





Colletotrichum acutatum complex isolated from apple flowers can cause bitter rot and *Glomerella* leaf spot

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ABSTRACT: The *Glomerella* leaf spot (GLS) and bitter rot (BR) are important apple diseases in Brazil, caused by species of *Colletotrichum*, which are usually related with quiescent infection. This study aimed to detect quiescent infections of *Colletotrichum* spp. in flowers and in unripe fruits from apple commercial orchard cultivars Eva and Gala. The pathogen was observed in various structures of the flower in both cultivars. In unripe fruits, the pathogen was recovered only from 'Eva'. Five isolates were obtained and were inoculated by mycelial (with and without wound) on ripe apple fruits and by conidia suspension (without wound) on ripe apple fruits and leaves of 'Gala'. On inoculated fruits by mycelial, the isolates induced symptoms of BR, and on inoculated fruits by conidia without wound the isolates induced symptoms of GLS. On inoculated leaves, isolates induced symptoms of GLS. The isolates were characterized by cultural, morphological and pathogenic aspects. Detection of *Colletotrichum* spp. in asymptomatic apple flowers is epidemiologically important, since the flowers can be a route for infection in unripe fruits. This is the first investigation of quiescent infection of this pathogen on apple flowers.

Key words: *Malus domestica*, epidemiology, quiescent infection.

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Colletotrichum species cause *Glomerella* leaf spot (GLS) and bitter rot (BR) on apples in Brazil. The GLS has caused high defoliation on plants of cultivar Gala and its clones, affecting the production of subsequent years, mainly in Southern Brazil. The cultivar Eva, cultivated in the state of Paraná since 1980s, although registered as resistant to GLS, showed symptoms typical of the disease in a field in years with high inoculum pressure (unpublished data). The disease was reported in Brazil (Leite Junior et al. 1988), USA (González and Sutton 1999), China (Wang et al. 2012), Canada (Grigg-McGuffin et al. 2014) and Uruguay (Casanova et al. 2017).

Symptoms on orchard are observed in leaves and fruits, and the infection is favored by prolonged leaf wetness and temperatures above 20 °C, although they can be observed from 12 °C (Katsurayama and Boneti 2010). The main symptoms are necrotic leaf spots of irregular size, with red/brown color, and can exceed 70% of leaf area (Moreira et al. 2019 a). The symptoms in fruits are round spots of brown color that usually do not progress to rot.

No data was found about pathogen infection on apple flowers, as well as studies about the identification of *Colletotrichum* species on flowers. The flower can be an important infection pathway, allowing the pathogen stay quiescent until the appearance of favorable conditions to their development and reproduction, as already reported in grape (Samuelian et al. 2012), strawberry (Debode et al. 2015) and sour cherry (Stensvand et al. 2017). *Colletotrichum* spp. can be quiescent in plants as appressoria or hyphae (Sinclair 1991) and the floral surfaces can shelter many plant pathogens (Pusey et al. 2009).

The knowledge of quiescent infections in the flower is important to adapt the disease management program, preventing future damages in the fruit production.

The aim of this study was to detect quiescent infections of *Colletotrichum* spp. in flowers and in unripe fruits from apple commercial orchard cultivars Eva and Gala.

Flowers from 'Gala' and 'Eva' were collected on commercial orchard at Campo Largo, Parana State (25°42'13.98"S and 49°54'61.43"W) before fungicide sprays, in August and September of 2011. In this orchard, the plants were conducted in the central leader with spacing of 4.0 × 1.0 m ('Gala') and 4.4 × 1.0 m ('Eva'), and were 13 years old.

Two methods were tested to confirm the presence of *Colletotrichum* infection: one of them was described by Luo et al. (2001) and the other was described by Mertely and Legard (2004). For both methods, 96 flowers of each cultivar were placed in gerbox (11 × 11 × 3.5 cm) with sterile filter paper moistened with sterile water, in six repetitions (16 flowers/gerbox). Following the method of Luo et al. (2001), the flowers were stored for three days at 25 °C, after transferred to a temperature of 4 °C during three days. According to Mertely and Legard (2004), flowers were submitted to temperatures of -15 to -20 °C in conventional freezer during 1 h, and after transferred to temperature of 25 ± 1 °C, in the dark, with 100% of humidity for 5 days of incubation.

The evaluations were performed with a stereomicroscope (20×) assessing the presence of necrosis and pathogen signs in the floral structures, confirming fungal morphology under an optical microscope (40×). The observed structures of *Colletotrichum* spp. were transferred to potato-dextrose-agar (PDA) and incubated at 25 ± 1 °C, in the dark. After seven days, the colonies were identified according to their cultural and morphological characteristics (Boneti et al. 1999). Data of flower infection were transformed in percentage of average incidence relative to 96 flowers per cultivar.

Unripe fruits of 'Gala' and 'Eva' (100 fruits per cultivar), were collected three weeks after the onset of plant fruiting, at the same commercial orchard (25°42'13.98"S and 49°54'61.43"W). The fruits were submitted to the overnight freezing incubation technique (ONFIT) (Luo and Michaelides 2001), where the surface disinfection was performed by the immersion of fruits in a solution of distilled water (2 L), sodium hypochlorite 0.525% (32 mL), ethanol 92.8% (32 mL) and Tween 80 (1 drop), followed by shaking for 5 min. The fruits were washed in distilled sterile water and dried on sterile paper. After that, they were placed in a freezer (-20 °C) for 15 h and then individually incubated at 25 °C in a moist chamber. The evaluation was daily during 10 days, observing the occurrence of necrosis and/or signals of *Colletotrichum* spp.

Five isolates obtained from flower quiescent infections (MdCaPR11-01, MdCaPR11-02, MdCaPR11-03, MdCaPR11-04, MdCaPR11-05) were evaluated according their colony characteristics at culture medium (PDA): daily mycelial diameter at different temperatures, size and shape of conidia, color above and below the Petri dish, and sectors formation, i.e., specific areas of mycelial growth changing color on a colony. These isolates were previously identified by Moreira et al. (2019 b) belonging to the *C. acutatum* complex, using molecular analyzes. Four isolates are *C. nymphaeae* (MdCaPR11-01=177, MdCaPR11-02=178, MdCaPR11-03=179, MdCaPR11-05=181) and one isolate is *C. limetticola* (MdCaPR11-04=180). The numbering in bold corresponds to the nomenclature used by Moreira et al. (2019 b).

For each isolate, mycelial plugs (3 mm) from eight-day-old colonies were transferred to the center of Petri dishes containing PDA. The isolates were incubated at temperatures of 6, 13, 18, 25, 29 and 33 ± 1 °C, in the dark. The experimental design was completely randomized, three replicates per treatment. The evaluation was performed daily for 14 days by measuring perpendicular diameters of the colonies. The optimum temperatures for mycelium growth of each isolate were also determined. Temperature data were adjusted to the generalized beta function (Bassanezzi et al. 1998). The adjustment was carried out with the R statistical software (R Development Core Team 2010).

The conidia morphology was evaluated preparing conidial suspensions from monosporic cultures grown in PDA and maintained for 15 days at 25 ± 1 °C, in the dark. The conidia evaluated characteristics were length (CC), width (LC), shape and CC/LC correlation. Twenty-five conidia per isolate were evaluated on optical microscope with a 40× objective.

To evaluate the colonies cultural characteristics, mycelial plugs (6 mm) of eight-day-old colonies were transferred to the center of Petri dishes containing PDA. The plates were maintained in the dark at 25 ± 1 °C during seven days. The experimental design was completely randomized, three replicates per isolate. The evaluations were performed daily for colony color (above and below), presence of spore mass and presence of sectors.

To verify the pathogenicity on leaves, four-months old apple seedlings of 'Gala' were used. Conidial suspension was obtained for colonies of 10 days old cultivated on PDA medium and the concentration was adjusted to 1.0×10^4 conidia·mL⁻¹ in sterile water. Leaves inoculation (without wound) was made by spraying the conidial suspension with manual sprayer (Wilbes, capacity: 340 mL; approximate flow rate: 10 mL·min⁻¹), until complete coverage of leaf surfaces. After inoculation the plants were maintained in a moist chamber (25 °C and 100% RH) during 48 h, and then transferred to the greenhouse. The control was sprayed with sterile and distilled water and maintained under the same conditions. Each isolate was inoculated on 20 leaves separated on two plants (10 leaves per replication). The evaluation was daily until 21 days after inoculation. The disease incidence was calculated in percentage and determined according to the number of leaves with lesion, relative to the total number of leaves.

The pathogenicity on fruits was tested by the inoculation of mycelial and conidia suspension. Inoculation of mycelial was made with plugs of 3 mm of diameter, placed on fruits with and without wound ('Gala'). Each wound was made with a needle (1 cm depth). Four fruits per treatment were inoculated, which were maintained in moist chamber at 25 °C and the lesion diameter measured daily, during 16 days after inoculation (DAI) for wounded fruits and during 21 DAI for unwounded fruits. The incubation period (time for onset of symptoms in 50% of the evaluated fruits) and the latent period (time for lesion sporulation in 50% of the evaluated fruits) of each isolate was quantified.

Inoculation with conidia suspension was performed at the same way and concentration described to leaves, spraying 10 mL of suspension/fruit ('Gala') unwound, four fruits per isolate. Fruits were maintained in moist chamber at 25 °C. The type of symptom was evaluated daily during 15 days.

The methods tested were effective to *Colletotrichum* spp. detection in apple flowers (Fig. 1A), with significant differences in the frequency of infected flowers. There was no interaction between methods and cultivars. The 'Eva' apple flowers

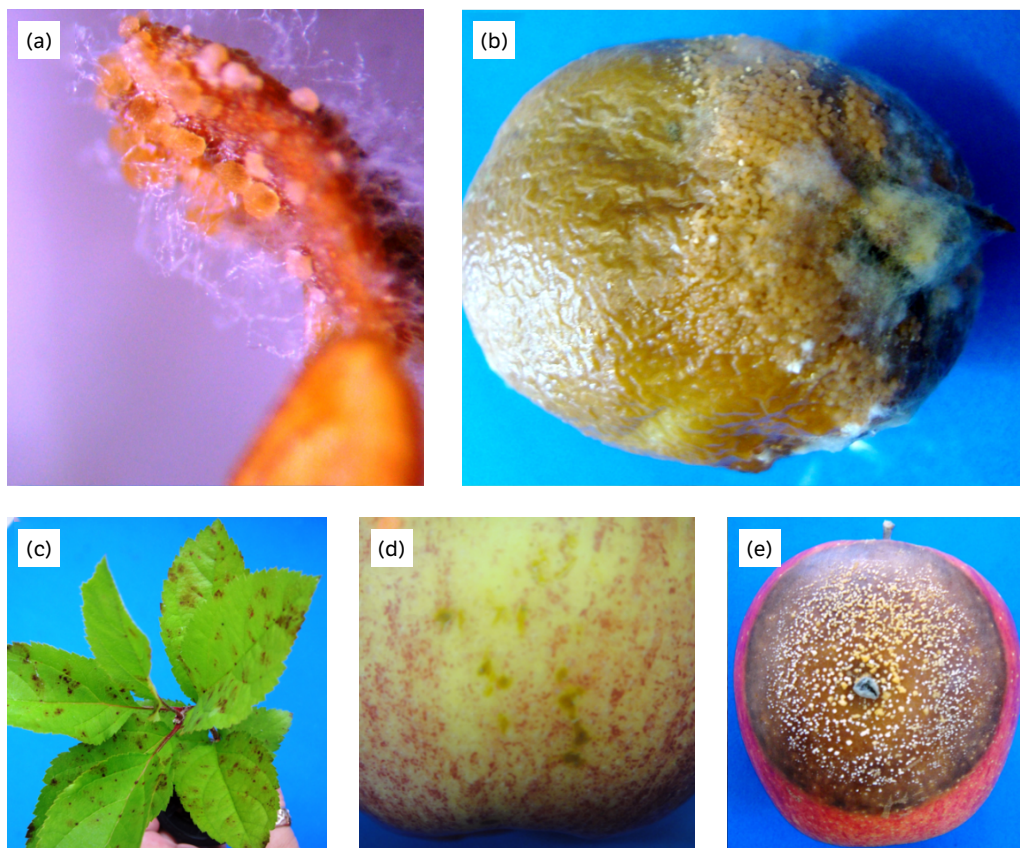


Figure 1. Detection of quiescent *Colletotrichum* spp. in the anther of apple flowers (a) and in apple fruit (b); Glomerella leaf spot symptoms reproduced in unwounded 'Gala' apple leaves (c) and in unwounded 'Gala' apple fruit; and (d) bitter rot symptom reproduced in wounded 'Gala' apple fruit (e). Apple leaves and fruit (C-E) were inoculated by *Colletotrichum nymphaeae* (MdCaPR11-02).

showed higher number of infected flowers to those observed in 'Gala', regardless of the methodology used, and the difference through cultivars was significant.

In 'Gala' apple flowers, the methodology of Luo et al. (2001) allowed pathogen detection in 3.13% of flowers, and, among these, 66.7% were in anthers and 33.3% in petals. The methodology of Mertely and Legard (2004) allowed the highest percentage of pathogen detection (12.5%) on flowers and the pathogen was observed in petals (66.7%), petiole (16.7%) anthers (8.3%) and both petals and petioles (8.3%).

In 'Eva' flowers, the methodology of Luo et al. (2001) allowed pathogen detection in 22.9% of flowers, at petals (63.6%), anthers (22.7%), stamens (4.6%) and both petals and anthers (9.1%). The methodology of Mertely and Legard (2004) allowed the highest percentage of pathogen detection (41.7%) on flowers and the pathogen was observed in petals (55.0%), anthers (12.5%), stamens (7.5%), sepals (5.0%), both petals and stamens (2.5%), both petiole and stamens (2.5%) and both petals and petiole (5.0%). The higher percentage in 'Eva' was probably due the less fungicides use at early blooming and during the previous season.

The methodology of Mertely and Legard (2004) showed to be more efficient in the detection of the pathogen on apple flowers, possibly for promoting greater tissue deterioration due to exposure of the negative temperatures (-15 to -20 °C) favoring the colonization by fungi present in a quiescent state.

The appearance of isolates of *C. acutatum* complex on different parts of the flower can be explained by the fact that this genus is not specific to particular floral tissue (Ngugi and Scherm 2006). Apple flowers may be infected also by other major pathogens of the crop, for example *Venturia inaequalis* (Becker et al. 1992).

The report of *C. acutatum* complex in apple flowers is unprecedented in Brazil, since to date the pathogen related to GLS was found in symptomatic leaves (Leite Junior et al. 1988), dormant twigs and buds of apple (Crusius et al. 2002; Hamada and May de Mio 2017), and fallen leaves on the ground during the dormancy period of the plants (Hamada and May de Mio 2017).

Detection of *Colletotrichum* spp. in asymptomatic apple flowers is epidemiologically important, since the flowers can be a route for infection by allowing the pathogen to remain dormant until the occurrence of favorable conditions for its development and reproduction, as occurs in cherry (Børve and Stensvand 2013) and peach crops (May de Mio et al. 2008). In apple, Andrews and Kenerley (1980) observed that other genera of fungi present in dormant buds may remain on flowers and new leaves.

The primary inoculum of GLS present in dormant buds of apple can remain on flowers after their opening without expressing symptoms, and consequently may be responsible for the symptoms observed in the mature fruit. This idea corroborates Everett et al. (2018) who related that the causal agent of BR (*C. acutatum*) can survive asymptotically in apple flowers in orchards in New Zealand.

'Gala' apple fruits did not show symptoms or signals of *Colletotrichum* spp. until the tenth day of evaluation. 'Eva' apple fruits showed pathogen sporulation (Fig. 1B) from fourth DAI (8%), and in the tenth DAI 25% of fruits showed abundant sporulation of *Colletotrichum* spp., mainly in the opposite region of pedicel.

The observation of *Colletotrichum* spp. in unripe fruits of 'Eva' strengthens the hypothesis that the primary inoculum can come from flowers. In addition, the quiescent infection in stigma was also detected only in 'Eva'. Failure in inoculum finding in unripe fruit of 'Gala' probably happened due to the fungicide spray carried out at the time of flowering to control GLS. In the case of 'Eva', flowers have not been subjected to fungicides treatments once it is an early cultivar and the blooming is, on average, 2 to 3 weeks before 'Gala'. The results of this work indicate the importance of reviewing the spraying program in apple orchards.

Detection of quiescent infection of *Colletotrichum* spp. in fruits has been reported by Biggs (1995) on apples ('Golden Delicious'), by Weber et al. (2015) on strawberries, and several other pathosystems. Infection of *Colletotrichum* species at different stages of apple fruit growth has not been studied yet.

The optimum growth temperature varied from 24.7 to 28.0 °C. According to the adjustment of beta generalized function, the maximum and minimum temperatures for growth of the isolates were 4.0 and 33.0 °C, respectively (Table 1).

Colletotrichum isolates showed little color variation between them and between the tested temperatures, all of which showed gray color (front) and olive gray (back), with abundant sporulation, which gave orange color to the colony center. None of the colonies showed the formation of sectors. However, the size of the conidia varied widely among isolates, with

Table 1. Optimum temperature for mycelium growth, pathogenicity, type of symptom, incubation period and days for sporulation (after inoculation) of *Colletotrichum acutatum* complex isolates from quiescent apple flower infections, on leaves (unwounded) and fruit (wounded and unwounded). Leaves inoculated by spraying suspension (1.0×10^4 conidia ml⁻¹) and fruits inoculated by mycelial disk.

Isolate*	Optimum temperature for mycelium growth (°C) ¹	Leaves			Wounded Gala fruit				Unwounded Gala fruit			
		Pathogenicity ²	Symptoms	IP ⁴	Pathogenicity ²	Symptoms	IP	LP ⁵	Pathogenicity ²	Symptoms	IP	LP
				(DAI) ⁶			(DAI)	(DAI)				
MdCaPR11-01	25.3	-	-	-	+	BR ⁷	4	8	-	-	-	-
MdCaPR11-02	24.7	+	GLS ³	3	+	BR	4	9	+	BR	14	16
MdCaPR11-03	27	+	GLS	3	+	BR	4	7	-	-	-	-
MdCaPR11-04	26.7	+	GLS	3	+	BR	4	-	-	-	-	-
MdCaPR11-05	28	+	GLS	5	+	BR	4	7	+	BR	15	16

*Four isolates are *C. nymphaeae* (MdCaPR11-01=**177**, MdCaPR11-02=**178**, MdCaPR11-03=**179**, MdCaPR11-05=**181**) and one isolate is *C. limeticola* (MdCaPR11-04=**180**). The numbering in bold corresponds to the nomenclature used by Moreira et al. (2019 b). ¹Adjustments on the generalized beta distribution according to Bassanezzi et al. (1998). ²Pathogenicity evaluated by the absence (-) or presence (+) of symptoms in inoculated plants or fruits. ³GLS = Glomerella leaf spot. ⁴IP = incubation period. ⁵LP = latent period. ⁶DAI = days after inoculation. ⁷BR = Bitter rot.

average length between 8.0 and 16.0 µm and average width ranging between 4.0 and 7.0 µm. Conidia morphology was similar to those described in the *C. acutatum* species complex, with both ends slightly acute.

The morphological and cultural characteristics evaluated are similar to those described for the *C. acutatum* complex, although the measure of conidia was not exactly the originally described by Simmonds (1965) for the species. This may be caused by environmental conditions that may interfere with the stability of the morphological characteristics (Freeman et al. 1998). The morphological and cultural characterization of the present study confirms the molecular characterization made by Moreira et al. (2019 b).

Typical symptoms of GLS (Fig. 1C) was caused by four isolates inoculated in 'Gala' apple leaves (Table 1). The average incidence (16 DAI) varied being 44 to 50%.

In the inoculation of wounded fruits with mycelial disk, all isolates were pathogenic and resulted in typical symptoms of BR (Fig. 1E); four isolates showed abundant sporulation on the lesion. The onset of symptoms occurred four DAI (incubation period). The onset of sporulation in wounded fruits was observed between seven and nine DAI (Table 1).

When inoculated on unwounded fruits (also with mycelium disk) two isolates were pathogenic, with the onset of symptoms 14 to 15 DAI. This caused typical symptoms of BR with sporulation on the lesions 16 DAI (Table 1). All isolates, when inoculated on fruits by spraying (unwounded), were pathogenic and caused typical symptoms of GLS (Fig. 1D) and the incubation period was 15 DAI.

Symptoms caused on unwounded leaves and fruits when inoculated by spraying were GLS typical symptoms, differing in time necessary for the expression of symptoms, that was higher in fruits. The biggest period necessary for the expression of symptoms due to the differences in the constitution of the leaves and peel of the fruit. According to Smith et al. (2006) the composition of the cuticle, particularly of the waxes, is specific to each species and plant organ and has directly influence in the germination of spores and the formation of appressoria. Moreira (2017)³ observed, through scanning electron microscopy, that spores of five species of *Colletotrichum* germinate in wounded apple fruits after 24 h but do not germinate on the intact surface of apple fruits, probably due to the wax layer.

When inoculated in fruits through the mycelium, isolates caused typical BR symptoms with abundant sporulation on the lesions. The different types of symptoms caused by isolates of *Colletotrichum* spp. on apples are not completely

³ Moreira, R. R. (2017). Etiologia da mancha foliar de Glomerella em macieira e sensibilidade do patógeno a fungicidas. 118p. (Thesis). Universidade Federal do Paraná, Curitiba. <https://acervodigital.ufpr.br/handle/1884/63651>

understood. One hypothesis for the occurrence of various symptoms caused by species of *C. acutatum* complex on apples is the concentration of conidia or ascospores, plant stress, specie of the pathogen species or cultivar characteristics can influence directly the type of symptom; however, studies need to be developed to prove these hypotheses.

Moreira et al. (2019 b) reported that *C. limeticola* from symptomatic leaves are pathogenic to apple leaves causing typical GLS symptoms. In the present study, *C. limeticola* (MdCaPR11-04) from flower was also pathogenic to apple leaves and it was still pathogenic to wounded apple fruits (Table 1). This is the first report of *C. limeticola* causing symptoms in apple fruit. However, no sporulation of this pathogen was observed in the fruit during the evaluation period. Therefore, epidemiological studies of this species should be the subject of other studies and surveys. The species most often found in flowers in this study was *C. nymphaeae*. This species was most frequently found in the states of Paraná and Rio Grande do Sul according to a survey by Moreira et al. (2019 b), in contrast to the state of Santa Catarina which predominantly has the species *C. fruticola* which belongs to the *C. gloeosporioides* complex.

According to the data of this study, *C. acutatum* complex cause quiescent infection in 'Gala' and 'Eva' flowers, and in unripe 'Eva' fruits. Flower infection monitoring is important to manage GLS, since it can be a route for infection unripe fruit.

CONCLUSION

Quiescent apple flower infection can be a route for future fruit infections. *Colletotrichum* spp. was detected in immature fruits of 'Eva' and isolates of *C. acutatum* complex obtained from apple flowers of 'Eva' and 'Gala' were pathogenic in fruits causing symptoms of bitter rot and Glomerella leaf spot and also pathogenic in leaves causing symptoms of Glomerella leaf spot.

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