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Microscopic analysis of colonization of *Colletotrichum abscissum* in citrus tissues

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ABSTRACT

Postbloom fruit drop (PFD), caused mainly by *Colletotrichum abscissum*, is one of the most severe citrus diseases and can causes up to 80% fruit loss in favorable climatic conditions. According to the literature, other *Colletotrichum* species colonize hosts using distinct strategies: intracellular hemibiotrophic or subcuticular intramural necrotrophic colonization. However, so far, for *C. abscissum* only the necrotrophic stage has been described and some aspects remain unclear in PFD disease cycle. To better understand the disease cycle, microscopy studies could be applied. However, even using eGFP strains (expressing green fluorescent protein), the results are unclear due to the autofluorescence of citrus leaves. To eliminate this problem and to study the interaction between *C. abscissum*-citrus we used a destaining and staining methodologies, and we observed that in leaves, even applying injury before inoculation, *C. abscissum* does not colonize adjacent tissues. Apparently, in the leaves the fungus only uses the nutrients exposed in the artificial lesions for growth, and then produces large amount of spores. However, in flowers, *C. abscissum* penetrated and colonized the tissues of the petals 12 h after inoculation. In the early stages of infection, we observed the development of primary biotrophic hyphae, suggesting this species as a hemibiotrophic fungus, with a short biotrophic phase during flower colonization followed by dominant necrotrophic colonization. In conclusion, the use of an eGFP strain of *C. abscissum* and a different methodology of destaining and staining allowed a better understanding of the morphology and mechanisms used by this citrus pathogen to colonize the host.

1. Introduction

Postbloom fruit drop (PFD) is one of the most severe diseases of citrus in Brazil and is characterized by reddish brown lesions on the petals and necrotic lesions on stigmas that result in premature fruit drop and retention of calyces (Feichtenberger et al., 2005; Marques et al., 2012; de Menezes et al., 2014; Silva-Junior et al., 2014). According to Goes et al., 2008, up to 80% of fruit losses can occur under favorable climatic conditions that include frequent rainy days during the bloom period. Although several species of the genus *Colletotrichum* are associated with citrus diseases (Guarnaccia et al., 2017), PFD disease is mainly caused by *Colletotrichum abscissum* (Pinho et al., 2015; Silva et al., 2017), and in low frequency by *Colletotrichum gloeosporioides sensu stricto* (Lima et al., 2011; Silva et al., 2017).

According to the literature, *Colletotrichum* species can colonize host plants using distinct strategies: intracellular hemibiotrophic or subcuticular intramural necrotrophic colonization (Bailey et al. 1992; Peres et al. 2005). In the hemibiotrophic lifestyle, the pathogen presents a short biotrophic phase (without lesions), followed by a necrotrophic phase and the development of lesions. On the other hand, in subcuticular intramural necrotrophic colonization, the fungus grows on the cuticle of the tissue and the wall of the epidermal and adjacent cells without penetration (Marques et al., 2012). However, in a study on the colonization of *C. acutatum* (currently *C. abscissum*) in petal tissues, Marques et al (2012) did not observe biotrophic colonization and suggested that this pathogen presents only the necrotrophic stage in

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citrus petals.

C. abscissum shows distinct development and penetration, even in the same host tissue, as reported by Zulfiqar et al. (1996) who observed that the conidia germinate on the petals and penetrate directly through the cuticle, without forming appressorium. On the other hand, Marques et al. (2012) reported that host penetration by the pathogen occurs more frequently after the appressorium formation. For the control of fungal diseases, an important step is to determinate the disease cycle, especially the molecular mechanisms of pathogen-host interaction, the strategy used by the fungus for dissemination, host colonization and pathogenicity (Braga et al., 2016). However, for *C. abscissum* there are some gaps in pathogenicity aspects, and some issues remain unclear in the disease cycle. The several outbreaks of PFD in areas where there was no prior history of the disease (Timmer and Zitko, 1993; Timmer et al., 1994), associated with the rapid spread of PFD, suggest a presence of pre-existing inoculum homogeneously distributed throughout the field, acting on the dissemination of this disease (Jelev et al., 2008).

The PFD cycle consists of the production of conidia in infected flowers, which are splashed on surrounding leaves and twigs and on the floral buds that remain after the blossom period (Agostini et al., 1992). Some reports suggest that *C. acutatum* (currently *C. abscissum*) can survive in citrus trees by producing appressoria, that remain attached to the surface of citrus leaves between flowering periods (Agostini and Timmer, 1994; Zulfiqar et al., 1996). Andrade et al. (2016) investigated the presence of *C. abscissum* and *C. gloeosporioides sensu stricto* as endophytes in citrus and weeds commonly found in citrus orchards (*Commelina benghalensis*, *Sida rhombifolia* and *Amaranthus deflexus*). The authors demonstrated that *C. gloeosporioides sensu stricto* is associated with citrus and weeds in the State of São Paulo (Brazil), but *C. abscissum* was not isolated as an endophyte from any of the plants investigated. However, another study using artificial inoculation of *C. abscissum* on the surface of weed leaves suggests that this species can colonize these plants, and may represent a source of inoculum (Frare et al., 2016).

In this scenario, it is still unclear whether *C. abscissum* is present in the endophytic form or as a latent pathogen in citrus leaves between bloom periods or even in weeds, and the source of the initial inoculum of *C. abscissum* remains unknown. It is also unclear whether *C. abscissum* colonizes citrus flowers using hemibiotrophic or necrotrophic lifestyles. To investigate the ability of *C. abscissum* to colonize citrus leaves and flowers, microscopy studies could be applied. However, even using eGFP strains (expressing green fluorescent protein), the images are not clear due to the autofluorescence of citrus leaves (Roshchina, 2012; Etxeberria et al., 2016).

To better understand the mechanisms of colonization and pathogenicity of *C. abscissum* in the citrus host, we used different methodologies, including leaf-clearing and immunolabbeling to eliminate the problems previously observed in the microscopic analysis of citrus phytopathogens.

2. Material and methods

2.1. Colletotrichum abscissum isolates

The strain Ca142 of *Colletotrichum abscissum* was isolated from PFD symptomatic flowers and used as a pathogenic strain in experiments in Brazil (de Menezes et al., 2014; Goulin et al., 2019). Protoplasts of strain Ca142 were transformed with the plasmid pIG1783 encoding different fluorescent proteins, according to Werner et al. (2007). A strain expressing eGFP (Ca142egfp1) was selected and used in the following experiments.

2.2. Fluorescent microscopy analyses

2.2.1. Inoculation of Ca142egfp1 on flowers and leaves of Citrus sinensis

The analysis of the infection process was carried out using detached flowers collected from citrus plants without PFD symptoms, located at the Federal University of Paraná, Curitiba, Paraná, Brazil, and attached leaves of two-year-old *C. sinensis* plants obtained from the germplasm collection of the Sylvio Moreira Citrus Center, Cordeirópolis, São Paulo, Brazil. Each citrus tissue (petal or leaf) received a 10 µL inoculum droplet of the 10^5 spores/mL suspension. The inoculated flowers were deposited in clear plastic boxes (volume of 500 ml, r = 5.5 cm, h = 8 cm) containing water-agar medium at room temperature according to Agostini et al. (1992) and Goulin et al. (2019) using a plastic box. The petals were analyzed 8, 12, 24, 36 and 48 h after inoculation. The leaves experiments were conducted with plants under controlled greenhouse conditions up to 60 days after inoculation, the inoculation of the spore solution was carried out using leaves with and without injury caused with an insulin needle. The analysis of colonization in the leaves were performed at 5, 10, 15, 30 and 60 days after inoculation. The experiments were conducted in five biological repetitions. For the negative control, 10 µL drops of saline solution were inoculated on citrus tissues.

2.2.2. Analysis of inoculated citrus petals

The evaluation of development of *C. abscissum* in flowers was performed by the analysis of macro and microscopic characteristics. The macroscopic analysis consists of the symptoms of the PFD disease in petals. For microscopic analysis, the petal was cut with a scalpel in the site where the spores were inoculated, transferred to microscope slides with antifade mediumFluoromount-G(ThermoFisher, 00-4958-02) and covered with a cover slip.

2.2.3. Immunochemistry for inoculated citrus leaves

We optimized a methodology for leaf, -due to the autofluorescence of the citrus leaves, to perform an immunostaining.. For that purpose, the leaves were immersed in acetic acid and alcohol solution (3:1, v/v) for at least 5 days. Since the destaining process results in the inactivation of GFP fluorescence, an antibody against GPF was used, as described below.

The leaf fragments were fixed in 0.8 mL of acetic acid-alcohol solution deposited in Petri dish (30 mm x 15 mm). Water was gradually added, decreasing 20% acetic acid-alcohol every three minutes until the acetic acid-alcohol concentration reached 20%.

The solubilization of the cuticle, digestion of cell walls, membrane permeabilization and the immunostaining was performed according Pasternak et al (2015) with some modifications.

For solubilization of the leaf cuticle, the solution from the previous step was replaced by 0.8 mL methanol (100%) and incubated for 10 min at 60 °C. Then, the alcohol was gradually decreased by adding 100 to 200 µl of water every 2 min until the final alcohol concentration reached ˜20%. The leaf fragments were washed twice for 5 min in water. For cell wall digestion the water was replaced by 0.8 mL of cell wall digestion solution (0.4% Driselase in 2 mM MES Buffer, pH 5.0) and incubated for 40 min at 37 °C. The cell wall digestion solution was replaced by MTSB buffer [15 g PIPES, 1.90 g EGTA, 1.22 g $MgSO_4 \cdot 7H_2O$ and 2.5 g KOH are dissolved in a total of 1000 ml water at pH 7.0 (adjusted with 10 M KOH)] and incubated for 4 min. For permeabilization of the membrane, the MTSB was removed and the leaf was submerged in 0.8 mL membrane permeabilization solution (3% IGEPAL, 10% DMSO *d*iluted in MTSB) and incubated for 20 min at 37 °C. The solution was washed out 4 times with MTSB (3 min each).

After solubilization of the plant tissue, the next steps were the immunolabelling of the GFP expressed by Ca142egfp. To minimize non-specific binding of the antibody a blocking reaction was performed by submerging the leaf fragment in 0.8 mL blocking buffer (2% of albumin fraction V BSA diluted in MTSB) for 30 min. The next step involved the immunostaining using the Mouse Anti-GPF Monoclonal Antibody (Cusabio, CSB-MA000051M0m), placing the leaf fragment in 0.1 mL of antibody solution (antibody diluted 1:500 in blocking solution) and incubating for 2 h at 37 °C. To remove the unbound antibody, the leaf was washed for 5 min with 0.1 mL of MTSB. Anti-GFP was detected by

incubating the leaf for 40 min in 0.1 mL goat anti mouse Alexa Fluor 488 (ThermoFischer, A-11001) diluted 1:300 in MTSB. The nuclei and cell-walls of plant and fungal cells were stained with 0.1 mL of propidium iodide (0.4 mg/L) and 0.1 mL of calcofluor white (0.4 mg/L) for 20 min. The leaf fragments were transferred to microscope slides with antifade medium Fluoromount-G and covered with a cover slip. Fluorescent images were obtained under a Nikon A1R MP + laser-scanning microscope (NIKON Instruments Inc., Japan). The excitation wavelength lasers used were 405 nm, 488 nm and 561 nm, and the emission filters (band pass) used were 450/50 nm, 525/50 nm and 595/50 nm, respectively for calcofluor white, Alexa Fluor 488 and propidium iodide. The images were analyzed using NIS-elements Analysis 4.20 software and Photoshop CS4. As a control the wild-type *C. abscissum* lacking the *gfp* gene was used.

2.3. Analysis of the lifestyle of Colletotrichum abscissum Ca142

2.3.1. Analysis of morphology of Ca142 using DAPI staining

Initially, we analyzed the morphological characteristics of *C. abscissum* using the onion epidermis, since, due to its single cellular layer, the visualization of the fungal structures is facilitated. DAPI staining of nuclei and appressoria was done as described by Chazotte (2011). *C. abscissum* Ca142 inoculation in onion tissues (fragments of 1 cm^2) was performed as previously described for leaf inoculation. 24 h after inoculation, the onion fragments were fixed in 3.7% formaldehyde for at least 10 min and washed in PBS (NaCl 8 g, KCl 0.2 g, Na_2PO_4 0.24 g, $CaCl_2$ $2H_2O$ 0.133 g, Na_2HPO_4 1.44 g. in 1000 mL) three times for 5 min. The cells were permeabilized by immersion in 0.2% Triton X-100 for 5 min and rinsed in PBS for 5 min, three times. For nucleus staining, the onion fragments were incubated for 5 min in DAPI solution (0.0005 mg/mL). The fragments were rinsed in PBS three times and deposited on slides. The fungal development was analyzed as described above.

2.3.2. Analysis of morphological development of Ca142 on citrus flowers

Healthy flowers of *C. sinensis* were harvested and placed in 7 x 12 cm plastic pots containing water-agar medium. Each petal was inoculated with 10 µL of *C. abscissum* spore solution, and incubated in BOD in 12 h photoperiod for 48 h at 24 ºC. After incubation, tThe petals were submerged for 24 h in ethanol-acetic acid (3:1) solution and then the petal was washed 3 times in water. For clearing the petals were immersed in successive solutions of ethanol (ethanol 50%, ethanol 70%, ethanol 85%, ethanol 100%) followed by solutions of ethanol methyl salicylate (ethanol-methyl salicylate 1:1, ethanol-methyl salicylate 1:3, methyl salicylate 100%) for 30 min each; according to the methodology adapted from Young et al. (1979). The petals were sectioned with a scalpel, transferred to excavated slides containing antifade medium and covered with a cover slip. The images were acquired and analyzed as described above.

3. Results and discussion

3.1. Colletotrichum abscissum penetrates floral petals of Citrus sinensis presenting a hemibiotrophic lifestyle

In the macroscopic analysis, 24 h after inoculation, we observed the beginning of fungal growth and the development of symptoms in flowers, with the emergence of water-soaked lesions (Fig. 1B). 48 h after inoculation, typical necrotic peach-to-orange lesions were observed (Fig. 1C), in agreement with the results of Agostini et al. (1992).

Despite the absence of visible symptoms 12 h after inoculation (Fig. 1A), the microscopic analysis of the petals showed spores of *C. abscissum* CA142egfp already germinated and hyphae penetrating the floral tissues (Fig. 1D). In agreement with the results of Marques et al. (2012), the penetration of the pathogen into the petals occurred more frequently after appressorium formation, although it has also been shown that *C. abscissum* infects the host without forming these structures (Zulfiqar et al., 1996; Marques et al., 2012).

Regarding the lifestyle of *C. abscissum*, Marques et al. (2012) reported only the necrotrophic phase in petals and the non-development of biotrophic hyphae. However, using confocal laser scanning microscopy, and analysis of different layers for the first time we observed the formation of biotrophic hyphae of *C. abscissum* within cells (Fig. 2). We also observed the germination of conidia of *C. abscissum* (Fig. 3A) and appressoria formation on the surface of the petals (Fig. 2). The penetration of the host occurs and then the formation of primary hyphae within the infected cell. The primary hyphae presented irregular and globular morphology, with 4 to 8 µm diameter (Fig. 2B–H), compatible with the establishment of the biotrophic phase described for other species of *Colletotrichum* (Oliveira and Deising, 2016). We also observed secondary hyphae that begin to develop from the primary hyphae and proliferate throughout the plant tissue. Necrotrophic hypha was regular and narrower than the biotrophic hyphae, measuring 1.5 to 2 µm in diameter (Fig. 2B–H). These findings suggest *C. abscissum* as hemibiotrophic, which establishes a biotrophic interaction with the host plant and subsequently switch to a destructive necrotrophic phase, as described for other pathogens (Vargas et al. 2012). The bleaching approach used (petal-clearing) in the present study may have facilitated the visualization of biotrophic hyphae in *C. abscissum* in citrus tissues for the first time.

The confocal microscopic analysis of the development of *C. abscissum* on the epidermis of onion using DAPI nucleus staining (blue) showed that *C. abscissum* spores and hyphae are uninucleated, whereas appressoria contain more than one nucleus (Fig. 3A–B). These data are in agreement with those described for other species of *Colletotrichum*, such as *C. gloeosporioides* and *C. truncatum*, which have more than 90% of uninucleated spores (Dale et al., 1988; Alahakoon et al., 1992; Jagtap and Sontakke, 2009).

3.2. Colonization of Citrus sinensis leaves by Ca142egfp1

The protocol used for the immunolocalization of Ca142egfp1 allowed to observe the fungal colonization in leaves and flowers of citrus and to differentiate the target organism from the other fungi, colonizing the sctructures, due to GFP fluorescence (Fig. 1 and 2). Therefore, the use of mutant strains expressing fluorescent proteins represents a reliable alternative to understand the lifestyle of endophytes and phytopathogens (Gomes-Figueiredo et al., 2010; Kato et al., 2017).

One of the limitations for the indiscriminate use of phytopathogens expressing fluorescent proteins for microorganism-host interaction studies is related to the autofluorescence of some plant tissues, such as citrus leaves (Momin et al., 2013). Faced with this limitation, we used acetic acid and alcohol to remove the autofluorescence of the leaves, which resulted in leaf destaining, but also in the inactivation of the fluorescence expressed by eGFP strain. To cincumvent this problem, an anti-GFP antibody was used to detect the mutant strain and then a secondary antibody associated with a fluorescent molecule was used, allowing visualization of the inoculated Ca142egfp1 strain (Fig. 4).

Some studies suggest that *C. acutatum* (currently *C. abscissum*) penetrates citrus leaves forming appressoria and penetration pegs, and subsequently causes a quiescent infection until the bloom period (Agostini et al., 1992; AAgostini and Timmer, 1994; Timmer et al., 1994). Intense colonization by *C. acutatum* (currently *C. abscissum*) in senescence leaves under laboratory conditions was observed by Zulfiqar et al. (1996), however, the authors also verified that this does not occur in orchard conditions. According to Agostini and Timmer (1994) and Zulfiqar et al. (1996) in citrus leaves *C. abscissum* survives only as an epiphyte. Agostini et al., 1992was able to isolate *C. acutatum* (currently *C. abscissum*) from citrus leaves, but at low frequency when compared to *C. gloeosporioides,* suggesting that quiescent *C. abscissum* infections are not frequent (Agostini et al., 1992).

Due to these different aspects, we investigated the ability of *C.*

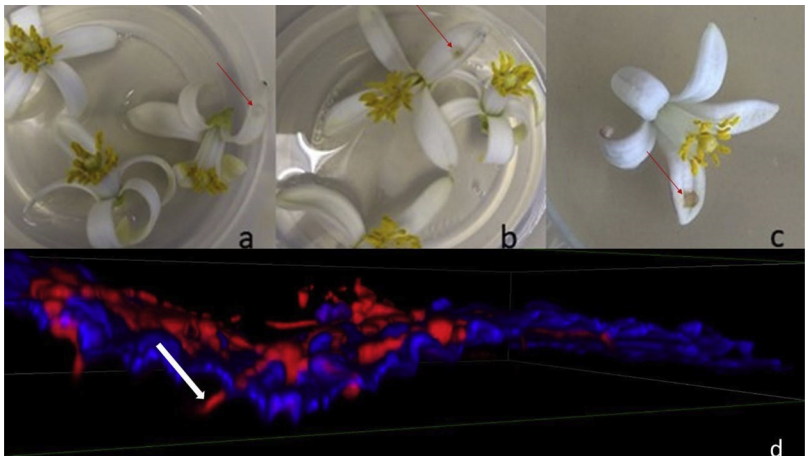

Fig. 1. Macroscopic (a–c) e microscopic (d) analysis of *Colletotrichum abscissum* 12 (a), 24 (b) and 48 (c) hours after inoculation of spore solution (10 µL of 10^5 spore/mL) on *Citrus sinensis* flowers (red arrows). a) non-symptoms; b) water-soaked lesions; c) classical postbloom fruit drop lesion; d) fluorescent microscopic analysis 12 h after inoculation showing the fungal hyphae expressing GFP (in red) penetrating the floral tissue (in blue).

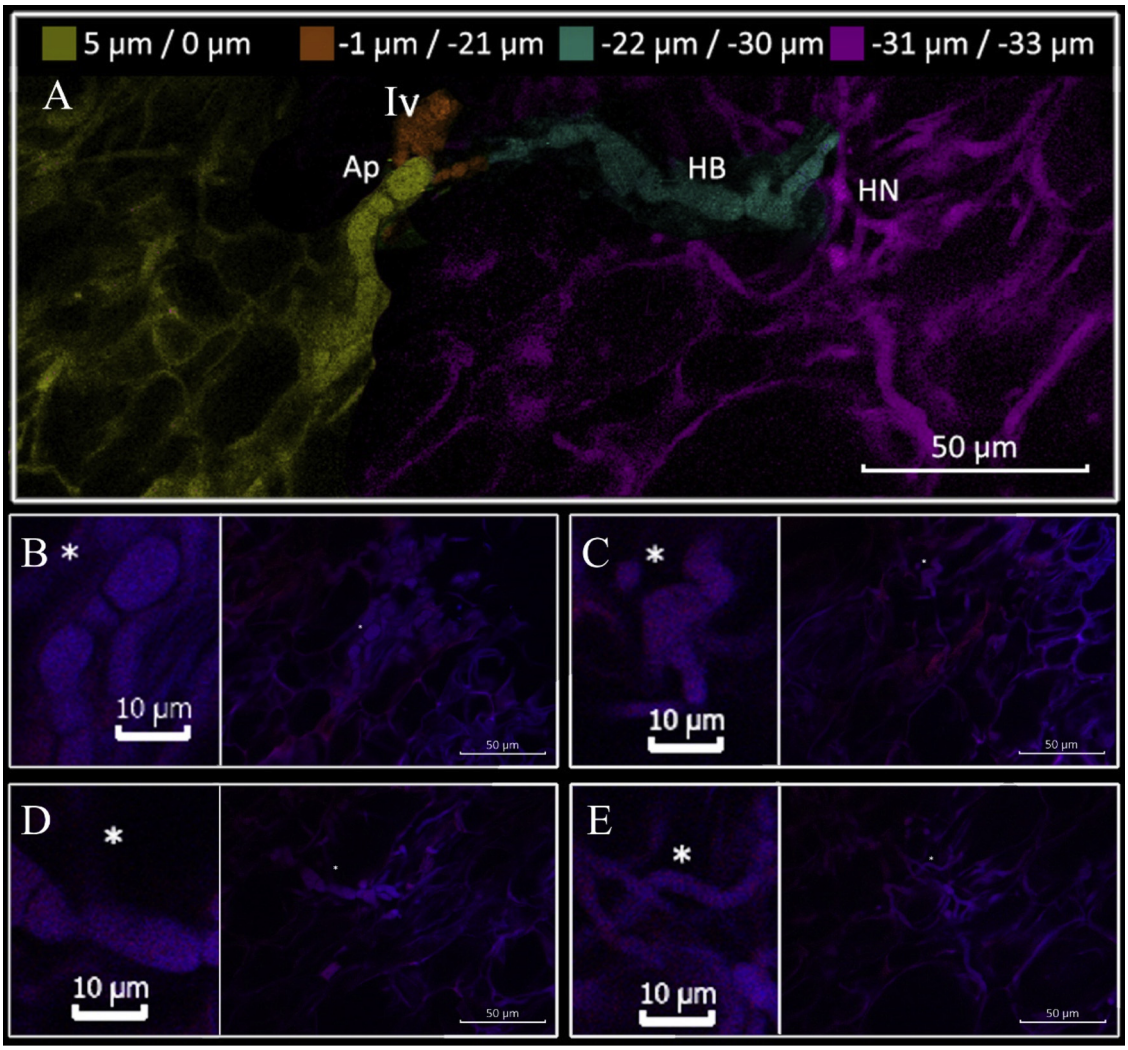

Fig. 2. Confocal Microscopic analysis, formed by superposition of image acquired in several layers showing the penetration and colonization of citrus petals by the fungus *Colletotrichum abscissum* 48 h after inoculation. A) in yellow an appressorium (Ap) formed on the surface of the petal, below it occurs the formation of vesicular infection (Iv), in orange, and the penetration of the first cells of the petals by the fungus (in green). Biotrophic hypha (BH) with irregular and globular morphology is marked in green, and in purple, the necrotrophic hypha (NH) is visible. B, C, D) Biotrophic hyphae of *C. abscissum* growing intracellularly and constricting passing through the cell wall through specific points of contact with other cells, with diameter from 4 µm to 8 µm. E) The necrotrophic hyphae are evident by their regular, narrower, and elongated form, with a diameter close to 2 µm. B–E) Images analyzed using the NIS-Element viewer 4.2 software and show the XY and XZ planes. The inset pictures show a magnification of the section indicated by the asterisk. XYZ coordinates are showed bottom in the picture with the distance in relation to the petal surface.

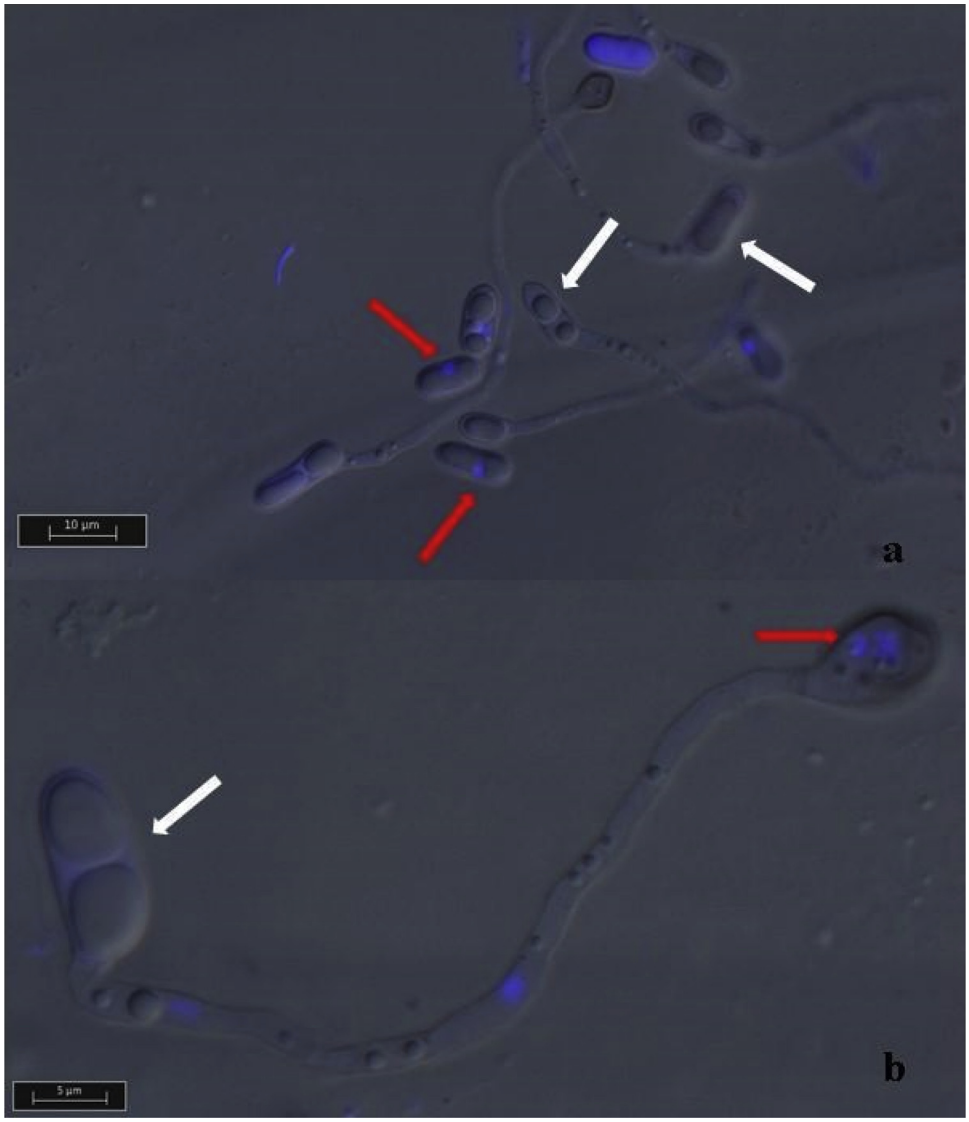

Fig. 3. Differential interference contrast (DIC) combined with confocal microscopy analysis showing growth of *Colletotrichum abscissum* on onion, 48 h after inoculation, stained with DAPI. a) uninucleate (blue dots) non-germinated spores (red arrows) and anucleate germinated spores (white arrows); b) anucleate germinated spore (white arrow), hypha with a single nucleus per cell and multinucleated appressorium (red arrow).

abscissum to colonize citrus tissues with and without artificial injury. In our analysis, *C. abscissum* grew on the surface of citrus leaves as an epiphytic fungus, however, no penetration and colonization of leaf tissue was observed (Fig. 4A–B). Even in the presence of a tissue injury, the fungus uses the plant material to grow and produces a large number of spores but does not spread to adjacent healthy tissues (Fig. 4B–C). These data corroborate with the hypothesis that appressorium production can allows this species to remain in a quiescent infection in citrus leaves, however, without colonization. It also indicates that tissue injuries may contribute to the spread of the fungus through the induction of spore production, which can be transported by rain (Agostini et al., 1992) or by honeybees (Gasparato et al., 2017). The absence of colonization of healthy tissues suggests that this is not the primary mechanism for fungal survival (Timmer and Zitko, 1993). These data are corroborated by the non-isolation of *C. abscissum* from disinfected citrus leaves (Andrade et al. 2017), since disinfection with hypochlorite (3%), or another compound that acts on the fungal cell-wall, will eliminate the pathogen, and it will not be recovered from the sample.

In addition, the use of nuclear and cell wall dyes using fluorescent molecules allows the study of fungal interactions, fungal development in plant tissues and several fungus-plant interaction studies. The use of different staining methods facilitates observation of the 3D images, or the observation of different planes at same time (Figs. 3 and 4) (Long et al., 2013). Another methodology used to observe fungal colonization of plant tissues involves the use of scanning electron microscopy (Read and Lord, 1991; Rawat and Tewari, 2010; Marques et al., 2018), however, this methodology does not differentiate endogenous fungi from the target species and, therefore, the use of axenic plants is required, which in some cases makes the analysis unfeasible or limiting.

4. Conclusions

The phytopathogen *C. abscissum* penetrated the citrus flowers 12 h after inoculation, and the presence of biotrophic hyphae in citrus flowers suggests this species as a hemibiotrophic fungus, with a short biotrophic phase followed by a dominant necrotrophic colonization. However, *C. abscissum* did not colonize citrus leaves, with or without injury. Apparently, the fungus uses only the nutrients released by the injuries to grow and then produces a large amount of spores, which may be an important mechanism for the dissemination of *C. abscissum*. The protocols used in this study allow us (i) to observe structures of plants and fungi simultaneously, (ii) to use in autofluorescent plants, due to

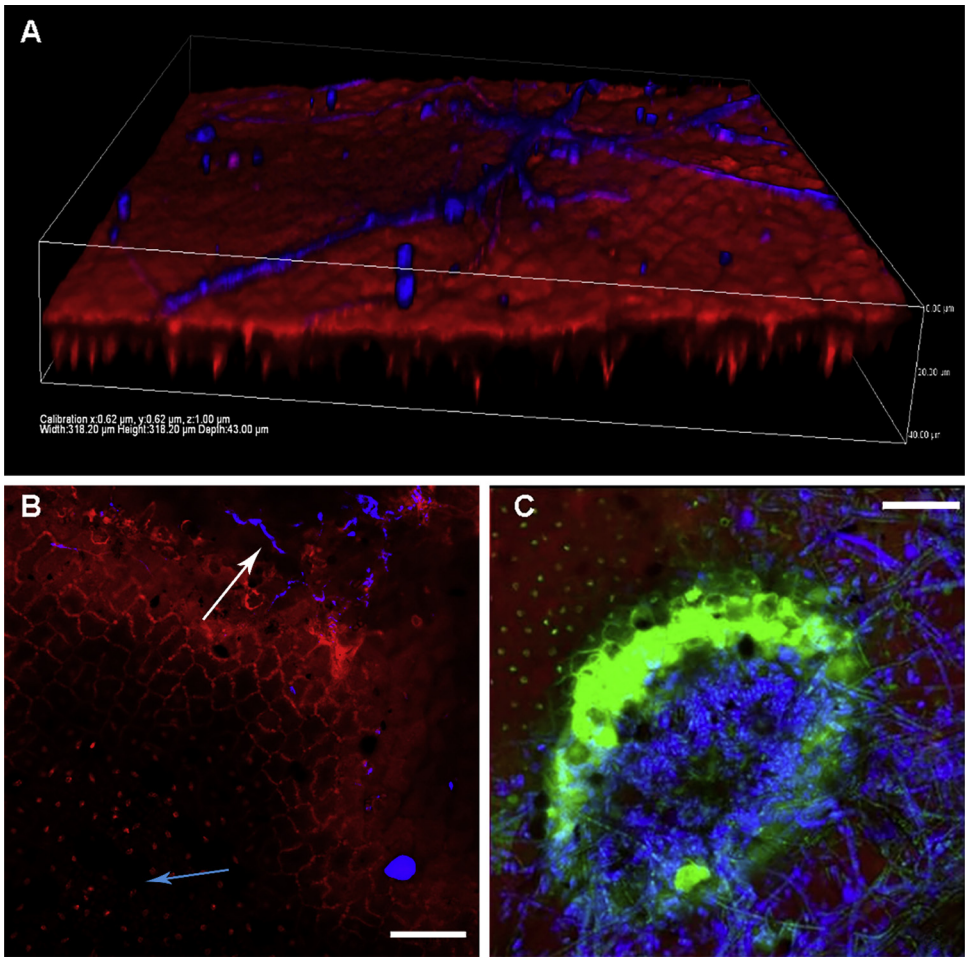

Fig. 4. Analysis of *Colletotrichum abscissum* growth in *Citrus sinensis* leaves without (a–b) and with artificial injury (c) 60 days after inoculation of 10 µL of a spore solution containing 10^5 conidia/mL. a) Superficial growth of *C. abscissum* (in purple) in citrus leaves (in red). b) Surface and in internal leaf tissues showing in blue the cell walls (stained with calcofluor white) and in red the nucleus (stained with DAPI). The mycelium of *C. abscissum* (in purple) is present only on the surface (white arrow) and no hypha was observed inside the foliar tissues (blue arrow), no fungal growth is observed in plant tissues adjacent to injury (blue arrow). c) Mycelial growth of *C. abscissum* expressing GFP (labeled with primary and secondary antibodies) and spores (in blue by staining with calcofluor white) in an artificial injury performed on the leaves before inoculation.

petal and leaf-clearing, (iii) differentiation between target species and microbiome, and (iv) visualization of the fungus-plant interaction in two and three dimensions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.micres.2019.05.005.

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