
Fallen leaves									
2011	116	18.6 (10.0–22.5)	9.0 (5.0–12.5)	Yes	White	White	No	4.43	C.a.
2011	117	14.1 (12.5–17.5)	6.0 (5.0–7.5)	Yes	Grey	Orange	Yes	6.07	C.a.
2011	119	13.9 (7.5–22.5)	8.8 (5.0–10.0)	Yes	White	Orange	No	5.90	C.a.
2011	141	16.6 (10.0–20.0)	5.8 (2.5–10.0)	Yes	Grey	Grey	No	5.18	C.a.
2011	142	12.5 (7.5–17.5)	7.3 (2.5–12.5)	Yes	White	Orange	No	5.70	C.a.

[a] Evaluated 30 days after inoculation on petri dishes with PDA.

[b] Evaluated seven days after inoculation on petri dishes with PDA.

[c] C.g. = *Colletotrichum* of the *gloeosporioides* complex; C.a. = *Colletotrichum* of the *acutatum* complex (Damm *et al*., 2012).

Table 3. Pathogenicity test to apple fruit (with and without injury) and apple leaves (without injury) cv. Gala, of the isolates of *Colletotrichum* spp. obtained during the dormant period of plants (June to August) from dormant buds, dormant twigs and fallen leaves on the projection of plant canopies. Winters of 2010 and 2011, Campo Largo municipality, Paraná, Brazil.

Years	Apple organs for pathogenicity test	Dormant buds			Dormant twigs			Fallen leaves		
		Number of isolates[a]	%		Number of isolates[a]	%		Number of isolates[a]	%	
			With Injury	Without injury		With Injury	Without injury		With Injury	Without injury
2010	Fruit	8	75.0	0	0	na	na	0	na	na
	Leaf	7	na[b]	0	0	na	na	0	na	na
2011	Fruit	14	92.8	42.9	22	100.0	63.6	24	88.9	11.1
	Leaf	7	na	71.4	5	na	100.0	11	na	100.0

[a] All isolates obtained from the survival assays were used for fruit inoculation, while isolates used for leaf inoculation were those isolates which had better sporulation.
[b] na: not assessed.

(42.9%) were also capable of causing BR in fruit without a wound. Also, the majority of isolates (71.4%) were pathogenic on inoculated leaves, causing typical symptoms of GLS (Table 3).

A comparison of pathogenicity of isolates from dormant twigs and fallen leaves, between years was not possible because no isolates were obtained from these parts in 2010. The 2011 isolates from dormant twigs showed the highest frequency of pathogenic individuals, as compared with those from dormant buds and fallen leaves on the ground of the same year. When inoculated on fruit with and without a wound, the 2011 dormant twig isolates caused symptoms of BR (100 and 63.6%, respectively), and when inoculated on leaves, they caused typical symptoms of GLS (100%) (Table 3).

Isolates from fallen leaves were pathogenic to fruits with and without wound causing symptoms of BR (88.9% with and 11.1% without wound). When these isolates were inoculated on leaves, 100% caused typical symptoms of GLS (Table 3).

Differences in pathogenicity among isolates from dormant buds collected in different years may occur because there is more than one species involved in the pathosystem. The morphological and cultural differences observed among isolates calls for a study to detect differences within isolates of the same complex. In Brazil, *C. gloeosporioides* and *C. acutatum* species are most frequently associated with GLS (Katsurayama *et al*., 2000b). In other countries including New Zealand (Johnston and Manning, 2005), Korea (Lee *et al*., 2007) and the United States (González *et al*., 2006), *C. acutatum* and *C. gloeosporioides* were reported to be pathogenic to apple, and were always associated with the occurrence of bitter rot on apple. More recently, Bragança (2013) detected five species associated with GLS, including *C. nymphae*, *Colletotrichum* sp., *C. fructicola*, *C. melonis* and *C. siamense*.

Isolates from *C. acutatum* species complex associated with fallen leaves on the ground were pathogenic to fruit (with and without wound) and leaves of apple cv. Gala, where they caused typical symptoms of BR and GLS, respectively. This is important to consider for disease management, as decaying leaves are a major source of primary inoculum of other pathogens such as *C. dematium* in mulberry (Yoshida and Shirata, 1999) and *Venturia* spp. in apple (Köhl *et al*., 2009; Caffier *et al*., 2012). Thus, use of measures to accelerate the decomposition of fallen leaves, may contribute significantly to the reduction of primary inoculum in orchards. These findings contradict a previous study (Crusius *et al*., 2002) that showed that fallen leaves left on the ground during winter were not a source of primary inoculum for *Colletotrichum* spp. causing GLS in the state of Rio Grande do Sul, Brazil.

Knowledge of the survival of the *C. acutatum* species complex in fallen leaves agrees with other data collected in the same orchard where most conidia of the pathogen were

Table 4. Accumulated precipitations (Pp in mm), number of days with rain (DR), number of consecutive days with rainfall (CDR), maximum, minimum and average temperatures (°C) and relative humidity (RH) recorded in Campo Largo municipality, Parana State, Brazil. Monthly data of the years 2010 and 2011.

Years	Months	Pp (mm)	DR	CDR	Temperatures (°C)			RH (%)
					Maximum	Minimum	Average	
2010	Mar	280.0	6	2–2	26.3	16.6	20.5	84.0
	Apr	180.0	5	4	23.7	13.3	17.8	83.0
	May	115.0	4	3	20.7	11.3	15.2	86.0
	Jun	60.0	3	2	20.3	9.5	14.1	82.0
	Jul	106.0	5	2	21.2	10.7	15.1	81.6
	Aug	32.0	2	2	21.4	8.6	14.2	75.7
2011	Mar	57.0	4	3	24.0	15.8	18.9	87.8
	Apr	0.0	0	0	24.2	14.8	18.7	83.3
	May	30.0	1	0	21.0	10.9	15.0	82.6
	Jun	145.0	4	2–2	20.1	7.7	12.9	80.1
	Jul	125.0	4	2	20.4	9.8	14.3	82.4
	Aug	309.0	6	2	20.7	10.0	14.6	79.0

captured near the lower parts of the plants (0.3 m from the ground) (Hamada and May-De-Mio, 2013). This indicates that the conidia trapped at lower heights mainly originate from decaying leaves, since the pathogen forms perithecia during winter and disseminate ascospores in the beginning of the vegetative stage.

Although *Colletotrichum* spp. are known to survive in soil in some pathosystems including anthracnose on pepper (Kang *et al*., 2009), this was not the case for GLS based on the methodology used in our study. This observation is reinforced by several authors who reported that the soil is not an inoculum source of *Colletotrichum* spp., due to the inability of the pathogen to survive in the soil environment (Ekefan *et al*., 2000; Freeman *et al*., 2002; Ripoche *et al*., 2008).

The variation of the number of isolates obtained in the sampled years was probably due to the timing of fungicide applications (Table 1) and the environmental conditions, especially the amount, intensity and frequency of rainfall (Table 4).The greater frequency of *C. acutatum vs. C. gloeosporioides* isolates complex in Paraná State has also been observed in recent surveys. In some orchards fungicides have been applied until the end of fruit harvest. Our results show that spraying fungicides shall be extended until natural leaf fall in order to reduce the initial inoculum in the orchard responsible for beginning the epidemic in the following season.

Treatment of plants with copper products, including copper hydroxide and copper sulfate, has been shown to reduce the amount of inoculum. The occurrence of *Colletotrichum* spp. in orchards during dormancy was previously reported in Rio Grande do Sul State (Crusius *et al*., 2002). Crusius *et al.* (2002) found that spraying with copper oxychloride reduced the amount of inoculum present in dormant buds by up to 94.1%, but did not eliminate it. An application of Bordeaux mixture in May and additional fungicide sprays in June are recommended to reduce the amount of inoculum in the orchard during the following autumn and winter.

Incidence of weather parameters

The cumulative rainfall amounts during the period from March to August were 773 mm in 2010 and 666 mm in 2011. The year 2010 presented the highest number of consecutive days with rain. The maximum temperature differences between hottest and coldest months each year during the study period were similar, with 6.4 and 6.0 °C in 2010 and 2011, respectively. The lowest relative humidity (RH) was 75.7 and 79.0% in 2010 and 2011, respectively, and the highest RH was 86.0 and 87.8%, respectively (Table 4).

We observed that the total amount of rainfall was less determining for GLS development than the frequency and duration of rains. Continuous rain over several days increased the survival of *Colletotrichum* spp. in winter. This has been similarly reported with *C. acutatum* on strawberry (Freeman, 2008), and with *C. kahawae* in coffee (Bedimo *et al*., 2010), where the greatest number of *Colletotrichum* spp. conidia occurred during the rainy season. In Brazil it was also observed that abundant continuous rainfall resulted in greater incidence of *Glomerella cingulata* in flower buds of dormant apple trees cv. Willie Sharp (Bernardi *et al*., 1983). This secondary, asymptomatic production of conidia is an important survival mechanism, similar to what was observed in *C. acutatum* on strawberry (Leandro *et al*., 2001).

Conclusion

Colletotrichum acutatum and *C. gloeosporioides* species complex that causes Glomerella leaf spot (GLS) disease was able to survive in dormant buds and dormant twigs of apple trees and was associated with fallen leaves on the ground. The pathogen was not detected in the soil underneath the tree canopy. Isolates obtained from all sources were pathogenic to apple trees cv. Gala leaves and fruit, with and without wound.

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